CYCLOOXYGENASE EXPRESSION IN HUMAN DIABETES

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

The work reported in this thesis was carried out solely by the candidate at RMIT University, through the School of Medical Sciences, Science, Engineering and Technology Portfolio, whilst in receipt of Postgraduate Research Scholarship (equivalent to RPA), since the official commencement date of Ph.D Candidature.

This thesis contains no material, which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains neither material previously published or written by another person nor experimental data from another person’s work except where due reference is made in the text of the thesis.

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Names need not to be mentioned for they will always be remembered and shall never be forgotten.
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**Achievement:**

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The 3rd Annual World Congress on the Insulin Resistance Syndrome (San Francisco USA)
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<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AGEs</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>enhancer-binding protein</td>
</tr>
<tr>
<td>CML</td>
<td>carboxyl methyl lysine</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DP</td>
<td>prostaglandin D receptor</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>EP</td>
<td>prostaglandin E receptor</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FP</td>
<td>prostaglandin F receptor</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GP</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>HEPES-BSS</td>
<td>HEPES buffered saline solution</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HUVECs</td>
<td>human umbilical endothelial cells</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>prostacyclin receptor</td>
</tr>
<tr>
<td>JNK/SAPK</td>
<td>Jun kinase/stress activated protein kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>micro BCA</td>
<td>micro bicinchoninic acid</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>MODY</td>
<td>maturity-onset diabetes of the young</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSR-A</td>
<td>macrophage scavenger receptor A</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide–adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide–adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NIDDM</td>
<td>non-insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>O(_2^+)</td>
<td>Superoxide anions</td>
</tr>
</tbody>
</table>
ONOO$^-$ peroxynitrite
PBS phosphate buffered saline
PG prostaglandin
PGG$_2$ prostaglandin G$_2$
PGH$_2$ prostaglandin H$_2$
PGI$_2$ prostacyclin
PLA$_2$ phospholipase A$_2$
PLC phospholipase C
PMSF phenylmethylsulphonylfluoride
RAGE receptors for AGEs
RIPA radioimmunoprecipitation buffer
RNS reactive nitrogen species
ROS reactive oxygen species
Ser serine residue
Sir2 Silent information regulator 2
STZ streptozotocin
TGF-$\beta$ transforming growth factor-$\beta$
TNF-$\alpha$ tumor necrosis factor-$\alpha$
TNS trypsin neutralizing solution
TP thromboxane receptor
TSA trichostatin A
TXA$_2$ thromboxane A$_2$
VCAM-1 vascular cell adhesion molecule-1
VNTR variable number of tandem repeats
VSMCs vascular smooth muscle cells
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CYCLOOXYGENASE EXPRESSION IN HUMAN DIABETES
Summary

Cyclooxygenase (COX) is the rate limiting enzyme that catalyses the production of prostanoids, which are crucial to vascular homeostasis. Evidence suggests that COX-mediated pathways play a role in aging and diabetes.

Aging is associated with endothelial dysfunction, which is characterized by impaired endothelial-dependent relaxation responses. The exact causes of age-related endothelial dysfunction are not clear. Previous animal studies by our laboratory at RMIT University reported enhanced COX expression with aging in rat aortas. Potentially, this may result in an imbalanced prostanoid production favoring the synthesis of vasoconstrictors such as TXA$_2$ and hence increase the risk of cardiovascular events in the aging population. Very little is known about the regulation of altered COX expression in aging. It has been suggested that histone hyperacetylation may be an important mechanism that regulates COX levels during the aging process as increased histone acetylation has been shown to occur with aging.

In the case of diabetes, studies have reported that the development of diabetes and its complications is associated with persistent inflammatory activity, evident with increased inflammatory markers in the circulation. COX-mediated pathways may be involved in this inflammatory process in diabetes. Furthermore, the formation of advanced glycation end products (AGEs) is accelerated in diabetes. AGEs can bind to receptors for AGEs (RAGE), which has also been suggested to play a role in inflammation in diabetes.

The present study aimed to investigate firstly, whether previously observed enhanced COX expression in aging is modulated by histone hyperacetylation and secondly, whether there are changes in COX and RAGE levels in platelets and monocytes in Type 1 and Type 2 diabetes.
The present study reported that
i) in cultured endothelial cells, COX expression was up-regulated in response to the treatment of a histone de-acetylase inhibitor, Trichostatin (TSA). COX up-regulation was accompanied by an increase in histone H4 acetylation status, suggesting that COX gene expression is modulated through histone acetylation/de-acetylation. This could be a potential mechanism by which increased COX expression in aging is regulated.

ii) an initial study in Zucker rats, an animal model that has the characteristics of Type 2 diabetes, found increased COX levels in platelets and monocytes, an observation that was not found in the human Type 2 diabetic study. This suggests that changes in COX levels in these inflammatory cells may be species-dependent. In addition to unaltered platelet COX-1 and monocyte COX-2 levels in Type 2 diabetes, the present human study further reported elevated plasma PGE$_2$, but not plasma TXB$_2$ in Type 2 diabetes, suggesting an overall increase in inflammatory activity in human Type 2 diabetic subjects. Treatment with non steroidal anti-inflammatory drugs (NSAIDs) in Type 2 diabetes reduced both COX expression and COX activity. This re-affirms the previous observation that inflammation was increased in human Type 2 diabetic subjects.

In addition, monocyte acetylated histone H4 expression was unaltered in human Type 2 diabetes, which was consistent with the lack of changes in monocyte COX-2 levels. This supports the hypothesis that COX expression is modulated through hyperacetylation of the core histones. The use of NSAIDs resulted in a reduction in monocyte acetylated histone H4 levels in Type 2 diabetes. To the best of our knowledge, this is a novel finding suggesting potentially COX expression may be regulated by NSAIDs via histone modifications.
iii) in human Type 1 diabetic subjects, the COX-2-mediated pathway was altered as indicated by increased monocyte COX-2 expression. In plasma, elevated PGE₂ levels were also observed in Type 1 diabetes. Treatment with NSAIDs in Type 1 diabetes reduced both COX expression in platelets and monocytes and COX activity shown by reduced plasma COX metabolite levels. These data suggest an overall increase in inflammatory activity in Type 1 diabetes.

Concurrent with increased monocyte COX-2 levels was an increase in acetylated histone H4 expression in monocytes in Type 1 diabetes, suggesting hyperacetylation of the core histones is associated with COX up-regulation. The use of NSAIDs down-regulated monocyte acetylated histone H4 levels in Type 1 diabetes. This is a novel finding suggesting that NSAIDs may regulate COX expression via histone acetylation/de-acetylation.

iv) RAGE levels were moderately increased in platelets and monocytes in both human Type 1 and Type 2 diabetic subjects. Increased RAGE levels were reduced by the treatment of NSAIDs in human diabetic subjects, suggesting RAGE may be a crucial player in the inflammatory process in diabetes. The ability of NSAIDs to down-regulate RAGE may be of importance in the management of diabetic complications.
CHAPTER 1
GENERAL INTRODUCTION
1.1 THE CIRCULATORY SYSTEM

The heart and blood vessels form the basis of the circulatory system whose function is to provide adequate blood supply to organs and tissues within the body. Several mechanisms including neural, endocrine and various local factors control the circulatory system by regulating blood flow to organs and tissues. For instance, by altering the rate and force of its contraction, these mechanisms modulate pumping function in the heart. Similarly, these mechanisms can alter blood vessel diameter hence, influencing vascular resistance, pressure and the rate of blood flow. Control of the circulatory system is essential in achieving a physiological homeostasis (Fox 1996; Guyton & Hall 2001).

1.1.1 FACTORS AFFECTING CARDIAC FUNCTION

Under the regulation of the central nervous system, the autonomic nerves exert their control over the heart by modulating its rate and force of contraction. The autonomic nervous system processes information collected by sensors, which respond to various changes in the body through autonomic reflexes. For instance, baroreceptors in arteries detect changes in blood pressure, chemoreceptors monitor oxygen, carbon dioxide and pH level in the bloodstream, visceral and cardiopulmonary reflexes respond to changes in blood flow and cardiac stretch/pressure, respectively (Sherwood 1989; Fox 1996). In response to the sensory information transmitted to the brain, the sympathetic nervous system releases neurotransmitters such as noradrenaline to enhance the rate and force of heart contraction via β–adrenoceptor activation (see Review by Xiao et al. 1999). In contrast, the parasympathetic nervous system opposes its effect by releasing acetylcholine, which in turn activates muscarinic receptors and reduces heart rate (see Review by Hill et al. 2001).

In addition to the autonomic nervous system, factors associated with the coronary and systemic circulation also influence the actions of the heart. Through both myogenic and metabolic mechanisms and neural input from the sympathetic nervous system, the coronary
circulation regulates the amount of blood supply to the heart whilst the systemic circulation
determines output resistance into which the heart is pumping (Fox 1996; Marieb 1998).

1.1.2 THE ARTERIAL CIRCULATION

The arterial circulation consists of large conduit arteries branching repeatedly into smaller
vessels, arterioles and capillaries. It is at the level of capillaries where nutrients and
low-pressure oxygen exchange takes place (see Review by Hill et al. 2001). The composition
of the arteries includes three layered walls, tunica adventitia, media and intima, surrounding a
hollow lumen with the tunica adventitia being the outermost layer. The tunica adventitia
consists mainly of collagen with lymphatic vessels, nerves and vasa vasorum (only in larger
arteries). Stimulation of nerves located in the tunica adventitia can result in the release of
vasoconstrictor and vasodilator neurotransmitters affecting vascular tone (see Review by
Fernández-Alfonso 2004). A large portion of the arterial wall comprises the elastic lamina
interspersed with smooth muscle cells and collagen, and is known as the tunica media. A
single layer of endothelial cells lying upon a basal elastin layer forms the innermost tunica
intima (see Review by Cohn 1999). The smooth muscle and endothelial cells are capable of
releasing various mediators and substances such as prostanoids that modulate vascular tone
(Berne et al. 1998; see Review by Luscher & Barton 1997).

1.1.3 CONTROL OF ARTERIAL CIRCULATION

Mechanisms that control blood vessel diameter are complex. Changes in blood vessel
diameter via neural, hormonal and other various factors ultimately affect the supply of
nutrients and oxygen to tissues and cells in various organs. The vascular smooth muscle cells
(VSMCs) also have a crucial influence on arterial blood flow as stimuli from hormones, local
mediators and neural transmitters can lead to a series of signal transduction cascades via
receptors on VSMCs (Berne et al. 1998).
1.1.3.1 The neural system

A network of neurons infiltrates the adventitial layer in most blood vessels. The dense neural network regulates the underlying medial smooth muscle layer by releasing neurotransmitters causing either a vasoconstriction or vasorelaxation response. While noradrenaline-activated $\alpha_1$- and $\alpha_2$-adrenoceptors are the principal sympathetic contractile influences in VSMCs, sympathetic nerves can also release a variety of substances such as neuropeptide Y and adenosine triphosphate (ATP), which can either directly cause a contractile response or achieve a contractile response through modulation of noradrenaline activity (see Review by Hill et al. 2001). In the case of vasodilatation it is regulated by both sensory neurons and vasodilator nerves. Sensory neurons can release peptides such as substance P, calcitonin gene-related peptide, neurokinin A and neurokinin B, which are all capable of inducing vasorelaxation. Vasodilator nerves on the other hand are found in some blood vessels. For instance, parasympathetic nerves are present in the pelvic viscera and produce acetylcholine and vasoactive intestinal peptide, leading to receptor-mediated vasorelaxation (see Review by Holzer & Lippe 1992).

1.1.3.2 The endocrine system

Hormonal influence from the endocrine system contributes greatly to the regulation of smooth muscle contractility in blood vessels and kidney. For example, adrenaline and noradrenaline are released into the blood stream in response to sympathetic nerve and adrenal medulla stimulation. They activate $\alpha$- and $\beta$-adrenoceptors hence the vasoconstriction and vasodilatation, respectively, of blood vessels (Marieb 1998). In the kidney, renin is released when arterial pressure is low. The release of renin leads to the conversion of angiotensinogen to angiotensin I, which is subsequently converted to angiotensin II in the endothelium. Angiotensin II is a potent vasoconstrictor (see Review by Touyz & Schiffrin 2000).
1.1.3.3 Regulatory factors

Endothelial cells release vasorelaxant factors including nitric oxide (NO), prostacyclin (PGI₂) and endothelium-derived hyperpolarizing factor (EDHF) in response to physiological and hormonal stimuli. While these vasorelaxation modulation factors relax VSMCs, endothelial cells also produce contracting factors such as endothelin, thromboxane A₂ (TXA₂) and angiotensin II. Modulation factors can also be released by smooth muscle and blood elements in the circulation, which further modulate and maintain vascular tone (Ramadan et al. 1990; see Review by Luscher & Barton 1997). PGI₂ and TXA₂ are products of cyclooxygenase (COX)-mediated pathways (for more details, see Section 1.2). Previous studies by our laboratory at RMIT University found that COX-mediated pathways were involved in the aging process in rat aortas (Kang et al. 2001). These changes may potentially result in an imbalanced prostanoid production favoring the synthesis of vasoconstrictors such as TXA₂ and contribute to endothelial dysfunction.

1.2 CYCLOOXYGENASE (COX) SIGNALING PATHWAYS

Cell membrane phospholipids can be cleaved by phospholipase A₂ (PLA₂) to release arachidonic acid, which is the precursor of a variety of signaling molecules that include prostaglandins and thromboxanes. Prostaglandins were first discovered in human semen in 1930 (Lawrence et al. 1999) with the biosynthesis mechanism outlined thirty-seven years later by Hamberg and Samuelsson (1967). The key event in the proposed mechanism involved the production of bicyclic peroxide (endoperoxide) from polyunsaturated fatty acid oxygenation (arachidonate) catalyzed by the enzyme termed prostaglandin endoperoxide H synthase, which is also known as cyclooxygenase (COX) (Hamberg & Samuelsson 1967; see Review by Lawrence et al. 1999). COX is coupled to a range of synthases. The products of this COX enzymatic pathway are prostanoids including various prostaglandins and thromboxanes, which play important roles in modulation of vascular tone in both physiological and pathophysiological conditions (see Figure 1-1).
**Figure 1-1**

**Prostanoid synthesis pathway**

Arachidonate is cleaved free by phospholipase A₂ (PLA₂) from the lipid membrane and is converted to PGH₂ via two major steps by COX enzymes: oxygenation of arachidonic acid into PGG₂ and reduction of PGG₂ into PGH₂. Isomerization or reduction of PGH₂ by terminal prostanoid synthases in tissues then results in the production of various prostanoids.
1.2.1 COX-MEDIATED PATHWAYS

The production of prostanoids via COX-mediated pathways is dependent on the availability of substrates and the activity of COX (see Figure 1-1 for the pathway). Three naturally occurring polyunsaturated fatty acids, trienoic (dihomo-γ-linoic) acid, tetraenoic (arachidonic) acid and pentaenoic acid, are substrates for COX enzymes. The predominant substrate however, is arachidonic acid since it is the most abundant in animal tissue (see Review by Crawford 1983).

Arachidonic acid is derived from membrane phospholipids predominantly by the action of phospholipase A$_2$ (PLA$_2$). Following the cleavage of arachidonic acid from the sn-2 position of the membrane phospholipids, COX catalyzes arachidonic acid, resulting in the formation of endoperoxide prostaglandin G$_2$ (PGG$_2$) (see Review by Crofford 2001). This reaction takes place in the COX active site of the catalytic domain where arachidonic acid enters the channel via the hydrophobic compartment created by the membrane-binding domain and forms a hairpin turn between C9 and C11. A tyrosyl radical from the oxidized Tyr-385 then removes the pro(S) hydrogen from C13 of arachidonic acid. Molecular oxygen forms the endoperoxide bridge connecting C9 and C11 followed by the ring closure between C8 and C12 resulting in a bicyclic cytopentyldioxygen prostaglandin structure (see Review by Garavito & DeWitt 1999).

The second molecular oxygen is then added to C15 producing PGG$_2$. PGG$_2$ then moves to the peroxidase subunit of the catalytic domain to be reduced to PGH$_2$. PGH$_2$ is the common precursor for many biologically active eicosanoids such as TXA$_2$, PGI$_2$ and other prostaglandins. The production of different prostanoids converted from PGH$_2$ depends on individual cell types and the presence of specific enzymes in those cells. For example, PGI$_2$ production is catalyzed by PGI$_2$ synthase in the endothelial cells. TXA$_2$ generation catalyzed by TXA$_2$ synthase is observed in the platelets. Several other prostaglandins including PGD$_2$, PGE$_2$ and PGF$_{2\alpha}$ are produced from PGH$_2$ mainly in the mast cells, macrophages and smooth muscle respectively (Reddy & Herschman 1996). Figure 1-1 details the biochemical pathways by which prostanoids are produced. Specific roles of prostanoids in the vasculature are further discussed in Section 1.3.
1.2.2 PROSTANOID RECEPTORS

Prostanoids produced from COX-mediated pathways exert their effects through the activation of prostanoid receptors. Prostanoid receptors belong to the rhodopsin superfamily of G protein-coupled receptors. There are eight types and subtypes of membrane prostanoid receptors in mammals from mouse to human (Table 1-1). They include the PGD (DP), PGF (FP), PGI (IP), TXA (TP) and four subtypes of PGE receptors (EP₁-₄). Prostanoid receptors can be divided into three categories based on their signal transduction and action. The DP, IP, EP₂ and EP₄ receptors have been termed the “relaxant” receptors, because they stimulate adenylate cyclase, resulting increased cyclic adenosine monophosphate (cAMP) levels via Gs to exert their inhibitory effects. In contrast, FP, TP and EP₁ receptors are coupled to phospholipase C via Gq to induce calcium mobilization hence they are referred to as “contractile” receptors. Among the prostanoid receptors, EP₃ receptors have the most complex molecular biology with the existence of seven isoforms through RNA splicing in humans (Adam et al. 1994). EP₃ receptors are usually coupled to Gi and inhibit cAMP production. EP₃, however can also increase cAMP production by coupling with Gs (see Review by Hatae et al. 2002). This vast diversity in receptor biology has further added complexity to COX-mediated signaling pathways.
<table>
<thead>
<tr>
<th>Prostanoid receptors</th>
<th>Activators</th>
<th>Species</th>
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<tbody>
<tr>
<td>DP</td>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Human, mouse</td>
<td>Hirata et al. 1994; Boie et al. 1995</td>
</tr>
<tr>
<td>EP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Mouse, human</td>
<td>Regan et al. 1994; Katsuyama et al. 1995</td>
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<tr>
<td>FP</td>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>Mouse, human, bovine, rat, sheep</td>
<td>Abramovitz et al. 1994; Kitanaka et al. 1994; Sakamoto et al. 1994; Sugimoto et al. 1994; Graves et al. 1995</td>
</tr>
<tr>
<td>IP</td>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Mouse, human, rat</td>
<td>Boie et al. 1994; Katsuyama et al. 1994; Nakagawa et al. 1994; Namba et al. 1994; Sasaki et al. 1994</td>
</tr>
<tr>
<td>TP</td>
<td>TXA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Mouse, rat, bovine</td>
<td>Namba et al. 1992; Abe et al. 1995; Muck et al. 1998</td>
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1.2.2.1 PGD receptors (DP)

DP receptor expression is the least abundant and least well characterized among the prostanoid receptors. It is expressed in platelets, lung, uterus, stomach, ileum and small intestine of humans (Hirata et al. 1994; see Review by Coleman et al. 1994). Despite its low receptor expression, the activation of DP receptor by its endogenous ligand, PGD$_2$, leads to increased cAMP and intracellular Ca$^{2+}$ levels (Wright et al. 2000). The activation of DP receptor by PGD$_2$ has also been implicated in many physiological processes including the induction of sleep (Matsumura et al. 1994), regulation of body temperature (Sri Kantha et al. 1994), inhibition of platelet aggregation (Negishi et al. 1993) and is a mediator of allergic disorders such as allergic rhinitis (Johnston et al. 1993).

1.2.2.2 PGE receptors (EP)

There are four subtypes of EP receptors identified, namely EP$_1$, EP$_2$, EP$_3$ and EP$_4$ receptors. Depending on the subtype, the activation of EP receptors by the endogenous ligand, PGE$_2$ can result in a diverse range of effects. EP$_1$ receptor has a limited tissue distribution and is found in the kidney, lung and stomach (Watabe et al. 1993). Their activation via calcium mobilization leads to constriction of smooth muscle in the gastrointestinal tract and trachea and together with EP$_3$ and EP$_4$, EP$_1$ receptor activation is associated with regulating kidney function particularly with water re-absorption (Breyer et al. 1993; Sugimoto et al. 1994).

In a similar fashion, EP$_2$ receptor expression is also less than abundant; in fact the EP$_2$ receptor is the least abundant among the PGE receptors. EP$_2$ receptor expression, however, can be effectively upregulated in response to stimuli. For instance, EP$_2$ expression is induced significantly by LPS in macrophage cell line (Negishi et al. 1995). The activation of this PGE receptor subtype, which is coupled to stimulation of cAMP, leads to vascular, bronchial and reproductive smooth muscle relaxation (Negishi et al. 1995).
With its capacity to couple to multiple G proteins including Gi, Gs and Gβγ, EP₃ receptor subtype has a more complex pharmacological profile than the other three EP subtypes (Hatae et al. 2002). EP₃ receptor is also widely distributed in many tissue types. For example, EP₃ mRNA is expressed in neurons of the myenteric ganglia, in fundic gland epithelial cells, both in parietal cells and in chief cells, in smooth muscle cells in longitudinal muscle throughout the gastrointestinal tract. Thus, EP₃ receptor activation has been linked to regulate smooth muscle contraction, particularly the vascular and uterine smooth muscle, reduce gastric acid secretion and hence affecting the cytoprotection in the gastrointestinal tract (Sugimoto et al. 1992; Sugimoto et al. 1994a; Morimoto et al. 1997).

The expression of EP₄ mRNA has been found in the neurons of the hypothalamus, lower brainstem (Momiyama et al. 1996) and in the gland of the gastric antrum (Morimoto et al. 1997). It is through cAMP stimulation that this Gs-protein-coupled EP₄ receptor subtype exerts relaxant effects on vascular smooth muscle including human cerebral artery (Davis et al. 2004).

1.2.2.3 PGF receptors (FP)

There are two FP receptor isoforms: FPₐ and FPₐ. The FPₐ isoform differs from the FPₐ isoform in that it lacks the last 46 amino acids at the C-terminus. Coupled to Gₙ, both receptor isoforms are activated by their endogenous ligand PGF₂α, although FP receptors can also be activated by PGF₂α, PGE₂ and PGD₂. Once engaged, FP receptors can activate phosphatidylinositol signaling pathways and the activation of protein kinase C (PKC) (Graves et al. 1995; Pierce et al. 1997; Pierce et al. 1999). The organ that most abundantly expresses FP mRNA is the corpus luteum (Powell et al. 1974). When the expression pattern of FP mRNA in luteal cells was investigated in a gonadotropin-primed pseudopregnant mouse model, it was found that the granulosa cells in the ruptured follicles began to express FP mRNA once ovulation was induced (Hasumoto et al. 1997). FP mRNA level then decreased
when the luteal cells underwent apoptosis, suggesting FP mRNA expression is influenced by the estrous cycle. In contrast, FP mRNA expression in the kidney, heart, stomach and lung of mouse does not change with the estrous cycle (Hasumoto et al. 1997).

**1.2.2.4 Prostacyclin receptors (IP)**

Distribution of IP mRNA has been examined in various mouse organs by Oida et al. (1995) in an in situ hybridization study. It was reported that IP mRNA was expressed in the kidney, megakaryocytes, smooth muscle of arteries, neurons of the dorsal root ganglion, matured thymocytes and splenic lymphocytes. This widespread localization of IP mRNA suggests IP receptors, activated by PGI₂ are involved in a diverse range of physiological processes. For instance, Murata et al. (1997) used carrageenan-induced paw swelling and acetic acid-induced writhing as models to demonstrate that IP receptors are involved in acute inflammation and pain, respectively. It was also reported that these IP-deficient mice, although viable, reproductive and normotensive, were susceptible to thrombosis. Detection of IP mRNA in megakaryocytes and the smooth muscle of arteries is consistent with the action of PGI₂ in the cardiovascular system, another example demonstrating the involvement of IP receptors in diverse physiological processes.

**1.2.2.5 Thromboxane receptors (TP)**

The TP receptor is primarily coupled to G₉, which activates phospholipase C (PLC) (Hirata et al. 1996). In human platelets, two TP isoforms, TPα and TPβ have been identified based on the differences in the C-terminal sequence (Kinsella 2001). Although both TPα and TPβ have the same G protein-dependent coupling to PLC and are both activated by TXA₂ and PGH₂, they do display differential regulation of cAMP (Walsh et al. 1998; Kinsella 2001).

TP receptors can be activated by the endogenous ligand, TXA₂, the precursor of TXA₂, PGH₂ and isoprostane, a prostaglandin-like substance produced from free radical oxidation of
arachidonic acid without the involvement of COX enzymes (Negishi et al. 1995; Kinsella et al. 1997). Once activated, TP receptors mediate numerous cellular responses including platelet aggregation, stimulation of VSMC growth and VSCM contraction (Morinelli et al. 1994; see Review by Narumiya et al. 1999). These potent effects are localized due to the instability and rapid metabolism of TXA₂ and PGH₂, which reduce their potential dangerous impacts on the systemic circulation. In addition to their well-known actions in the cardiovascular and respiratory systems, TP receptors may also play a role in the immune system. Namba et al. (1992) found that TP mRNA was abundantly expressed in thymus and spleen of mice. Furthermore, a study using the radio-ligand binding assay by Ushikubi et al. (1993) found that immature thymocytes express TP receptors at a density as high as that in platelets. Once T-cells mature, TP receptor expression then declines with the exception of peripheral T-cells, which still express high levels of TP receptors. Thus, TP receptors may in fact be involved in thymocyte differentiation and development.

1.2.3 COX ISOFORMS

There are at least two COX isoforms that have been identified and they are COX-1 and COX-2. COX enzymes are encoded by separate genes located in human chromosome 9 and 1, respectively (see Reviews by Smith & DeWitt 1996; Hla et al. 1999). COX-1 and COX-2 are found in the lumen of the endoplasmic reticulum (ER) where they catalyze prostanoid production. The prostanoids are released from the cell and act on G-protein-coupled receptors. Although COX-2 is found in the cytoplasm, it is also present in the nuclear envelope. Nuclear-located COX-2 synthesizes prostanoids that may act through the nucleoplasmic or nuclear membrane targets related to cell differentiation and replication (Parfenova et al. 2001).

Studies of the structure of COX have revealed the two isoforms share approximately 60% homology. Structurally, COX is a homodimeric, heme-containing, glycosylated protein
comprising three domains, an epidermal growth factor domain, a membrane-binding domain consisting of a spiral of four amphipathic helices and a globular catalytic domain (see Reviews by Smith et al. 1996; Garavito & DeWitt 1999; Marnett et al. 1999). The catalytic domain further contains two subunits, the COX active site and the peroxidase active site separated by the heme prosthetic group (see Reviews by Garavito & DeWitt 1999; Marnett et al. 1999). COX is a monotopic integral protein and it interacts with the ER membrane by penetrating its membrane-binding domain into only one leaflet of the lipid bilayer (see Review by Garavito & DeWitt 1999). In addition to anchoring to the lipid bilayer, the membrane-binding domain also creates a hydrophobic compartment that forms the mouth of the COX active site. The active site is a long, hydrophobic channel linked with a residue Tyr-385 (Picot et al. 1994). Within the channel, two polar residues are found, Arg-120 and Ser-530. Arg-120 is located about midway between the mouth and the apex of the COX active site and Ser-530 is the site acetylated by aspirin, a COX inhibitor (Loll et al. 1995). Arg-120 is important for COX-1 activity, because it interacts with the carboxylic moieties of most non-steroidal anti-inflammatory drugs such as aspirin, which are known to inhibit COX activity (Picot et al. 1994). Arg-120 also interacts with the carboxylic moieties of arachidonic acid (Mancini et al. 1995), which is crucial for prostanoid synthesis.

1.2.3.1 COX expression

COX-1, a 69-kD protein which in cultured cells is expressed at constant levels throughout the cell cycle, is termed a constitutive isoform. COX-1 has a “house-keeping” role such as maintaining gastric integrity and renal function. In contrast, COX-2 is an inducible isoform whose expression under normal physiological conditions is not usually detected, although very often it is co-expressed in the same cell in which COX-1 is expressed (see Review by Smith et al. 1996). COX-2 expression, however, can be upregulated transiently in response to a variety of agents such as hormones, growth factors, bacterial endotoxin, tumor promoters and pro-inflammatory stimuli (see Review by Garavito & DeWitt 1999). The ability of
anti-inflammatory cytokines interleukin (IL)-4, IL-12 and glucocorticoids to suppress COX-2 expression, but not COX-1 demonstrates the important role that COX-2 plays during inflammation (see Review by Herschman 1996).

COX-2 is also found constitutively expressed, although in limited tissue types only; notably in brain (Yamagata et al. 1993), testes (Neeraja et al. 2003), tracheal epithelia (Walenga et al. 1996) and the macula densa in kidney (Harris et al. 1994; see Reviews by Smith et al. 1996; Garavito & DeWitt 1999). COX-2 is also responsible for a variety of physiological stimuli and plays a role in postsynaptic signaling of excitatory neurons (Yamagata et al. 1993; Kaufmann et al. 1996), ovulation, fertilization and implantation in ovary (Lim et al. 1997), regulating salt, volume and blood pressure homeostasis in kidney (Harris et al. 1994). Furthermore, mice that lack COX-2 develop nephropathy (Morham et al. 1995), suggesting that the COX-2 isoform plays a physiological role in the renal system.

With the advancement of molecular biology, emerging data indicate the possible existence of a third COX isoform based on its unique selectivity for paracetamol (Willoughby et al. 2000; see Review by Botting 2000). Recently this isoform has been found to be an alternative splicing of COX-1 with its biological role still under investigation (Chandrasekharan et al. 2002; Senior 2002; Schwab et al. 2003). This isoform has been termed as both COX-3 and COX-1 variant, which has caused some confusion in the literature. As possibly more COX isoforms will be identified in the future, a more consistent system to name newly identified isoforms is urgently needed.

1.2.3.2 COX regulation

COX-1 is known as the constitutive isoform, because it is constantly expressed throughout the cell cycle and the COX-1 gene lacks a TATA box, which is crucial for regulation (Yokoyama & Tanabe 1989; see Review by DeWitt & Meade 1993). Little is known about the regulation
of COX-1 expression, although several putative transcription factor-binding sites such as Sp-1, AP-2 and GATGA-1 have been identified on the COX-1 gene (Wang et al. 1993), suggesting the presence of potential regulatory mechanisms. A study by Taniura et al. (2002) has reported that in neurons, COX-1 expression can be regulated at the transcriptional level via histone acetylation, possibly through Sp-1 binding site on COX-1 promoter. More details on histone regulation mechanisms are discussed in Chapter 2.

Unlike COX-1, COX-2 expression is regulated by a wide variety of stimuli including oncogenic factors (see Review by Wu 1995), mitogens such as phorbol ester (Schuette & LaPointe 2000), cytokines such as IL-1α and tumor necrosis factor-α (TNF-α) (Nakagawa et al. 1999; Vlahos & Stewart 1999), hypoxia (Ji et al. 1998), growth factors (Kawaguchi et al. 1995) and glucocorticoids such as dexamethasone (Newton et al. 1997). Given the diversity of the stimuli involved, the signaling pathways associated with COX-2 regulation and the interactions between transcription factors and COX-2 promoter are also complex. For example, growth factors including epidermal growth factor, platelet-derived growth factor and fibroblast growth factor up-regulate COX-2 expression via the tyrosine kinase signaling pathway (see Review by O’Banion 1999). In human fibroblasts and vascular endothelial cells, IL-1β-induced COX-2 expression has been shown to be associated with p38 mitogen-activated protein kinase pathways, which are known to regulate cellular processes including inflammation, cell differentiation, cell growth and cell death (Ridley et al. 1997). On the COX-2 promoter region, transcriptional activation sites such as nuclear factor-κB (NF-κB) and NF-IL-6 (C/ERPβ) sites are the targets for TNF-α activation subsequently up-regulation of COX-2 in mouse osteoblastic cells (Yamamoto et al. 1995). New mechanisms such as histone acetylation/de-acetylation have also been shown to regulate COX-2 expression in macrophages (Park et al. 2004), airway smooth muscle (Nie et al. 2003), colon cancer (Tong et al. 2004), inflammation and diabetes (Miao et al. 2004). It is clear that the range of stimuli that regulate COX-2 expression is diverse and the signaling pathways
involved are complex.

1.2.4 COX ACTIVITY

Although there is about 60% homology between the two COX isoforms within a species, the activity and expression of the different COX isoforms are differentially regulated. Several distinctions can be made in regards to the activity of the COX isoforms. Firstly, COX activity requires low levels of lipid peroxides for activation and then continued activation of the enzyme is achieved autocatalytically by the newly generated PGG$_2$. There is a difference in the threshold for hydroperoxide activation between COX-1 and COX-2 in which COX-2 requires 10-fold lower concentrations of hydroperoxide for activation. This low hydroperoxide activation requirement may explain how these two isoforms are able to function independently of each other within the same cell. Secondly, the requirement for substrate is also different between two COX isoforms. At low concentrations of arachidonic acid (500 to 1000 nmol/L) the enzymatic activity of COX-1 is lower compared with that of COX-2. Thus, under conditions of low arachidonate concentrations, COX-2 activity allows for a greater prostanoid synthesis (Spencer et al. 1998, see Review by Smith et al. 2000).

Although a direct effect of NO on COX activity in an isolated enzyme preparation has not been demonstrated, there are reports suggesting that COX activity may be influenced by NO. For instance, NO has been shown to increase COX activity in various cell types including vascular endothelial cells (Davidge et al. 1995). It has also been suggested that COX activation by NO may occur via intermediary pathways, for example, peroxynitrite, a product resulting from interaction between NO and superoxide anions, can activate COX (Landino et al. 1996). Indeed, the influence of COX activity by NO is complex and the result may vary depending on the COX isoforms, quantity and/or source of NO (Davidge et al. 1999; Onodera et al. 2000). This is supported by the findings in which exogenous NO donors enhance COX-1 activity in non-activated bovine aortic endothelial cells. In contrast, exogenous NO
donors led to decreased COX-2 activity in bovine aortic endothelial cells activated by serum or phorbol ester (Onodera et al. 2000). Details of the NO influence on COX activity are further discussed in Section 1.3.3.

The COX-mediated pathway has been identified as one of the sources of free radical production. It was found that the primary source of oxygen free radicals in piglet cerebral cortex with ischemia/reperfusion is due to the release of superoxide anions formed by the activation of COX (Armstead et al. 1988). Based on these observations, it has been suggested that there may be a feed-forward loop for COX activation via lipid peroxidation and/or peroxynitrite formation (Figure 1-2).

On the other hand, even though COX activation is a source of superoxide anions, specific induction of COX-2 has been shown to limit oxidative damage caused by hydrogen peroxide in cardiomyocytes due to production of PGI₂ (Adderley & Fitzgerald 1999). It has been therefore suggested that COX-2 induction may be beneficial at sites of endothelial injury to replace the protective actions of COX-1 (see Review by Mitchell & Evans 1998), although future studies are needed to confirm this suggestion.
Superoxide anions (O$_2^-$) released from COX pathway initiate membrane lipid peroxidation. When O$_2^-$ react with NO, peroxynitrite (ONOO$^-$) is produced, which deplete NO reserve. ONOO$^-$ and/or lipid peroxides can also activate COX resulting in enhanced production of PGH$_2$ and TXA$_2$, as well as a feed-forward mechanism contributing further to O$_2^-$ production. Meanwhile, PGH$_2$ and TXA$_2$ bind to the same receptor causing a vasoconstriction. Although not required usually, COX activity (possibly via O$_2^-$ production) can initiate isoprostane synthesis which are products of a free radical–mediated pathway from arachidonate. In addition, prostacyclin (PGI$_2$) is reduced due to extensive lipid peroxidation and/or ONOO$^-$ (which preferentially inhibits PGI$_2$ synthase activity), leading to reduced vasorelaxation.
1.3 COX AND VASCULAR FUNCTION

COX enzymes play a crucial role in maintaining homeostasis in the vasculature. This is achieved through modulation of vascular tone, platelet adhesion and aggregation via the production of prostanoids such as PGI$_2$ and TXA$_2$. More details are discussed below.

1.3.1 LOCALIZATION OF COX IN VASCULATURE

COX enzymes are found both in the endothelium and smooth muscle cells, although endothelial cells contain up to 20 times more COX than smooth muscle cells (DeWitt et al. 1983). Using immuno-labeling microscopy, COX-1 and COX-2 have been found to be present in equal proportions in the luminal surface of ER and in the inner and outer membranes of the nuclear envelope in human umbilical endothelial cells (HUVECs) (Spencer et al. 1998). Interestingly, PGI$_2$ synthase and COX-1 were found to co-localize in the nuclear envelope and ER in bovine aortic endothelial cells. This relationship did not exist between COX-2 and PGI$_2$ synthase in bovine aortic endothelial cells (Liou et al. 2000).

Some cells express both COX-1 and COX-2, a perplexing observation, however, several explanations have been put forward. A review by Smith and DeWitt (1996) suggested that COX-1 may be part of an ER prostanoid biosynthesis system. Prostanoids produced via this system may function extracellularly as paracrine factors, acting through G protein-coupled receptors on the cell surface to maintain house-keeping responses. The roles that COX-2 play, in contrast, are more complex. It is thought that two sub-populations of COX-2 exist. The first COX-2 sub-population co-localizes with COX-1 on the luminal surface of the ER, which augments COX-1-mediated effects or takes on the role of COX-1 in cells lacking COX-1. The second COX-2 sub-population is part of a nuclear prostanoid synthesis system. This latter sub-population is located in the luminal surface of the inner membrane of the nuclear envelope, catalyzing prostanoids that target cell differentiation and replication (see Review by Smith & DeWitt 1996). It is also possible that the temporal localization of COX expression
simply means that the constitutive COX-1 isoform regulates house-keeping functions that do not require COX-2, whose expression is only induced under special conditions. The notion that these two isoforms act independently of each other has also been suggested. It was found that COX-1 and COX-2 use different arachidonate substrate pools and their prostanoid productions are coupled to different extracellular stimuli, possibly also different phospholipase systems (Reddy & Herschman 1996; see Review by Murakami et al. 1995). It is, however, possible that the two COX isoforms function at different locations within the same cells, because subtle differences do exist to differentiate COX-1 from COX-2 even though COX-1 and COX-2 appear to be localized in the same sub-cellular locations. For instance, COX-2 is more concentrated on the nuclear envelope than COX-1 and histochemical staining for enzyme activity in intact cells showed that the staining attributable to COX-1 was primarily in the cytoplasm whereas staining attributable to COX-2 was both in the cytoplasm and over the surface of the nucleus (Morita et al. 1995).

1.3.2 PRODUCTS OF COX IN VASCULATURE

COX enzymes linked to a variety of synthases including PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, PGI$_2$, and TXA$_2$ synthases can produce a wide variety of prostanoids, which are important vascular regulators. Under normal physiological conditions, COX-1-linked PGI$_2$ is the main prostanoid product released by non-activated endothelial cells, which is responsible for maintaining vasorelaxation and preventing platelet aggregation (Moncada et al. 1976; Dusting et al. 1977).

Although VSMCs also have the ability to release PGI$_2$, the concept that the endothelium is the main source of PGI$_2$ production by vessel wall was reinforced by MacIntyre et al. (1978) who demonstrated that incubation of aortic medial smooth muscle or aortic adventitial fibroblasts with arachidonic acid did not prevent platelet aggregation. In 1984, Dejana et al. reported for the first time that PGI$_2$ production was induced by IL-1 from vascular cells. Many other studies later also confirmed that pro-inflammatory cytokines induce PGI$_2$ production and/or
enhance the effects of other agonists including histamine, thrombin and calcium ionophore, all of which result in increased PGI\(_2\) release by endothelial cells. More importantly, these agents/substances are also associated with COX-2 expression (Albrightson \textit{et al}. 1985; Kawakami \textit{et al}. 1986; Zavoico \textit{et al}. 1989; Breviario \textit{et al}. 1990; Bull & Dowd 1990; Camacho \textit{et al}. 1998). At the time the assumption was that PGI\(_2\) is the main prostanoid generated by endothelial cells when COX-2 is over expressed. But when the synthesis of other prostanoid types was also evaluated, it was found that PGI\(_2\) was not the prostanoid being produced in most abundance in endothelial cells after exposure to IL-1. In fact after IL-1\(\beta\) treatment of HUVECs, PGE\(_2\), PGF\(_2\alpha\) and PGD\(_2\) were produced via the COX-2-mediated pathway while PGI\(_2\) synthase was inactivated. This was confirmed by Camacho \textit{et al}. (1998) who found that over-expression of COX-2 actually reduced the ability of endothelial cells to produce PGI\(_2\). This reduction was more pronounced when an excess of substrate was provided. This phenomenon was partially explained by speedy PGI\(_2\) synthase inactivation in IL-1\(\beta\)-treated cells compared with the resting cells (Camacho & Vila 2000) possibly due to accelerated suicide inactivation (Wade \textit{et al}. 1995). The reduction in PGI\(_2\) synthesis could also be NO-dependent. In HUVECs, IL-1\(\beta\) treatment did not affect PGI synthase mRNA levels, but PGI\(_2\) synthase activity was partially inactivated. With the treatment of various scavengers and inhibitors, it was postulated that NO, peroxynitrite and hydroxyl radicals were involved in this process (Mehl \textit{et al}. 1999; Camacho & Vila 2000; Zou \textit{et al}. 2000).

PGE\(_2\) has a role in diverse physiological and pathological processes due to its receptors being able to couple to complex signaling transduction pathways (details see Section 1.2.2.1). Depending on tissue types and stimuli, PGE\(_2\) can either mediate a constriction or relaxation of blood vessels, stimulate or inhibit water and sodium re-absorption in the kidneys and play a role in both pro- and anti-inflammatory reactions (see Reviews by Negishi \textit{et al}. 1995; James \textit{et al}. 2001).
Although PGD$_2$ is mainly produced by mast cells upon immunological challenge (Lewis et al. 1982), PGD$_2$ production has been detected in endothelial cells in response to hypoxia (Michiels et al. 1993). PGD$_2$ has the ability to act on thromboxane receptors in the airways causing bronchial smooth muscle to constrict hence; PGD$_2$ plays a role in the pathogenesis of asthma (McKenniff et al. 1988; Coleman & Sheldrick 1989). In contrast, PGD$_2$ is a vasodilator in vascular smooth muscle when acting through PGD$_2$ receptors (Giles et al. 1989).

PGF$_{2\alpha}$ plays a crucial role in both renal and reproductive functions by regulating contractile activities in both vascular and non-vascular tissues and luteolysis when pregnancy fails to take place (see Reviews by Smith 1992; Negishi et al. 1995). In the vasculature, PGF$_{2\alpha}$ has been released in response to cardiac tissue injury causing coronary vasoconstriction and is linked to the occurrence of coronary vasospasm (Cannon 1984; Rigel & Shetty 1997; Dallas & Khalil 2003).

The discovery of TXA$_2$, which has an opposing biological role to PGI$_2$, later prompted a hypothesis to explain the interactions between platelets and the vascular wall. TXA$_2$, mainly produced by platelets, is a potent vasoconstrictor and stimulates platelet aggregation. Enhanced production of TXA$_2$ subsequently causes platelet hyperactivity thus increasing the risk of thrombosis, which is discussed further in Section 3.4.1.2.

### 1.3.3 COX AND AGING

Aging, a process accompanied by alterations in both structure and function of tissues and organs, is a risk factor that contributes to the development of cardiovascular disease (Australian Institute of Health and Welfare 1999). Aging is associated with endothelial dysfunction, which is characterized by impaired endothelial-dependent relaxation to
acetylcholine (more details see Section 2.1). The exact causes of age-related endothelial
dysfunction are not clear, but it is clear that the balance between vasoconstriction and
vasodilatation mediated by various vasoactive mediators is altered (Reckelhoff et al. 1994).
Studies have shown that COX-mediated pathways are involved in endothelial dysfunction in
the aging process. Previous animal studies by our laboratory at RMIT University reported an
increase in both COX-1 and COX-2 expression with aging in rat aortas (Kang et al. 2001).
Potential consequences of enhanced COX expression in aging may result in an imbalanced
prostanoid production favoring the synthesis of vasoconstrictors such as TXA₂ and hence
increase the risk of cardiovascular events in aging population. More details on COX
involvement in the aging is discussed in Chapter 2.

1.3.4 COX AND DIABETES
Diabetes mellitus, characterized by hyperglycemia and by disturbances of carbohydrate, fat
and protein metabolism, is associated with a deficiency in insulin secretion from the
pancreatic β cells, defective insulin action, or both. Two major types of diabetes are Type 1
and Type 2, the former type dependent on daily insulin treatment for survival. Long term
consequences of diabetes are the development of micro- and macro-vascular complications in
the eyes, kidneys, nerves, heart and blood vessels (see Reviews by Barcelo 1996; Schuster &
Duvuuri 2002). Although the etiology of diabetes is unknown, it is clear that the cause of the
disease is not due to a simple factor. In fact, the etiology of diabetes is complex, involving
genetic factors, which are further precipitated by environmental factors (see Chapter 3 for a
detailed review).

Increasing evidence suggests that the development of diabetes and its complications is
associated with persistent inflammatory activity, which is shown by increased inflammatory
markers such as TNF-α, CRP and IL-6 in the circulation (Yudkin et al. 1999). It has been
suggested that COX-mediated pathways may be involved in this inflammatory process in
diabetes. For example, TXA$_2$ and PGE$_2$ released by platelets and monocytes due to the action of COX-1 and COX-2, respectively are important prostanoids involved in thrombosis and atherosclerosis development in diabetes (for more details, see Section 3.4). COX expression has been shown to alter in both Type 1 and Type 2 diabetic animal models. For instance, both COX-1 and COX-2 was increased in diabetic rat kidney (Nasrallah et al. 2003). In contrast, reduced COX-1 and increased COX-2 in renal cortex of diabetic rats was also reported (Komers et al. 2005). In vasculature, COX-2, but not COX-1 expression was down-regulated in diabetic rat aortas (Takahashi et al. 2004) whilst platelet COX-1 was increased in diabetic rats (Raju et al. 2006). More details on the involvement of COX in Type 1 and Type 2 diabetes are discussed in Chapters 4 and 5, respectively.

In addition to altered COX-mediated pathways, the formation of advanced glycation end products (AGEs) is also enhanced in diabetes when glucose forms irreversible cross-links with the amino groups on the macromolecules such as proteins, lipids and nucleic acids (Brownlee et al. 1988). AGEs can bind to receptors for AGEs (RAGE) and activate complex signaling pathways involving p21$^{ras}$, erk 1/2 kinases and NF-$\kappa$B (Kislinger et al. 1999). Strong evidence further suggests that RAGE plays a role in inflammation (Hou et al. 2004), which is often observed in diabetes. Interestingly, it was reported that RAGE was able to induce COX-2 expression in plaque macrophages isolated from diabetic mice (Bucciarelli et al. 2002). An increase in both RAGE and COX-2 expression was also found in atherosclerotic plaques in human diabetic subjects (Cipollone et al. 2003), suggesting RAGE may also be implicated in the development of diabetic complications. More details on the involvement of RAGE in diabetes are discussed in Chapter 6.
1.4 OVERALL AIMS

Given that little is known about COX regulation, which has been found to be increased previously in aging and its possible involvement in diabetes, the first part of the study focused on COX-mediated pathways followed by the investigation of RAGE-mediated pathways, which may contribute to the development of vascular complications in diabetes. The overall aims of the study were to examine:

i) the regulatory mechanisms of COX by studying histone acetylation status in endothelial cells, platelets and monocytes;

ii) possible changes in COX-1 and COX-2 levels in platelets and monocytes, respectively in diabetes; and

iii) possible changes in RAGE levels in platelets and monocytes in diabetes.
CHAPTER 2
MODULATION OF COX EXPRESSION
BY HISTONE ACETYLATION
2.1 INTRODUCTION

2.1.1 AGING AND ENDOTHELIAL FUNCTION

The endothelium is very important in the preservation of blood vessel integrity. It acts as a modulator in a variety of processes including coagulation, platelet activation, vascular tone, angiogenesis and chemotaxis (see Review by Luscher & Barton 1997). Endothelial cells produce numerous substances such as enzymes, vasoactive substances, mitogens and chemotactic factors, which are crucial in modulating endothelial functions (Ramadan et al. 1990). For example, endothelial cells produce nitric oxide (NO), which is a potent vasodilator and also inhibits platelet aggregation. The stimulus for NO production is an increase in intracellular Ca\(^{2+}\) in the endothelial cells, which activates NO synthase. The increase in intracellular Ca\(^{2+}\) can be brought about by receptor activation such as acetylcholine muscarinic receptors and bradykinin receptors, or through distention of the endothelium. In addition, the endothelium can produce endothelin-1, which is a potent peptide constrictor (see Review by Luscher & Barton 1997). Prostanoids are also important in endothelial function and the endothelium is an important source of PGI\(_2\), which is a potent vasodilator and inhibits platelet aggregation. The endothelium, thus acts to modulate vascular tone and inhibit platelet aggregation. Another potent vasodilator is EDHF, which is probably a Ca\(^{2+}\)-activated potassium channel activator or a prostanoid (Urakami-Harasawa et al. 1997), which hyperpolarizes and relaxes VSMC predominantly through activation of Ca\(^{2+}\)-dependent K\(^+\) channels. This EDHF response was reduced in the arteries of aging rats (Goto et al. 2004), although conflicting results have been reported (Alvarez de Sotomayor et al. 1999).

Aging is a risk factor that contributes to the development of cardiovascular disease (Australian Institute of Health and Welfare 1999), which is accompanied by alterations in both structure and function of tissues and organs. Aging is associated with endothelium injury or dysfunction as seen by impaired endothelial-dependent relaxation to acetylcholine in the coronary artery of dogs and rats (Donoso et al. 1994), aorta of rabbits (Ragazzi et al. 1995)

The exact causes of age-related endothelial dysfunction are not clear, but current theories have surrounded the mechanisms mediated by NO and oxidative stress. NO is synthesized from L-arginine via NO synthase. In aging, there is a decrease in L-arginine availability coupled with changes in activity or expression of endothelial NO synthase (Reckelhoff et al. 1994), which may contribute to a reduction in NO production. The generation of reactive oxygen species (ROS) including superoxide production is increased in aging (see Review by Terman & Brunk 2006). These species can react with NO to not only reduce its bioavailability (see Review by Schulz et al. 2004) and protective effects, but also, as in the case of superoxide, producing peroxynitrite to cause widespread cellular damage through protein nitration (Bouloumié et al. 1997; see Review by Stadtman 2001). COX-mediated pathways may also be involved in the process of aging. In addition to COX being a potential source of ROS (see Review by Adibhatla & Hatcher 2005), COX metabolites such as TXA$_2$ and PGI$_2$ can also play an important role in maintaining endothelial function (more details are discussed in Section 2.1.1).

**2.1.1.1 COX and the aging process**

COX enzymes play a crucial role in modulating endothelial function. PGI$_2$ synthesized by the endothelium acts on PGI$_2$ receptors resulting in increased cAMP in VSMCs and vasorelaxation. In contrast, COX-mediated generation of TXA$_2$ production in platelets leads to platelet aggregation and vasoconstriction though decreasing cAMP and mobilization of intracellular Ca$^{2+}$ in VSMCs. A balance in prostanoid production via these two COX-mediated pathways is crucial to vascular homeostasis.
Evidence suggests that COX-mediated pathways are altered in the aging process. An early study by Koga et al. (1989) investigated the relaxation responses to acetylcholine in aortas isolated from young (4 to 6 weeks), adult (3 to 6 months) and old (12 to 35 months) hypertensive and normotensive rats. In endothelium-intact vessels, high concentration of acetylcholine resulted in vasoconstriction in adult and old hypertensive and old normotensive rats, which was inhibited by the non-selective COX inhibitor, indomethacin. Davidge et al. (1996) also tested the hypothesis that increased arachidonate metabolism was associated with an impairment of endothelium-dependent vascular function in the mesenteric arteries of female Sprague-Dawley rats between 20 and 40 weeks old. In this study, phenylephrine-induced constriction of mesenteric arteries was reduced by COX inhibition in the 40-week-old rats, but not 20-week-old rats. Furthermore, 40 week-old mesenteric arteries showed a reduction in the sensitivity to the methacholine-mediated relaxation response compared to 20 weeks. This was restored with COX inhibition or blockade of TXA2 receptors. Furthermore, aortas from the 40-week-old rats had a higher expression of COX enzymes. These data suggest that COX-mediated pathways are altered in the blood vessels of aging female Sprague-Dawley rats.

COX-2, but not COX-1 expression has been shown to increase with aging in rat hearts (Kim et al. 2001). Since COX-2 up-regulation has been associated with inflammation, it is not surprising that Casolini et al. (2002) also reported an increased production of pro-inflammatory markers including IL-1β, TNF-α and PGE2 with age in the hippocampus. The link between inflammation and aging via COX-mediated pathways was evident when chronic administration of celecoxib, a selective COX-2 inhibitor, resulted in the reduction of these pro-inflammatory markers in the hippocampus. The effect of enhanced COX-2-mediated activity in the aging process may, however, be greater than originally anticipated. For instance, increased oxidative stress is associated with aging resulting in oxidative modification of macromolecules and endothelial dysfunction (see Review by
Stadtman 2001). Potentially, increased COX-2 activity may provide an additional oxidative source further promoting endothelial injury. In comparison to COX-2, changes in COX-1 in the aging process are less well defined. Among a few studies, one reported COX-2, but not COX-1 expression was altered in aging rat aortas and mesenteric arteries (Stewart et al. 2000) while two others reported age-related increases in both COX-1 and COX-2 expression in these blood vessels (Heymes et al. 2000; Matz et al. 2000).

Despite the effort, much is still unknown in regards to regulation of COX synthesis, particularly with COX-1 since the COX-1 gene lacks a TATA box (Smith et al. 1996). Interestingly, for the first time in 2002, Taniura and his colleagues reported that COX-1 expression was up-regulated by histone hyperacetylation, induced by the use of histone de-acetylase inhibitors in human astrocytes. Several other groups have reported that histone acetyltransferase activity is increased in aging mice, rats and elderly human subjects (Sarg et al. 1999; Li et al. 2002; Sourlingas et al. 2002). It is clear that histone acetylation may regulate COX expression during the aging process.

2.1.2 HISTONES
Histones, discovered over a century ago, are small proteins that act as the structural scaffold for the organization of nuclear DNA into chromatin. A histone has an N-terminus, a globular domain, and a C-terminus. There are four core histones, H2A, H2B, H3 and H4. These share a common motif known as the histone fold, which consists of a long central helix flanked on either side by a loop and a short helix. In addition to the central histone fold, histones H2A and H3 also contain additional helices at the N-terminus; whereas histone H2B contains an additional helix at the C-terminus (see Review by Ramakrishnan 1997). Two copies each of the four core histones form an octameric protein, which is wrapped around by 146 base pairs of DNA in approximately two turns, forming the nucleosome, the simplest level of organization found in chromosomes. Each of the core histones exists as part of a dimer, which
is their most stable structural entity. The association of an H3-H4 dimer to form a tetramer, in the initial step in nucleosome assembly, determines nucleosome positioning (Dong & van Holde 1991; Hayes et al. 1991; Karantza et al. 1995). The final association of an H2A-H2B dimer on the each side of the (H3-H4)\textsubscript{2} tetramer completes the formation of the histone octamer. A fifth linker histone H1 then binds to the nucleosome and organizes nucleosomes into a high-order structure, the 30-nm filament (Luger et al. 1997; see Review by Ramakrishnan 1997).

The N-terminal tails of the core histones are lysine-rich. These tails extend out beyond the nucleosome core particle and are subject to a range of post-translational modifications by the actions of enzymes in both the nucleus and cytoplasm. Although most histone modifications occur at the N-terminal tails, the C-terminal tails and globular domains can still be modified. The most commonly studied and best understood modifications are acetylation, phosphorylation, methylation and to a lesser extent ubiquitination, addition of ubiquitin. These modifications allow processes such as transcription, DNA repair, recombination and replication to be regulated (see Reviews by Bradbury 1992; Wade et al. 1997).

**2.1.2.1 Histone modifications**

Chromatin structures are strictly regulated by histones. It is through these modifications that histones are able to influence the accessibility of transcription factors to DNA.

**2.1.2.1.1 Acetylation**

Acetylation and de-acetylation is the best understood histone modification, which occurs on the epsilon amino group of lysine residues of all four core histones. The highly conserved histone H3 lysines at positions 9, 14, 18 and 23, and histone H4 lysine residues 5, 8, 12 and 16, are frequently targeted for modification (Roth et al. 2001). Catalyzed by histone acetyltransferases, acetylated histones are usually associated with transcriptionally active
chromatin and de-acetylated histones with inactive chromatin (Allfrey et al. 1964; Review by Ng & Bird 2000). Histone acetyltransferases are transcriptional coactivators/adaptors, which interact with DNA-bound activators leading to recruitment of the transcriptional apparatus. For example, when the N-terminal tail of histone H4 contains 4 lysine residues at lysines 5, 8, 12 and 16 is acetylated by histone acetyltransferases, the positive charge on the basic side chain of the lysine residue is eliminated reducing its interaction with the negatively charged phosphate backbone of the DNA. In general, it is believed that by weakening the interaction between histone H4 tail and DNA, the remodeling of chromatin required for gene transcription is facilitated (see Review by Wade et al. 1997). Histone acetyltransferases exist in large complexes consisting of several subunits. These subunits may help to target promoters, provide substrate specificity and possess regulatory functions. In addition to histones, some transcription activators, such as p53 are also substrates of histone acetyltransferases. Therefore, hyperacetylation is correlated strongly with increases in gene expression (Figure 2-1).

In addition to gene transcription, histone acetylation also plays key roles in processes such as replication, nucleosome assembly, higher-order chromatin packing and interactions of non-histone proteins with nucleosomes (see Review by Grant & Berger 1999).
Histone acetylation/de-acetylation and gene expression (adapted from Chang & Min 2002)

Histones are hyperacetylated and hypoacetylated at the N-terminal tails by histone acetyltransferases and de-acetylases. Histone hyperacetylation results in the opening of nucleosomal structures (open state), which gives transcription factors better access to transcription sites and therefore, is associated with active gene transcription. In contrast, histone hypoacetylation leads to the “closed” state of DNA structures preventing the binding of transcription factors to DNA and is associated with gene silence.
2.1.2.1.2 Phosphorylation

Despite the wealth of information about the large number of histone acetyltransferases and histone de-acetylases, much less is known about other histone modifications. Histone phosphorylation, however, also plays a role in processes such as transcription, DNA repair, apoptosis and chromosome condensation (see Review by Cheung et al. 2000). Histone phosphorylation of serine residues in the amino termini of all four core histones and in multiple regions of histone H1 is an important regulatory mechanism during cell division (Goto et al. 1999; de la Barre et al. 2000; Review by Thomson et al. 1999). Histone phosphorylation also regulates transcriptional activation during heat-shock responses in *Drosophila* (Nowak & Corces 2000). In quiescent fibroblasts treated with epidermal growth factor, serine 10 of histone H3 N-tail was phosphorylated rapidly, coincident with the induction of early response genes including c-*fos* and c-*jun*. The key enzyme involved in this phosphorylation is the Rsk-2 kinase, and cells derived from Rsk-2-deficient Coffin-Lowry Syndrome patients do not undergo serine 10 phosphorylation or c-*fos* induction in response to the epidermal growth factor (Sassone-Corsi et al. 1999).

The actual mechanism by which histone phosphorylation contributes to transcriptional activation is not well understood. It is, however, being proposed that the addition of negatively charged phosphate groups neutralizes the histone basic charge hence, possibly reduces their affinity for DNA. Interestingly, it has been found that in some cases phosphorylation can enhance the activity of several histone acetyltransferases to serine 10-phosphorylated substrates (Cheung et al. 2000a; Lo et al. 2000). Thus, it is likely that histone phosphorylation works in conjunction with histone acetylation in order to modulate transcription.
Histone H3 phosphorylation is also known to facilitate DNA repair. The phosphorylation process starts after activation of DNA-damage signaling pathways. A conserved motif found in the C-terminus of yeast histone H2A and the mammalian histone H2A variant H2A is rapidly phosphorylated upon exposure to DNA-damaging agents. Instead of the usual serine 10, another residue was involved in this modification. Serine 139 is phosphorylated by phosphatidylinositol-3-OH kinase, Mec1, in yeast in response to damage and is crucial to the repair of DNA (Rogakou et al. 1999; Downs et al. 2000).

### 2.1.2.1.3 Methylation

Histone methylation, a process described in 1964, can occur on both lysine and arginine residues (Murray 1964). It was not until the histone H3 arginine-specific histone methyltransferase CARM1 was found to interact and cooperate with the steroid-hormone-receptor coactivator GRIP-1 in transcriptional activation that a direct link between methylation and transcription was established (Chen et al. 1999).

Studies have indicated that lysines 4, 9 and 27 of histones H3 and lysine 20 of histone H4 are frequently preferentially methylated (Strahl et al. 1999). Once these lysines are modified, they have the ability to be mono-, di- or tri- methylated, which dramatically increases potential complexity to the posttranslational status of H3 and H4 tails.

Certain histone methyltransferases have been classified as gene repressors. These methyltransferases include human SUV39H1, murine Suv39h1 and fission yeast Clr4 proteins, which methylate lysine 9 of histone H3 selectively (Rea et al. 2000; Nakayama et al. 2001). It has been found that over-expression of SUV39H1 increased the formation of heterochromatin, a transcriptionally inactive region of chromatin, in a normally genetically active location (Melcher et al. 2000).
2.1.2.1.4 Ubiquitination

Ubiquitination, a process involving the addition of ubiquitin to a protein, which acts as a signal to protein degradation by proteasome and results in the inactivation of the protein, is the least understood of the histone modifications. Histones H1, H3, H2A, and H2B can be ubiquitinated *in vivo*, but the most prevalent are histones H2A and H2B. Histone ubiquitination occurs on the C-terminal tails of affected histones. In yeast, for example, the loss of the ubiquitination site on the C-terminus of histone H2B leads to defects in mitosis and meiosis (Robzyk *et al.* 2000). In some cases, enzymes that are capable of facilitating other histone modifications also possess ubiquitination ability. The transcription factor TAFII250, a histone acetyltransferase, has the ability to both ubiquitinate and acetylate histones (Pham & Sauer 2000). Therefore, it is not unreasonable to anticipate the complex interactions between histone modifications, which can affect the chromosomal environment profoundly.

2.1.2.2 Histone acetylation/de-acetylation and the aging process

The cellular aging process involves accumulation of DNA damage in response to oxidative stress (Sedelnikova *et al.* 2004) and resulting in shortening of telomeres (see Reviews by Campisi 2001; Harrington & Robinson 2002). Since histones, through post-translational modifications, have the ability to control and regulate gene expression, it is reasonable to hypothesize that histones also contribute to aging progression. Among the four modifications, histone acetylation/de-acetylation has received the most attention and has been associated with the aging process in both insects and mammals.

Several studies have shown that histone de-acetylases are involved in aging. It is believed that histone de-acetylases silence aging genes and hence prolong the life span. Kang *et al.* (2002) reported that feeding Drosophila with a general histone de-acetylase inhibitor, phenylbutyrate increased the lifespan of the flies. In this study, treating Drosophila with phenylbutyrate altered the expression of several hundreds of genes. While hundreds of genes such as fatty
acid synthase, cycline-dependent kinase 9 and glyceraldehyde-3-phosphate dehydrogenase-1 were repressed by phenylbutyrate, a large number of genes reported to enhance longevity were induced. These include superoxide dismutase (Parkes et al. 1998; Sun & Tower 1999), glutathione S-transferase and cytochrome P450 (Dudas & Arking 1995; see Review by Mannervik 1985).

Lin and colleagues in 2000 reported another histone de-acetylase, which is involved in the aging process. Silent information regulator 2 (Sir2) is a NAD$^+$-dependent protein de-acetylase (Imai et al. 2000), which contains a silencing protein, Sir2p. Sir2 was found to control longevity in lower eukaryotes such as *Saccharomyces cerevisiae* and *Caenorhabditis elegans* as increased Sir2 activity extended the lifespan in these organisms (Kaeberlein et al. 1999; Tissenbaum & Guarente 2001). Through histone modifications at telomeres, the Sir2 protein also played a critical role in heterochromatic gene silencing, ribosomal DNA clusters, and mating-type loci in yeast (see Review by Lustig 1998). A mammalian Sir2-homologue, sirtuin family consisting of seven protein members, was identified in humans (Cohen et al. 2004). Limited data is available regarding biological functions or cellular localizations of the sirtuin family. It is possible, however, that the sirtuin family, which shares the Sir2 catalytic domain (see Reviews by Blander & Guarente 2004; North & Verdin 2004) may regulate the aging process in a similar fashion in both humans and in lower eukaryotes.

Histone de-acetylases act on histones to silence aging genes. Histone de-acetylase inhibitors such as trichostatin A (TSA), cause hyperacetylation of histones and are associated with terminal differentiation, a process that is tightly coupled with cell arrest and cell death (see Review by Yoshida et al. 1995).
Despite reports showing a positive link between increased histone acetylation and aging, decreased histone acetylation in aging has also been demonstrated. For example, Matuoka et al. (2001) reported that in human diploid fibroblasts, nicotinamide induced rapid and reversible reversion of aging phenotypes in terms of cell morphology and senescence-associated β-galactosidase activity. It was found that histone H4 acetylation and histone acetyltransferase activity were lowered in aged cells in comparison to elevated histone acetyltransferase activity seen in nicotinamide-exposed cells. This reduction in histone acetyltransferase activity in aging is not an isolated observation. When Li et al. (2002) studied two particular proteins, cyclic adenosine monophosphate-regulated enhancer-binding protein (CBP) and its homologue p300 that both possess intrinsic histone acetyltransferase activities, in various tissues of fetal, young and old mice, it was found that the histone acetyltransferase activities of CBP and p300 actually decreased with aging in liver, muscle and testes. In contrast, the histone acetyltransferase activities of these proteins remained to be relatively stable in most of other tissues with aging. Furthermore, the histone acetyltransferase activities of CBP and p300 were high in the brain and liver of fetal and newborn mice compared to the old animals. These findings suggest that alteration in histone acetylation with aging is species- and tissue-specific, which may explain why contradictory findings were reported as data showing increased histone acetylation with aging were observed in flies. Despite contradictory/conflicting findings on the level of histone modifications in aging, evidence to date has indicated that acetylation of histones play an important role in the process of aging.
2.2 AIMS

Previous studies from our laboratory have shown that in aortas of 54-week-old Spraque-Dawley rats, there was an increase in both COX-1 and COX-2 levels compared to those of 8-week-old aortas (Kang et al. 2001). Increased COX-1 and COX-2 levels were also observed in the platelets and mononuclear cells, respectively, with aging in Spraque-Dawley rats with the same age groups (Kang et al. 2001a). Changes in COX protein expression in the aging process may result in an imbalance of prostanoid production and thus impair endothelial functions. The underlying regulatory mechanisms to these changes in COX levels, in particular with COX-1 expression in aging are not well understood. Studies have suggested that histone acetylation/de-acetylation is altered in aging and this may be a mechanism by which COX enzymes are regulated.

Little information about the possible role of histones in regulating COX is available. The aim of this study was to investigate if increased COX expression in endothelial cells were modulated by histone modification. We hypothesized that hyperacetylation of histone H4 contributes to up-regulation of COX proteins, which have been previously observed in the aging process.
2.3 METHODS

2.3.1 CELL CULTURE OF ENDOTHELIAL CELLS

2.3.1.1 Cell culture of murine SVR endothelial cell line
SVR cells, a murine pancreatic islet endothelial cell line, were donated by Dr. Ian Darby (RMIT University). The cell line was cultured in 6-well plates containing Dulbecco's modified Eagle's medium (DMEM) with supplementation: 4 mM L-glutamine adjusted to contain 18 mM NaHCO₃, 25 mM glucose, 20% fetal bovine serum (FBS), gentamicin sulfate (50 µg/mL) and streptomycin (100 µg/mL) (Invitrogen, CA, USA). SVR cells were grown to 100% confluence in a 37°C, 5% CO₂ humidified incubator before ready for experiment (see Section 2.3.1.3 for details).

2.3.1.2 Cell culture of human umbilical vein endothelial cell line (HUVEC)
Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science (MD, USA). Prior to cell culture, 6-well plates were coated with 0.1% (v/v) gelatin (Sigma, St Louis, USA) in phosphate-buffered saline (PBS) containing 1 mM KH₂PO₄, 154 mM NaCl, and 5.7 mM Na₂HPO₄ (Invitrogen, CA, USA) for 30 minutes at 37°C. Gelatin-coated plates were washed twice with pre-warmed PBS (37°C) before seeding HUVECs. Cells were cultured using Clonetics® Endothelial Cell System (catalogue No. CC-3214) developed by Cambrex Bio Science (MD, USA), supplement pack containing 2% FBS, 0.4% bovine brain extract and 1 ml/L of each of the following: human epidermal growth factor, hydrocortisone, GA-1000 (aqueous solution of gentamicin Sulfate and amphotericin-B). Cells were grown to 100% confluence in a 37°C, 5% CO₂ humidified incubator before ready for experiment (see Section 2.3.1.3 for details).

2.3.1.3 Histone de-acetylase inhibitor treatment
When endothelial cells were grown to 100% confluence, they were treated with either the histone de-acetylase inhibitor, trichostatin A (TSA, 100 nM in 0.1% DMSO; Sigma, St Louis,
USA) or 0.1% (v/v) DMSO for 48 hours. A third group of cells was left untreated. After 48 hours, medium was removed and cells were washed twice with ice-cold PBS (Invitrogen, CA, USA) before being collected for histone extraction and Western blotting (see Section 2.3.1.4 Cell lysis and protein extraction). Another set of cells treated with TSA was reserved for cell viability test/count (see Section 2.3.1.5 Cell viability test).

2.3.1.4 Cell lysis and protein extraction

To prepare for cell collection for Western blotting experiments, endothelial cells were placed on ice and washed with ice cold PBS to remove media. Modified radioimmunoprecipitation (RIPA) buffer (10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% Deoxycholate, 5 mM EDTA, 150 mM NaCl), which contained a mixture of protease inhibitors (1 mM phenylmethylsulfonylfluoride (PMSF), 10 μM leupeptin, 20 μM pepstatin) was used for cell lysis and protein extraction. Cells were incubated in ice cold RIPA buffer (100 μL per well) for 1 minute and were scraped off the well. Scraped cells were suspended in RIPA buffer and transferred to an eppendorf tube and centrifuged at 15,000 g for 3 minutes. The pellet was discarded and supernatant was removed for protein assays.

2.3.1.5 Cell viability test

2.3.1.5.1 SVR endothelial cell trypsinization

SVR endothelial cells were incubated with trypsin/EDTA solution (0.25% trypsin/0.03% EDTA solution, 37°C; Invitrogen, CA, USA) for two minutes. Detachment of cells was monitored under a microscope. After two minutes, 10 mL of culture medium with 20% FBS was placed in the culture flask to neutralize the action of trypsin. The cell suspension was centrifuged at 220 g for 5 minutes followed by aspiration of supernatant after the spin. The pellet was re-suspended in DMEM with FBS and supplements and the cell suspension was ready for subculturing and the trypan blue exclusion assay for cell viability.
2.3.1.5.2 HUVEC trypsinization

To collect HUVECS, a ReagentPack™ (catalogue No. CC-5034) from Clonetics® Endothelial Cell System (Cambrex Bio Science, MD, USA) was used. This reagent pack included trypsin/EDTA (trypsin 0.025%/EDTA 0.01%), trypsin neutralizing solution (TNS) and HEPES buffered saline solution (HEPES-BSS). HUVECS were rinsed with 5 mL of room temperature HEPES-BSS followed by the incubation of cells with 2 mL of trypsin/EDTA solution, which was pre-warmed to 37°C. The cell layer was examined microscopically to ensure trypsinization continued until approximately 90% of the cells were rounded up and released from the culture surface. To neutralize the action of trypsin, room temperature TNS (4 mL) was added to the cell suspension, which was collected and centrifuged at 220 g for 5 minutes to pellet the cells. Most of the supernatant was aspirated except for 100-200 μl, which was used to loosen the pellet. The cell suspension was then diluted with growth medium (5 mL, details see Section 2.3.1.2) and was ready for subculturing and trypan blue exclusion assay.

2.3.1.5.3 Trypan blue exclusion assay

Approximately 20 μL of cell suspension was mixed with an equal amount of trypan blue vital stain solution (0.4%; Cambrex Bio Science, MD, USA) in an eppendorf tube. Using the pipette, the cell suspension (10 μL) was transferred to the edge of the hemacytometer. The suspension spread evenly by capillary action, which covered the area within the chamber of the hemacytometer. The chamber was divided by grid lines forming nine 1 mm squares with each of them being surrounded by three grid lines. Trypan blue vital stain allowed for differentiation between viable cells and non-viable cells as non-viable cells were stained blue. Using the 10x objective of a microscope, cells within all four corner grids were counted and averaged to obtain number of cells \((n)\). For every 1 mm square, cells that lay on the top and the left-hand lines of each square were counted, but not those on the bottom or right-hand lines. This prevented cells from being counted twice. For standard subculture, counts between
100 and 300 cells per mm² are ideal. If necessary, the cell suspension was further diluted with PBS or HEPES-BSS depending on cell types and re-counted to ensure accuracy of counting was not affected by over populated cell number. When only few cells (<100/mm²) were present, additional squares surrounding the central square were counted. Cell viability was then calculated using the following equation:

\[
\text{% cell viability} = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained plus unstained)}} \times 100
\]

### 2.3.2 HISTONE EXTRACTION

A separate set of cells was grown to 100% confluence in gelatin-coated 6-well plates as detailed previously. Cells were washed twice with ice-cold PBS and lysed with 1 mL of histone extraction lysis buffer (10 mM Tris-HCl, pH 6.5, 50 mM sodium bisulfite, 10 mM MgCl₂, 10 mM sodium butyrate, 8.6% sucrose, 1% Triton X-100) and homogenized by repeated pipetting. The homogenates were centrifuged at 1,000 × g for 5 min at 4 °C, and the pellets were washed with 0.5 mL of suspension buffer (10 mM Tris, pH 8.0, 13 mM EDTA). The pellets were then re-suspended in 125 µL of ice-cold distilled water, sulphuric acid was added to a final concentration of 0.4 N (100 µL). Subsequently, lysates were incubated on ice for 1 h followed by centrifugation at 10,000 g for 5 min. The supernatants were precipitated with 10 volumes of acetone at -20 °C overnight. The precipitated histones were collected by centrifugation, dried with a stream of nitrogen, and re-suspended in histone extraction lysis buffer. Protein concentration was measured by using the micro BCA protein assay reagent.

### 2.3.3 WESTERN BLOTTING

#### 2.3.3.1 Protein assay

The total protein concentration of the endothelial cell suspension was determined by using the micro bicinchoninic acid (micro BCA) assay system from the Pierce micro BCA kit (Pierce, Rockford, IL, USA). The suspension was diluted 400X with modified RIPA lysis buffer (see Section 2.3.1.4). Protein standard concentrations ranging from 5 to 20 µg/mL were prepared
from the stock of 2 mg/mL bovine serum albumin protein (Pierce, Rockford, IL, USA) and
diluted with lysis buffer. Blanks were prepared with lysis buffer without any protein content.
The micro BCA reagents were mixed and added to protein samples, standards and blanks
according to the manufacturer’s protocol followed by incubation at 60 °C for 1 hour before
determination of total protein content with a micro plate reader at 562 nm absorbance
wavelength.

2.3.3.2 Western blot in cultured endothelial cells (acetylated histone H4)
The samples were diluted with homogenizing buffer and reducing buffer (Section 2.3.3.1).
Samples and molecular weight markers (Sigma, St Louis, USA) were denatured at 100 °C for
3 minutes on a heating block. Samples in duplicate and molecular weight markers with a final
total protein concentration of 10 μg per well were electrophoresed on a 15%
SDS-polyacrylamide gel (200 V, 40 minutes) and transferred onto nitrocellulose membranes
(100 V, 70 minutes). The membranes were then blocked with washing buffer containing 1%
(w/v) skim milk powder for 1 hour followed by incubation with anti-rabbit polyclonal
anti-acetyl histone H4 antibody (Upstate, NY, USA) overnight. Next day the membranes were
washed 5 times with washing buffer and were incubated with an anti-acetyl histone secondary
antibody for 1 hour at room temperature. Selective acetylated histone H4 protein levels were
visualized by enhanced chemiluminescence, quantified by densitometry using the
ChemiDoc™ XRS system (Biorad, CA, USA) and values were expressed as a percentage of
internal control.

2.3.3.3 Western blot in endothelial cell lines (COX)
The samples were diluted with homogenizing buffer (50 mM Tris base, pH 7.6, 1.0% Triton
X-100, 1 mM EGTA, 2 mM MgCl, 1 mM PMSF, 10 μM leupeptin, 20 μM pepstatin) and
reducing buffer (1 mg/mL bromophenol blue, 20% glycerol, 100 mM dithiothreitol, 2%
sodium dodecyl sulphate, pH 6.8). Samples and molecular weight markers (Sigma, St. Louis,
USA) were placed in eppendorf tubes and denatured at 100 °C for 3 minutes on a heating block. Samples were loaded in duplicate with a final total protein concentration of 10 μg per well. The protein samples and molecular weight markers were electrophoresed on a 10% SDS-polyacrylamide gel (200 V, 40 minutes), transferred onto nitrocellulose membranes (100 V, 70 minutes). The membranes were then blocked with washing buffer containing 1% (w/v) skim milk powder (20nM Tris base, 137 mM NaCl, pH 7.5, 1% Tween-20) at room temperature for 1 hour followed by incubation with selective COX-1 and COX-2 antibodies (Cayman Chemical, Ann Arbor, MI, USA; BD Biosciences, San Jose, CA, USA) overnight. Next day the membranes were washed 5 times with washing buffer and were incubated with anti-COX-1 and anti-COX-2 secondary antibodies for 1 hour at room temperature. COX protein levels were visualized by enhanced chemiluminescence, quantified by densitometry using the ChemiDoc™ XRS system (Biorad, CA, USA) and values were expressed as a percentage of internal control.

2.3.4 DRUGS AND CHEMICALS

Drugs were obtained from the following sources: ColorBurst protein markers, leupeptin, pepstatin A, phenylmethylsulfonylfluoride (PMSF); Sigma (St. Louis, USA). Polyclonal rabbit anti-murine COX-1 antibody, monoclonal mouse anti-ovine COX-1 antibody; Cayman Chemical (Ann Arbor, MI, USA). Monoclonal mouse anti-rat COX-2 antibody; BD Biosciences (San Diego, CA, USA). Anti-rabbit acetyl-histone H4 (lys 16) antibody; Upstate (NY, USA). Peroxidase conjugated swine anti-rabbit immunoglobulins, peroxidase conjugated goat anti-mouse immunoglobulins; Chemicon International (Temecula, CA, USA).

Chemicals were obtained from the following sources: Ethanol; Selby-Biolab (Melbourne, Victoria, Australia). Bromophenol blue, calcium chloride (CaCl₂), dithiothreitol, methanol, developer solution, fix solution, D-glucose, glycerol, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), sodium bicarbonate
(NaHCO$_3$), N, N, N’, N’-tetramethylethylenediamine (TEMED), sodium chloride (NaCl); Sigma (St. Louis, USA). Magnesium sulphate (MgSO$_4$), potassium chloride (KCl), sulphuric acid; Ajax Chemicals (Auburn, NSW, Australia). Tris-base; Boehringer Mannheim (IN, USA). EDTA, KH$_2$PO$_4$, Na$_2$HPO$_4$; BDH Chemical (Kilsyth, Victoria, Australia). Glycine, sodium dodecyl sulphate; ICN Biomed (Aurora, OH, USA). Ammonium persulphate, bis-polyacrylamide; Biorad Laboratories (Hercules, CA, USA).

2.3.5 STATISTICAL ANALYSES

All values are given as the mean ± standard error of mean (S.E.) and $n$ indicates the number of observations. Differences in the percentage densities of Western blot bands were analyzed using 1-way ANOVA (repeated measure) and post-hoc test (Tukey’s multiple comparison tests) whenever applicable. SPSS (SPSS Inc., IL, USA) and Prism (GraphPad Software, Inc., CA, USA) statistical packages were used in statistical analyses.
2.4 RESULTS

2.4.1 ENDOTHELIAL CELL VIABILITY

2.4.1.1 Murine SVR endothelial cell viability
Murine SVR endothelial cells were cultured to 100% confluence followed by treatment with a histone de-acetylase inhibitor, TSA for 48 hours. To assess the effects of TSA, which was prepared in 0.1% DMSO on cell viability, murine SVR endothelial cells were subjected to cell count and trypan blue exclusion assay procedures. There was no difference in cell numbers and cell viability between the control, DMSO- and TSA-treated groups (Figure 2-2). Cell images for each study group were taken to show the cells were proliferative and healthy and continued to grow to confluence (Figure 2-2).

2.4.1.2 HUVEC viability
HUVECs were cultured to 100% confluence followed by the treatment with a histone de-acetylase inhibitor, TSA for 48 hours. Cell count and trypan blue exclusion assay procedures were then performed to assess cell viability in response to TSA treatment. There was no difference in cell numbers and cell viability between the control, DMSO- and TSA-treated groups (Figure 2-3). Cell images for each study group were taken to show the cells were proliferative and healthy and continued to grow to confluence (Figure 2-3).
SVR endothelial cell viability assessment

Murine SVR endothelial cells were grown to 100% confluence before being treated with a histone de-acetylase inhibitor, TSA (100 nM). After 48 hours cells were trypsinized for cell count (Panel A) and trypan blue exclusion assay (Panel B). Panel C: Images of murine SVR endothelial cells after 48h TSA treatment. Murine SVR endothelial cells were grown to 100% confluence before being treated with a histone de-acetylase inhibitor, TSA (100 nM). From left to right: Control group was incubated in culture medium without any treatment. DMSO group was treated with 0.1% DMSO for 48 hours. TSA group was treated with 100 nM TSA in 0.1% DMSO for 48 hours. Values represent the mean, S.E. and number of independent experiments. There was no significant difference in cell numbers and cell viability between all groups, $p>0.05$, 1-way ANOVA (repeated measures ).
HUVEC viability assessment

HUVECs were grown to 100% confluence before being treated with a histone de-acetylase inhibitor, TSA (100 nM). After 48 hours cells were trypsinized for cell count (Panel A) and trypan blue exclusion assay (Panel B). Panel C: Images of HUVECs after 48h TSA treatment. HUVECs were grown to 100% confluence before being treated with a histone de-acetylase inhibitor, TSA (100 nM). From left to right: Control group was incubated in culture medium without any treatment. DMSO group was treated with 0.1% DMSO for 48 hours,. TSA group was treated with 100 nM TSA in 0.1% DMSO for 48 hours. Values represent the mean, S.E. and number of independent experiments. There was no significant difference in cell numbers and cell viability between all groups, $p>0.05$, 1-way ANOVA (repeated measures).
2.4.2 WESTERN BLOT METHOD VALIDATION

2.4.2.1 Western blot validation for acetylated histone expression in endothelial cell lines

2.4.2.1.1 Specificity of primary antibody (acetylated histone H4)

The specificity of the acetylated histone H4 primary antibody (Upstate, NY, USA) was tested on the samples collected from cultured murine SVR endothelial cells and HUVECs. Western blotting for acetylated histone H4 protein was performed with the omission of the acetylated histone H4 primary antibody but in the presence of the secondary antibody. Western blots presented in Figure 2-4 show that omission of acetylated histone H4 primary antibody failed to detect acetylated histone H4 protein in murine SVR endothelial cells. The same results were obtained in HUVECs (Figure 2-4).

2.4.2.1.2 Molecular weight (acetylated histone H4)

To ensure the bands detected on the gels corresponded to acetylated histone H4 protein, molecular weight standards were run parallel to all samples. Detected protein bands shown in Figure 2-4 was compared with the manufacturer’s values of molecular weight standards corresponding to approximately 10 kD, which is the established molecular weight for acetylated histone H4 protein.

2.4.2.1.3 Sample loading determination (acetylated histone H4)

A series of protein concentrations was performed in selected samples to determine the most suitable sample loading concentration. In murine SVR endothelial cells and HUVECs, it was found that 10 μg of total protein from cultured cell samples produced one clear 10 kD band of acetylated histone H4 protein expression (Figure 2-4). Acetylated histone H4 expression at protein load above 10 μg became over-saturated and the density reading failed to show a linear relationship corresponding to increased protein loading concentration (Figure 2-4).
2.4.2.1.4 Internal standard (acetylated histone H4)

In every gel, an internal standard, acetylated histone H4 (Upstate, NY, USA) was run parallel to all samples. To normalize possible density differences between gels, the densities of all acetylated histone H4 bands were expressed as a percentage of the respective acetylated histone H4 standard band, which was standardized at 100%.

2.4.2.2 Western blot validation for COX expression in endothelial cell lines

2.4.2.2.1 Specificity of primary antibodies (COX)

The specificity of the COX primary antibodies was tested on the samples collected from cultured murine SVR and human umbilical vein endothelial cells. Western blotting for COX-1 and COX-2 protein was performed with the omission of the COX primary antibodies but in the presence of the secondary antibodies. Western blots presented in Figure 2-5 show that omission of COX primary antibodies failed to detect COX protein in murine SVR endothelial cells. The same results were observed in HUVECs (Figure 2-6).

2.4.2.2.2 Molecular weight (COX)

In order to verify that the bands detected on the gels corresponded to COX-1 and COX-2 proteins, molecular weight standards were run parallel to all samples. Detected protein bands (Figures 2-5 and 2-6) were compared with the manufacturer’s values of molecular weight standards corresponding to approximately 70 kD (69 kD for COX-1 and 72 kD for COX-2; see Review by Davidge 2001), which is the established molecular weight for both COX-1 and COX-2 proteins (see Reviews by Smith et al. 1996; Golden & Abramson 1999).

In HUVECs, Western blotting failed to detect COX-2 protein expression although COX-2 protein was expressed in the internal standard suggesting the failure of COX-2 protein detection was not due to unsuccessful experimental protocol (Figure 2-6).
2.4.2.2.3 Sample loading determination (COX)

A series of protein concentrations was performed in selected samples to determine the most suitable sample loading concentration. In murine SVR endothelial cells, it was found that 10 μg of total protein from cultured cell samples produced one clear 69 kD band of COX-1 and 72 kD band of COX-2 protein expression, respectively (Figure 2-5). COX expression at protein loads above 10 μg became over-saturated and the density reading failed to show a linear relationship corresponding to increased protein loading concentration (Figure 2-5).

In HUVECs, it was found that 10 μg of total protein from cultured cell samples produced one clear 69 kD band of COX-1 protein expression, respectively (Figure 2-6). COX expression at protein loads above 10 μg became over-saturated and the density reading failed to show a linear relationship corresponding to increased protein loading concentration (Figure 2-6).

2.4.2.2.4 Internal standard (COX)

In every gel, an internal COX standard prepared from 8 week-old, male Sprague-Dawley rats was run parallel to all samples. To normalize possible density differences between gels, the densities of all COX-1 and COX-2 bands were expressed as a percentage of the respective COX standard band, which was standardized at 100%. 
Western blot validation: acetylated histone H4 protein levels in endothelial cells

Panels A and C: A signal for acetylated histone H4 in murine SVR and human endothelial cells, respectively, was evident at 10 kD according to molecular weight markers. Panels B and D: Acetylated histone H4 immuno-reactivity was verified by negative controls, which were performed with omission of the acetylated histone H4 primary antibody but in the presence of the secondary antibody. Lane a: internal standard, lanes b + c: duplicate samples from control group, lanes d + e: duplicates samples from DMSO group (0.1%), lanes f + g: duplicate samples from TSA group (100 nM). A series of sample loading concentrations was performed to determine the final protein loading for acetylated histone H4 in murine SVR (Panels E and F) and human endothelial cells (Panels G and H). Lanes h to k represent 5, 10, 15 and 20 μg protein loading. A final protein loading of 10 μg per lane was chosen, because this was the concentration at which the minimal amount of sample was required to produce a reliable and clear band. Higher protein loading resulted in the risk of signal saturation.
Figure 2-5

Western blot validation: COX protein levels in endothelial cells (SVR)

Panels A and C: A signal for COX-1 and COX-2, respectively in murine SVR endothelial cells was evident at 70 kD according to molecular weight markers. Panels B and D: COX immuno-reactivity was verified by negative controls, which were performed with omission of the COX primary antibodies but in the presence of the secondary antibodies. Lane a: internal standard, lanes b + c: duplicate samples from TSA group (100 nM), lanes d + e: duplicates samples from DMSO group (0.1%), lanes f + g: duplicates from control group. A series of sample loading concentrations was performed to determine the final protein loading for COX-1 (Panels E and F) and COX-2 (Panels G and H) in murine SVR endothelial cells, respectively. Lanes h to k represent 5, 10, 15 and 20 \( \mu \)g protein loading. A final protein loading of 10 \( \mu \)g per lane was chosen, because this was the concentration at which the minimal amount of sample was required to produce a reliable and clear band. Higher protein loading resulted in the risk of signal saturation.
Western blot validation: COX protein levels in endothelial cells (HUVECs)

Panels A and C: A signal for COX-1 and COX-2, respectively in HUVECs was evident at 70 kD according to molecular weight markers. Panels B and D: COX immuno-reactivity was verified by negative controls, which were performed with omission of the COX primary antibodies but in the presence of the secondary antibodies. COX-2 expression was not present in un-stimulated HUVECs. Lanes a + b: duplicates from control group, lanes c + d: duplicates samples from DMSO group (0.1%), lanes e + f: duplicate samples from TSA group (100 nM), lane g: internal standard. Panels E and F: A series of sample loading concentrations was performed to determine the final protein loading for COX-1 in HUVECs, respectively. Lanes h to k represent 5, 10, 15 and 20 μg of protein loading. A final protein loading of 10 μg per lane was chosen, because this was the concentration at which the minimal amount of sample was required to produce a reliable and clear band. Higher protein loading resulted in the risk of signal saturation.
2.4.3 ACETYLATED HISTONE EXPRESSION IN TSA-TREATED ENDOTHELIAL CELLS

2.4.3.1 Acetylated histone expression in TSA-treated endothelial cells

The level of acetylated histone H4 was assessed in cultured murine and human endothelial cells treated with the histone de-acetylase inhibitor, TSA. In murine SVR endothelial cells, TSA treatment resulted in increased acetylated histone H4 levels in the TSA-treated group compared with the DMSO-treated group. Acetylated histone H4 levels remained unchanged between the control and DMSO-treated cells (Figure 2-7).

Similar results were found in HUVECs. There was no difference in acetylated histone H4 expression in the control group compared with the DMSO-treated group. Acetylated histone H4 levels, however, were increased significantly in TSA-treated group compared with DMSO-treated group in response to 48-hour TSA treatment (Figure 2-7).

2.4.4 COX EXPRESSION IN TSA-TREATED ENDOTHELIAL CELLS

2.4.4.1 COX-1 expression in TSA-treated endothelial cells

There was no significant difference in COX-1 expression between the control and DMSO-treated group. In contrast, COX-1 expression increased significantly in the TSA-treated group compared with DMSO-treated group in both murine SVR and human umbilical vein endothelial cell lines (Figure 2-8).

2.4.4.2 COX-2 expression in TSA-treated endothelial cells

There was no difference in COX-2 expression between the control and DMSO-treated groups in the murine SVR endothelial cells. COX-2 expression was found to increase in response to TSA treatment compared to those cells treated with DMSO only. In HUVECs, COX-2 expression was not detectable in any of the groups regardless of the treatment (Figure 2-8).
Figure 2-7

Quantitative densitometry of acetylated histone H4 protein expression in cultured endothelial cells

Analysis of acetylated histone H4 expression in cultured endothelial cells treated with TSA (100 nM). The control group was incubated in culture medium without any treatment. The DMSO group was treated with 0.1% DMSO for 48 hours. The TSA group was treated with 100 nM TSA in 0.1% DMSO for 48 hours. Panel A: acetylated histone H4 protein expression was measured in murine SVR endothelial cells. Panel B: acetylated histone H4 protein expression was measured in HUVECs. A histone H4 standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of independent experiments. * represents significant difference from DMSO group, $p<0.01$, 1-way ANOVA (repeated measure; Tukey’s multiple comparison test).
Quantitative densitometry of COX protein expression in cultured endothelial cells

Analysis of COX expression in cultured endothelial cells treated with TSA (100 nM). The control group was incubated in culture medium without any treatment. The DMSO group was treated with 0.1% DMSO for 48 hours. The TSA group was treated with 100 nM TSA in 0.1% DMSO for 48 hours. Panel A: COX-1 protein expression was measured in murine SVR endothelial cells. Panel B: COX-2 protein expression was measured in murine SVR endothelial cells. Panel C: COX-1 protein expression was measured in HUVECs. A rat COX standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of independent experiments. * represents significant difference from DMSO group, $p<0.01$, 1-way ANOVA (repeated measure; Tukey’s multiple comparison test).
2.5 DISCUSSION

Previous studies from our laboratory found enhanced COX-1 and COX-2 expression in aging rat aortas. Functionally, when the endothelium was removed in 54-week rat aortas, an increase in vasoconstriction of the blood vessel was observed compared with those of 8-week rat aortas, a process which was found to be both COX-1- and COX-2-dependent (Kang et al. 2001). Further study in human male subjects found monocyte COX-2 expression was increased with aging (Kang et al. 2003). Very little is known about the underlying mechanisms by which increased COX levels are regulated in aging. Evidence has suggested that histone H4 acetylation is increased with aging in human lymphocytes after TSA treatment (Sourlingas et al. 2002; Kypreou et al. 2004). The aim of the current study was to test the hypothesis that changes in COX-1 and COX-2 expression in the endothelium is modulated by histone hyperacetylation, a post-translational modification that is frequently associated with active gene transcription. Murine SVR and human umbilical vein endothelial cell lines were cultured and treated with a specific histone de-acetylase inhibitor, TSA. Acetylated histone H4 and COX levels were assessed using Western blotting techniques.

2.5.1 ACETYLATED HISTONE H4 EXPRESSION IN TSA-TREATED ENDOTHELIAL CELLS

TSA, a specific histone de-acetylase inhibitor is known to increase histone acetylation levels (Yoshida et al. 1990). The present study reported that histone H4 extracted from TSA-treated murine and human endothelial cells had a higher level of histone acetylation in comparison to non-TSA-treated cells. This verified the efficacy of TSA as a histone de-acetylase inhibitor. Further studies were then performed to determine whether histone hyperacetylation was associated with changes in COX expression in endothelial cells in response to TSA treatment.
2.5.2 COX PROTEIN EXPRESSION IN TSA-TREATED ENDOTHELIAL CELLS

The present study tested the hypothesis that up-regulation of COX-1 expression was associated with histone acetylation/de-acetylation mechanism in endothelial cell lines. Enhanced COX-1 expression in both murine and human endothelial cell lines was found after 48 hour TSA treatment, suggesting COX-1 expression may be affected by histone acetylation. This finding is in agreement with the findings of Taniura et al. (2002) using neural cells. Enhanced COX-1 expression was found in normal human astrocyte cells and glioma cell lines after treatment with TSA for 24 and 48 hours. Taniura et al. further suggested that TSA upregulates COX-1 level by acting on the two Sp1-binding sites and the proximal Sp1-binding site located at the COX-1 promoter.

In the case of COX-2, the current study reported that COX-2 levels were enhanced in response to TSA in the murine SVR endothelial cell line, a phenomenon not observed in HUVECs. In fact, COX-2 expression was not detected in HUVECs in either TSA-treated and -untreated groups, an observation verified by the positive COX-2 internal standard, suggesting it was not due to failure of experimental protocol. This finding is consistent with the report by Caughey et al. (2001), who noted that un-stimulated HUVECs only expressed COX-1, but not COX-2. In another report, Norata et al. (2004) found that COX-2 expression in un-stimulated HUVECs was very low. The detection of enhanced COX-2 expression in the tumor cell line but not in un-stimulated HUVECs after TSA treatment suggests that histone acetylation may only promote COX-2 expression in some cells, which have an intrinsic capacity to express COX-2.

Increasing evidence suggests that COX-mediated pathways are altered during the aging process. The COX-2-mediated pathway has been shown to be increased with aging indicated by enhanced production of PGE\textsubscript{2} in the hippocampus of rats. This was reduced with the treatment of celecoxib, a selective COX-2 inhibitor (Casolini et al. 2002). Previous study
from our group at RMIT University also demonstrated an age-dependent increase in monocyte COX-2 expression in healthy human male subjects (Kang et al. 2003). The biological effects of changes in COX-mediated pathways in aging may be profound and are discussed in Section 2.1.1.1.

The data from the present study show that COX-1 and COX-2 expression was increased after histone hyperacetylation in endothelial cells. This is a potential explanation for increased COX in aging since in aging there is histone hyperacetylation. For instance, studies have reported that histone acetyltransferase activity is increased in aging mice, rats and elderly human subjects (Sarg et al. 1999; Li et al. 2002; Sourlingas et al. 2002). Furthermore, histone H4 acetylation was found to increase in response to TSA treatment in human lymphocytes with advancing age (Sourlingas et al. 2002; Kypreou et al. 2004). In contrast, an early study in rats showed that histone H4 acetylation was reduced with aging in neurons (Pina et al. 1988). Although it is unclear why conflicting data have been reported, it is possible that the effect of histone acetylation in aging is cell/tissue/species-specific.

2.6 CONCLUSIONS
The present study found firstly, that COX-1 expression in both murine and human endothelial cells was increased in response to TSA incubation, which was accompanied by an increase in histone H4 acetylation status. During aging, increased COX-1 expression may also be regulated through enhanced histone acetylation. Secondly, COX-2 expression was increased in a murine tumor cell line after TSA treatment with concomitant increased histone H4 acetylation. In contrast, COX-2 expression was not detected in un-stimulated HUVECs and TSA treatment had no effect on COX-2 expression in this case. The fact that COX-2 was only up-regulated in the murine tumor cell line, but not in un-stimulated HUVECs after TSA treatment suggests that pathological process such as tumor growth and inflammation may serve as triggers that enhance histone acetylation resulting in increased COX-2 expression.
CHAPTER 3
DIABETES REVIEW
3.1 HISTORY OF DIABETIC DISEASES

The earliest known record of diabetes mellitus was documented in the Ebers Papyrus, an Egyptian document in 1500 B.C. Described by physician Hesyra, the article noted that diabetes was a chronic systemic disease with a symptom of frequent urination (see Review by King & Rubin 2003). The term “diabetes” was first noted by Araetus of Cappodocia around 81-133 A.D. (see Review by Majumdar 2001). Thomas Willis in 1675 re-discovered, after the ancient Indians, the sweetness of urine and blood of diabetic individuals, hence the word “mellitus” was introduced. Willis’ observation was then confirmed by Dobson in 1776, who identified the presence of excess sugar in urine and blood as the cause of their sweetness. Eventually, the role of the pancreas in the pathogenesis of diabetes was discovered by Mering and Minkowski in 1889, which led eventually, to the extraction of insulin by Frederick Banting and John Macleod in 1921, who later received the Nobel Prize in 1923 for their contribution (see Reviews by Majumdar 2001; Ahmed 2002; King 2003; King & Rubin 2003).

Diabetes mellitus is a group of chronic metabolic diseases characterized by hyperglycemia and by disturbances of carbohydrate, fat and protein metabolism. It is associated with absolute or partial deficiency in insulin secretion from the pancreatic β cells of the islets of Langerhans and/or defective insulin action (Barcelo 1996; see Review by Schuster & Duvuuri 2002). Insulin, acting on insulin receptors in the membrane of cells, promotes the uptake of glucose from the circulation into muscle and adipose tissue through the glucose transporter proteins. According to the World Health Organization (1994), the two major types of diabetes mellitus are insulin-dependent diabetes mellitus (or Type 1 diabetes) and non-insulin-dependent diabetes mellitus (or Type 2 diabetes). Type 1 diabetes, characterized by defective insulin production, is most common in children and young adolescence whereas Type 2 diabetes, characterized largely by defective insulin action, tends to appear in adults over forty. Among the two, Type 2 diabetes is the more common form, accounting for about 85% of all cases.
3.2 TYPE 2 DIABETES MELLITUS

Type 2 diabetes mellitus, also known as non-insulin dependent diabetes mellitus, is the most common form of diabetes and occurs with increasing frequency with age. The condition is often associated with insulin resistance, a condition in which the cells fail to respond to insulin, and always with either relative or absolute insulin deficiency (see Reviews by Efendic & Luft 1986; Zimmet 1992; Gerich 1998). When insulin secretion is insufficient, glucose uptake in muscle is reduced and the liver increases glucose production. Adipose tissue also enhances free fatty acid mobilization. All of these contribute to prolonged elevation of glucose levels in the circulation after meals in Type 2 patients. Insulin resistance can also promote the development of Type 2 diabetes. As the tissue sensitivity to insulin is reduced, the pancreas compensates by increasing in insulin production. Hence, hyperinsulinemia and insulin resistance are frequently found even before the clinical onset of Type 2 diabetes (see Reviews by Zimmet 1989; Charles et al. 1991; Leboritz 1998). Eventually, hyperinsulinemia leads to insulin deficiency particularly in those patients whose pancreatic β cells have limited secretory reserve. Although the metabolic disturbance is complex, Type 2 diabetes individuals usually do not require insulin treatment for survival.

3.2.1 ETIOLOGY OF TYPE 2 DIABETES

The etiology of Type 2 diabetes is complex. Genetic inheritance is strongly associated with the disease development, which is further precipitated by environmental factors.

3.2.1.1 Genetic influence

Genetic factors are important in the etiology of Type 2 diabetes. A single-gene disorder known as maturity-onset diabetes of the young (MODY), characterized by autosomal dominant inheritance and an age of onset of twenty five years or younger (Froguel et al. 1993; Hanis et
al. 1996; see Reviews by Fajan 1989; Ledermann 1995) has been found to account for 2-5% of the Type 2 diabetic population. MODY genes have been localized on chromosomes 7, 12 and 20 (Bell et al. 1991; Froguel et al. 1993; Vaxillaire et al. 1995) and mutations in these genes can lead to progressive pancreatic β cell failure, decreased glucose-regulated secretion of insulin and decreased glucose stimulation of insulin gene transcription leading to defective insulin production (Byrne et al. 1996 and see Reviews by Polonsky 1995; Hattersley 1998).

In contrast to the rare case of MODY, there is a poorly defined genetic component in Type 2 diabetes etiology for 70-85% of Type 2 diabetic population and the degree of genetic influence may vary according to ethnicity and geography. The genes involved in Type 2 diabetes may also vary. For instance, there is a great difference in the two diabetes susceptibility genes for Type 2 diabetes identified. NIDDM 1, found in Mexican Americans (Hanis et al. 1996) is different from NIDDM 2, which was found in Finnish families (Mahtani et al. 1996). Although the pathogenesis of the majority of Type 2 diabetes cases is unclear, it is generally agreed that in addition to the genetic influence, there is a strong environmental component involved (see Section 3.2.1.2 and Reviews by Hamman 1992; Kahn 1994; Bouchard 1995; Yki-Järvinen 1995).

### 3.2.1.2 Environmental influence

Whilst the genetic influence is an important component in the development of Type 2 diabetes (see Section 3.2.1.1), environmental factors such as nutrition, obesity and physical inactivity also influence the clinical expression of genetic defects and are often required for the clinical expression of the polygenic defects, a term used to describe the need for the simultaneous presence of several abnormal genes for disease development. Barker et al. (1993) reported a higher prevalence of insulin resistance, obesity and Type 2 diabetes in adults who had a low birth weight and postulated a link with fetal malnutrition.
Ravussin *et al.* (1994) observed the lifestyle of Pima Indians who live in Arizona. They have a sedentary lifestyle with high calorie diets and are also quite obese with an extremely high prevalence of Type 2 diabetes. In contrast, Pima Indians who live in the mountains of northern Mexico have an intense and active lifestyle with low calorie diets. The prevalence of Type 2 diabetes in this tribe is low and approximately equal to the general Mexican population. This suggests that diet and exercise are strongly linked to Type 2 diabetes.

### 3.3 TYPE 1 DIABETES MELLITUS

Insulin-dependent diabetes mellitus or Type 1 diabetes is an autoimmune disease. It is characterized by an inflammatory process, with the presence of T lymphocytes, B lymphocytes and macrophages (Imagawa *et al.* 1999) in the pancreas and selective destruction of the pancreatic β cells (Tabatabaie *et al.* 2000). The consequence of pancreatic β cell destruction is a failure of insulin production by the pancreas resulting in elevated blood glucose levels. As a result, Type 1 diabetic patients depend on daily insulin therapy.

#### 3.3.1 ETIOLOGY OF TYPE 1 DIABETES

The development of Type 1 diabetes cannot be explained simply by one single factor. The gradual progression of the disease involves several stages including genetic predisposition, environmental factors, active autoimmunity, progressive pancreatic β cell destruction and presentation of the symptoms of Type 1 diabetes (see Review by Devendra *et al.* 2004), which are further discussed below.

#### 3.3.1.1 Genetic pre-disposition

Individuals with Type 1 diabetes have a genetic pre-disposition to the disease as the familial occurrence of Type 1 diabetes has been long observed. According to Redondo *et al.* (2001), a sibling of Type 1 diabetes has a risk of developing Type 1 diabetes about 12 to 100 fold greater than the risk in the general population. The best studied genes in Type 1 diabetes are
the insulin gene and the ones found in the human leukocyte antigen (HLA) region. HLA region, a primary region of susceptibility for Type 1 diabetes, is located in chromosome 6 and contains more than 150 genes. Genes that are found in the class II sub-region of HLA such as DR, DQ and DP loci account for 40 to 50 percent of the diabetes risk that people inherit from their parents. It has been documented that at least 90% of patients who develop Type 1 diabetes have either DR3, DQ2 or DR4, DQ8 haplotypes, closely linked alleles inherited as a unit. In contrast, less than 40% of normal controls have these haplotypes (Noble et al. 1996; Dubois-Laforgue et al. 1997; Atkinson & Eisenbarth 2001, see Review by Dorman & Bunker 2000).

The variable number of tandem repeats (VNTR), a repeated section of DNA forming the insulin gene, which is the region of DNA that codes for the protein insulin may also be involved in the genetic pre-disposition of Type 1 diabetes. Different numbers of DNA repeats forms either the smaller VNTR regions containing only 26 to 63 DNA repeats or the long VNTR regions containing 140 to 200 repeats. Owerbach & Gabbay (1993) suggested that a person with two short VNTR regions is two to five times more susceptible to the development of Type 1 diabetes than a person with least one long VNTR.

### 3.3.1.2 Environmental influence

Genetic pre-disposition of Type 1 diabetes is profoundly influenced by environmental factors triggering the disease. Data obtained from studies of identical twins with Type 1 diabetes have confirmed this observation. Srikanta and colleagues (1983) found that when one twin had Type 1 diabetes, the other twin developed the condition only half of the time. If the cause of Type 1 diabetes were purely genetic, both identical twins would always have Type 1 diabetes.
One environmental trigger is thought to be the infection by picornaviruses such as Coxsackie B virus as a possible etiology of Type 1 diabetes by inducing more subtle, long-term changes in the pancreatic β cells. In 1994, Tian and colleagues reported that Coxsackie viruses trigger Type 1 diabetes in genetically susceptible mice, but not in those that had a different genetic profile. Another study in the same year showed that the autoimmune reaction observed in Type 1 diabetes was triggered by a similarity between a Coxsackie virus protein and a protein in the pancreas, subsequently shown by Kaufman and others to be an enzyme called glutamic acid decarboxylase (GAD) (Kaufman et al. 1992; Atkinson et al. 1994). GAD is found on the surface of insulin-producing pancreatic β cells and a small segment of GAD is structurally similar to a segment of a Coxsackie B protein. It has been suggested that when the immune system attacks a Coxsackie infection, it also inadvertently attacks GAD leading to destruction of insulin-producing pancreatic cells (Atkinson et al. 1994).

3.3.1.3 Autoimmunity

Autoimmunity, a condition in which the immune system produces antibodies against the body's own tissues, plays a role in Type 1 diabetes etiology. Environmental factors can trigger the production of auto-antibodies such as GAD (also known as 64-K) antibody and the ICA 512 antibody causing damage to the pancreatic β cells (see Review by Devendra & Eisenbarth 2003). Maternal exposure to viral infections such as mumps, rubella, Coxsackie B4 during pregnancy (Dahlquist et al. 1995; Hyoty et al. 1995), exposure to cow’s milk in infancy, nitrosamines (Akerblom et al. 2002) or vitamin D supplementation (EURODIAB Substudy 2 Study Group et al. 1999) have all been shown to trigger an autoimmune response resulting in a profound inflammatory response against the pancreatic β cells leading to insulin deficiency.

In addition to autoimmunity to the pancreatic β cells, Type 1 diabetic subjects are often associated with other autoimmune conditions as determined by the presence of specific antibodies. Data from the Belgian Diabetes Registry showed that the prevalence of thyroid
peroxidase auto-antibodies is 22% in patients with Type 1 diabetes. In addition, transglutaminase IgA auto-antibodies are detected in approximately 1 in 10 patients with Type 1 diabetes. More than half of these patients have coeliac disease on intestinal biopsy. Approximately 1 in 50 people with Type 1 diabetes have 21-hydroxylase autoantibodies, and approximately 25% of these patients progress to Addison's disease (see Review by Devendra et al. 2004).

3.4 CONSEQUENCES OF DIABETES MELLITUS

The chronic hyperglycemia of diabetes mellitus is associated with long-term damage, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Vascular complications are the leading causes of morbidity and mortality in diabetic patients and diabetes is regarded as a significant risk factor for macro- and micro-vascular events. The macrovascular complications include coronary artery disease, atherosclerosis and peripheral vascular disease whereas the microvascular complications include retinopathy, nephropathy and neurovascular abnormalities associated with autonomic neuropathy (see Review by Schuster & Duvuuri, 2002). The increased risk of vascular complications in diabetes may be related to inflammation, increased generation of AGEs and arachidonic acid metabolism.

3.4.1 INFLAMMATION AND DIABETES MELLITUS

Inflammation, a part immune response, involves a series of events in response to infection or irritation, which results in removal of the irritants, limitation on tissue damage and the initiation of tissue repair. The process involves a variety of blood and tissue derived mediators including cytokines, growth factors and other agents including prostanoids (for more details for the effects of prostanoids in the vasculature, see Section 1.3.2). At the cellular level, inflammation is initiated when injured tissues release histamine, bradykinin and serotonin, which leads to vasodilatation and increased permeability. The blood vessel wall then becomes coarse, which slows down the blood flow and allows the leukocytes to move out of the blood
vessels and into the injured tissue. These leukocytes act as phagocytes surrounding, engulfing, destroying foreign substances and preventing the spread of an infection while cleaning away cellular debris. In the case of chronic inflammation, cytokines such as IL-1 and TNF-α released by macrophages at the site of injury will act on endothelial cells leading to up-regulation vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, which are capable of binding to leukocytes, subsequently resulting in further recruitment of neutrophils and monocytes, activation of T and B cells and adherence of leukocyte to the endothelium (see Review by Frijns & Kappelle 2002).

Inflammation is though to be involved in the development of diabetes based on four lines of evidence. First, non-obese individuals without previous history of glucose abnormalities who rapidly develop diabetes often have islet cell antibodies and antibodies to glutamic acid decarboxylase, both of which are autoimmune responses against the pancreatic β cell (Tuomi et al. 1993; Tuomilehto et al. 1994; Pietropaolo et al. 2000). Second, inflammatory markers such IL-6 have been found to be increased in Type 2 diabetic individuals compared with those without diabetes (Pickup et al. 1997). Further, in response to increased cytokine concentrations, the liver can produce acute phase protein such as C-reactive protein (CRP), an indicator of the presence of low-grade inflammation. Indeed, elevated levels of sialic acid, a measure of CRP were detected in Type 2 diabetic individuals (Pickup et al. 1997). Third, inflammation mediators can interfere with insulin signaling pathways. For instance, inflammatory cytokines such as TNF-α have been shown to increase insulin resistance and impair insulin signaling pathways in cells (Uysal et al. 1997; Cheung et al. 1998). Last, inflammation as measured by an enhanced CRP level has been associated with the insulin resistance syndrome, and metabolic dysfunction in lipid and carbohydrate metabolism, sedentary life style, hypertension as well as other genetic factors, which contribute to the development of diabetes (Festa et al. 2000; Frohlich et al. 2000). Evidence suggests that enhanced inflammatory activity in diabetes is implicated in the development of chronic
diabetic complications and may be associated with hyperactivity in platelets, which is discussed below in Section 3.4.1.2.

3.4.1.1 Atherosclerosis and diabetes mellitus

Atherosclerosis involves the formation of atheroma or atherosclerotic plaques and hardening of arterial blood vessels resulting in tissue damage due to restriction of blood flow. The exact cause of atherosclerosis is unclear, however, evidence suggests that deposition of lipids in the arterial intima initiates the development of atherosclerotic plaque. Small lipid deposits at sub-endothelial layer triggers an inflammatory response, attracting monocytes from the bloodstream to the arterial wall. Monocyte-derived macrophages then ingest oxidized cholesterol forming large foam cells. Lipid-laden foam cells eventually die and further propagate the inflammatory response. Low density lipoproteins continuously deliver oxidized cholesterol into the blood vessel wall, a process that also sustains inflammatory process and attracts more macrophages. Fatty deposits accumulate underneath the intima forming a plaque, which has a protective fibrous cap over it. The presence of atherosclerotic plaques can narrow the blood vessel, damage the endothelium, compromise the activity of the vessel to dilate/constrict and disrupt blood flow to vital organs such as the brain and heart causing stroke and ischemic heart disease (see Review by Stary et al. 1994).

The number, size and complexity of atherosclerotic plaques increases in diabetes indicated by increased lipids in the core region and increased infiltration of macrophages and lymphocytes present in plaque region (Jander et al. 1998; King et al. 1998). This may have important consequences. Matrix metalloproteinases (MMPs), proteolytic enzymes capable of degrading extracellular matrix components are up-regulated by cytokines released from peripheral blood monocytes and monocyte-derived macrophages (Moreau et al. 1999; Xu et al. 1999) and may decrease plaque stability (Galis et al. 1994). Enhanced MMP-9 activity in unstable carotid plaques (Loftus et al. 2000) and increased expression of active MMP-2 and MMP-9 has also
been reported in vulnerable regions of human carotid plaques in association with macrophages (Galis et al. 1994). The secretion of MMP-2 and MMP-9 by macrophages in human atherosclerotic plaques has been shown to occur through a PGE$_2$/cAMP–dependent pathway (Corcoran et al. 1994), possibly via COX enzymes and PGE$_2$ synthases associated with NFk-B activation (Cipollone et al. 2001). Thus macrophage COX may have a role in plaque stability and given enhanced numbers of macrophages in diabetes, plaques may predispose diabetic patients to increased cardiovascular risks.

3.4.1.2 Platelet hyperactivity and diabetes mellitus

Platelets are small anucleate discoid cells found in the circulation with their main function being to limit leaks through blood vessel walls by forming a thrombus. In response to injury, von Willebrand factor, a clotting factor attaches to sub-endothelium exposing multiple intrinsic binding sites for the platelet specific membrane Glycoprotein (GP) Ib. Once von Willebrand factor-GPIb binding takes place, it stimulates platelet shape changes and disintegration exposing GP IIb-IIIa binding sites for further attachment of von Willebrand factor and fibrinogen. Platelets then undergo a series of events to form a thrombus. First, dense granules in the platelets release ADP, a substance that promotes platelet aggregation and disintegration. TXA$_2$, serotonin, phospholipids and lipoproteins are also released by the platelets to sustain platelet activation and promote the coagulation cascade. Second, translocation of P-selectin from the membrane of α-granules to the plasma membrane takes place (Stenberg et al. 1985) and α-granules release the coagulation proteins including fibrinogen, thrombospondin, fibronectin, factor V and factor VIII. The phospholipid phosphatidylserine, also known as platelet factor 3, is found on the surface of the disintegrating platelet, serving as an essential base for the initiation of soluble proteins in the coagulation pathway. When tissue factors form a complex with factor VII in the presence of Ca$^{2+}$, factors IX and X are activated resulting in the conversion of thrombin from prothrombin. Fibrin is released from fibrinogen, a process initiated by thrombin. Fibrin then polymerises to
form the fibrin clot. Factor XIII, activated by thrombin and Ca$^{2+}$, stabilizes this clot by forming covalent bonds between the fibrin molecules (see Review by Ashby et al. 1990).

Platelet activity can be measured by platelet activation markers such as CD31, CD36, CD49b, CD62P and CD63. In diabetes, an increase in the expression of these activation markers on the surface of the platelets has been detected. These platelet activation markers were reduced in the platelets of Type 2 diabetic subjects with a good glycemic control regime (Eibl et al. 2004). Several mechanisms have been proposed to explain platelet hyperactivity. First, chronic hyperglycemia has been identified as a causal factor of in vivo platelet activation and hypereactivity in diabetes (Bridges et al. 1965; Davì et al. 1997; Davì et al. 1999). Second, hyperglycemia promotes non-enzymatic glycation of platelet membrane proteins leading to structural changes in proteins, as well as alterations of membrane lipid dynamics (see Review by Winocour 1992). Consequently, platelet receptor expression increases due to altered dynamics of platelet membrane (Tschoepe et al. 1995), potentially affecting receptor accessibility to ligands (Watala et al. 1996). Third, hyperglycemia can induce oxidative stress and thus increase peroxidation of arachidonic acid to form biologically active isoprostanes without the action of COX, creating a feedback loop sustaining platelet activation (Davì et al. 1999). Furthermore, increased oxidative stress promotes the endothelium towards a pro-thrombotic state by inhibiting endothelial thrombomodulin, a cofactor for a potent anticoagulant called protein C (Glaser et al. 1992). Therefore, the implications of hyperactive platelets in diabetes can be serious. With enhanced platelet activation, platelets are prone to aggregation and adhesion, which promote thrombosis development and increases the risk of ischemic heart diseases.
3.4.2 ADVANCED GLYCATION END PRODUCTS (AGES) AND DIABETES MELLITUS

AGEs are formed from the non-enzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. Precursor AGEs form covalent crosslinks with these macro molecules changing their structure thus affecting the functioning of cellular matrix, basement membranes, and vessel-wall (see Review by Raj et al. 2000). AGEs also interact with a variety of cell-surface AGE-binding proteins, resulting in either cell degradation or cellular activation and pro-oxidant, pro-inflammatory events (see Review by Peppa et al. 2003). In diabetes, the hyperglycemic environment promotes the formation of AGEs, which is evident in elevated serum AGE level in diabetic children and adolescents with or without vascular complications (Jakus & Rietbrock 2004).

The formation of AGEs is linked to chemical modification of proteins by glucose and is thought to play a part in the pathogenesis of diabetic complications including atherosclerosis, nephropathy, neuropathy and retinopathy (see Review by Jakus & Rietbrock 2004). For instance, glycation of platelet membrane proteins has been associated with a change in the structure of platelet membrane resulting in decreased ligand-membrane interactions in diabetic patients (Watala et al. 1996). More details on the roles of AGE in diabetes are discussed in Chapter 6.

3.4.3 ARACHIDONIC ACID METABOLISM AND DIABETES MELLITUS

Arachidonic acid signaling pathway involves the production of prostanoids by PLA2, COX and prostanoid synthases. Prostanoids produced by this pathway mediate numerous physiological processes including vasoconstriction and vasodilatation, platelet aggregation and renal re-absorption (for more details see Section 1.3).
In diabetes, increasing evidence suggests that arachidonic acid metabolism is involved in the promotion of diabetic vascular complication development. PGE\textsubscript{2} and TXA\textsubscript{2} production increased in human mesangial cells and aortic endothelial cells, respectively after glucose incubation (Cosentino et al. 2003; Kiritoshi et al. 2003), whilst a decrease in PGI\textsubscript{2} production was observed in HUVECs in response to glucose incubation (Cosentino et al. 2003). Furthermore, a study by Shanmugam et al. (2004) found that treating THP-1 monocytes with high concentrations of glucose led to a significant 3 to 5 fold induction of COX-2 mRNA and protein expression but not COX-1 mRNA. Enhanced levels of COX-2 mRNA were also found in isolated peripheral blood monocytes from Type 1 and Type 2 diabetic patients. In contrast, there was no COX-2 mRNA expression detected from normal subjects. These studies suggest that high levels of glucose can alter the prostanoid profile through the arachidonic acid pathway. Potentially, changes in the arachidonic acid pathway and prostanoid profile can result in a series of downstream events including alteration in vascular tone (see Section 1.3.2 for the effects of prostanoids in the vasculature), platelet and monocyte activity, all of which promote diabetic vascular complication development (see Section 3.4 for more details).

Given that diabetic subjects are at risk of vascular complications, however, the underlying mechanisms involved in the development of diabetic vascular complications are not clear, although AGE- and COX-mediated signaling pathways may be involved with inflammation as a common theme. The following three chapters discussed possible changes in these pathways in both Type 1 and Type 2 diabetes.
CHAPTER 4
ROLES OF COX IN TYPE 2 DIABETES
4.1 TYPE 2 DIABETES MELLITUS

Type 2 diabetes is characterized by a high level of glucose in the circulation, which is caused by insufficient insulin secretion by the pancreatic $\beta$ cells, increased insulin resistance in the tissue or both (for more details, see Section 3.2 and Reviews by Efendic & Luft 1986; Zimmet 1992; Gerich 1998). The chronic diabetic complications in Type 2 diabetes include retinopathy, nephropathy, neuropathy, diseases of blood vessels and ischemic heart conditions (for more details, see Section 3.4).

4.1.1 COX-MEDIATED PATHWAYS IN TYPE 2 DIABETES

Increasing evidence suggests that insulin resistance and progressive pancreatic $\beta$ cell failure, key events in the development of Type 2 diabetes is associated with persistent inflammatory activity indicated by elevated inflammatory markers such as TNF-$\alpha$, CRP and IL-6 (Yudkin et al. 1999). Further animal studies found that these inflammatory markers interfered with insulin signaling pathways. For instance, IL-6 was capable of modifying glucose-stimulated insulin release from isolated pancreatic $\beta$ cells in rodents (Sandler et al. 1990) and decreased insulin-stimulated glycogen synthesis in cultured hepatocytes (Kanemaki et al. 1998). TNF-$\alpha$ has also been shown to induce insulin resistance through mechanisms such as direct inhibitory effects on the glucose transporter protein GLUT4, the insulin receptor, and insulin receptor substrates (Hotamisligil & Spiegelman 1994). One of the consequences of Type 2 diabetes is the development of chronic diabetic complications including atherosclerosis. Data derives from experimental and epidemiological studies have shown that low-grade inflammation plays an important role in the initiation and progression of atherosclerotic plaque development. For example, CRP, an acute phase reactant produced by the liver has been found to induce monocyte chemoattractant protein and cellular adhesion molecule expression in human endothelial cells (Pasceri et al. 2001), and mediate low density lipoprotein uptake by macrophages (Zwaka et al. 2001). It is clear that both Type 2 diabetes and its complications may share a common link, namely inflammation.
COX expression, particularly COX-2, can be elevated by inflammatory cytokines (see Section 1.2.3.2). COX isoforms are rate-limiting enzymes that catalyze arachidonic acid leading to the eventual production of various prostanoids, which can alter vascular reactivity, platelet aggregation and atherosclerotic plaque formation and stability. COX-1, expressed at constant levels throughout the cell cycle, is termed a constitutive isoform and has a “house-keeping” role (see Review by DeWitt & Meade 1993). In contrast, inducible COX-2, whose expression under normal physiological conditions is not usually detected, is up-regulated in response to a variety of agents such as hormones, growth factors, pro-inflammatory stimuli and inflammation (see Reviews by Herschman 1996; Smith et al. 1996; Garavito & DeWitt 1999). Prostanoids produced by COX pathways are crucial to numerous physiological processes including platelet aggregation, vasoconstriction and vasodilatation (for more details, see Section 1.3). TXA$_2$ released by platelet and PGE$_2$ released by monocytes due to the action of COX-1 and COX-2, respectively are important prostanoids involved in thrombosis and atherosclerosis development (for more details, see Section 3.4). Given that inflammation is elevated in Type 2 diabetes and the ability of COX-mediated prostanoids to be involved in thrombosis and atherosclerosis, it was of interest to investigate if COX-mediated pathways were altered in platelets and monocytes of Type 2 diabetes.

In animal studies, COX-mediated pathways have been found to change in Zucker rats, an insulin resistant animal model that resembles the characteristics of Type 2 diabetes. For example, both COX-1 and COX-2 expression in platelets was increased significantly in obese Zucker rats compared with the lean controls (Raju et al. 2006). Renal COX-2, but not COX-1 expression was also up-regulated in obese Zucker rats (Komers et al. 2005). Less data are available in humans, however, the limited available data have indicated that COX-mediated pathways may be altered in human Type 2 diabetic subjects. For instance, enhanced levels of COX-2 mRNA were found in isolated peripheral blood monocytes from Type 2 diabetic patients whereas COX-2 mRNA expression was not detected in human non-diabetic subjects.
(Shanmugam et al. 2004). High glucose treatment of human THP-1 monocytes resulted in NF-κB activation (Miao et al. 2004), which is known to stimulate COX-2 expression. This appeared to be linked to enhanced histone acetylation as increased histone acetylation was observed near the promoter region of COX-2 in monocytes extracted from human Type 2 diabetic subjects compared with non-diabetic controls (Miao et al. 2004). These animal and human studies suggest that in Type 2 diabetes COX-mediated pathways are altered. Altered COX-mediated activities may affect the production of prostanoids, potentially influencing vascular tone, platelet and monocyte activity, all of which play important roles in the development of diabetic vascular complications.

4.2 AIMS

There is some evidence that COX-mediated pathways are involved in the development and progression of Type 2 diabetes and its complications, but the data is not conclusive. The aim of this study was to investigate possible changes of COX-mediated pathways in Type 2 diabetes. The specific aims were:

1) to measure changes in COX expression in the platelets and monocytes isolated from female Zucker diabetic rats of 25 weeks of age,
2) to investigate COX-mediated pathways in the platelets and monocytes isolated from human subjects by assessing COX protein and plasma COX metabolite levels,
3) to determine whether COX expression in Type 2 diabetes is associated with histone H4 hyperacetylation in monocytes.
4.3 METHODS

4.3.1 COX LEVELS IN PLATELETS AND MONOCYTES IN ZUCKER RATS

4.3.1.1 Collection of blood sample (Zucker rats)

The present study involved the use of animals. Samples were collected from female Zucker rats and kindly given by Professor Julianne Reid and her research unit at RMIT University, Melbourne. Female Zucker rats, 25 weeks old, were anesthetized with carbon dioxide followed by decapitation. Whole blood (approximately 10 mL) was collected by draining the blood from the neck of the rats into a centrifuge tube containing citrate buffer (129 mM sodium citrate, pH 7.4) as anticoagulant (1 part citrate buffer: 9 parts blood). The collected blood sample was divided equally for platelet or monocyte isolation.

4.3.1.2 Isolation of platelets and monocytes (Zucker rats)

The collected blood sample (see Section 4.3.1.1) was centrifuged at 150 g for 10 minutes at 4°C to isolate the platelet-rich plasma. The platelet-rich plasma was collected into an eppendorf tube followed by centrifugation at 200 g for 15 minutes at 4°C using a microcentrifuge to sediment platelets. The supernatant was then removed at the end of the spin and sedimented platelets were washed with excess Tris-citrate buffer (50 mM Tris-HCl, 137 mM NaCl, 5.4 mM KCl, 1 mM dextrose, pH 7.4, with added citrate buffer; see Section 4.3.1.1) then centrifuged at 200 g for 10 minutes at 4°C. The washing procedure was repeated before re-suspending the platelet pellet in PBS-based lysis buffer (1 mM KH$_2$PO$_4$, 154 mM NaCl, and 5.7 mM Na$_2$HPO$_4$, 0.5% Nonidet P-40, 0.01% EDTA, pH 7.4), with added protease inhibitors (1 mM PMSF, 0.2 mM leupeptin and 50 μM pepstatin A). Each sample was aliquoted into individual eppendorf tubes and stored at -80°C.

The rat monocytes were isolated from the collected blood sample (see Section 4.3.1.1) with the mixer flotation technique. In a 50 mL centrifuge tube, 5 mL of whole blood sample was mixed with 0.625 mL of a density gradient medium, OptiPrep™ (Nycomed Pharma, Oslo,
Norway). To facilitate monocytes flowing to the surface, 0.5 mL of TBS-buffered saline (10 mM Tricine-NaOH, 0.85% NaCl (w/v), pH 7.4) was layered onto the top of the blood mixture. The mixture was centrifuged at 1,300 g for 30 minutes at 4°C. Monocytes were then collected from the meniscus downwards to about 0.5 cm from the cell pellet and were transferred into another centrifuge tube. To completely remove platelets, the collected materials were diluted with an equal volume of TBS-buffered saline, which was layered over an equal volume of diluted OptiPrep™ solution (5 volume OptiPrep™ and 22 volume TBS-buffered saline). Samples were centrifuged at 350 g for 15 minutes at 4°C. The platelets form a wide band just below the interface; the entire liquid phase was aspirated and the monocyte pellet was then re-suspended in lysis buffer and protease inhibitors as described in platelet sample preparation above. Each sample was aliquoted into individual eppendorf tubes and stored at -80°C.

4.3.1.3 Protein assay (Zucker rats)

The total protein concentration of the isolated platelet and monocyte samples were determined by using the micro bicinchoninic acid (micro BCA) assay system from the Pierce micro BCA kit (Pierce, Rockford, IL, USA). The re-suspended samples were diluted 400X with lysis buffer (see Section 2.3.3.1). According to the manufacturer’s protocol, the micro BCA reagents were mixed and added to protein samples, standards and blanks followed by incubation at 60°C for 1 hour before determination of total protein content with a micro plate reader at 562 nm absorbance wavelength. Protein standard concentrations ranging from 5 to 20 μg/mL were prepared from the stock of 2 mg/mL bovine serum albumin protein (Pierce, Rockford, IL, USA) and diluted with lysis buffer. Blanks were prepared with lysis buffer without any protein content.

4.3.1.4 Western blot: COX protein (Zucker rats)

The samples were diluted with lysis buffer and reducing buffer (1 mg/mL bromophenol blue, 20% glycerol, 100 mM dithiothreitol, 2% sodium dodecyl sulphate, pH 6.8) to produce
equivalent protein concentrations (usually 40 μg/mL). Samples and molecular weight markers (Sigma, St Louis, USA) were placed in eppendorf tubes and denatured at 100°C for 3 minutes on a heating block. Samples were loaded in duplicate onto a 10% polyacrylamide gel with a final total protein concentration of 10 μg per well. The protein samples and molecular weight markers were electrophoresed, transferred onto nitrocellulose membranes and incubated with selective COX-1 and COX-2 antibodies using the same Western blot protocol for the detection of COX protein expression as in Section 2.3.3. The density of all Western blot bands was analyzed using the ChemiDoc™ XRS system (Biorad, CA, USA).

4.3.2 COX LEVELS IN PLATELETS AND MONOCYTES IN TYPE 2 DIABETES

4.3.2.1 Human subjects (Type 2 diabetes)

Twenty five (25) non-diabetic control (14 males, 11 females) and 52 Type 2 diabetic (28 males, 24 females) human subjects were recruited at St. Vincent’s Hospital (Melbourne, Australia). The diagnosis of diabetes was made based on plasma glucose levels of venous plasma using the diagnostic guideline provided by the Australian Diabetes Society (a single fasting plasma glucose level of ≥ 7 mmol/L or 2 hour post-glucose or casual postprandial plasma glucose level of ≥ 11.1 mmol/L; Colman et al. 1999). All participants were informed of the study by an endocrinologist, Dr. Alicia Jenkins, who was a collaborator of this study. Human subjects were required to sign an approved written informed consent prepared by St Vincent’s Hospital, Melbourne (HREC-A 024/00 Glycoxidation, HDL and diabetes angiopathy). The plain language statement that details the study of the human subjects is attached in Appendix. In addition, 14 non-diabetic samples from healthy male human subjects collected during a previous project at RMIT University (HREC 15/01 Majewski: Age-dependent alterations in cyclooxygenase in platelets and human monocytes) and stored away at -80°C were also included in this study. In my role I had no data, which linked any results to individual patients by name.
4.3.2.2 Collection of human blood samples (Type 2 diabetes)

The volunteer recruitment and blood sample collection was performed by the endocrinologist, Dr. Alicia Jenkins at St Vincent’s Hospital (Melbourne, Australia). Human subjects were asked to donate approximately 50 mL of whole blood where possible by a single venepuncture. In some cases, human subjects were only asked to donate 37 mL of blood sample based on Dr. Jenkins’ professional opinion. For each sample, approximately 5 mL of blood were sent to the Pathology Department of the Hospital for the determination of hemoglobin A1C, cholesterol and triglyceride levels if the human subjects did not already have their blood profile measured. The remaining blood sample was mixed with citrate buffer as anticoagulant (see Section 4.3.1.1) and was divided equally for the isolation of platelets and monocytes. In some cases, plasma sample was available for collection and was stored at -80°C for COX metabolite study.

4.3.2.3 Isolation of human platelets and monocytes (Type 2 diabetes)

The isolation of human platelets and monocytes was performed using OptiPrep™ solution according to the manufacturer’s standard protocol. To isolate platelets, in a centrifuge tube a 10 mL blood sample was layered over 10 mL density barrier (5 volume OptiPrep™ and 22 volume of Solution B: 0.85% (w/v) NaCl, 20 mM HEPES-NaOH, pH 7.4, 1 mM EDTA). The sample was centrifuged at 350 g for 15 min at 4°C in a swinging-bucket rotor and the rotor was allowed to decelerate without the brake. The autologous plasma and the platelet-containing band were harvested from the broad turbid band below the interface. The collected material was then centrifuged at 400 g for 10 minutes at 4°C before re-suspending the pellet in lysis buffer (see Section 4.3.1.2 for lysis buffer recipe) and being processed and stored at -80°C as detailed in Section 4.3.1.2. To isolate human monocytes, a 10 mL blood sample was mixed gently but thoroughly (by repeated inversion) with 1.25 mL OptiPrep™ in a 50 mL centrifuge tube. To facilitate monocyte isolation, 0.5 mL of TBS (0.85% (w/v) NaCl, 10 mM Tricine-NaOH, pH 7.4) was layered on top and the whole preparation was centrifuged.
at 1,500 g for 30 min at 4°C. Human monocytes were then purified, collected and processed as outlined in Section 4.3.1.2 for further analysis.

4.3.2.4 Protein assay (Type 2 diabetes)
Determination of human sample protein concentration was performed using the Biorad microBCA system as outlined in Section 4.3.1.3.

4.3.2.5 Western blot: COX protein (Type 2 diabetes)
Human samples were loaded in duplicate with a final total protein concentration of 10 μg per well. The protein samples and molecular weight markers were electrophoresed, transferred onto nitrocellulose membranes and incubated with selective COX-1 and COX-2 antibodies using the same Western blot protocol for the detection of COX protein expressions as in Section 4.3.1.4. The density of all Western blot bands was analyzed using the ChemiDoc™ XRS system (Biorad, CA, USA).

4.3.3 PLASMA COX METABOLITE STUDY IN TYPE 2 DIABETES
The Biotrak EIA system (Amersham Bioscience, UK) was chosen to measure plasma TXB₂ and PGE₂. Plasma samples contain interfering compounds, which make the already low quantity of TXB₂ metabolite that is difficult to measure directly by the EIA method. To avoid this, plasma TXB₂ metabolite had to be extracted from plasma sample prior to measurement whereas the level of plasma PGE₂ was directly determined by the Biotrak EIA kit.

4.3.3.1 Solid phase extraction of COX metabolites (Type 2 diabetes)
COX metabolites can be extracted from plasma samples using Solid phase extraction procedures with Amprep C2 ethyl minicolumn (100 mg, Amersham Bioscience, UK).
4.3.3.1.1 Plasma TXB$_2$ extraction (Type 2 diabetes)

Extraction of plasma TXB$_2$ was performed according to the manufacturer’s protocol using Amprep C2 minicolumn (Amersham Bioscience, UK). Briefly, Amprep C2 minicolumn was conditioned and activated by 2 mL methanol and 2 mL milliQ water followed by 1 mL acidified plasma sample (2M HCl, pH 3). The minicolumn was then washed in sequence with 5 mL of milliQ water, 10% ethanol and hexane to elute away interference. Analyte was then collected with 500 μL elution solvent, methyl formate, which was evaporated under nitrogen. The extracted TXB$_2$ was determined with the Biotrak EIA system (see Section 4.3.3.2 for details).

4.3.3.1.2 Solid phase extraction validation (Type 2 diabetes)

In order to verify the solid phase extraction procedure and analyte recovery, a known amount of PGE standard (PGE$_2$, 1 ng/mL; Sigma, St Louis, USA) was passed through a conditioned minicolumn in place of the acidified plasma sample as described in Section 4.3.3.1.1. PGE$_2$ standard was collected with the elution solvent, methyl formate in four different volumes (500 μL, 1, 1.5 and 2 mL). Extracts were then dried under nitrogen followed by the determination of PGE$_2$ recovery by the Biotrak EIA system.

4.3.3.2 Amersham Biotrak EIA system (Type 2 diabetes)

The PGE$_2$ EIA Biotrak System allows a direct determination of plasma PGE$_2$ levels without the need for PGE$_2$ extraction. The measurement of extracted TXB$_2$ metabolites and plasma PGE$_2$ were performed using the TXB$_2$ and PGE$_2$ EIA Biotrak System, respectively (Amersham Bioscience, UK). Briefly, the Biotrak EIA system is based on the competition between unlabelled TXB$_2$ or PGE$_2$ and a known quantity of peroxidase-labeled TXB$_2$ or PGE$_2$ for a given number of binding sites on a TXB$_2$ or PGE$_2$ specific antibody. The amount of peroxidase-labeled ligand bound by the antibody is inversely proportional to the concentration of added unlabelled ligand. Peroxidase-labeled TXB$_2$ or PGE$_2$ bound to the antibody is
immobilized by the secondary antibody which was pre-coated to the wells of the EIA system. The addition of a tetramethylbenzidine/hydrogen peroxide single pot substrate allows the determination of the amount of peroxidase-labeled TXB\(_2\) or PGE\(_2\). The reaction is then terminated by the addition of sulphuric acid (1 M, 100 μL/well, Sigma, St Louis, USA). The optical density was read at 450 nm immediately.

### 4.3.4 ACETYLATED HISTONE H4 LEVELS IN TYPE 2 DIABETES

**4.3.4.1 Isolation of histones from human monocytes (Type 2 diabetes)**

Histones were isolated from human monocytes as previously described in Section 2.3.2

**4.3.4.2 Western blot: acetylated histone H4 protein (Type 2 diabetes)**

Histone samples extracted in Section 4.3.4.1 were electrophoresed on a 15% SDS-PAGE gel and labelled with anti-rabbit polyclonal anti-acetyl histone H4 antibody (Upstate, NY, USA) according to standard Western blot procedures outlined in Section 2.3.3.2.

### 4.3.5 DRUGS AND CHEMICALS

Dextrose, HCl, methyl formate, Nonidet P-40, sodium citrate, Tricine-NaOH; BDH Chemical (Kilsyth, Victoria, Australia). PGE\(_2\), sulphuric acid; Sigma (St Louis, USA). OptiPrep\(^{TM}\); Nycomed Pharma (Oslo, Norway). Other drugs and chemicals used in the preparation of buffers/solutions and present Western blot experiments were the same as that used in Chapter 2 and thus, were obtained from the same sources (see Section 2.3.4).

### 4.3.6 STATISTICAL ANALYSES

All values are given as the mean ± standard error of mean (S.E.) and \(n\) indicates the number of observations. Differences in the percentage densities of Western blot bands were analyzed using Student’s \(t\)-test (unpaired and paired), Mann-Whitney test, 1-way ANOVA and post-hoc tests (Tukey’s, Games-Howell and Dunn’s multiple tests), Pearson Chi-Square and Fisher’s
exact test whenever applicable. SPSS (SPSS Inc., IL, USA) and Prism (GraphPad Software, Inc., CA, USA) statistical packages were used in statistical analyses.

4.3.7 ANIMAL AND HUMAN ETHICS APPROVAL

All procedures used for animal blood/tissue collection were approved by the Animal Ethics Committee of RMIT University (Melbourne, Australia) and the investigation conforms to the Australian code of practice for the care and use of animals for scientific purposes published by the National Health and Medical Research Council of Australia. All procedures used for human blood collection were approved by the Human Research Ethics Committee of St. Vincent’s Hospital (Melbourne, Australia; HREC-A 024/00 Glycoxidation, HDL and diabetes angiopathy) in accordance with the National Statement on Ethical Conduct in Research Involving Humans (June 1999) produced by the National Health and Medical Research Council of Australia. A separate set of human samples from another study was included in the present study. The collection of these samples was approved by the Human Research Ethics Committee of RMIT University (Melbourne, Australia; HREC 15/01 Majewski: Age-dependent alterations in cyclooxygenase in platelets and human monocytes).
4.4 RESULTS

4.4.1 COX LEVELS IN PLATELETS AND MONOCYTES IN ZUCKER RATS

4.4.1.1 Western blotting method validation (Zucker rats)

4.4.1.1.1 Specificity of COX primary antibodies (Zucker rats)

The specificity of the COX primary antibodies (murine anti-ovine COX-1 monoclonal antibody, Cayman, MI, USA and mouse anti-COX-2 monoclonal antibody, BD Biosciences, CA, USA) was tested by incubating samples with the secondary antibody only. The absence of COX bands confirmed the specificity of the antibodies to targeted COX proteins (Figure 4-1).

4.4.1.1.2 COX expression at correct molecular weight (Zucker rats)

In order to verify that the bands on the gels were corresponding to COX-1 and COX-2 proteins, molecular weight standards were run parallel to all samples (Figure 4-1). Detected protein bands were compared with the manufacturer’s values of molecular weight standards corresponding to approximately 70 kD (69 kD for COX-1 and 72 kD for COX-2; see Davidge 2001), which is the established molecular weight for both COX-1 and COX-2 proteins (see Reviews by Smith et al. 1996; Golden & Abramson 1999).

4.4.1.1.3 Sample loading determination (Zucker rats)

A series of protein concentrations (5 to 20 μg/lane) was performed in selected samples to determine the most suitable sample loading concentration. It was found that 10 μg of total protein from platelet and monocyte samples produced one clear 69 kD band of COX-1 and 72 kD band of COX-2 protein expression, respectively (Figure 4-1).
4.4.1.4 Internal standard (Zucker rats)

On every gel, a COX standard prepared from 8 week-old, male Sprague-Dawley rats was run parallel with all samples. To normalize possible density differences between gels, the densities of all COX-1 and COX-2 bands were expressed as a percentage of the respective platelet or monocyte standard band, which was standardized at 100%.

4.4.1.2 Western blot: COX levels in platelets and monocytes in Zucker rats

Immunoblotting studies shown in Figure 4-2 summarize COX-1 and COX-2 protein levels in the respective platelet and monocyte samples isolated from lean and obese female Zucker rats. It was observed that the platelets and monocytes isolated from obese Zucker rats expressed higher platelet COX-1 and monocyte COX-2 protein levels compared to lean Zucker rats (Figure 4-2).
Western blot validation: COX protein levels in platelets and monocytes (Zucker rats)

Western blot validation for platelet COX-1 and monocyte COX-2 in female Zucker rat. Panels A and C: A signal for COX-1 and COX-2, respectively, was evident at 70 kD according to molecular weight markers. Panels B and D: COX-1 and COX-2 immuno-reactivity was verified by negative controls, which were performed with omission of the COX primary antibodies but in the presence of the secondary antibodies. Lane a: internal standard, lanes b + c: duplicate samples from lean Zucker rats, lanes d + e: duplicates samples from obese Zucker rats. A series of sample loading concentrations was performed to determine the final protein loading for COX-1 (Panels E and F) and COX-2 (Panels G and H). Lanes f to i represent 5, 10, 15 and 20 μg protein loading. A final protein loading of 10 μg per lane was chosen, because this was the concentration at which the minimal amount of sample was required to produce a reliable and clear band. Higher protein loading resulted in the risk of signal saturation.
Figure 4-2

Quantitative densitometry of COX proteins in Zucker rats

Panel A: Platelet COX-1 protein expression was measured in lean and obese Zucker rats. Panel B: Monocyte COX-2 protein expression was measured in lean and obese Zucker rats. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands were expressed as a percentage of the corresponding standard control. In all cases, 10 μg of total protein of platelet or monocyte samples was used. Values represent the mean, S.E. and number of Zucker rats. * significant difference from lean values, p<0.001, unpaired Student’s t-test; # significant difference from lean values, p<0.05, Mann Whitney test.
4.4.2 COX-MEDIATED PATHWAYS IN TYPE 2 DIABETES

4.4.2.1 Basis parameters of human Type 2 diabetic subjects

Human subject selection was made by Dr. Alicia Jenkins at St. Vincent’s Hospital, Melbourne, Victoria for another research project, which had broad selection criteria. The present study did not exclude any samples collected including samples from smokers and hypertensive human subjects in control groups. The goal of this was to have a sample population that resembled the characteristics of the general population. One exception was COX inhibition. Human subjects on non-steroidal anti-inflammatory drugs were separated from the rest of the sample population and treated separately. Thus the three groups in the present study were: 39 human control subjects (28 males, 11 females), 28 human Type 2 diabetic subjects (13 males, 15 females) and 23 human Type 2 diabetic subjects on NSAIDs (14 males, 9 females). A summary of the physiological parameters, blood examination and pharmacological interventions of these human subjects is detailed in Table 4-1 and Table 4-2. Human Type 2 diabetic subjects were older than human control subjects and were characterized by greater weight, BMI, serum glucose and hemoglobin A1c levels. White blood cell counts were significantly greater in human Type 2 diabetic subjects who were not on NSAIDs compared with human control subjects. Human Type 2 diabetic subjects taking NSAIDs had lower levels of high density lipoprotein and hemoglobin compared with the control group, although the readings were within normal ranges. The incidence of vascular complications, hypertension and hyperlipidemia was higher in human Type 2 diabetic subjects, particularly human subjects taking NSAIDs compared with human non-diabetic subjects (Table 4-2).
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Type 2 diabetes</th>
<th>Type 2 diabetes with NSAIDs</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n=39 )</td>
<td>( n=28 )</td>
<td>( n=23 )</td>
<td></td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>28/11</td>
<td>13/15</td>
<td>14/9</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37.9±2.2</td>
<td>60.5±2.6*</td>
<td>67±2.8*</td>
<td>-</td>
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<tr>
<td>Height (cm)</td>
<td>174±1.7</td>
<td>166.9±3.6</td>
<td>162.8±3.6*</td>
<td>-</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74.5±3.7</td>
<td>91.7±3.9*</td>
<td>94.3±4.3*</td>
<td>-</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.5±1</td>
<td>32±2.1</td>
<td>40.3±2.2*</td>
<td>18.5-24.9</td>
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<tr>
<td>Serum glucose (mmol/L)</td>
<td>4.9±1</td>
<td>12.1±1.2*</td>
<td>11.5±1.1*</td>
<td>3.0-7.7</td>
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<tr>
<td>Hemoglobin A1C (%)</td>
<td>5.2±0.3</td>
<td>8.2±0.3*</td>
<td>8.0±0.3*</td>
<td>4.0-6.0</td>
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<td>Cholesterol (mmol/L)</td>
<td>4.9±0.2</td>
<td>5.0±0.2</td>
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<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.8±0.3</td>
<td>2.2±0.3</td>
<td>2.3±0.3</td>
<td>&lt;2.0</td>
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<tr>
<td>High density lipoprotein (mmol/L)</td>
<td>1.4±0.1</td>
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<td>1.1±0.1*</td>
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<td>Low density lipoprotein (mmol/L)</td>
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<td>2.9±0.2</td>
<td>2.3±0.2</td>
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<td>26±20</td>
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<td>Hemoglobin (g/L)</td>
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<td>140.2±3.5</td>
<td>130.9±4*</td>
<td>130-180</td>
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<td>Red blood cells (x 10¹²/L)</td>
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<td>4.6±0.2</td>
<td>4.5±0.2</td>
<td>4.6-6.2</td>
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<tr>
<td>Packed cell volume</td>
<td>0.4±0</td>
<td>0.4±0</td>
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<tr>
<td>Mean corpuscular volume (fL)</td>
<td>91.1±1</td>
<td>92.2±1.9</td>
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<td>Mean corpuscular hemoglobin (pg)</td>
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<td>30.8±0.7</td>
<td>29.6±0.8</td>
<td>27-32</td>
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<tr>
<td>White cell count (x10⁹/L)</td>
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<td>Neutrophils (x10⁹/L)</td>
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<td>4.0±0.5</td>
<td>2.0-8.0</td>
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<td>Lymphocytes (x10⁹/L)</td>
<td>1.8±0.1</td>
<td>2.3±0.3</td>
<td>2.2±0.3</td>
<td>1.0-4.0</td>
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<td>Monocytes (x10⁹/L)</td>
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<td>0.5±0.1</td>
<td>0.6±0.1</td>
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<td>Eosinophils (x10⁹/L)</td>
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<td>0.2±0</td>
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<td>0±0</td>
<td>0±0</td>
<td>&lt;0.2</td>
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</table>

\( n \) indicates the number of subjects. Some subjects did not have a full blood examination.

* \( p<0.05 \), significant difference from the control group (1-way ANOVA, Tukey’s or Games-Howell multiple comparison).
Table 4-2
Clinical characteristics of human subjects in Type 2 diabetic study

<table>
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<tr>
<th></th>
<th>control</th>
<th>Type 2 diabetes</th>
<th>Type 2 diabetes with NSAIDs</th>
<th>Across group</th>
<th>Between Type 2 diabetes</th>
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<td>Smokers</td>
<td>2</td>
<td>7</td>
<td>3</td>
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<td>8</td>
<td>12</td>
<td>$p &lt;0.05$</td>
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<tr>
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<td>6</td>
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<td>7</td>
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</tr>
<tr>
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<td>4</td>
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<td>13</td>
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<td>$p &lt;0.05$</td>
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<td>$p &lt;0.05$</td>
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<td>0</td>
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</tbody>
</table>

$n$ indicates the number of subjects. Some subjects did not have a full blood examination.
4.4.2.2 Experimental protocol validations

4.4.2.2.1 Specificity of Western blot: COX (humans)

COX-1 and COX-2 protein bands were determined and verified against their corresponding molecular weight at approximately 70 kD. The specificity of the COX primary antibodies was further assessed by incubating samples with the secondary antibodies only. The absence of COX bands at 70 kD in this case confirmed the specificity of the primary antibodies to COX proteins (Figure 4-3). A series of sample protein concentration was performed to determine the suitable loading concentrations. Density analysis indicated that 10 μg of total protein was the loading requirement for detecting a clear COX protein band that was not over saturated (Figure 4-3).

4.4.2.2.2 Western blot data reproducibility: COX (humans)

Type 2 diabetic study was done concurrently with Type 1 diabetic study. Data for Type 1 diabetes will be the subject of Chapter 5. All validation studies for both studies were performed using samples collected from Type 1 diabetes. COX protein levels were measured in two samples collected from 6 human Type 1 diabetic subjects at separate occasions (Section 5.3.1.2). The values obtained were very consistent indicating both assay accuracy and reproducibility after time of COX levels in the same human subjects (Figure 4-4).
Figure 4-3

Western blot validation: COX protein levels in platelets and monocytes (humans)

Western blot validation for platelet COX-1 and monocyte COX-2 in humans. Panels A and C: A signal for COX-1 and COX-2, respectively, was evident at 70 kD according to molecular weight markers. Panels B and D: COX-1 and COX-2 immuno-reactivity was verified by negative controls, which were performed with omission of the COX primary antibodies but in the presence of the secondary antibodies. Lane a: internal standard, lanes b + c: duplicate samples from human control subjects, lanes d + e: duplicates samples from Type 2 diabetic human subjects. A series of sample loading concentrations was performed to determine the final protein loading for COX-1 (Panel E and F) and COX-2 (Panels G and H). Lanes f to i represent 5, 10, 15 and 20 μg protein loading. A final protein loading of 10 μg per lane was chosen, because this was the concentration at which the minimal amount of sample was required to produce a reliable and clear band. Higher protein loading resulted in the risk of signal saturation.
Figure 4-4

Reproducibility of COX protein expression in human Type 1 diabetic subjects

COX expression in human platelets and monocytes from individual human Type 1 diabetic subjects taken on separate occasions (sets A and B). Panel A: Platelet COX-1 protein expression measured in repeated samples from 6 human Type 1 diabetic subjects. Panel B: Monocyte COX-2 protein expression measured in repeated samples from 6 human Type 1 diabetic subjects. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. $p>0.05$, paired Student’s $t$-test.
4.4.2.2.3 **Solid phase extraction of COX metabolite validation: recovery of analyte (humans)**

Figure 4-5A summarizes the validation of the metabolite extraction method. Eluting analyte with 500 μL methyl formate produced 87.7% metabolite recovery whereas larger eluting solvent volumes (1.5 and 2 mL methyl formate) produced a slightly better metabolite recovery (90.6 and 90.7%, respectively). The time required to evaporate methyl formate more than 500 μL, however, was longer than 30 minutes per sample. This extended drying time was not practical and may also have affected the integrity of extracted metabolites. Given this consideration, an eluting volume of 500 μL methyl formate was satisfactory. No correction for recovery was performed, but the variability was very minimal and repeat analyses were reproducible (Figure 4-5B).

4.4.2.2.4 **EIA method: Data reproducibility (humans)**

Plasma levels of TXB₂ and PGE₂ were measured in repeated samples collected from 6 human Type 1 diabetic subjects at separate occasions (see Section 5.3.1.2). The values obtained were very consistent indicating both EIA assay accuracy and reproducibility after time of plasma TXB₂ and PGE₂ levels in the same human subjects (Figure 4-5B).
Method validation and reproducibility of plasma COX metabolites

COX metabolite extraction method was studied using a known standard to assess the recovery of the extraction method. Data accuracy and reproducibility was further studied by measuring COX metabolites at plasma level from individual human Type 1 diabetic subjects taken on separate occasions (sets A and B). Panel A: A PGE$_2$ standard (1 ng/mL) was used to estimate the recovery of the extraction method. The standard was applied to a conditioned C2 minicolumn followed by washing away interference and eluting analyte with various volumes of methyl formate. Values represent the mean, S.E. and number of independent experiments. * represents significant difference from 0.5 mL; 1-way ANOVA (Tukey’s multiple comparison test). Panel B: Plasma TXB$_2$ was measured in repeated samples obtained from 6 human Type 1 diabetic subjects. Panel C: PGE$_2$ was measured in repeated samples obtained from 6 human Type 1 diabetic subjects. $p>0.05$, paired Student’s $t$-test.
4.4.2.3 Influence of ages on sample population (Type 2 diabetes)

There was a difference in age between control and Type 2 diabetic groups (37.9±2.2 and 60.5±2.6 years old, respectively. \( p<0.001 \), unpaired Student’s \( t \)-test, Table 4-1). Correlation analyses were performed to analyze the relationship between COX expression and age of the human subjects. Platelet COX-1 was not correlated with age. Monocyte COX-2, in contrast, was correlated with advancing age (Figure 4-6).

4.4.2.3.1 Age-matching procedures

To correct for age-dependent effects on COX expression, ages for both groups was matched so that control and Type 2 diabetic age ranges were the same. This meant that older human diabetic subjects (>66 years old) were excluded as were younger human control subjects (<29 years old). Then each human control subject was age-matched with the closest human Type 2 diabetic subject and both human subjects then eliminated from the matching pool. This age-matching process continued until all human control subjects were matched with an individual Type 2 diabetic subject. The result of this process was closely matched mean ages of 50.0±2.4 and 53.0±2.6 years old, which were not significantly different (Table 4-3). The process, however, eliminated 29 human subjects from the original pool.
Figure 4-6

Correlation analysis on COX expression and age of human subjects (Type 2 diabetes)

The relationship between COX expression and age of the human subjects was investigated. Panel A: Scatter plot of the densities of COX-1 human platelet samples in relation to age. Panel B: Scatter plot of the densities of COX-2 human monocyte samples in relation to age. $R^2$ represents correlation coefficient. * represent significant correlation, $p<0.05$, Pearson test.
Table 4-3
Physiological parameters of human subjects in Type 2 diabetic study (age-matched)

<table>
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<th>Control</th>
<th>Type 2 diabetes</th>
<th>Normal range</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n=19</td>
<td>n=19</td>
<td></td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>11/8</td>
<td>9/10</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50±2.4</td>
<td>53±2.6</td>
<td>-</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>171±1.5</td>
<td>170±7.3</td>
<td>-</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76±23.7</td>
<td>95±6.2*</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>26±1.2</td>
<td>33±3.7*</td>
<td>18.5-24.9</td>
</tr>
<tr>
<td>Serum glucose (mmol/L)</td>
<td>5.1±0.3</td>
<td>13±3.1*</td>
<td>3.0-7.7</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)</td>
<td>5.2±0.1</td>
<td>8.2±0.4*</td>
<td>4.0-6.0</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.3±0.2</td>
<td>5±0.3</td>
<td>&lt;5.5</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>2.2±0.5</td>
<td>2.3±0.4</td>
<td>&lt;2.0</td>
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<tr>
<td>High density lipoprotein (mmol/L)</td>
<td>1.5±0.1</td>
<td>1.2±0.1</td>
<td>&gt;1.0</td>
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<td>Low density lipoprotein (mmol/L)</td>
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<td>3±0.3</td>
<td>&lt;3.5</td>
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<td>Platelets (x10^9/L)</td>
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<td>278±23</td>
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<tr>
<td>Hemoglobin (g/L)</td>
<td>142±0.1</td>
<td>140±3</td>
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<tr>
<td>Red blood cells (x 10^12/L)</td>
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<td>4.7±0.2</td>
<td>4.6-6.2</td>
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<tr>
<td>Packed cell volume</td>
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<td>0.4±0</td>
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<td>Mean corpuscular volume (FL)</td>
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<td>91±2</td>
<td>80-98</td>
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<tr>
<td>Mean corpuscular hemoglobin (pg)</td>
<td>31±0.3</td>
<td>30±0.7</td>
<td>27-32</td>
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<td>White cell count (x10^9/L)</td>
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<td>8.3±1*</td>
<td>4.0-11.0</td>
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<td>Neutrophils (x10^9/L)</td>
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<td>5.2±0.8</td>
<td>2.0-8.0</td>
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<td>Lymphocytes (x10^9/L)</td>
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<td>2.4±0.2*</td>
<td>1.0-4.0</td>
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<td>Monocytes (x10^9/L)</td>
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<td>0.5±0</td>
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<td>Eosinophils (x10^9/L)</td>
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<td>0.2±0</td>
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<tr>
<td>Basophils (x10^9/L)</td>
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<td>0±0</td>
<td>&lt;0.2</td>
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</table>

n indicates the number of subjects. Some subjects did not have a full blood examination.

* p<0.05, significant difference from control group (unpaired Student’s t-test).
Table 4-4
Clinical characteristics of human subjects in Type 2 diabetic study (age-matched)

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<td>complication</td>
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<td>8</td>
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</table>

$n$ indicates the number of subjects. Some subjects did not have a full blood examination.
4.4.2.4 COX levels in Type 2 diabetes

Initial analyses on non age-matched human subjects revealed a significant increase in monocyte COX-2 expression in human Type 2 diabetic subjects compared with human control subjects while platelet COX-1 protein expression remained unchanged (Figure 4-7). In age-matched sub-analyses, previously increased monocyte COX-2 expression disappeared and there were no differences and in age-matched human subjects platelet COX-1 levels were not significantly different (Figure 4-8). Further analysis on physiological parameters showed that platelet COX-1 expression was elevated in human subjects with hyperlipidemia (Figure 4-9). COX-1 levels in platelets were significantly higher in human non-diabetic subjects with hyperlipidemia whereas COX-1 levels were not affected by hyperlipidemia in Type 2 diabetes (Figure 4-9).

4.4.2.5 Plasma COX metabolites in Type 2 diabetes

The level of COX metabolites, TXB$_2$ and PGE$_2$ was measured in the plasma. It was found that plasma PGE$_2$ level, but not TXB$_2$ level was significantly higher in human Type 2 diabetic subjects compared with human control subjects in non age-matched groups (Figure 4-7). This elevation in plasma PGE$_2$ level in human Type 2 diabetic subjects appeared to be age-independent as shown in age-matched analyses where PGE$_2$ levels were still elevated (Figure 4-8).
Figure 4-7

Quantitative densitometry of COX protein expression and plasma metabolites in human subjects
(Type 2 diabetes, non age-matched)

Non age-matched analysis of COX expression in human platelets and monocytes and plasma COX metabolites. Panel A: Platelet COX-1 protein expression was measured in control and human Type 2 diabetic subjects. Panel B: Monocyte COX-2 protein expression measured in control and human Type 2 diabetic subjects. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Panel C and Panel D: Plasma level of TXB$_2$ and PGE$_2$ were measured using EIA. Values represent the mean, S.E. and number of human subjects. * significant difference from human control subjects, $p<0.01$, Mann Whitney test; N.S. represents not significant, $p>0.05$, unpaired Student’s $t$-test or Mann Whitney test.
Figure 4-8

Quantitative densitometry of COX protein expression and plasma metabolites in human subjects (Type 2 diabetes, age-matched)

Age-matched analysis of COX expression in human platelets and monocytes and plasma COX metabolites.

Panel A: Platelet COX-1 protein expression was measured in control and human Type 2 diabetic subjects. Panel B: Monocyte COX-2 protein expression measured in control and human Type 2 diabetic subjects. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Panel C and Panel D: Plasma level of TXB$_2$ and PGE$_2$ were measured using EIA. Values represent the mean, S.E. and number of human subjects. * significant difference from human control subjects, $p<0.01$, Mann Whitney test; N.S. represents not significant, $p>0.05$, unpaired Student’s $t$-test or Mann Whitney test.
Figure 4-9

Quantitative densitometry of platelet COX-1 protein expression in human subjects
(Type 2 diabetes, hyperlipidemia, non age-matched)

Non age-matched analysis of COX-1 expression in human platelets. Panel A: Platelet COX-1 protein expression was measured in human subjects with and without hyperlipidemia. Panel B: Sub-analysis of platelet COX-1 protein expression was measured in human subjects with and without hyperlipidemia. A rat platelet standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of human subjects. * significant difference from human subjects without hyperlipidemia, \( p<0.05 \), unpaired Student’s \( t \)-test; # significant difference from human control subjects without hyperlipidemia, \( p<0.05 \), 1-way ANOVA (Tukey’s multiple comparison test).
4.4.3 ACETYLATED HISTONE H4 LEVELS IN MONOCYTES IN TYPE 2 DIABETES

4.4.3.1 Method validations

A series of method validations was performed to ensure the data produced by Western blot were accurate and reproducible.

Acetylated histone H4 bands were determined and verified against the molecular weight at 10 kD. The specificity of the primary histone antibody was further assessed by incubating samples with the secondary antibody only. The absence of histone H4 bands at 10 kD confirmed the specificity of the primary antibody to histone H4 proteins (Figure 4-10). A series of loadings was performed with selected samples to ensure the accuracy of target protein detected (Figure 4-10).

Histone H4 protein expression measured in two samples collected from 6 human Type 1 diabetic subjects at separate occasions (see Section 5.3.1.2 for the recruitment of human Type 1 diabetic subjects). The values obtained were very consistent suggesting both Western blot procedure accuracy and reproducibility over time of acetylated histone H4 in samples collected from the same human subjects (Figure 4-11).

4.4.3.2 Acetylated histone H4 levels in Type 2 diabetes

Initial analyses on non age-matched human subjects did not reveal any significant change in monocyte acetylated histone expression in human Type 2 diabetic subjects compared with human control subjects (Figure 4-12). Furthermore, histone H4 acetylation in monocytes isolated from human Type 2 diabetic subjects remained unchanged compared with age-matched human control subjects (Figure 4-12).
Western blot validation: acetylated histone H4 protein levels in monocytes (humans)

Western blot validation for monocyte acetylated histone H4 in humans. Panel A: A signal for acetylated histone H4 was evident at 10 kD according to molecular weight markers. Panel B: Acetylated histone H4 immuno-reactivity was verified by negative controls, which was performed with omission of the acetylated histone H4 primary antibody but in the presence of the secondary antibody. Lane a: internal standard, lanes b + c: duplicate samples from human control subjects, lanes d + e: duplicates samples from Type 2 diabetic human subjects. Panels C and D: A series of sample loading concentrations was performed to determine the final protein loading for acetylated histone H4. Lanes f to i represent 5, 10, 15 and 20 μg protein loading. A final protein loading of 10 μg per lane was chosen, because this was the concentration at which the minimal amount of sample was required to produce a reliable and clear band. Higher protein loading resulted in the risk of signal saturation.
Reproducibility of histone H4 acetylation in human Type 1 diabetic subjects

Monocyte histone H4 acetylation levels were measured from individual human Type 1 diabetic subjects taken on separate occasions (sets A and B). A rat histone standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. \( p > 0.05 \), paired Student’s \( t \)-test.
Figure 4-12

Quantitative densitometry of histone H4 acetylation in human subjects (Type 2 diabetes)

Level of histone H4 acetylation in human monocytes. Panel A: Non age-matched analysis of acetylated histone H4 in human monocytes. Panel B: Age-matched analysis of acetylated histone H4 in human monocytes. A rat histone H4 standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of human subjects. N.S. represents not significant, $p>0.05$, Mann Whitney test or unpaired Student’s $t$-test.
4.4.4 EFFECT OF NSAIDS ON TYPE 2 DIABETES

4.4.4.1 Effect of NSAIDs on COX, TXB$_2$, PGE$_2$ and acetylated histone H4 levels in Type 2 diabetes

Human Type 2 diabetic subjects with NSAID treatment regime showed a significant reduction in platelet COX-1 and monocyte COX-2 expression compared to human Type 2 diabetic subjects without NSAIDs (Figure 4-13). There was a decrease in plasma TXB$_2$ and PGE$_2$ levels in human Type 2 diabetic subjects taking NSAIDs compared to human Type 2 diabetic subjects not taking NSAIDs (Figure 4-13). Monocytes of human Type 2 diabetic subjects taking NSAIDs expressed markedly lower acetylated histone H4 proteins compared to non age-matched human Type 2 diabetic subjects not taking NSAIDs (Figure 4-14).
Figure 4-13

Quantitative densitometry of COX protein expression and plasma metabolites in the presence of NSAIDs in human subjects (Type 2 diabetes, non age-matched)

Non age-matched analysis of COX expression in human platelets and monocytes and plasma COX metabolites in the presence of NSAIDs. Panel A: Platelet COX-1 protein expression was measured in human Type 2 diabetic subjects with and without NSAIDs. Panel B: Monocyte COX-2 protein expression measured in human Type 2 diabetic subjects with and without NSAIDs. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Panel C and Panel D: Plasma level of TXB$_2$ and PGE$_2$ were measured using EIA. Values represent the mean, S.E. and number of human subjects. * and ^ significant difference from Type 2 diabetes not taking NSAIDs, $p<0.05$, 1-way ANOVA (Dunn’s multiple comparison test); # significant difference from Type 2 diabetes not taking NSAIDs, $p<0.05$, 1-way ANOVA (Tukey’s multiple comparison test); N.S. represents not significant from Type 2 diabetes not taking NSAIDs, $p>0.05$, 1-way ANOVA (Dunn’s multiple comparison test).
Figure 4-14
Quantitative densitometry of histone H4 acetylation in human subjects in the presence of NSAIDs in human subjects (Type 2 diabetes, non age-matched)

The level of histone H4 acetylation in monocytes was measured in non age-matched human Type 2 diabetic subjects with and without NSAIDs. A rat histone H4 standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of human subjects. * significant difference from Type 2 diabetes without NSAIDs, $p<0.001$, 1-way ANOVA (Dunn’s multiple comparison test).
4.5 DISCUSSION

The two questions of this current study were whether COX-1 and COX-2 expression was altered in Type 2 diabetes and if so, could this be associated with altered histone acetylation? The animal model used was obese Zucker rats, which has several of the elements of Type 2 diabetes including hyperglycemia, obesity, dyslipidemia and hypertension (see Review by Kasiske et al. 1992). The human subjects were Type 2 diabetic patients from a hospital clinic. The two cell types studied were platelets and monocytes.

4.5.1 COX LEVELS IN PLATELETS AND MONOCYTES IN ZUCKER RATS

4.5.1.1 COX-1-mediated pathway in platelets in Zucker rats

Obese Zucker rats have been extensively used as a model of Type 2 diabetes as they exhibit several characteristics of Type 2 diabetes including hyperglycemia, obesity, dyslipidemia and hypertension. In the present study, COX-1 levels in platelets were measured in obese Zucker rats and found to be elevated compared to lean Zucker rats (Figure 4-2). Similar finding has also been reported by Raju et al. (2006) in the platelets of obese Zuker rats. Hyperactive platelets have been reported by Schafer et al. (2004) in the young Zucker rats with impaired glucose tolerance and increased COX-1 levels in platelets may potentially result in enhanced production of prostanoids such as TXA$_2$ and platelet aggregation (Liu et al. 2004). Potentially, changes in prostanoid production may result in an increased risk in thrombosis due to enhanced platelet activation and aggregation, a phenomenon that was not observed in COX-1 knock-out mice (Yu et al. 2005).

4.5.1.2 COX-2-mediated pathway in monocytes in Zucker rats

COX-2 is the rate-limiting enzyme in prostanoid production in monocytes. In diabetes, COX-2 up-regulation in monocytes is often associated with enhanced monocyte infiltration into the intima and macrophage-induced inflammation processes (Martin-Ventura et al. 2005). In the present study, monocyte COX-2 levels were measured and found to be elevated in
obese Zucker rats compared with the lean rats (Figure 4-2). In obese Zucker rats, enhanced COX-2 expression in the renal cortex and micro-vessels was found to contribute to renal damage (Komers et al. 2005; Dey et al. 2004; Dey et al. 2004a). Increased monocyte COX-2 levels observed in the current study may further exacerbate the renal injury often seen in obese Zucker rats. In monocytes, elevated COX-2 levels can also lead to increased PGE$_2$ production. COX-2-mediated release of PGE$_2$ plays a role in inflammatory processes. For instance, PGE$_2$ increases blood flow to inflamed area by dilating blood vessels and stimulates the migration of leukocytes through capillary walls. Furthermore, COX-2-mediated release of PGE$_2$ has been suggested to play a role in destabilizing atherosclerotic plaque via MMP pathways (for more details, see Section 3.4.1.1). Therefore, an up-regulation of COX-2 in monocyte in obese Zucker rats indicates an elevated inflammatory activity and could account for the more severe degree of atherosclerosis in obese Zucker rats compared to lean Zucker rats (see Review by Kasiske et al. 1992).

4.5.2 COX-MEDIATED PATHWAYS IN TYPE 2 DIABETES

4.5.2.1 Effect of age on COX-mediated pathways in Type 2 diabetes

Studies in humans are less controlled than animal studies and in the present study, because the blood samples were being obtained from an open-ended study with few exclusion criteria, post-hoc analyses were performed to try and refine the population. One important factor is age, which in animal studies had been shown to increase COX-1 and COX-2 with advancing age (Heymes et al. 2000; Armstead et al. 2003; Siironen et al. 2004). In the present study, an important finding was that COX-2 levels in monocytes increased with age in human Type 2 diabetic subjects (Figure 4-6). Therefore, sub-analysis was done using an age-matched procedure to take this into account.
4.5.2.2 COX-1-mediated pathway in platelets in Type 2 diabetes

The present study showed that platelet COX-1 levels were unaltered in Type 2 diabetes with and without age-matching (Figures 4-7 and 4-8). To the best of our knowledge, platelets COX levels in human Type 2 diabetic subjects were not previously measured, although an early study by Abbate et al. (1988) reporting enhanced TXA₂ synthesis by platelets in human Type 2 diabetic subjects after thrombin stimulation. The animal study reported in Section 4.5.1.1 showed that COX-1 in platelets was increased in female diabetic Zucker rats, data from this current human study did not agree with data from the animal model. This suggests that changes in platelet COX-1 may be species-dependent.

Several studies indicated that platelets are more hyperactive in diabetes (see Section 3.4.1.2 for more details) and a good glycemic control has been shown to reduce platelet activation markers in human Type 2 diabetic subjects (Eibl et al. 2004). Hyperglycemia has been proposed to be a causal factor for in vivo platelet activation. For instance, excess glucose contributes to non-enzymatic glycation of platelet lipid membrane, which may affect the activation of receptors on the surface of platelet lipid membrane (for more details, see Chapter 6). High levels of glucose can also increase oxidative stress via polyol pathway (see Section 6.1.3), which may be responsible for enhanced peroxidation of arachidonic acid and increased isoprostane production, which is known to cause persistent platelet activation (see Review by Ferroni et al. 2004). The current study, however, suggests that changes in platelet COX-1 is most likely not involved in hyperactivity of platelets in Type 2 diabetes.

The current data were also sub-analyzed according to disease and age. Hyperlipidemia is a condition characterized by an elevated lipid level including cholesterol, phospholipids and triglycerides in the bloodstream. The present study found that human subjects with hyperlipidemia had a significantly higher level of platelet COX-1 compared with human control subjects (Figure 4-9). Further sub-analysis showed that increased platelet COX-1
levels were clearly elevated with hyperlipidemia in control and diabetic groups, although this was not significant in human Type 2 diabetic subjects presumably due to the low numbers (Figure 4-9). A study by Davi et al. (1997) found enhanced COX-1-mediated activity in platelets as indicated by an increased TXA₂ synthesis in hypercholesterolemic subjects. Belton et al. (2003) further reported elevated COX-1 expression in the vascular lesions during atherosclerosis development was reported in apolipoprotein E-deficient mice. Changes in lipid levels are known to promote thrombosis by affecting the activity of platelets (Di Minno et al. 1986), fibrinolytic factors (Lowe et al. 1991; Levin et al. 1994) and coagulation proteins (Drake et al. 1991; Hoffman et al. 1994). Indeed, increased cholesterol levels increase the risk of arterial thrombotic events in patients with atherosclerosis (Pitt et al. 1995). Potentially, alteration in COX-1 levels due to hyperlipidemia, a condition commonly observed in Type 2 diabetes may contribute to the development of macro-vascular complications. The present results for the first time show a link between hyperlipidemia and COX-1, a factor which could promote platelet aggregation. It may also be an indication for the use of COX inhibitors in hyperlipidemia.

The present study measured plasma TXB₂. In Type 2 diabetes, plasma TXB₂ was unaltered with and without age-matching, although a trend of increased plasma TXB₂ was observed in human Type 2 diabetic subjects (Figures 4-7 and 4-8). Plasma TXB₂ level, however, is not always indicative of platelet COX-1 level and activity, because plasma TXB₂ can derive from various sources. These sources include endothelial cells (Kobayashi et al. 2005), Kupffer cells (Xu et al. 2005), lung epithelial cells (Khan et al. 2002), mast cells (Mita et al. 1999), monocytes and macrophages (Yamazaki et al. 2002; Eligini et al. 2006). When platelet COX-1 activity was measured, Vericel et al. (2004) demonstrated that basal TXB₂ significantly increased in resting platelets from human Type 2 diabetic subjects. When stimulated with thrombin, platelets isolated from human Type 2 diabetic subjects were capable of producing high level of TXA₂ (Abbate et al. 1988), suggesting hyperactivity in
platelets in Type 2 diabetes. The present study would suggest that this is not due to increased platelet COX-1 levels.

4.5.2.3 COX-2-mediated pathway in monocytes in Type 2 diabetes

Very little is known about COX-2 in Type 2 diabetes. Increasing evidence has revealed increased inflammation in Type 2 diabetes and inflammation may be an important component in diabetic complication development (Bluher et al. 2005; Lee et al. 2005), our study hence investigated if COX-2 expression in monocytes was elevated in Type 2 diabetes as elevated COX-2 occurs in inflammation (see Section 1.2.3.2).

Initial analyses on non age-matched data indicate that monocyte COX-2 expression was significantly increased in human Type 2 diabetic subjects (Figure 4-7). This increased in COX-2 expression appeared age-dependent and subsequent analyses in age-matched human subjects failed to detect any changes in COX-2 level in human Type 2 diabetic subjects compared with age-matched human control subjects (Figure 4-8). In contrast, COX-2 mRNA levels were increased in peripheral blood monocytes from human Type 2 diabetic subjects (Shanmugam et al. 2004). This non age-matched study, however, did not measure the actual expression of COX-2 protein and only had a very small sample size (four human control and two human Type 2 diabetic subjects, respectively). Therefore, it is difficult to interpret the results and establish the relationship between increased COX-2 mRNA and actual functional protein expression. In another study involved age-matched human subjects, COX-2 up-regulation was observed in monocyte-derived macrophages at the site of atherosclerotic plaques in Type 2 diabetes (Cipollone et al. 2003). Potentially, changes in COX-2 expression in these cells can lead to an activation of numerous signalling transduction pathways. For example, through PGE2-mediated EP4 signaling pathway, enhanced COX-2 is associated with the induction of MMPs, proteases that digest extracellular matrix and destabilize atherosclerotic plaques (Cipollone et al. 2005; Lu & Wahl 2005). The age-dependence of
COX-2 expression in monocytes, which the present study observed is worth noting as it has been reported previously (Kang et al. 2003) and may be a potential factor in cardiovascular complications of aging.

The present study found that plasma PGE$_2$ increased significantly in Type 2 diabetes in age-matched analysis (Figure 4-8). This increase in plasma PGE$_2$ level in Type 2 diabetes suggests that inflammatory activity is elevated in Type 2 diabetes. The exact source of enhanced plasma PGE$_2$ production is unclear as several sources of plasma PGE$_2$ have been identified. These sources include endothelial cells (Uracz et al. 2002), platelets (Abbate et al. 1988), lung epithelial cells (N'guessan et al. 2006), alveolar macrophages (Kuhn et al. 1995) and monocyte-derived macrophages at the site of atherosclerotic plaques (Zhou & von Eckardstein 2002). It has also been reported that stromal pre-adipocytes expressed high level of COX-2 protein and released PGE$_2$ after stimulation by adiponectin, a hormone secreted by adipocytes that regulates glucose and lipid metabolism (Yokota et al. 2002). Obesity is a common problem among human Type 2 diabetic subjects, it is possible that pre-adipocytes via adiponectin may be the alternative source of plasma PGE$_2$ production in Type 2 diabetes.

Regardless the source of the elevated plasma PGE$_2$, PGE$_2$ production has been associated with destabilizing atherosclerotic plaques (Martin-Ventura et al. 2005; Gomez-Hernandez et al. 2006), diabetic retinopathy (Sennlaub et al. 2003) and nephropathy (Kiritschi et al. 2003) possibly through increased MMP activity (Cipollone et al. 2005; Lu & Wahl 2005) and increased oxidative stress (Tabatabaie et al. 2003). Therefore, this may be an important factor in diabetic complications.

**4.5.3 HISTONE H4 ACETYLATION IN TYPE 2 DIABETES**

We have demonstrated previously in Section 2.4.4 that both COX-1 and COX-2 proteins were up-regulated in endothelial cells treated with TSA, a histone de-acetylase inhibitor, suggesting
COX expression may be associated with histone hyperacetylation (see Section 2.4.3). In this section, we studied whether acetylated histone H4 expression changed in monocytes of human Type 2 diabetic subjects and if such a change was associated with monocyte COX-2 expression.

4.5.3.1 Histone H4 acetylation in monocytes in Type 2 diabetes

Histones are small proteins that are subject to extensive post-translational modifications on the N-terminus. Hyperacetylation of the core histones, which loosens DNA strands and allows transcription factors better access to DNA is commonly associated with transcriptionally active regions within the genome, while histone de-acetylation is believed to be involved in the repression of genes (see Reviews by Pazin & Kadonaga 1997; Imhof & Wolffe 1998).

The current study found that in age-matched human subjects, monocyte acetylated histone H4 expression was not altered in Type 2 diabetes compared with the control group (Figure 4-12). The lack of changes in acetylated histone H4 is consistent with lack of changes in monocyte COX-2 (see Section 4.4.2.4). The current result, however, differed from that of a study by Miao et al. (2004), which showed that in cultured THP-1 monocytes and human blood monocytes isolated from Type 2 diabetic, increased COX-2 levels was associated with histone H3 and H4 hyperacetylation at the promoter of COX-2 genes. It is, however, important to note that this particular study only recruited two controls and two human Type 2 diabetic subjects following the initial cell culture work. With such a limited n number, a valid conclusion cannot be made. In the present study, the age-matched analysis in the present study contained 19 subjects per control and Type 2 diabetic groups.

Enhanced histone acetyltransferase activity and histone acetylation, however, have also been observed in diabetes in various other experimental conditions and cell types. For example, treatment of high level of glucose in mouse insulinoma 6 cells, an islet tumor cell line that
produces excessive insulin led to histone H4 hyperacetylation at the insulin gene and glucose transporter-2 gene promoter (Mosley & Ozcen 2003). In isolated islets, transcription factors including β-2/NeuroD1 and Pdx-1, which are involved in glucose-stimulated insulin gene expression, have also been shown to interact with the histone acetyltransferase p300 (Mosley at al. 2004). To the best of our knowledge, the present work was the first with a reasonable sample size investigating monocyte histone acetylation status in Type 2 diabetes and the results were negative.

It is important to note that we failed to observe an age-depend increase in monocyte histone H4 acetylation in human Type 2 diabetes (Figure 4-12) despite an age-dependent increase in monocyte COX-2 expression (Figure 4-7). Although previous studies suggest a link between increased COX levels and histone hyperacetylation in aging (see Chapter 2), it is possible that this relationship may be cell/tissue-dependent. Hence modulation of COX expression via histone acetylation requires further investigation.

4.5.4 EFFECT OF NSAIDS ON TYPE 2 DIABETES

NSAIDs have long been used clinically to reduce pain and inflammation. Most NSAIDs except for aspirin block COX active site preventing the binding of the enzyme to the substrate, arachidonic acid (Vane 1996). Aspirin inhibits COX enzymatic activity permanently by covalently binding to a serine residue (Ser\textsuperscript{529} and Ser\textsuperscript{516} for human COX-1 and COX-2, respectively) located in the COX channel of the enzyme (Roth & Majerus 1975; Lecomte et al. 1994). By inhibiting COX activity, NSAIDs are able to reduce platelet aggregation and have been used commonly to prevent ischemic heart diseases (see Review by Patrono et al. 2005). NSAIDs can be divided into three categories: traditional NSAIDs that inhibiting both COX-1 and COX-2 activities, salicylates with aspirin being the most well-known and COX-2 selective NSAIDs. In the case of aspirin, inhibition of platelet COX-1 activity prevents platelet aggregation, reduces the occurrences of thrombosis and hence decreases myocardial
infarction (see Review by Patrono et al. 2005). Type 2 diabetes is a strong risk factor for cardiovascular disease and is associated with the development of atherosclerosis and significant death through stroke and myocardial infarction (see Review by Garber 2000). This present study aimed to investigate if the use of NSAIDs in human Type 2 diabetic subjects would affect COX-1 and COX-2 protein levels.

4.5.4.1 Effect of NSAIDs on COX-mediated pathways in Type 2 diabetes

The current study reported that the use of NSAIDs significantly reduced platelet COX-1 and monocyte COX-2 protein expression in Type 2 diabetes (Figure 4-13). No work has been reported in regards to COX levels in platelets or monocytes in Type 2 diabetes, although the effect of NSAIDs on COX in other cell types has been observed. For example, in endothelial cells, IL-stimulated COX expression was down-regulated by non-selective COX inhibitor, naproxen and COX-1 selective inhibitor, aspirin (Wu et al. 1991; Zyglewska et al. 1992). These studies, however, did not specify which COX isoform was down-regulated by NSAIDs. To the best of our knowledge, the present finding that platelet COX-1 levels were reduced by NSAIDs in human Type 2 diabetic subjects was a novel finding and it may contribute to these anti-COX effects of these drugs.

Platelets play a crucial role in atherosclerotic plaque formation and human Type 2 diabetic subjects have higher basal platelet activity (see Review by Ferroni et al. 2004). Consequently, this increases the chance of developing thrombosis and other cardiovascular diseases in Type 2 diabetes. Low dose NSAIDs, particularly aspirin has long been used to lower and prevent ischemic heart diseases (see Review by Patrono et al. 2005). The ability of NSAIDs not merely to inhibit COX activity, which was reflected by decreased plasma TXB\textsubscript{2}, but also to lower platelet COX-1 expression in human Type 2 diabetic subjects suggests an additional regulatory mechanism for COX-1 by NSAIDs, which may be important in delaying or preventing the macro-vascular complication in Type 2 diabetes.
Increased COX-2 expression has been associated with increased inflammation and atherosclerosis development (Martin-Ventura et al. 2005; Gomez-Hernandez et al. 2006), conditions that are common in human Type 2 diabetic subjects. NSAIDs have been used to reduce inflammation and prevent thrombosis by inhibiting COX activity. The present study found that monocyte COX-2 expression was reduced significantly in human Type 2 diabetic subjects taking low dose NSAIDs compared with human Type 2 diabetic subjects not taking NSAIDs.

The use of NSAIDs has been shown to affect COX-2 expression in various experimental settings. NSAIDs such as indomethacin, piroxicam and flurbiprofen partially inhibit serum-induced COX-2 mRNA in murine osteoblastic cells (Pilbeam et al. 1995). Sodium salicylate also down-regulated COX-2 expression in human endothelial cells (Xu et al. 1999a). In contrast, Meade et al. (1999) reported increased COX-2 transcription in mammary epithelial cells by NSAIDs and suggested that this up-regulation occurred via a peroxisome proliferator response element, a defined nucleotide sequence crucial to gene transcription, which is found at the sites of COX-2 promoter region. Furthermore, Gao et al. (2004) reported COX-2 expression up-regulation by aspirin and indomethacin in endometrial cancer cell lines. It is unclear why conflicting data have been reported. Factors such as differences in cell line and type of NSAIDs used may account for the different results.

In addition to changes in COX levels, the present study also reported a reduction in both plasma TXB$_2$ and plasma PGE$_2$ by NSAIDs in Type 2 diabetes (Figure 4-13). A small study by Capone et al. (2004) found that COX-1 and COX-2 had a different response to NSAIDs when aspirin and naproxen were tested in nine healthy human subjects for six days. Aspirin and naproxen reduced platelet COX-1 activity, indicated by both plasma TXB$_2$ and urinary excretion of TXB$_2$ level, in a similar fashion. In contrast to low-dose aspirin, naproxen
significantly lowered LPS-induced PGE\(_2\) in the plasma, an index of monocyte COX-2 expression and activity. This confirms again that the use of different type of NSAIDs affects COX-mediated pathways differently. As previously explained, plasma TXB\(_2\) and PGE\(_2\) can come from various cellular sources (see Sections 4.5.2.2. and 4.5.2.3 for more details), a reduction in COX metabolites by NSAIDs in the current finding suggests a global decrease in COX expression and activity.

The ability of NSAIDs to affect COX-1- and COX-2-mediated pathways levels may be of clinical significance in management of Type 2 diabetes, because both platelets and monocytes play a crucial role in the development of vascular complications such as thrombosis, atherosclerosis and ischemic heart diseases in Type 2 diabetes (for more details, see Section 3.4). Cipollone et al. (2001) has shown that enhanced COX-2 expression in macrophages located at the site of human atherosclerotic plaques is associated with reduced in plaque stability via MMP induction. A similar finding was reported in plaque-derived macrophages in Type 2 diabetes (Cipollone et al. 2003). Furthermore, compared with human control subjects, inflammation, oxidative stress and platelet hyperactivity was significantly elevated in human Type 2 diabetic subjects as indicated by increased levels of plasma CRP, urinary isoprostane and platelet-mediated release of TXB\(_2\), respectively (Santilli et al. 2006). Enhanced platelet activity in Type 2 diabetes was re-affirmed with increased expression of soluble CD40 ligand (Santilli et al. 2006), which is a transmembrane protein structurally similar to TNF-\(\alpha\) and is expressed on the surface of activated platelets. Soluble CD40 ligand promotes the secretion of chemokines and expression of adhesion molecules by endothelial cells, thereby facilitating the binding of leukocytes to activated platelets and endothelium and contributing to thrombus formation (Henn et al. 1998). Both platelet-mediated released TXB\(_2\) and soluble CD40 ligand were reduced in human Type 2 diabetic subjects after a week of aspirin use, with slow recovery over 10 days after aspirin withdrawal (Santilli et al. 2006). Therefore, the present study suggests that the use of NSAIDs in Type 2 diabetes may be beneficial.
4.5.4.2 Effect of NSAIDs on histone H4 acetylation in Type 2 diabetes

Histone acetylation in general is associated with active gene transcription. Core histone acetylation loosens DNA strands and allows the better access of transcription factors to the DNA resulting in gene up-regulation (see Review by Ng & Bird 2000). This section of the study investigated if the use of NSAIDs affected monocyte histone acetylation in Type 2 diabetes. It was found that the use of NSAIDs lowered monocyte acetylated histone H4 expression in Type 2 diabetes (Figure 4-14). This is consistent with reduced monocyte COX-2 level in human Type 2 diabetic subjects taking NSAIDs (see Section 4.5.4.1) supporting the hypothesis that COX expression was regulated by histone hyperacetylation (see Chapter 2 for more details). Human COX-2 promoter region, which are responsible for the induction of human COX-2 gene, contains multiple regulatory elements including NF-κB, CCAAT/enhancer-binding protein binding sites and cAMP-response element (Tazawa et al. 1994). It has been found that human COX-2 gene induction by NF-IL-6β was regulated by p300, a coactivator and an integrator for gene transcription with histone acetyltransferase activity (Wang et al. 2006). Our finding suggests that changes in the level of the core histone acetylation may be associated with down-regulation of monocyte COX-2 in human Type 2 diabetic subjects taking NSAIDs. This novel finding may potentially be a new mechanism by which NSAIDs regulate COX expression.

4.6 CONCLUSIONS

The current study reported that COX levels were up-regulated in platelets and monocytes of Zucker rats, an observation that was not found in human Type 2 diabetic study. This suggests that changes in COX levels in these inflammatory cells may be species-dependent. In addition to unaltered platelet COX-1 and monocyte COX-2 levels in Type 2 diabetes, the human study also reported elevated plasma PGE₂, but not plasma TXB₂ in Type 2 diabetes, suggesting an overall increase in inflammatory activity in Type 2 diabetes. Treatment with NSAIDs in Type 2 diabetes reduced both platelet COX-1 and monocyte COX-2 levels. Plasma TXB₂ and PGE₂
levels were also decreased in human Type 2 diabetic subjects taking NSAIDs. This re-affirms the previous observation that inflammation was increased in human Type 2 diabetic subjects.

Further analysis showed acetylated histone H4 expression in monocytes in Type 2 diabetes was unaltered, which was concurrent with lack of changes in monocyte COX-2 levels suggesting COX expression may be associated with hyperacetylation of the core histones in Type 2 diabetes. The use of NSAIDs resulted in a reduction in monocyte acetylated histone H4 levels in Type 2 diabetes. To the best of our knowledge, this is a novel finding suggesting potentially COX regulation by NSAIDs may involve post-translational modification of the core histones. Further studies are required to better understand the relationship between histone modifications and COX expression.
CHAPTER 5
ROLES OF COX IN TYPE 1 DIABETES
5.1 TYPE 1 DIABETES MELLITUS

Type 1 diabetes, also known as insulin-dependent diabetes mellitus or juvenile-onset diabetes, accounts for approximately 10-15% of the diabetic population. Type 1 diabetes appears at any age, although most Type 1 diabetic patients are diagnosed before 40 years of age. It is a chronic disease of carbohydrate, fat and protein metabolism due to the inability of the pancreas to produce insulin, a hormone that regulates blood glucose levels by promoting glucose uptake into cells. In Type 1 diabetes, the body's immune system destroys the insulin-producing β cells in the pancreas during an inflammatory process with an infiltration of inflammatory cell types such as T lymphocytes, B lymphocytes and macrophages and it is classified as an auto-immune disease (Imagawa et al. 1999; Tabatabaie et al. 2000). Human Type 1 diabetic subjects require daily insulin injections to manage their blood glucose levels.

5.1.1 COX-MEDIATED PATHWAYS IN TYPE 1 DIABETES

COX-mediated pathways involve the release of arachidonic acid from the lipid membrane by the action of PLA₂, production of prostanoids via COX and tissue prostanoid synthases (for more details see Section 1.2). Evidence suggests that COX-mediated pathways are involved in the development of Type 1 diabetes. To create a diabetic animal model that has similar characteristics of Type 1 diabetes, streptozotocin (STZ), a glucosamine-nitrosourea compound that causes DNA damage and toxic to the pancreatic β cell, is commonly used to induce injury of the pancreas. In the STZ-treated diabetic rats, Takahashi et al. (2004) reported that LPS-induced COX-2 expression decreased in the aortas compared with the control rats and this was accompanied by a reduction in PGI₂ production. COX-1 levels remained stable in aortas isolated from both diabetic and control rats. In contrast to these reports, Nasrallah (et al. 2003) found both COX-1 and COX-2 isoforms were up-regulated in kidney cells in a STZ-induced diabetic rat model. Western blotting revealed a 4-fold increase in both COX isoforms at 4 to 6 week of diabetes with a 6-fold increase in PGE₂ synthase expression noted in the outer medullary region of kidney of 6-week old diabetic rats. Culturing cells from rat
inner medullary collecting duct with high levels of glucose for 4 days also resulted in up-regulation of both COX isoforms. Although PGE$_2$ synthase expression did not alter, increased PGE$_2$ production was found in the culture (Nasrallah et al. 2003). In another study using cultured monocytes, COX-2 mRNA and protein expression, but not COX-1 was up-regulated in response to glucose treatment (Shanmugam et al. 2004). Although conflicting data have been reported, it is clear that COX-mediated pathways are altered in Type 1 diabetes, particularly with changes in COX-2 expression, which can be induced by inflammatory stimuli (For details of the inflammatory processes, see Section 3.4.1). Increased inflammation is indicated by the presence of inflammatory markers such as TNF-α, IL-6 and CRP, which are elevated in human Type 1 diabetic subjects with a history of cardiovascular diseases (Jager et al. 1999; Schalkwijk et al. 1999; Kilpatrick et al. 2000; Lechleitner et al. 2000). Alterations in COX-mediated pathways potentially upset the balance of prostanoid production such as TXA$_2$ and PGI$_2$, which are known to regulate blood vessel tone and platelet activity and participate in the development of thrombosis and atherosclerosis (for more details see Section 3.4).

5.2 AIMS

Accumulating evidence suggests the involvement of COX-mediated pathways in the development and progression of Type 1 diabetes and its complications. The aim of this study was to investigate the changes in COX-mediated pathways in Type 1 diabetes. The specific aims were:

1) to measure possible changes in COX-mediated pathways in the platelets and monocytes isolated from human subjects by assessing COX protein and plasma COX metabolite levels and

2) to determine whether COX expression in Type 1 diabetes is associated with histone H4 hyperacetylation in monocytes.
5.3 METHODS

5.3.1 COX LEVELS IN PLATELETS AND MONOCYTES IN TYPE 1 DIABETES

5.3.1.1 Subjects (Type 1 diabetes)

Twenty five (25) non-diabetic Control (14 males, 11 females) and 19 Type 1 diabetic (10 males, 9 females) subjects were recruited at St. Vincent’s Hospital (Melbourne, Australia). All participants were informed about the details of the study by an endocrinologist, Dr. Alicia Jenkins, who was an external collaborator of this study. Subjects were required to sign an approved written informed consent prepared by St Vincent’s Hospital, Melbourne (HREC-A 024/00 Glycoxidation, HDL and diabetes angiopathy). The plain language statement that details the study to the subjects is attached in Appendix. In addition, 14 non-diabetic samples from healthy male subjects collected during previous project at RMIT University (HREC 15/01 Majewski: Age-dependent alterations in cyclooxygenase in platelets and human monocytes) and stored away at -80°C were also included in this study. In my role I had no data, which linked any results to individual patients by name.

5.3.1.2 Collection of human blood samples (Type 1 diabetes)

The volunteer recruitment and blood sample collection was performed by the endocrinologist, Dr. Alicia Jenkins at St Vincent’s Hospital (Melbourne, Australia). For more details see Section 4.3.2.2.

5.3.1.3 Isolation of human platelets and monocytes (Type 1 diabetes)

The isolation of human platelets and monocytes was performed using OptiPrep™ solution according to the manufacturer’s protocol, which was described in Section 4.3.2.3.

5.3.1.4 Protein assay (Type 1 diabetes)

Determination of human sample protein concentration was performed using the Biorad microBCA system as outlined in Section 4.3.2.4.
5.3.1.5 Western blot: COX protein (Type 1 diabetes)

Samples were loaded in duplicate with a final total protein concentration of 10 μg per well. The protein samples and molecular weight markers were electrophoresed, transferred onto nitrocellulose membranes and incubated with selective COX-1 and COX-2 antibodies using the same Western blot protocol for the detection of COX protein levels as in Section 4.3.5.5. The density of all Western blot bands was analyzed using the ChemiDoc™ XRS system (Biorad, CA, USA).

5.3.2 PLASMA COX METABOLITE STUDY IN TYPE 1 DIABETES

The Biotrak EIA system (Amersham Bioscience, UK) was chosen to measure plasma TXB₂ and PGE₂. For more details see Section 4.3.3.

5.3.2.1 Solid phase extraction of COX metabolites (Type 1 diabetes)

COX metabolites were extracted from plasma samples using Solid phase extraction procedures with Amprep C2 ethyl minicolumn (100 mg, Amersham Bioscience, UK). Prior to plasma TXB₂ extraction, the solid phase extraction method was validated with a known amount of amount of PGE standard (PGE₂, 1 ng/mL; Sigma, St Louis, USA). The extraction validation and extraction method for plasma TXB₂ was detailed in Section 4.3.3.1.

5.3.2.2 Amersham Biotrak EIA system (Type 1 diabetes)

The PGE₂ EIA Biotrak System allows a direct determination of plasma PGE₂ levels without the need for PGE₂ extraction. The measurement of extracted TXB₂ metabolites and plasma PGE₂ were performed using the TXB₂ and PGE₂ EIA Biotrak System, respectively (Amersham Bioscience, UK). See Section 4.3.3.2 for more details on the EIA Biotrak System.
5.3.2.2.1 EIA method: Data reproducibility (humans)

Reproducibility of the Biotrak EIA system assessed by measuring repeated samples obtained from 6 human Type 1 diabetic subjects.

5.3.3 ACETYLATED HISTONE H4 LEVELS IN TYPE 1 DIABETES

5.3.3.1 Isolation of histones from human monocytes (Type 1 diabetes)

Histones were isolated from human monocytes as previously described in Section 2.3.2.

5.3.3.2 Western blot: acetylated histone H4 protein (Type 1 diabetes)

Histone samples extracted in Section 5.3.3.1 were electrophoresed on a 15% SDS-PAGE gel and labelled with anti-rabbit polyclonal anti-acetyl histone H4 (Upstate, NY, USA) according to standard Western blot procedures outlined in Section 2.3.3.2.

5.3.4 DRUGS AND CHEMICALS

Drugs and chemicals used in the present experiments were the same as those used in Chapters 2 and 4 and thus, were obtained from the same sources (see Sections 2.3.4 and 4.3.5).

5.3.5 STATISTICAL ANALYSES

All values are given as the mean ± standard error of mean (S.E.) and $n$ indicates the number of observations. Differences in the percentage densities of Western blot bands were analyzed using unpaired Student’s $t$-test, Mann-Whitney test, 1-way ANOVA and post-hoc tests (Tukey’s, Games-Howell and Dunn’s multiple tests), Pearson Chi-Square and Fisher’s exact test whenever applicable. SPSS (SPSS Inc., IL, USA) and Prism (GraphPad Software, Inc., CA, USA) statistical packages were used in statistical analyses.
5.3.6 HUMAN ETHICS APPROVAL

All procedures used for human blood collection were approved by the Human Research Ethics Committee of St. Vincent’s Hospital (Melbourne, Australia; HREC-A 024/00 Glycoxidation, HDL and diabetes angiopathy) in accordance with the National Statement on Ethical Conduct in Research Involving Humans (June 1999) produced by the National Health and Medical Research Council of Australia. A separate set of human samples from another study was included in the present study. The collection of these samples was approved by the Human Research Ethics Committee of RMIT University (Melbourne, Australia; HREC 15/01 Majewski: Age-dependent alterations in cyclooxygenase in platelets and human monocytes).
5.4 RESULTS

5.4.1 COX-MEDIATED PATHWAYS IN TYPE 1 DIABETES

5.4.1.1 Basis parameters of human Type 1 diabetic subjects

Subject selection was made by Dr. Alicia Jenkins at St. Vincent’s Hospital, Melbourne, Victoria for another research project, which had broad selection criteria. The present study did not exclude any samples collected including samples from smokers and hypertensive subjects in the control group. The goal of this was to have a sample population that resembled the characteristics of the general population. The exception was subjects on non-steroidal anti-inflammatory drugs (NSAIDs). Thus the four groups in the present study were: 39 human control subjects (28 males, 11 females), 19 human Type 1 diabetic subjects (10 males, 9 females), 6 human Type 1 diabetic subjects on NSAIDs (2 males, 4 females). Six human Type 1 diabetic subjects had a second sample taken and this was used for assay validation. A summary of the physiological parameters, blood examination and pharmacological interventions of these subjects is detailed in Table 5-1 and Table 5-2. Human Type 1 diabetic subjects were of similar age as human control subjects and were characterized by elevated serum glucose and hemoglobin A1c levels. All human subjects with Type 1 diabetes were treated with insulin. Hemoglobin levels were significantly lower in human Type 1 diabetic subjects not taking NSAIDs compared with human control subjects, although the readings were within normal ranges. The incidence of vascular complications, hypertension and depression/schizophrenia was higher in human Type 1 diabetic subjects compared with human non-diabetic subjects (Table 5-2).
Table 5-1
Physiological parameters of human subjects in Type 1 diabetic study

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Type 1 diabetes</th>
<th>Type 1 diabetes with NSAIDs</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>28/11</td>
<td>10/9</td>
<td>2/4</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37.9±2.4</td>
<td>34.6±3.4</td>
<td>42.5±6</td>
<td>-</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>174±5.2</td>
<td>154.5±8.2</td>
<td>169.7±15.7</td>
<td>-</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74.5±23</td>
<td>77.4±3.7</td>
<td>80.4±6.4</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.5±0.9</td>
<td>25.5±1.4</td>
<td>24.6±2.6</td>
<td>18.5-24.9</td>
</tr>
<tr>
<td>Basophils (x10⁹/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils (x10⁹/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes (x10⁹/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (x10⁹/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils (x10⁹/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets (x10⁹/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>4.9±1.1</td>
<td>12.7±1.5*</td>
<td>10.4±3*</td>
<td>3.0-7.7</td>
</tr>
<tr>
<td>Hemoglobin A1C (%)</td>
<td>5.2±0.2</td>
<td>8.9±0.2*</td>
<td>8±0.4*</td>
<td>4.0-6.0</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.9±0.2</td>
<td>4.3±0.2</td>
<td>4.6±0.4</td>
<td>&lt;5.5</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.8±0.3</td>
<td>1.5±0.3</td>
<td>1.45±0.5</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>High density lipoprotein (mmol/L)</td>
<td>1.4±0.1</td>
<td>1.5±0.1</td>
<td>1.35±0.2</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>Low density lipoprotein (mmol/L)</td>
<td>2.8±0.2</td>
<td>2.1±0.2</td>
<td>2.6±0.4</td>
<td>&lt;3.5</td>
</tr>
<tr>
<td>Platelets (x10⁹/L)</td>
<td>245±12</td>
<td>276±16</td>
<td>308±32</td>
<td>150-400</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>145.5±2.3</td>
<td>133.8±3.3*</td>
<td>133.8±6.4</td>
<td>130-180</td>
</tr>
<tr>
<td>Red blood cells (x 10¹²/L)</td>
<td>4.8±0.1</td>
<td>4.5±0.1</td>
<td>5±0.3</td>
<td>4.6-6.2</td>
</tr>
<tr>
<td>Packed cell volume</td>
<td>0.4±0</td>
<td>0.4±0</td>
<td>0.4±0</td>
<td>0.4-0.54</td>
</tr>
<tr>
<td>Mean corpuscular volume (fL)</td>
<td>91.4±1.1</td>
<td>89.1±1.5</td>
<td>81±2.9</td>
<td>80-98</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin (pg)</td>
<td>30±5.0</td>
<td>30±5.0</td>
<td>27±1</td>
<td>27-32</td>
</tr>
<tr>
<td>White cell count (x10⁹/L)</td>
<td>6.2±0.3</td>
<td>7.4±0.4</td>
<td>7.6±1.2</td>
<td>4.0-11.0</td>
</tr>
<tr>
<td>Neutrophils (x10⁹/L)</td>
<td>3.6±0.2</td>
<td>4.7±0.3</td>
<td>4.9±0.7</td>
<td>2.0-8.0</td>
</tr>
<tr>
<td>Lymphocytes (x10⁹/L)</td>
<td>1.8±0.1</td>
<td>2±0.2</td>
<td>2.1±0.3</td>
<td>1.0-4.0</td>
</tr>
<tr>
<td>Monocytes (x10⁹/L)</td>
<td>0.5±0</td>
<td>0.5±0</td>
<td>0.45±0.1</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Eosinophils (x10⁹/L)</td>
<td>0.2±0</td>
<td>0.2±0</td>
<td>0.2±0.1</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>Basophils (x10⁹/L)</td>
<td>0±0</td>
<td>0±0</td>
<td>0.1±0</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

n indicates the number of subjects. Some subjects did not have a full blood examination.

*p<0.05, significant difference from the control group (1-way ANOVA, Tukey's or Games-Howell multiple comparison).
Table 5-2
Clinical characteristics of human subjects in Type 1 diabetic study

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Type 1 diabetes</th>
<th>Type 1 diabetes with NSAIDs</th>
<th>Chi-square/Fisher's exact test Across group</th>
<th>Between Type 1 diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=39</td>
<td>n=19</td>
<td>n=6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>28/11</td>
<td>10/9</td>
<td>2/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micro-vascular</td>
<td>0</td>
<td>5</td>
<td>3</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Retinopathy</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Nephropathy</td>
<td>0</td>
<td>5</td>
<td>3</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Neuropathy</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Macro-vascular</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Ischemic heart</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>0</td>
<td>19</td>
<td>6</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Metformin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfonylurea</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrate</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Anti-coagulants</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>COX-2 selective</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Other NSAIDs</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Other medications</td>
<td>0</td>
<td>5</td>
<td>3</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression/schizophrenia</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other conditions</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

n indicates the number of subjects. Some subjects did not have a full blood examination.
5.4.1.2 Experimental protocol validations

5.4.1.2.1 Western blot validation: COX (humans)

Several validation procedures were in place to verify COX-1 and COX-2 protein bands detected. These validations included (for more details on Western blot validation, see Section 4.4.2.2):

i) establishing COX proteins at correct molecular weight with the use of molecular weight standard (Figure 4-3),

ii) COX primary antibody specificity testing by incubating target proteins with secondary antibodies only (Figure 4-3),

iii) establishing the suitable sample loading concentration (Figure 4-3),

iv) normalizing densitometry readings between blots with inclusion of an internal standard on every gel and

v) data reproducibility was also assessed in repeated samples from 6 human Type 1 diabetic subjects (Figure 4-4).

5.4.1.2.2 COX metabolite validation: recovery of analyte and EIA method data reproducibility (humans)

A solid phase extraction method was used to extract TXB\(_2\) from plasma samples. Extracted plasma was then measured with the Biotrak EIA system. Validation procedures for both the extraction procedure and EIA system are summarized in Figure 4-5.

5.4.1.3 Influence of ages on sample population (Type 1 diabetes)

There was no significant difference in age between control and Type 1 diabetic groups (37.9±2.4 and 34.6±3.4 years old, respectively), although monocyte COX-2 was weakly correlated with age (Figure 5-1).
Correlation analysis on COX expression and age of human subjects (Type 1 diabetes)

The relationship between COX levels and age of the subjects was investigated. Panel A: Scatter plot of the densities of all COX-1 human platelet samples in relation to age. Panel B: Scatter plot of the densities of all COX-2 human monocyte samples in relation to age. $R^2$ represents correlation coefficient. * represent significant correlation, $p<0.05$, Pearson $R^2$ test.
5.4.1.4 COX levels in Type 1 diabetes

Monocyte COX-2 levels, but not platelet COX-1 levels were significantly increased in human Type 1 diabetic subjects compared with human control subjects (Figure 5-2). Further sub-analyses according to physiological parameters of the subjects showed that COX-1 levels were altered in subjects with hypertension and hyperlipidemia.

In human subjects with hypertension, platelet COX-1 was significantly elevated compared with human subjects without hypertension (Figure 5-3A). Insufficient sample size, however, prevented further conclusive analyses after sub-grouping, although there was a trend showing increased platelet COX-1 levels in human Type 1 diabetic subjects with hypertension compared with human Type 1 diabetic subjects without hypertension (Figure 5-3B).

COX-1 levels were also significantly enhanced in subjects with hyperlipidemia (Figure 5-4A) and further analyses on sub-groups showed that platelet COX-1 levels were elevated in human control subjects with hyperlipidemia compared with human control subjects without hyperlipidemia (Figure 5-4B).

5.4.1.5 Plasma COX metabolites in Type 1 diabetes

The level of COX metabolites, TXB$_2$ and PGE$_2$ was measured in the plasma. It was found that plasma PGE$_2$, but not TXB$_2$ was significantly higher in human Type 1 diabetic subjects compared with the human control subjects (Figure 5-2).
Figure 5-2

Quantitative densitometry of COX protein levels and plasma metabolites in human subjects
(Type 1 diabetes)

Analysis of COX levels in human platelets and monocytes and plasma COX metabolites. Panel A: Platelet COX-1 protein levels were measured in human control and human Type 1 diabetic subjects. Panel B: Monocyte COX-2 protein levels were measured in human control and human Type 1 diabetic subjects. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Panel C and Panel D: Plasma levels of TXB₂ and PGE₂ were measured using EIA. Values represent the mean, S.E. and number of subjects. * significant difference from human control subjects, p<0.05, unpaired Student’s t-test; # significant difference from human control subjects, p<0.05, Mann Whitney test; N.S. not significant difference from human control subjects, p>0.05, unpaired Student’s t-test or Mann Whitney test.
Figure 5-3

Quantitative densitometry of platelet COX-1 protein levels in human subjects
(Type 1 diabetes, hypertension)

Analysis of COX-1 levels in human platelets. Panel A: Platelet COX-1 protein levels were measured in subjects with and without hypertension. Panel B: Sub-analysis of platelet COX-1 protein levels was performed in subjects with and without hyperlipidemia. A rat platelet standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of subjects. * significant difference from subjects without hypertension, p<0.01, unpaired Student’s t-test; N.S. not significant difference from Type 1 diabetes without hypertension, p>0.05, 1-way ANOVA (Tukey’s multiple comparison test).
Quantitative densitometry of platelet COX-1 protein levels in human subjects (Type 1 diabetes, hyperlipidemia)

Analysis of COX-1 levels in human platelets. Panel A: Platelet COX-1 protein levels were measured in subjects with and without hyperlipidemia. Panel B: Sub-analysis of platelet COX-1 protein levels was performed in subjects with and without hyperlipidemia. A rat platelet standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of subjects. * significant difference from subjects without hyperlipidemia, \( p < 0.01 \), unpaired Student’s \( t \)-test; # significant difference from Type 1 diabetes without hyperlipidemia, \( p < 0.05 \), 1-way ANOVA (Tukey’s multiple comparison test).
5.4.2 ACETYLATED HISTONE H4 LEVELS IN MONOCYTES IN TYPE 1 DIABETES

5.4.2.1 Western blot validation: acetylated histone H4 (humans)

Several validation procedures were in place to verify acetylated histone H4 protein bands detected. These validations included (more details on Western blot validation, see Section 4.4.3.1):

vi) establishing acetylated histone H4 protein at correct molecular weight with the use of molecular weight standard (Figure 4-10),

vii) histone H4 primary antibody specificity testing by incubating target proteins with secondary antibodies only (Figure 4-10),

viii) establishing the suitable sample loading concentration (Figure 4-10),

ix) normalizing densitometry readings between blots with inclusion of an internal standard on every gel and

x) data reproducibility was also assessed in repeated samples from 6 human Type 1 diabetic subjects (Figure 4-11).

5.4.2.2 Acetylated histone H4 levels in Type 1 diabetes

In Type 1 diabetes, acetylated histone H4 levels were significantly increased in monocyte compared with those of the human control subjects (Figure 5-5).
Figure 5-5

Quantitative densitometry of histone H4 acetylation in human subjects (Type 1 diabetes)

Analysis of acetylated histone H4 levels in human monocytes. A rat histone H4 standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of subjects. * significant difference from human control subjects, $p<0.001$, Mann Whitney test.
5.4.3 EFFECT OF NSAIDS ON TYPE 1 DIABETES

5.4.3.1 Effect of NSAIDs on COX, TXB₂, PGE₂ and acetylated histone H4 levels in Type 1 diabetes

Human Type 1 diabetic subjects with NSAID treatment regime showed a significant reduction in platelet COX-1 and monocyte COX-2 levels compared to human Type 1 diabetic subjects without NSAIDs (Figure 5-6).

There was a significant reduction in PGE₂, but not plasma TXB₂ levels in human Type 1 diabetic subjects taking NSAIDs compared to human Type 1 diabetic subjects not taking NSAIDs (Figure 5-6).

Monocytes of human Type 1 diabetic subjects taking NSAIDs expressed markedly lower acetylated histone H4 proteins compared to human Type 1 diabetic subjects not taking NSAIDs (Figure 5-7).
Figure 5-6

Quantitative densitometry of COX protein levels and plasma metabolites in human subjects in the presence of NSAIDs (Type 1 diabetes)

Analysis of COX levels in human platelets and monocytes and plasma COX metabolites in the presence of NSAIDs. Panel A: Platelet COX-1 levels were measured in human control, human Type 1 diabetic subjects with and without NSAIDs. Panel B: Monocyte COX-2 levels were measured in human control, human Type 1 diabetic subjects with and without NSAIDs. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Panel C and Panel D: Plasma levels of TXB2 and PGE2 were measured using EIA. Values represent the mean, S.E. and number of subjects. * significant difference from Type 1 diabetes not taking NSAIDs, $p<0.05$, 1-way ANOVA (Dunn’s comparison test); N.S. not significant difference from Type 1 diabetes not taking NSAIDs, $p>0.05$; # significant difference from control; ^ significant difference from Type 1 diabetes not taking NSAIDs significant difference from Type 1 diabetes not taking NSAIDs, $p<0.001$, 1-way ANOVA (Tukey’s multiple comparison test).
Fig 5-7

Quantitative densitometry of histone H4 acetylation in human subjects in the presence of NSAIDs (Type 1 diabetes)

Acetylated histone H4 expression was measured in human control, human Type 1 diabetic subjects with and without NSAIDs. A histone H4 standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of subjects. * significant difference from human control subjects, \( p < 0.01 \); # significant difference from Type 1 diabetes not taking NSAIDs, \( p < 0.001 \), 1-way ANOVA (Dunn’s multiple comparison test).
5.5 DISCUSSION
The purpose of the current study was to investigate whether COX-1 and COX-2 levels were altered in platelets and monocytes of Type 1 diabetes and if so, could this be associated with altered histone acetylation?

5.5.1 COX-MEDIATED PATHWAYS IN TYPE 1 DIABETES

5.5.1.1 Effect of age on COX-mediated pathways in Type 1 diabetes

Human subjects in the present study were recruited from a hospital clinic with few exclusion criteria. Post-hoc analyses were performed to try and refine the population. One important factor is age, which in animal studies had shown that COX-1 and COX-2 increase with advancing age (Heymes et al. 2000; Armstead 2003). In the present study, COX protein levels did not change with aging in either platelets or monocytes isolated, although monocyte COX-2 expression was weakly correlated with age (Figure 5-1).

5.5.1.2 COX-1-mediated pathway in platelets in Type 1 diabetes

The present study revealed that COX-1 levels in platelets were unaltered in Type 1 diabetes (Figure 5-2). Platelet COX-1 levels in human Type 1 diabetic subjects have not been measured previously. Further, there are no animal studies indicating changes in COX-1 in platelets in Type 1 diabetes, although kidney COX-1 is elevated in STZ diabetic rats (Nasrallah et al. 2003). Several studies showed that platelets of Type 1 diabetes are more reactive (Davi et al. 2003; Hu et al 2004), but if this is the case elevated COX-1 expression is not involved.

The current data were also sub-analyzed according to disease and age. It was found that COX-1 protein levels in platelets was up-regulated in hypertensive human subjects compared with non-hypertensive human subjects (Figure 5-3A). An in vivo study by Davi et al. (1997) found that hypertension was associated with platelet activation as indicated by enhanced
TXA₂ synthesis suggesting enhanced COX-1-mediated pathway in platelets. Further conclusive analyses after sub-grouping in the present study, however, could not be made due to insufficient sample size, although there was a trend showing increased platelet COX-1 levels in human Type 1 diabetic subjects with hypertension compared with human Type 1 diabetic subjects without hypertension (Figure 5-3B).

It was also found that subjects with hyperlipidemia had significantly higher levels of platelet COX-1 compared with human control subjects (Figure 5-4A). Further sub-analysis on non-diabetic subjects showed increased platelet COX-1 levels were associated with hyperlipidemia, although this was not observed in human Type 1 diabetic subjects (Figure 5-4B). The precise effect of hyperlipidemia-dependent increase in COX-1 levels is not clear, but changes in lipid levels are known affect the activity of platelets (Di Minno et al. 1986). A study by Davi et al. (1997) found enhanced COX-1-mediated activity in platelets indicated by increased TXA₂ synthesis was associated with hypercholesterolemia. Therefore, potentially, alteration in COX-1 levels in subjects with hyperlipidemia may promote thrombosis development (Pitt et al. 1995).

In addition to platelet COX-1 levels, the present study also measured plasma TXB₂. Plasma TXB₂ was unaltered in Type 1 diabetes and was not correlated with COX-1 levels in platelets (Figure 5-2). Similar findings were reported previously in that no difference in the plasma levels of TXB₂ was found between Type 1 diabetic and human control subjects (Arisaka et al. 1986; Mourits-Andersen et al. 1986). Plasma TXB₂ levels, however, is not always indicative of COX-1 levels and activity in platelets as plasma TXB₂ can come from various sources as described in Section 4.5.2.2. Unaltered plasma TXB₂ levels in Type 1 diabetes in this case suggests overall COX-1 levels were not changed. This, however, does not mean that the COX-1-mediated pathway is unaltered in platelets in Type 1 diabetes as a direct functional assessment of platelet COX-1 enzymatic activity is required to determine this. In the literature,
several reports found platelets were more hyperactive in Type 1 diabetes. Early studies by Udvardy et al. (1987) and Abbate et al. (1988) showed that platelets isolated from human Type 1 diabetic subjects produced more TXB$_2$ when stimulated with thrombin compared with those of human control subjects. Similar findings were also reported in resting platelets in Type 1 diabetes (Vericel et al. 2004). Platelet hyperactivity contributes to the development of thrombosis and may in this case increase the risk of cardiovascular complications in Type 1 diabetes.

5.5.1.3 COX-2-mediated pathway in platelets in Type 1 diabetes

The COX-2 isoform is up-regulated in inflammation and is implicated in Type 1 diabetes, which was re-affirmed by increased COX-2 expression in mouse islet-infiltrated macrophages (Luo et al. 2002). The present study investigated changes in human monocyte COX-2 expression in Type 1 diabetes and reported that monocyte COX-2 levels were increased in Type 1 diabetes (Figure 5-2). A similar finding was reported in human monocyte (Litherland et al. 1999; Litherland et al. 2003). Furthermore, COX-2 mRNA was also found to be induced by glucose in cultured THP-1 monocyte cells and in peripheral blood monocytes from human Type 1 diabetic subjects, although only four control and three human Type 1 diabetic subjects were involved in this investigation (Shanmugam et al. 2004).

The current study reported that in Type 1 diabetes, plasma PGE$_2$ levels were elevated compared with the control group (Figure 5-2). This is supported by early studies by Chase et al. (1979) and Arisaka et al. (1986) who reported increased plasma PGE$_2$ in children with diabetes, although no difference in the plasma levels of PGE$_2$ between Type 1 diabetic and human control subjects has also been reported (Mourits-Andersen et al. 1986). The exact source of this increased plasma PGE$_2$ levels is unknown as plasma PGE$_2$ can come from various sources (see Section 4.5.2.3 for more details). Enhanced plasma PGE$_2$ production may affect renal functions as PGE$_2$ is a major prostanoid in the kidney. In the kidney, PGE$_2$
selectively activates at least three distinct EP receptors with each EP receptor preferentially couples to different signal transduction pathways (for more details on EP receptors, see Section 1.2.2.2). Activation of these EP receptors leads to changes in the regulation of vascular smooth muscle tone, glomerular filtration, renin release and tubular salt and water transport in kidney (see Review by Breyer et al. 1996).

Although the exact source was unknown, elevated plasma PGE$_2$ in the current study suggested an overall alteration on COX-2-mediated activity and possibly an elevated inflammatory activity in Type 1 diabetes. The potential biological effects associated with these changes can be profound and are discussed in Section 4.5.2.3.

5.5.2 HISTONE H4 ACETYLATION IN TYPE 1 DIABETES

We have demonstrated previously in Section 2.4.3 that up-regulation of both COX-1 and COX-2 protein expression in endothelial cells was associated with histone H4 hyperacetylation. The current study investigated whether histone H4 acetylation changed in monocytes isolated from human Type 1 diabetic subjects and if the levels of histone H4 acetylation were associated with monocyte COX-2 expression.

5.5.2.1 Histone H4 acetylation in monocytes in Type 1 diabetes

Histone hyperacetylation, a post-translational modification of histones, is associated with active gene transcription. Histone acetylation loosens histone structure and DNA strands making DNA more accessible to transcription factors (see Reviews by Pazin & Kadonaga 1997; Imhof & Wolffe 1998). The current study found that in monocytes acetylated histone H4 expression was increased in Type 1 diabetes compared with the control group (Figure 5-5). This increased acetylated histone H4 is associated with increased COX-2 levels in monocytes in Type 1 diabetes, suggesting the possibility that COX expression in human Type 1 diabetes is modulated through histone modifications (see Chapter 2 for more details). In support of the
current finding, enhanced histone acetyltransferase activity and histone acetylation in islets cells (Mosley & Ozacn 2003; Mosley at al. 2004) and in cultured THP-1 monocytes (Miao et al. 2004) have been reported. To the best of our knowledge, the current study was the first with a meaningful sample size to investigate monocyte histone H4 acetylation status in Type 1 diabetes.

5.5.3 EFFECT OF NSAIDS ON TYPE 1 DIABETES

NSAIDs have long been used clinically to reduce pain and inflammation by inhibiting COX activity (for more details on the action of NSAIDs on COX enzymes, see Section 4.5.4). By inhibiting COX activity, NSAIDs are able to reduce platelet function and prevent ischemic heart diseases (see Review by Vane 1971; Patrono et al. 2005). Type 1 diabetes is associated with micro- and macro-vascular complications with tissue damage in eyes, kidneys, blood vessels and nerves. These chronic diabetic vascular complications may involve mechanisms mediated by COX enzymes (see Section 3.4). The present study aimed to investigate whether the use of NSAIDs in human Type 1 diabetic subjects would change COX-1 and COX-2 protein levels and if so, was this regulated through histone acetylation modification. This preliminary study did not include human non-diabetic subjects on NSAIDs. Further recruitment of these subjects is required to accurately analyze and interpret the data collected.

5.5.3.1 Effect of NSAIDs on COX-mediated pathways in Type 1 diabetes

The current study reported that the use of NSAIDs significantly lowered platelet COX-1 and monocyte COX-2 in Type 1 diabetes (Figure 5-6). COX down-regulation by NSAIDs has been previously reported. Wu et al. (1991) and Zyglewska et al. (1992) found that COX levels were reduced by naproksen and aspirin in human endothelial cells. In the case of monocyte COX-2, Pilbeam et al. (1995) showed that indomethacin, piroxicam and flurbiprofen were able to partially down-regulate mRNA COX-2 expression in murine osteoblastic cells. A decrease in COX-2 expression by sodium salicylate was also found in human endothelial cells.
(Xu et al. 1999). In contrast, COX-2 expression was up-regulated by NSAIDs in mammary epithelial cells (Meade et al. 1999) and endometrial cancer cell lines (Gao et al. 2004). Factors such as differences in cell line and type of NSAIDs studied may account for the different results.

The present study also found that NSAID use decreased plasma TXB$_2$ and PGE$_2$, but in the case of TXB$_2$ this was not statistically significant, presumably due to low sample numbers (Figure 5-6). A small study by Capone et al. (2004) found that COX-1 and COX-2 had a different response to NSAIDs when aspirin and naproxen were tested in nine healthy human subjects for six days. Aspirin and naproxen reduced platelet COX-1 activity, indicated by both plasma TXB$_2$ and urinary excretion of TXB$_2$ levels, in a similar fashion. In contrast to low-dose aspirin, naproxen significantly lowered LPS-induced PGE$_2$ in the plasma, an index of monocyte COX-2 activity. This confirms again that the use of different types of NSAIDs affects COX-mediated pathways differently.

Platelets and monocytes play an important role in the development of vascular complications such as thrombosis and atherosclerosis. In diabetes, platelets are more hyperactive (Li et al. 2001) and particularly platelets of human Type 1 diabetic subjects with micro complications expressed higher levels of P-selectin and soluble CD40 ligand (Yngen et al. 2004). P-selectin, an adhesion molecule, is located in membranes of $\alpha$-granules in resting platelets and redistributed to the cell surface upon platelet activation (Stenberg et al. 1985; Berman et al. 1986; see Review by Hogg 1992). Soluble CD40 ligand, a transmembrane protein structurally similar to TNF-$\alpha$, is expressed on the surface of activated platelets CD40 ligand. Both P-selectin and soluble CD40 ligand promote the secretion of chemokines and expression of adhesion molecules by endothelial cells, thereby facilitating the binding of leukocytes to activated platelets and endothelium and contributing to thrombus formation (Henn et al. 1998; see Review by Hogg 1992). Anti-thrombotic therapy such as aspirin may be able to prevent
over-expression of these platelet surface proteins by reducing the activation of platelets through blocking platelet COX-1 activity. Consequently, the use of NSAIDs clinically may lower the occurrences of thrombosis and ischemic heart diseases in Type 1 diabetes.

The COX-2-mediated pathway is implicated in Type 1 diabetes with increased COX-2 expression found in the monocytes of human Type 1 diabetic subjects (Litherland et al. 1999). Most NSAIDs currently available are non-selective COX inhibitors and they are effective anti-inflammatory agents. COX-2 selective inhibitors in particular, however, play a crucial role in the inflammatory process. NSAIDs may reduce vascular and leukocyte inflammatory components and therefore interfere with the development of atherothrombotic disease, a condition that may involve increased COX-2 expression (Luo et al. 2002; Martin-Ventura et al. 2005; Gomez-Hernandez et al. 2006). The present study showed the ability of NSAIDs to down-regulate COX-2 levels in monocytes, possibly through inhibition of NF-κB (Yoneda et al. 2003) in Type 1 diabetes. This suggests the importance of NSAIDs in management of Type 1 diabetes.

5.5.3.2 Effect of NSAIDs on histone H4 acetylation in Type 1 diabetes

The core histones are subjected to extensive post-translational modifications, which result in changes in DNA structure. Histone acetylation, a process that loosens DNA strands and allows the access of transcription factors to the DNA, is commonly associated with active gene transcription (Allfrey et al. 1964; see Review by Wade et al. 1997). This section of the study investigated whether the use of NSAIDs affected monocyte histone acetylation in Type 1 diabetes. It was found that the use of NSAIDs lowered monocyte acetylated histone H4 expression in Type 1 diabetes (Figure 5-7). This is consistent with reduced monocyte COX-2 levels in human Type 1 diabetic subjects taking NSAIDs (see Section 5.5.3.1), supporting the hypothesis that COX expression was regulated by histone hyperacetylation (see Chapter 2 for more details). This is further supported by Wang et al. (2006) who reported that in cultured
cells, histone acetyltransferase, p300, up-regulated COX-2 gene by acetylating NF-IL6β, a transcription factor that activates COX-2 promoter region. To the best of our knowledge, the down-regulation of acetylated histone H4 expression in monocytes by NSAIDs in Type 1 diabetes is a novel finding. This may potentially be an important mechanism by which NSAIDs regulate COX expression.

5.6 CONCLUSIONS

This human study reported monocyte COX-2, but not platelet COX-1 increased in Type 1 diabetes compared with the control group. Elevated plasma PGE$_2$, but not plasma TXB$_2$ was also observed in Type 1 diabetes, suggesting an overall increase in inflammatory activity in Type 1 diabetes. Treatment with NSAIDs in Type 1 diabetes reduced both platelet COX-1 and monocyte COX-2 levels. Plasma TXB$_2$ and PGE$_2$ levels were also decreased in human Type 1 diabetic subjects taking NSAIDs. This re-affirms the previous observation that inflammation was increased in human Type 1 diabetic subjects.

Further analysis showed increased acetylated histone H4 expression in monocytes in Type 1 diabetes, which was concurrent with enhanced COX-2 expression in monocytes, suggesting hyperacetylation of the core histones is associated with COX up-regulation. The use of NSAIDs down-regulate monocyte acetylated histone H4 levels in Type 1 diabetes. To the best of our knowledge, this is a novel finding suggesting potentially NSAIDs may down-regulate COX expression via histone acetylation/de-acetylation.
CHAPTER 6
CHANGES OF RECEPTORS FOR ADVANCED GLYCATION END PRODUCTS (RAGE) IN DIABETES
6.1 INTRODUCTION

The strong association between hyperglycemia in diabetes mellitus and the development of chronic diabetic complications including both micro and macro vascular dysfunction has been shown in the results of an intervention study in both Type 1 (Diabetes Control and Complications Trial Research Group 1993) and Type 2 (UKPDS 1998) diabetic patients. Despite much research, the mechanisms by which chronic hyperglycemia results in functional changes and tissue injury are yet to be clarified, although several mechanisms have been proposed. These include enhanced glucose metabolism through the polyol pathway, activation of PKC by hyperglycemia, increased oxidative stress, enhanced inflammation and increased formation of AGEs. These mechanisms have some overlapping links and are discussed below (for details of enhanced inflammation in diabetes, see Section 3.4.1).

6.1.1. POLYOL PATHWAY

Most cells require the action of insulin for glucose entry into the intracellular compartments; the few exceptions are vascular endothelial cells of the retina, kidney, and nervous tissue. These insulin-independent cells allow a free interchange of glucose between the extracellular and intracellular spaces regardless of insulin’s action. The insulin-independent diffusion of glucose ensures normal physiologic cellular functions to proceed during the euglycemic state, and the homeostasis is then maintained by the enzyme aldose reductase, which metabolizes excess glucose into sorbitol via the polyol pathway (see Review by Swidan & Montgomery 1998). Under normal circumstances, the amount of glucose metabolized by aldose reductase is minimal as the enzyme has a low affinity for glucose. Sorbitol production in the insulin-independent cells, however, increases dramatically when excess glucose enters the polyol pathway during hyperglycemic states (Figure 6-1). When this happens there is a concomitant decrease in nicotinamide–adenine dinucleotide phosphate (NADPH). Sorbitol is then oxidized by sorbitol dehydrogenase to fructose, coupled with the reduction of oxidized nicotinamide–adenine dinucleotide (NAD⁺) to the reduced form nicotinamide–adenine
dinucleotide (NADH) (Figure 6-1 and see Reviews by Swidan & Montgomery 1998; Brownlee 2001). Changes in the polyol pathway may impact on various metabolic reactions. For instance, NADPH is important in keeping glutathione in its reduced form. Reduced glutathione together with glutathione peroxidase converts dangerous reactive oxygen species such hydrogen peroxide to water (for more details on oxidative stress, see Section 6.1.3). Subjects who could not make adequate NADPH were found to be at an increased risk of oxidative damage from glutathione insufficiency (Cathcart 1985). Excess glucose can also result in an increased production of sorbitol, which cannot diffusible through cell membranes easily. The accumulation of sorbitol causes osmosis stress leading to imbalances in intracellular homeostasis. Together with enhanced glycation of macromolecules (for more details, see Section 6.1.4), increased sorbitol may contribute to the development of diabetic complications (see Reviews by Kinoshita 1986; Chung et al. 2003).

This series of biochemical activities provides a ground for metabolic disturbances resulting from altered enzyme activity due to a decreased availability of unbound cytosolic NADPH or an increased availability of NADH (see Reviews by Brownlee 2001; Sheetz & King 2002). Accumulation of sorbitol, fructose and unbound cytosolic NADH potentially can result in microvascular injury via mechanisms such as sorbitol-induced osmotic stress, increased AGE and prostaglandin production, and alterations in PKC activity (see Reviews by Brownlee 2001; Tilton 2002).
Polyol Pathway

Aldose reductase metabolizes excess glucose, resulting in an increase in sorbitol production. This process takes place with a concomitant decrease in nicotinamide–adenine dinucleotide phosphate (NADPH) before sorbitol is oxidized by sorbitol dehydrogenase to fructose. This action results in a decrease in glutathione, an important molecule in the defence of oxidative stress. Oxidized nicotinamide–adenine dinucleotide (NAD⁺) is then reduced to nicotinamide–adenine dinucleotide (NADH), subsequently producing advanced glycation end product (AGE) precursors such as methylglyoxal. AGE precursors are then further re-arranged and consequently become irreversible AGEs.
If the polyol pathway is important, selective inhibitors should prevent some of the consequences of high glucose. *In vivo* studies involving polyol pathway inhibitors have been, for the most part, disappointing. In diabetic rats, inhibition of aldose reductase, an enzyme that converts glucose to sorbitol has been shown to prevent diabetes-induced nephropathy (Mauer *et al.* 1989), neuropathy (Cameron *et al.* 1986) and retinopathy (Robinson *et al.* 1989). Zenarestat, a potent aldose reductase inhibitor was studied in a fifty two-week, randomized, placebo-controlled, double-blind clinical trial by Greene *et al.* (1999). Nerve conduction velocity and small-diameter sural nerve density were assessed in patients with mild to moderate diabetic peripheral neuropathy. It was found that zenarestat decreased diabetic peripheral neuropathy in these patients (Greene *et al.* 1999). Long term studies involving aldose reductase inhibition, however, have shown contradictory results. Engerman *et al.* (1994) conducted a five-year study in dogs and found aldose reductase inhibition did not prevent diabetic retinopathy or thickening of the capillary basement membrane in the retina or kidney, respectively. While some clinical trials showed the effectiveness of aldose reductase inhibitors in improving diabetic complications, results from most other clinical trials of aldose reductase inhibition have been less impressive due to inadequate efficacy and/or safety concerns (Sorbinil Retinopathy Trial Research Group 1990; Oates *et al.* 1999). In short, while experimental data provide support to the notion that sorbitol production may contribute to micro-vascular dysfunction observed in diabetes, most human studies to date have failed to demonstrate a clinical benefit of reducing sorbitol levels.

### 6.1.2 PKC AND ITS ISOFORMS

PKC is a large family of at least 12 isoforms of the serine and threonine kinase family. In the vasculature, several PKC isoforms are present, however, in a diabetic rat model there is a preferential activation of PKC β2 in the aorta, heart, and retina, and PKC β1 in the glomeruli (Inoguchi *et al.* 1992; Koya *et al.* 1997). Phospholipases C and D, produce diacylglycerol (DAG) from phosphatidylinositides and phosphatidylcholine, respectively. Elevated DAG
levels then leads to activation of PKC, a process which is known to maintain important functions in vascular cells including regulating permeability, contractility, extracellular matrix integrity, cell growth, angiogenesis, cytokine actions, and leukocyte adhesions, all of which are abnormal in diabetes (see Reviews by Nishizuka 1992; Nishizuka 1995). Excess glucose can be transported intracellularly, mainly by the glucose transporter GLUT-1, and lead to increased DAG production and PKC activation (Kaiser et al. 1993 and see Reviews by Greene et al. 1987; Baynes 1991; Williamson et al. 1993; Derubertis & Craven 1994; Sharma & Ziyadeh 1995; King et al. 1997).

Hyperglycemia-induced PKC activation has been shown to increase platelet-derived growth factor-β receptor expression in smooth muscle cells, endothelial cells and monocyte/macrophages (Kawno et al. 1993; Inaba et al. 1996). PKC activation can also increase the expression of transforming growth factor-β (TGF-β), which has been implicated in the development of mesangial expansion and basement membrane thickening in diabetes (MacKay et al. 1989; Nakamura et al. 1992; Suzuki et al. 1993; Yamamoto et al. 1993; Pankewycz et al. 1994; Sharma et al. 1994). TGF-β activates gene expression of proteoglycans and collagen and decreases the synthesis of proteolytic enzymes that degrade matrix proteins and therefore, is one of the most important growth factors regulating extracellular matrix production (Nabel et al. 1993). Up-regulation of TGF-β has been linked to thickening of the capillary basement membrane, one of the early structural abnormalities observed in almost all tissues in diabetes (Williamson et al. 1984). These structural alterations, however, were slowed down when TGF-β antibodies were applied to the renal tissue of diabetic rats and in cultured mesangial cells exposed to high glucose levels (Ziyadeh et al. 1994; Sharma et al. 1996). LY333531, a PKC β selective inhibitor has been shown to reduce expression of TGF-β and extracellular matrix proteins such as fibronectin and type IV collagen in renal glomeruli of diabetic rats (Koya et al. 1997; Koya et al. 2000), biochemical and molecular changes associated with early renal dysfunctions in nephropathy in diabetic
models. Stringent assessment of PKC inhibition, however, is required as to date, long-term studies involving PKC inhibitors have not been possible due to their non specificity for other kinases resulting in serious toxic effects.

6.1.3 OXIDATIVE STRESS

Another hypothesis, which has been proposed to explain the development of diabetic complications, involves oxidative stress, a persistent imbalance between the production of highly reactive molecular species and antioxidant defences (see Review by Rosen et al. 2001). Oxidative stress can result from increased production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS), decreased anti-oxidative defence and failure to repair oxidative damage. ROS or RNS can either be free radicals, reactive anions containing oxygen or nitrogen atoms, or molecules containing oxygen or nitrogen atoms that can either produce free radicals or are chemically activated by them.

The precise sources of or the mechanisms involving enhanced oxidative stress in diabetes are unclear, although polyol and PKC pathways may be involved. Satoh et al. (2005) showed that increased NADH was associated with enhanced ROS production in the kidneys of STZ-induced diabetic rats. The use of specific PKC and NADPH inhibitors successfully lowered systemic oxidative stress in vivo in both Zucker and STZ-induced diabetic rats (Sonta et al. 2004). Under normal circumstances, increased free radical production is neutralized by the action of superoxide dismutase, catalase or glutathione peroxidase, which form the anti-oxidant defence mechanisms (see Reviews by Fiers et al. 1999; Hayes & McLellan 1999; Nicholls & Budd 2000). In diabetes, these mechanisms may be impaired (Weidig et al. 2004) and favor the action of reactive molecular species, which have the ability to directly cause macromolecular damage by oxidizing and damaging DNA, proteins and lipids (see Reviews by Nishikawa et al. 2000; Rosen et al. 2001). The oxidative stress hypothesis, therefore, may be the common link between proposed mechanisms of diabetic complications (i.e. sorbitol
formation and PKC activation), resulting in vasculopathy in diabetes.

In the vasculature, ROS can alter the production of cytokines and growth factors regulating the vascular endothelium, affect lipid oxidation status and enhance platelet hyperreactivity. For example, ROS-induced activation of PKC isoforms results in the generation of TGF-β, endothelin, and vascular endothelial growth factor from the vascular endothelium. PKC activation in this instance also alters the expression of fibronectin, hence affecting the integrity of the basement membrane (Williams & Schrier 1992 and see Review by King et al. 1996). By activating the NF-κB pathway, inducing gene transcription, ROS are also able to increase cytokine production such as TNF-α and adhesion molecules and potentiate the formation of atherosclerotic lesions (see Reviews by Thurberg BL, Collins 1998; Christman et al. 2000). Signaling pathways such as NH2-terminal Jun kinases/stress activated protein kinases (JNK/SAPK), p38 mitogen-activated protein (MAP) kinase and hexosamine are also reported to be modulated by ROS (see Reviews by Marshall et al. 1991; Kyriakis & Avruch 1996; Barnes & Karin 1997). Therefore, in addition to directly oxidizing macromolecules, ROS also acts through numerous intracellular signaling pathways hence, affecting gene expression and consequently cellular damage results. Oxidative stress thus, plays a critical role in the etiology of diabetic complications.

6.1.4 FORMATION OF AGES

The formation of AGEs, as detailed in Figure 6-2, is the result of non-enzymatic glycosylation of proteins or lipids when a high level of glucose is present, a “browning” effect observed made by Maillard almost a century ago, also known as a Schiff’s base (Maillard 1912). A Schiff’s base is further rearranged to form the more stable Amadori-type product and eventually the irreversible AGEs (Brownlee et al. 1988).
**Figure 6-2**

Formation of AGEs (diagram adapted from Sulochana et al. 2001)

AGE formation begins with reversible condensation of the aldehyde group of glucose with a macromolecule such as a protein forming a Schiff’s base. A Schiff’s base is re-arranged to form a stable Amadori product and eventually irreversible AGEs such as carboxyl methyl lysine (CML).
AGEs can be found in plasma and various cell types including monocytes, red blood cell, vessel walls and kidney. Of the large number and complex family, carboxyl methyl lysine (CML) (Ikeda et al. 1996) and pentosidine (Sell & Monnier 1989) are the predominant AGEs found in human. Although a hyperglycemic environment favors the formation of AGEs, diabetes mellitus is not the pre-requisite for such a formation. Studies have shown that AGE cross-linking is a natural process in aging and can also take place in conditions such as Alzheimer’s disease (Picklo et al. 2002). Once formed, AGE cross-linking affects the biochemistry of proteins such as reducing enzyme activity (Facchiano et al. 2002), changing biophysical properties of proteins and affecting protein interactions with other enzymes (Verzijl 2002; Badenhorst 2003). AGEs can also bind to specific receptors including RAGE, AGE-R2, and AGE-R3. Hence, they play an important role in cell signaling processes.

Increasing evidence suggests that the engagement of AGEs to their receptors, RAGE plays a pivotal role in the accelerated vascular dysfunction observed in diabetes.

6.1.4.1 Cell surface AGE binding proteins

Whilst glycation per se may alter protein activity in a general sense, there appears to be specific pathways, which are activated by glycated macromolecules. Using radio-labelled AGE proteins, proteins that bind to AGEs have been identified, isolated and purified from cell membranes of various cell types including monocytes, lymphocytes, erythrocytes and endothelial cells (see Review by Kim et al. 2005).

AGE binding proteins appear to be very heterogenous, because they have different molecular weights depending on the cell type (see Review by Hudson et al. 2002). Further study by Schmidt et al. (1994) has resulted in the characterisation of AGE binding proteins from endothelial cells. The main component of the cellular binding site is an integral membrane protein belonging to an immunoglobulin superfamily known as RAGE. A second lactoferrin-like polypeptide, which is highly homologous with lactoferrin binds
non-covalently to RAGE forming the cellular AGE binding site (Schmidt et al. 1994). More AGE receptors have been discovered and the list of AGE binding proteins now includes OST-48 (AGE-R1), 80K-H (AGE-R2), galectin-3 (AGE-R3), the macrophage scavenger receptor A (MSR-A), and lysozyme (see Review by Vlassara 1996). Despite the large number of AGE receptors, RAGE is the best characterized AGE receptor and appears to play a central role in AGE cell signaling pathways (see Review by Schmidt et al. 2001).

6.1.4.1.1 Receptors for RAGE

RAGE is a member of the immunoglobulin superfamily of cell surface molecules. Immunohistochemistry and in situ hybridization studies have shown that RAGE is expressed in endothelial cells, vascular smooth muscle cells, macrophage, T-lymphocytes, mesangial cells and astrocytes (Brett et al. 1993; Ritthaler et al. 1995). Cloned from bovine lung in 1992, RAGE has a molecular weight of 35 Kd in a truncated form (Neeper et al. 1992). The extracellular portion of the receptor consists of one V-type, two C-type domains and a short cytoplasmic tail. This highly charged cytoplasmic tail may be responsible for the recruitment of cellular effectors by binding to signal transduction molecules in the cytoplasm once the ligand occupies the receptor (Neeper et al. 1992; Schmidt et al. 1992; Schmidt et al. 1994). There is a high degree of RAGE homology in human, rat and cow. The slight differences in glycosylation sites and susceptibility to proteases may contribute to their different pharmacological parameters (Renard et al. 1997; Renard et al. 1999). There are also similarities in homology between RAGE and some other members of the immunoglobulin superfamily. For instance, the cytosolic tail of RAGE at the C terminus is the most homologous with CD20, a B cell activation marker. Its place in the immunoglobulin superfamily means RAGE may participate in the host response to environmental perturbation (Li & Schmidt 1997).
The signaling transduction pathways involving RAGE are complex. It has been shown that AGE-RAGE interactions can result in the generation of oxidative stress and activation of p21\textsuperscript{ras}, erk 1/2 kinases and NF-κB in endothelial cells, monocytes, and vascular smooth muscle cells (Kislinger \textit{et al.} 1999). Consequently, this series of events leads to expression of pro-inflammatory mediators. The role of RAGE in mediating inflammation was further demonstrated by the suppression of the effects of AGEs when RAGE was blocked with soluble RAGE, the extracellular ligand-binding domain of the receptor or by transient transfection of cDNA encoding cytosolic tail-deleted RAGE into RAGE-bearing cells (Neeper \textit{et al.} 1992; Schmidt \textit{et al.} 1992). More importantly, it was found that AGE-RAGE interaction itself can provide a positive feedback loop by enhancing RAGE expression, which further sustains RAGE up-regulation. (Soulis \textit{et al.} 1997). This positive feedback loop of AGE-RAGE interaction has been proposed to set the stage for chronic cellular activation and tissue damage (Schmidt \textit{et al.} 1999).

\textbf{6.1.4.1.2 Cellular interaction of AGEs: central role of RAGE in diabetes}

In the presence of diabetes, cells and tissues are constantly exposed to AGEs from the circulating blood and consequently results in AGE accumulation. The cross-links between AGEs and proteins/tissues can lead to disturbances in vascular homeostasis. For example, in a diabetic rat model following AGE-RAGE engagement, endothelial cells reduce in size resulting in increased endothelial permeability (Wautier \textit{et al.} 1996), which is an early sign of the development of diabetic vasculopathy (Viberti 1983). RAGE can be blocked by the soluble form of RAGE, which consists of only the extracellular ligand-binding domain of RAGE. Soluble RAGE can reverse previously observed endothelial impairment (Wautier \textit{et al.} 1996). The use of antioxidants further reduced AGE-mediated endothelial hyper-permeability, suggesting AGE-mediated vascular dysfunction was in part oxidative-stress related (Wautier \textit{et al.} 1996).
The binding of AGEs to endothelial RAGE also results in the expression of vascular cell adhesion molecule-1 (VCAM-1) by endothelial cells stimulated by recruitment of monocytes to the vessel wall. Monocytes then infiltrate the blood vessel wall and release cytokines and growth factors when activated, setting the stage for the development of atherosclerosis (Schmidt et al. 1995). In fact, several studies have suggested that RAGE may be a key component in the progression of atherosclerosis and its acceleration observed in diabetic animal models (Park et al. 1998; Kislinger et al. 1999; Kislinger et al. 2001). In human studies, atherosclerotic plaques showed infiltration of RAGE-expressing cells in the expanded intima (Brett et al. 1993).

Accumulating evidence strongly indicates a close relationship between RAGE and inflammation. Enhanced expression of monocyte RAGE has been shown to be involved in systemic inflammation during chronic kidney disease. In a study including 102 non-diabetic subjects with chronic kidney disease, Hou et al. (2004) found that monocyte RAGE expression increased with the severity of chronic kidney disease and RAGE expression was strongly correlated with plasma levels of pentosidine, a marker for AGE formation and various other substances including TNF-α, the monocyte activation marker, neopterin and CRP. When stimulated with AGEs, these monocytes showed an increase in the number and affinity of specific AGE binding sites together with a higher production of TNF-α. This was reduced by a RAGE antibody (Hou et al. 2004). Interestingly, Bucciarelli et al. (2002) has reported a link between RAGE and the COX-2 pathway, which is also known to play a role in the process of inflammation. It was found that COX-2 expression in plaque macrophages isolated from diabetic mice was up-regulated by RAGE. Potentially, it is possible that RAGE may augment and amplify COX-mediated matrix metalloproteinase release and worsen diabetic complications.
6.2 AIMS

Given that AGE-RAGE interaction plays a critical role in diabetes, a better understanding of RAGE expression in platelets and monocytes may provide a better means to understand diabetic vasculopathy. The aims of the current study were to investigate the protein level of RAGE in platelets and monocytes isolated from both human Type 2 and Type 1 diabetic subjects.
6.3 METHODS

6.3.1 RAGE LEVELS IN PLATELETS AND MONOCYTES IN HUMAN DIABETIC SUBJECTS

6.3.1.1 Human subjects and blood sample collection (Type 2 diabetes)

Twenty five (25) human non-diabetic control (14 males, 11 females) and 52 human Type 2 diabetic (28 males, 24 females) subjects were recruited at St. Vincent’s Hospital (Melbourne, Australia). The volunteer recruitment and blood sample collection was performed by the endocrinologist, Dr. Alicia Jenkins (Melbourne, Australia; see Section 4.3.2.2). In addition, 14 non-diabetic samples from healthy male subjects collected during previous project at RMIT University and stored away at -80°C were also included in this study.

6.3.1.2 Human subjects and blood sample collection (Type 1 diabetes)

Twenty five (25) human non-diabetic control (14 males, 11 females) and 19 Type 1 diabetic (10 males, 9 females) subjects were recruited by Dr. Alicia Jenkins at St. Vincent’s Hospital (Melbourne, Australia; see Section 4.3.2.2). In addition, 14 non-diabetic samples from healthy male subjects collected during previous project at RMIT University and stored away at -80°C were also included in this study.

6.3.1.3 Isolation of human platelets and monocytes

The isolation of human platelets and monocytes was performed using OptiPrep™ solution according to the manufacturer’s protocol, which was described in Section 4.3.2.3.

6.3.1.4 Protein assay (humans)

Determination of human sample protein concentrations was performed using the Biorad microBCA system as outlined in Section 4.3.1.3.
6.3.1.5 Western blot: RAGE protein (humans)

Human samples were loaded in duplicate with a final total protein concentration of 10 μg per well. The protein samples and molecular weight markers were electrophoresed, transferred onto nitrocellulose membranes and incubated with anti-mouse monoclonal anti-RAGE antibody using the same Western blot protocol for the detection of COX protein expressions as in Section 4.3.1.4. The density of all Western blot bands was analyzed using the ChemiDoc™ XRS system (Biorad, CA, USA).

6.3.2 DRUGS AND CHEMICALS

Anti-mouse monoclonal anti-RAGE antibody, peroxidase conjugated goat anti-mouse immunoglobulins; Chemicon International (Temecula, CA, USA). All other drugs and chemicals used in the present experiments were the same as that used in Chapters 2 and 4 and thus, were obtained from the same sources (see Sections 2.3.4 and 4.4.5).

6.3.3 STATISTICAL ANALYSES

All values are given as the mean ± standard error of mean (S.E.) and n indicates the number of observations. Differences in the percentage densities of Western blot bands were analyzed using unpaired Student’s t-test, Mann-Whitney test, 1-way ANOVA and post-hoc tests (Tukey’s, Games-Howell and Dunn’s multiple tests), Pearson Chi-Square and Fisher’s exact test whenever applicable. SPSS (SPSS Inc., IL, USA) and Prism (GraphPad Software, Inc., CA, USA) statistical packages were used in statistical analyses.

6.3.4 HUMAN ETHICS APPROVAL

All procedures used for human blood collection were approved by the Human Research Ethics Committee of St. Vincent’s Hospital (Melbourne, Australia; HREC-A 024/00 Glycoxidation, HDL and diabetes angiopathy) in accordance with the National Statement on Ethical Conduct in Research Involving Humans (June 1999) produced by the National Health
and Medical Research Council of Australia. A separate set of human samples from another study was included in the present study. The collection of these samples was approved by the Human Research Ethics Committee of RMIT University (Melbourne, Australia; HREC 15/01 Majewski: Age-dependent alterations in cyclooxygenase in platelets and human monocytes).
6.4 RESULTS

6.4.1 RAGE LEVELS IN PLATELETS AND MONOCYTES IN TYPE 2 DIABETES

6.4.1.1 Basis parameters of human Type 2 diabetic subjects

Human subject selection was made by Dr. Alicia Jenkins at St. Vincent’s Hospital, Melbourne (Section 4.3.2.2). The present study did not exclude any samples collected in order to have a sample population that resembled the characteristics of the general population. One exception was COX inhibition. Human subjects on non-steroidal anti-inflammatory drugs (NSAIDs) were separated from the rest of the sample population and treated separately. Thus the three groups in the present study were: 39 human control subjects (28 males, 11 females), 29 human Type 2 diabetic subjects (13 males, 15 females) and 23 human Type 2 diabetic subjects on NSAIDs (14 males, 9 females). A summary of the physiological parameters, blood examination and pharmacological interventions of these subjects is detailed in Table 4-1 and Table 4-2 (Section 4.4.2.1).

6.4.1.2 Western blot validations (humans)

6.4.1.2.1 Specificity of Western blot: RAGE (humans)

RAGE protein bands were determined and verified against their corresponding molecular weight at approximately 48 kD. The specificity of the primary RAGE antibody was further assessed by incubating samples with the secondary antibody only. The absence of RAGE bands at 48 kD in this case confirmed the specificity of the primary antibody to RAGE proteins (Figure 6-3). A series of sample protein concentrations was performed to determine the suitable loading concentration. Density analysis indicated that 10 μg of total protein was the minimum loading requirement for detecting a clear RAGE protein band that was not over saturated (Figure 6-3).
Figure 6-3

Western blot validation: RAGE protein levels in platelets and monocytes (humans)

Western blot validation for platelet and monocyte RAGE in human subjects. Panels A and C: A signal for platelet RAGE and monocyte RAGE, respectively, was evident at 48 kD according to molecular weight markers. Panels B and D: RAGE immuno-reactivity was verified by negative controls, which were performed with omission of the RAGE primary antibody but in the presence of the secondary antibody. Lanes a + b, c + d: duplicate samples from human Type 2 diabetic subjects, lanes e + f, g + h: duplicates samples from human control subjects, lane i: internal standard. A series of sample loading concentrations was performed to determine the final protein loading for platelet RAGE (Panels E and F) and monocyte RAGE (Panels G and H). Lanes j to m represent 5, 10, 15 and 20 μg protein loading. A final protein loading of 10 μg per lane was chosen, because this was the concentration at which the minimal amount of sample was required to produce a reliable and clear band. Higher protein loading resulted in the risk of signal saturation.
6.4.1.2.2 Western blot data reproducibility: RAGE (humans)

RAGE protein expression was measured in two samples collected from 6 human Type 1 diabetic subjects on separate occasions (Section 5.3.1.2). The values obtained were very consistent indicating both assay accuracy and stability of RAGE levels in the same subjects (Figure 6-4).

6.4.1.3 Influence of age on sample population (Type 2 diabetes)

There was a difference in age between human control and Type 2 diabetic groups (37.9±2.2 and 60.5±2.6 years old, respectively. Table 4-1, Section 4.4.2.1). Correlation analyses were performed to analyze the relationship between RAGE expression and age of human Type 2 diabetic subjects. There was no significant correlation between platelet RAGE or monocyte RAGE expression and advancing age (Figure 6-5).

RAGE gene expression, however, has been shown to decrease (Casselmann et al. 2004; Simm et al. 2004) in aging human hearts. Simm et al. (2004) further assessed RAGE protein expression and found that it was increased with aging in human hearts. This inverse relationship suggested that RAGE may be regulated at the post-transcriptional rather than transcriptional level. In order to assess potential age-dependent effects on RAGE expression in the current study, age-matching was performed according to the procedure detailed in Section 4.4.2.3. This age-matching process continued until all human control subjects were matched with an individual Type 2 diabetic subject. The result of this process was closely matched mean ages of 50.0±2.4 for human controls (n=19) and 53.0±2.6 years old for Type 2 diabetic patients (n=19), which were not significantly different. A summary of the physiological parameters, blood examination and pharmacological interventions of age-matched subjects is detailed in Table 4-3 and Table 4-4 (see Section 4.4.2.2.1).
Figure 6-4

Reproducibility of RAGE protein expression in human Type 1 diabetic subjects

RAGE expression in human platelets and monocytes from individual Type 1 diabetic subjects taken on separate occasions (sets A and B). Panel A: Platelet RAGE protein expression measured in repeated samples from 6 human Type 1 diabetic subjects. Panel B: Monocyte RAGE protein expression measured in repeated samples from 6 human Type 1 diabetic subjects. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands were expressed in the percentage of the corresponding standard control. *p*>0.05, paired Student’s *t*-test.
Figure 6-5

Correlation analysis on RAGE expression and age of human subjects (Type 2 diabetes, non age-matched)

The relationship between RAGE expression and age of the subjects was investigated. Panel A: Scatter plot of the densities of all platelet RAGE samples in relation to age. Panel B: Scatter plot of the densities of all monocyte RAGE samples in relation to age. $R^2$ represents correlation coefficient. $p>0.05$, Pearson Correlation.
6.4.1.4 RAGE levels in Type 2 diabetes

Initial analyses on non age-matched subjects revealed a significant increase in both platelet and monocyte RAGE expression in human Type 2 diabetic subjects compared with human control subjects. The increased RAGE level in platelets was, however, age-dependent as shown in age-matched analysis (Figure 6-6C) whereas monocyte RAGE levels remained to be significantly elevated in age-matched Type 2 diabetes (Figure 6-6D).

RAGE expression was not correlated with COX expression in either platelets or monocytes (Figure 6-7) and RAGE was not significantly correlated with plasma hemoglobin A1C (Figure 6-8).
Figure 6-6

Quantitative densitometry of RAGE protein expression in Type 2 diabetes

Density analysis of RAGE expression in human platelets and monocytes. Panel A: Non age-matched analysis of platelet RAGE protein expression in human control and human Type 2 diabetic subjects. Panel B: Non age-matched analysis of monocyte RAGE protein expression in human control and human Type 2 diabetic subjects. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Panel C: Age-matched analysis of platelet RAGE protein expression in control and human Type 2 diabetic subjects. Panel D: Age-matched analysis of monocyte RAGE protein expression in human control and human Type 2 diabetic subjects. Values represent the mean, S.E. and number of subjects. * significant difference from human control subjects, $p<0.01$, Mann Whitney test; N.S. not significant, unpaired Student’s $t$-test.
Figure 6-7

Non-age matched correlation analysis on RAGE expression and COX expression of human subjects (Type 2 diabetes)

The relationship between RAGE and COX expression in human control and human Type 2 diabetic subjects was investigated. Panel A: Scatter plot of the densities of all platelet RAGE samples in relation to platelet COX-1 expression. Panel B: Scatter plot of the densities of all monocyte RAGE samples in relation to monocyte COX-2 expression. $R^2$ represents correlation coefficient. $P>0.05$, Pearson Correlation.
Figure 6-8

Non-age matched correlation analysis on RAGE expression and hemoglobin A1c of human subjects (Type 2 diabetes)

The relationship between RAGE and hemoglobin A1c in human control and human Type 2 diabetic subjects was investigated. Panel A: Scatter plot of the densities of all platelet RAGE samples in relation to hemoglobin A1c. Panel B: Scatter plot of the densities of all monocyte RAGE samples in relation to hemoglobin A1c. $R^2$ represents correlation coefficient. * significant correlation, $p<0.05$, Pearson Correlation.
6.4.2 RAGE LEVELS IN PLATELETS AND MONOCYTES IN TYPE 1 DIABETES

6.4.2.1 Basis parameters of human Type 1 diabetic subjects

Human subject selection of Type 1 diabetic study was based on broad selection criteria. Most human subjects were included in the study analyses with one exception that human subjects on NSAIDs were separated from the rest of the sample population and treated separately. Thus the three groups in the present study were: 39 human control subjects (28 males, 11 females), 19 human Type 1 diabetic subjects (10 males, 9 females) and 6 human Type 1 diabetic subjects on NSAIDs (2 males, 4 females). A summary of the physiological parameters, blood examination and pharmacological interventions of these subjects is detailed in Table 5-1 and Table 5-2 (see Section 5.4.1.1).

6.4.2.2 Western blot validations (humans)

Several validation procedures were in place to verify that bands detected corresponded to RAGE protein. These procedures have been discussed in details in previous Section 6.4.1.2.

6.4.2.3 Influence of ages on sample population (Type 1 diabetes)

There was no significant difference in age between human control and human Type 1 diabetic subjects (37.9±2.4 and 34.6±3.4 years old, respectively. Table 5-1, Section 5.4.1), which was also shown in correlation analysis (Figure 6-9), although RAGE expression has been shown to alter in human heart with aging, as previously discussed (Casselmann et al. 2004; Simm et al. 2004).
6.4.2.4 RAGE levels in Type 1 diabetes

The present study showed a significant increase in both platelet and monocyte RAGE expression in human Type 1 diabetic subjects compared with human control subjects (Figure 6-10). Correlation analyses showed a weak correlation between RAGE expression and plasma hemoglobin A1C (Figure 6-11). RAGE expression was not correlated with COX expression in either platelets or monocytes (Figure 6-12). COX levels were not correlated with hemoglobin A1C (Figure 6-13).
Correlation analysis on RAGE expression and age of human subjects (Type 1 diabetes)

The relationship between RAGE expression and age of the subjects was investigated. Panel A: Scatter plot of the densities of all platelet RAGE samples in relation to age. Panel B: Scatter plot of the densities of all monocyte RAGE samples in relation to age. $R^2$ represents correlation coefficient. $p>0.05$, Pearson Correlation.
Figure 6-10

Quantitative densitometry of RAGE protein expression in Type 1 diabetes

Density analysis of RAGE expression in human platelets and monocytes. Panel A: Platelet RAGE protein level in human control and human Type 1 diabetic subjects. Panel B: Monocyte RAGE protein expression in human control and human Type 1 diabetic subjects. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of subjects. * significant difference from human control subjects, \( p < 0.0001 \), Mann Whitney test; ** significant difference from human control subjects, \( p < 0.0001 \), unpaired Student’s t-test.
Correlation analysis on RAGE expression and plasma hemoglobin A1c of human subjects (Type 1 diabetes)

The relationship between RAGE and plasma hemoglobin A1c in human control and human Type 1 diabetic subjects was investigated. Panel A: Scatter plot of the densities of all platelet RAGE samples in relation to platelet COX-1 expression. Panel B: Scatter plot of the densities of all monocyte RAGE samples in relation to monocyte COX-2 expression. R² represents correlation coefficient. * significant correlation, p<0.05, Pearson Correlation.
Figure 6-12
Correlation analysis on RAGE expression and COX expression of human subjects (Type 1 diabetes)

The relationship between RAGE and COX expression in human control and human Type 1 diabetic subjects was investigated. Panel A: Scatter plot of the densities of all platelet RAGE samples in relation to platelet COX-1 expression. Panel B: Scatter plot of the densities of all monocyte RAGE samples in relation to monocyte COX-2 expression. R² represents correlation coefficient. * significant correlation, p<0.05, Pearson Correlation.
Figure 6-13

Correlation analysis on monocyte COX-2 expression and plasma hemoglobin A1C of human subjects (non age-matched)

The relationship between monocyte COX-2 expression and plasma hemoglobin A1C in human control and diabetic subjects was investigated. Panel A: Scatter plot of the densities of all monocyte COX-2 samples in relation to plasma hemoglobin A1C. Panel B: Scatter plot of the densities of all monocyte COX-2 samples in relation to plasma hemoglobin A1C. $R^2$ represents correlation coefficient. $p>0.05$, Pearson Correlation.
6.4.3 EFFECT OF NSAIDS ON RAGE LEVELS IN HUMAN DIABETIC SUBJECTS

6.4.3.1 Effect of NSAIDs on RAGE levels in Type 2 diabetes

Human Type 2 diabetic subjects with NSAID treatment regime showed a significant reduction in platelet and monocyte RAGE expression compared to human Type 2 diabetic subjects not taking NSAIDs (Figure 6-14).

6.4.3.2 Effect of NSAIDs on RAGE protein levels in Type 1 diabetes

Human Type 1 diabetic subjects with NSAID treatment regime showed a significant reduction in platelet and monocyte RAGE expression compared to human Type 1 diabetic subjects not taking NSAIDs (Figure 6-15).
Figure 6-14

Quantitative densitometry of RAGE protein expression in the presence of NSAIDs in human subjects (Type 2 diabetes, non age-matched)

Non age-matched analysis of RAGE expression in Type 2 diabetes in the presence of NSAIDs. Panel A: Platelet RAGE protein expression was measured in human Type 2 diabetic subjects with and without NSAIDs. Panel B: Monocyte RAGE protein expression measured in human Type 2 diabetic subjects with and without NSAIDs. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of subjects. * significant difference from Type 2 diabetes not taking NSAIDs, $p<0.05$, 1-way ANOVA (Dunn’s multiple comparison test).
Figure 6-15
Quantitative densitometry of RAGE protein expression in the presence of NSAIDs in human subjects (Type 1 diabetes)
Analyses of RAGE expression in Type 1 diabetes in the presence of NSAIDs. Panel A: Platelet RAGE protein expression was measured in human Type 1 diabetic subjects with and without NSAIDs. Panel B: Monocyte RAGE protein expression measured in human Type 1 diabetic subjects with and without NSAIDs. A rat platelet or monocyte standard control was run with each Western blot and the density of all sample bands was expressed as a percentage of the corresponding standard control. Values represent the mean, S.E. and number of subjects. * significant difference from Type 2 diabetes not taking NSAIDs, $p<0.05$, 1-way ANOVA (Dunn’s comparison test); # significant difference from Type 2 diabetes not taking NSAIDs, $p<0.05$, 1-way ANOVA (Tukey’s multiple comparison test).
6.5 DISCUSSION

Non-enzymatic glycosylation macromolecules such as proteins, enzymes and lipids results in the formation of irreversible AGEs (Maillard 1912; Brownlee et al. 1988). The consequence of AGE formation is reduced enzyme activity, alteration in protein structures and altered protein interactions (Facchiano et al. 2002; Verzijl 2002; Badenhorst 2003). Increasing evidence suggests that the engagement of AGES to specific receptors such as RAGE plays a pivotal role in the accelerated vascular dysfunction observed in diabetic animal models (Park et al. 1998; Kislinger et al. 1999; Kislinger et al. 2001).

The present study investigated the presence of RAGE in diabetes since RAGE up-regulation can be induced by AGES (Schmidt et al. 1999; Buenting et al. 2001). The two cell types chosen in this study were platelets and monocytes, which were isolated from human Type 1 and Type 2 diabetic subjects. Previous work has shown elevation in RAGE in a variety of animal tissues including the VSMCs, endothelial cells, heart and aorta (Sun et al. 1998), lacrimal glands (Alves et al. 2005) of STZ-treated rats, a Type 1 diabetic animal model. In humans, mononuclear cells of Type 2 diabetic patients with vascular complications also have elevated RAGE (Buchs et al. 2004), but otherwise there have been few human studies in platelets or monocytes.

6.5.1 RAGE LEVELS IN TYPE 2 DIABETES

6.5.1.1 Platelet RAGE in Type 2 diabetes

The result of the present study found that RAGE levels in platelets were significantly, but only slightly increased (15%) in Type 2 diabetes. After age-matching, however, increased RAGE levels were no longer statistically significant (Figure 6-6). RAGE levels were only weakly correlated with age (Figure 6-5) therefore, the lack of significance is most likely due to the decrease in sample size in the age-matching process, particularly since the elevation prior to age-matching was modest. To the best of our knowledge, the present study was the
first to measure platelet RAGE levels in Type 2 diabetes with meaningful sample sizes. Previously only a small study by Buenting et al. (2001) reported the presence of RAGE in platelets in both human diabetic and non-diabetic subjects ($n=9$ and $n=2$, respectively), but no meaningful conclusions could be made.

There is clear evidence of platelet hyperactivity in Type 2 diabetes (Section 3.4.1.2), but it is unclear whether increased RAGE levels in platelets could play a role since platelets are devoid of genetic material and the most prominent RAGE actions are related to changes in gene expression (Yan et al. 1994). There is, however, a possibility that the pre-platelet stage of megakaryocytes may be affected. This may alter platelet architecture and signaling pathways, which persist in matured platelets. Alternatively, lack of nuclear material does not necessarily preclude up-regulation of proteins and receptors in platelets. There is evidence for enhanced expression of proteins such as P-selectin, CD 40 ligand, and receptors such as thromboxane receptor $\alpha$ in platelets, which are pre-formed and stored in the $\alpha$ granules in platelets. Upon activation, these proteins/receptors are then redistributed to the surface of the platelets (Stenberg et al. 1985; Berman et al. 1986; Henn et al. 1998; Nurden et al. 2003).

### 6.5.1.2 Monocyte RAGE in Type 2 diabetes

The present study showed that human Type 2 diabetic subjects have significantly increased monocyte RAGE levels compared with human control subjects (13%). Age-analysis found that monocyte RAGE expression remained significantly elevated in human Type 2 diabetic subjects compared with age-matched human control subjects (Figure 6-6). There was no correlation between age and RAGE expression in monocytes (Figure 6-5). In the literature, several studies reported that RAGE was up-regulated in diabetes. For example, Buchs et al. (2004) observed increased RAGE expression in peripheral blood mononuclear cells of human Type 2 diabetic subjects with vascular complications compared to human diabetic subjects without complications. Other studies also observed increased RAGE levels in various
tissue/cell types. For example, RAGE was up-regulated in endothelium, VSMCs, monocyte-derived macrophages and cardiac myocytes of a bovine diabetic model (Brett et al. 1993). In STZ-induced Type 1 diabetic mice, RAGE expression was found to be up-regulated in the aorta and kidney compared with non-diabetic animal (Kislinger et al. 2001).

Enhanced RAGE expression may be crucial to the development of atherosclerosis in diabetes. Several animal studies have reported that treatment of diabetic mice with advanced atherosclerosis with soluble RAGE (a RAGE inhibitor) suppressed the development of atherosclerosis (Park et al. 1998; Wendt et al. 2006). Monocyte infiltration and conversion to macrophages are pivotal steps in atheroma formation and hence monocyte RAGE may be important. The engagement of RAGE-bearing monocytes by AGE ligands can lead to chemotaxis (Schmidt et al. 1993) and monocyte infiltration to the endothelial monolayer (Kirstein et al. 1990; Vlassara et al. 1992). In atherosclerotic plaques isolated from human Type 2 diabetic subjects, Cipollone et al. (2003) observed an enhanced macrophage and T cell infiltration compared to non-diabetic plaques accompanied by increased RAGE expression. Further immuno-staining showed that active macrophages in these plaques actually expressed RAGE. Cipollone and colleagues further reported increased activation of NF-κB in diabetic plaques and this showed a strong concordance with RAGE expression, although further studies are needed to determine a direct relationship.

Interestingly, increased RAGE expression in monocytes may potentially link to COX-mediated pathways as Cipollone et al. (2003) found that increased RAGE was co-localized with increased COX-2, PGE synthase and MMP expression in the activated macrophages derived from diabetic atherosclerotic plaques. Another study by Bucciarelli et al. (2002) showed that COX-2 antigen levels were reduced in the aorta of diabetic mice in the presence of soluble RAGE. The present study, however, did not observe a correlation between monocyte RAGE and monocyte COX-2 expression (Figure 6-7). It is important to note that
the present study did not study macrophages and the differences between the two cell types may account for the different experimental findings.

6.5.2 RAGE LEVELS IN TYPE 1 DIABETES

6.5.2.1 Platelet RAGE in Type 1 diabetes

Increasing evidence suggests that RAGE up-regulation in some cell types is involved in long-term diabetic complications in Type 1 diabetes. For example, in animal models, RAGE is implicated in diabetic nephropathy (Jensen et al. 2006) possibly through induction of TGF-β and cytokines that promote the trans-differentiation of epithelial cells to form myofibroblasts resulting in eventual diabetic nephropathy (Oldfield et al. 2001). A large study involving eight hundred and thirty eight subjects further found that 82 Ser allele of the RAGE gene is a risk allele for developing advanced nephropathy (Prevost et al. 2005).

The present study found an increase in RAGE levels in platelets isolated from human Type 1 diabetic subjects (38%, Figure 6-10). Age was not a confounding factor as RAGE was not correlated with age in these subjects (Figure 6-9). Of all cells studied (platelets and monocytes) in both Type 1 and Type 2 diabetic patients, this was the largest change observed. Whether this plays a role in platelet hyperactivity in diabetes remains to be seen.

6.5.2.2 Monocyte RAGE in Type 1 diabetes

The current study reported an increase (23%) in RAGE expression in monocytes isolated from human Type 1 diabetic subjects (Figure 6-10). This increase was not affected by the age of the subjects (Figure 6-9). Burke et al. (2004) found that an increased presence of RAGE expressed macrophages around and within necrotic cores of atherosclerotic plaques in human Type 1 diabetic subjects, which is consistent with the current observation, although another study by Miura et al. (2004) reported that monocyte expression of RAGE mRNA did not significantly differ between human control and human Type 1 diabetic subjects.
A study in murine RAW 264.7 macrophage cell line reported up-regulation of COX-2 expression by AGEs (Lin et al. 2002). A similar study was also reported by Shanmugam et al. (2003). S100b, a specific RAGE ligand, was found to induce COX-2 mRNA and protein expression, but not COX-1 in cultured human monocytes isolated from human Type 1 diabetic subjects. The elevated expression of RAGE in monocytes in the current study was not correlated with monocyte COX-2 expression (Figure 6-12). Further, there was no correlation between monocyte COX-2 expression and AGEs as measured by plasma hemoglobin A1C (an AGE) (Figure 6-13). Despite lack of correlations, enhanced RAGE expression in monocytes as seen in the present study, which may further differentiate into macrophages, may contribute to the development of macro-vascular complications in diabetes (see Section 6.5.1.2).

### 6.5.2.3 AGE production and RAGE expression in diabetes

One clinical measure of AGEs is plasma hemoglobin A1C. In the present human study, this was elevated in both Type 1 and Type 2 diabetic subjects (Tables 4-1 and 5-1). AGEs have been suggested to stimulate RAGE expression (Schmidt et al. 1999). Changes in RAGE expression observed in the current study were small making it difficult to see any correlation between plasma hemoglobin A1C and RAGE (Figures 6-8 and 6-11). However, in the case of platelet RAGE in Type 1 diabetes where RAGE expression increased by 38% (the maximum effect seen in any cell, Figure 6-10), a relationship to plasma hemoglobin A1C was evident with an R² of 0.38 (Figure 6-11).

### 6.5.3 EFFECT OF NSAIDS ON RAGE LEVELS IN HUMAN DIABETIC SUBJECTS

Accelerated atherosclerosis and micro-vascular diseases such as retinopathy and nephropathy are common complications associated with diabetes (Kannel & McGee 1979; see Review by Schuster & Duvuuri 2002). The underlying mechanisms of these chronic diabetic complications have been discussed in details in Section 3.4 and in the introduction of this chapter, Section 6.1. One of the mechanisms involves the signaling of RAGE. RAGE has
been implicated in chronic inflammation. Ligands such as AGEs can bind to RAGE and result in increased IL-2 expression and cell proliferation in lymphocytes and increased IL-1β and TNF-α in mononuclear phagocytes (Hofmann et al. 1999; see Review by Schafer & Heizmann 1996). NSAIDs, notably aspirin, are successfully used clinically to reduce pain and inflammation in chronic conditions such as arthritis and in some cases to reduce the risk of cardiovascular diseases in high risk groups including diabetes by inhibiting the activity of COX enzymes (see Review by Vane & Botting 2003; for more details see Section 4.5.4). This preliminary study investigated the effect of NSAIDs on RAGE levels in both Type 1 and Type 2 diabetes.

6.5.3.1 Effect of NSAIDs on RAGE levels in human diabetic subjects

RAGE levels in platelets and monocytes in human diabetic subjects receiving NSAID treatment were significantly lower than that of human non-diabetic controls indicating that NSAIDs may reduce RAGE expression (Figures 6-14 and 6-15). The mechanisms involved in down-regulation of RAGE by NSAIDs are unclear, although fluorescent-induced AGE formation has been shown to be down-regulated in rat tail tendon by ibuprofen (Menzel & Reihsner 1996) and similarly AGE formation in human corneal and scleral collagen with age was reduced by aspirin (Malik & Meek 1994). These data suggest that potentially RAGE expression could be down-regulated through reduced AGE/RAGE interaction, which may be of importance in lowering the development of diabetic complications.

To the best of our knowledge, a reduction of RAGE level in platelets and monocytes in diabetic subjects by NSAIDs is a novel finding. Down-regulation of RAGE can potentially affect a variety of downstream signaling transduction pathways involving NF-κB, MMP, TGF-β and TNF-α, which have been shown in various cell types including monocytes and mesangial cells (Throckmorton et al. 1995; Li & Schmidt 1997; Bierhaus et al. 2001; Cipollone et al. 2003; see Review by Collins 1993). The current findings suggest that the use
of NSAIDs may reduce the risk of cardiovascular events in diabetic subjects through these RAGE-mediated signaling pathways

6.6 CONCLUSIONS

Moderate to small increases in RAGE levels were observed in platelets and monocytes in both Type 1 and Type 2 diabetes. Increased RAGE levels were reduced by the treatment of NSAIDs in human diabetic subjects, suggesting RAGE may be in fact a crucial player in the inflammatory process in diabetes. The ability of NSAIDs to down-regulate RAGE may be of importance in the management of diabetic complications.
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APPENDIX
10 June, 2004

Dr A Jenkins
Dept of Medicine

Dear Dr Jenkins

HREC PROTOCOL NO: HREC-A 024/00
‘Glycooxidation, HDL and diabetes angiopathy.’
Dr A Jenkins, Prof J Best, Dr K D’Souza, Dr P Colman, Dr D Prior, Dr A Wilson, Dr K Rowley & Dr C Nelson

Thank you for your letter of 20 May 2004 requesting an extension for the above project for four years in order to recruit a further 250 participants.

Approval has been granted to extend this project until 10 June 2008 using the participant information and consent form version 3 dated 03/03.

Yours sincerely

Jill Hambling
Secretary, Human Research Ethics Committee
Participant Information and Consent Form
024/00 Version 3 Dated 03/03
Site: St Vincent's Hospital

Full Project Title: Glycoxidation, HDL and Diabetic Angiopathy
Principal Researcher: Alicia Jenkins
Associate Researcher(s): James Best, Peter Colman, Andrew Wilson, Craig Nelson, Kevin Rowley, David Prior and Karen D'Souza

This Participant Information and Consent Form is 9 pages long. Please make sure you have all the pages.

1. Your Consent
You are invited to take part in this research project.

This Participant Information contains detailed information about the research project. Its purpose is to explain to you as openly and clearly as possible all the procedures involved in this project before you decide whether or not to take part in it.

Please read this Participant Information carefully. Feel free to ask questions about any information in the document. You may also wish to discuss the project with a relative or friend or your local health worker. Feel free to do this.

Once you understand what the project is about and if you agree to take part in it, you will be asked to sign the Consent Form. By signing the Consent Form, you indicate that you understand the information and that you give your consent to participate in the research project.

You will be given a copy of the Participant Information and Consent Form to keep as a record.

2. Purpose and Background
The purpose of this project is to investigate whether levels of various substances in the blood or urine, or related variations in your genes (DNA) are associated with blood vessel damage in diabetes. You may also be asked to have an ultrasound of your heart as studies by other researchers suggest that the pattern of heart muscle contraction and relaxation may be different in diabetes.

Previous experience has shown that only about one in three or four people with diabetes develop complications due to damage to the large blood vessels, such as in the heart, and to small blood vessels, such as in the kidney and eyes. This damage can result in heart attacks, strokes, poor circulation to the legs, kidney failure and loss of vision. Recent studies suggest that the pattern of heart muscle contraction and relaxation is related to levels of potentially toxic substances in the blood that are being measured in this project. While we know that some
inherited factors, poor blood sugar control, smoking and high blood pressure are risk factors for the development of diabetes complications, we do not have a good enough range of blood, urine or genetic (DNA) tests to predict early on whether people with diabetes are prone or resistant to such complications. Knowledge of types of DNA or of blood or urine factors that are associated with or predict blood vessel or heart damage will also suggest new treatments to prevent or slow down complications. Using ultrasound equipment we also aim to determine if heart muscle function is different in people with diabetes.

You are invited to participate in this research project because you are 18 years of older and either have diabetes, or are a healthy person without diabetes with whom we will compare results of people with diabetes.

The results of this research may also be used to help researchers Drs' Andrew Wilson, Craig Nelson and Karen D’Souza to obtain a degree.

A total of 200 people with diabetes and 200 people without diabetes will participate in this project.

3. Procedures

After a 10 - 12 hr overnight fast (during which water is permitted), up to 50cc of blood (one eighth that taken by the Red Cross for blood donation or three and half tablespoons) will be removed from a vein in the arm through a needle. You will also be asked to provide a urine sample. The duration of participation in this study is that which is necessary to obtain a single blood sample and urine sample, and will take approximately twenty to thirty minutes. Where possible the blood and urine testing will be added to tests that your doctor would be requesting as part of your normal care. Your health record may be reviewed by one of the doctors involved in the research to confirm your diagnosis and medications. Some routine analyses including levels of blood sugar control, kidney function, and blood fats will be performed, and you will be provided with the results of these tests. If you wish we will also provide you with the results of the specialized tests that we will do, but the significance of the results with regard to current your future health is uncertain. That is why we are conducting the research.

We will measure levels of various substances in your blood and urine, and variations in your DNA that may (in the future) help doctors to predict which people with diabetes may be at high risk of kidney and heart damage. We also request permission to store any remaining DNA from your blood sample for up to twenty years, so that it can be used in future research by our research group or our collaborators in diabetic and heart research. The remaining DNA will be destroyed immediately if you do not wish to allow us to store your DNA for future related research. If you do permit storage, and your sample is not used by twenty years then we will destroy the DNA. If you do not agree to DNA collection you may still participate in the other aspects of the study.

We will compare levels of various factors measured in people with vs without diabetes, in people with diabetes with and without complications (such as kidney damage) and will relate results to factors such as length of diabetes and to measures of blood sugar control.
Some research participants will have an ultrasound of the heart. This test, which takes about an hour, involves a painless ultrasound test of the heart while lying down and at rest. No fasting, needles or drugs are required. This test measures the pattern and strength of heart contraction, and does not determine the presence or absence of coronary artery disease. The test will be performed by a doctor or by a certified cardiac technician. This test can be performed on the same day as the above blood and urine tests, or if you prefer, on another day. This part of the study is optional. Not participating in this aspect of the project does not exclude participation in the rest of the project.

4. Possible Benefits

Possible benefits include recognition of heart disease risk factors and knowledge of your recent blood sugar control as you will be provided with the results of tests of your blood fats, blood sugar, and kidney function. If you have an ultrasound test of your heart a mild previously undetected heart valve problem, such as a leaky valve, may be identified and further consultation with a cardiologist (heart specialist) would be recommended. In general, the results of these tests may benefit medical science by helping to understand the reasons why some people with diabetes are more susceptible to developing complications than others. The results may also suggest means, such as improved blood sugar and blood fat control and antioxidant supplementation which may slow the development of blood vessel damage. Thus, some significant benefits from the project may only accrue to members of society with diabetes in the future and NOT to the individuals taking part in the project. We cannot guarantee or promise that you will receive any benefits from this project.

5. Possible Risks

Possible risks, side effects and discomforts include the inconvenience of attending for blood sampling and urine collection. This inconvenience will be minimized by including the sample collection for research along with that that your doctor is requesting for your usual health care.

There are the following risks and/or discomforts associated with blood sampling:
1. There may be a slight pain when the arm is stuck with the needle.
2. A bruise may be left temporarily at the spot where the arm is stuck. The risk is approximately 1/100.
3. There is a slight chance of inflammation of the vein and/or clot formation. The risk is approximately 1/5000.
4. There is a risk of feeling faint. The risk is approximately 1/100.
5. There is a risk of allergy to band-aids.

To minimize these risks an experienced health care professional will take the blood sample in a sterile manner while you are lying down. Cotton wool and a bandage or band-aid will be applied after.

There is also the risk that you may be upset by the finding of high blood sugars, a bad blood fat (cholesterol) profile or kidney function. Medical treatment is available for all of these conditions. A doctor will review the results of the tests for which there is known risk to health and treatment available. You will be advised of
any abnormal results and offered copies of results to be sent to your treating doctor(s) or given suggestions of where and how to have abnormal test results followed up.

If you have an ultrasound, you may be uncomfortable with having to expose your chest. A hospital gown will be worn to preserve modesty.

There may be additional unforeseen or unknown risks.

6. Alternatives to Participation
There is no alternative means by which we can conduct this research.

7. Privacy, Confidentiality and Disclosure of Information
In addition to details provided by you on the day of sample collection we may require additional information from your usual treating doctors. For e.g. we may need to review your St Vincent's Hospital record.

Blood and urine samples for routine analyses will be sent to the Chemical Pathology Department with full identification (e.g. name, address, date of birth, hospital record number if applicable) so that these results can be given to you if desired and utilized (with your permission) by your treating doctors for your usual health care.

Results of these tests and the special tests performed by our research laboratory will be entered into a secured electronic data-base that is accessible only by the investigators. The DNA will be coded such that only the Principal Investigator will be aware of your identity. DNA tests performed will not be made available to prove or disprove parenthood.

When data is being analyzed and presented (such as at conferences and in research journal publications) there will be no identifiers that will enable anyone to trace the results back to you. Results of groups of people rather than individuals will be presented.

Any information obtained in connection with this project and that can identify you will remain confidential. Results of routine testing will only be disclosed to you and at your request to your treating doctors with your permission, except as required by law. If you give us your permission by signing the Consent Form, we plan to discuss results of routine tests only with you and if you wish with your treating doctors. We plan to discuss results of the study group at medical research conferences and publish in medical journals. In any publication, information will be provided in such a way that you cannot be identified.

Data will be stored in a locked office and password protected data-base. Once all sample analysis and manuscripts have been prepared then data will be kept for at least seven years after final publication, in keeping with usual research practice.
We do not foresee any commercial development of the research results.

8. **New Information Arising During the Project**
   During the research project, new information about the risks and benefits of the project may become known to the researchers. If this occurs, you will be told about this new information. This new information may mean that you can no longer participate in this research. If this occurs, the person(s) supervising the research will stop your participation. In all cases, you will be offered all available care to suit your needs and medical condition.

9. **Results of Project**
   Results of the routine health care tests will be provided to you by telephone or by post or at your next clinic visit. Results of the overall project will be made available by a form letter sent to all participants and an article in the St Vincent’s Hospital Diabetes clinic newsletter (which you can request to be sent even if you do not attend the clinic).

10. **Further Information or Any Problems**
    If you require further information or if you have any problems concerning this project, you can contact the principal researcher Dr Alicia Jenkins, Dr James Best, Dr Kevin Rowley or Dr David Prior. The contact numbers of these responsible researchers are 03 9288 2745 for Dr’s Jenkins, Best and Rowley and 03 9288 4423 for Dr Prior during the day, or 0407229375 for Dr Jenkins, and after hours for all investigators via the hospital switchboard 03 9288 2211 or to Dr Jenkins 0407229375.

11. **Complaints**
    If you have any complaints about any aspect of the study or the way in which it is being conducted you may contact the Patient Representative at St Vincent’s Health on Telephone: 9288 2211. You will need to tell the Patient Representative the name of the person who is noted above as principal investigator.

12. **Participant’s Rights**
    If you have any questions about your rights as a research participant, then you may contact

    **Name:**  Jill Hambling  
    **Position:**  Grants and Research Office  
    **Telephone:**  9288 3930  

    You will need to tell Ms Hambling the name of the Principal Investigator, Dr Alicia Jenkins.
13. Participation is Voluntary

Participation in any research project is voluntary. If you do not wish to take part you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with St Vincent’s Hospital.

Before you make your decision, a member of the research team will be available to answer any questions you have about the research project. You can ask for any information you want. Sign the Consent Form only after you have had a chance to ask your questions and have received satisfactory answers.

If you decide to withdraw from this project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to inform you if there are any health risks or special requirements linked to withdrawing. We do not envisage this to be the case as this is a single visit study.

14. Ethical Guidelines

This project will be carried out according to the National Statement on Ethical Conduct in Research Involving Humans (June 1999) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

The ethical aspects of this research project have been approved by the Human Research Ethics Committee of St Vincent’s Hospital.

15. Reimbursement for your costs

You will not be paid for your participation in this project.

However, you will be provided with breakfast and may also be reimbursed for any travel or parking costs that you incur as a result of participating in this trial.
Consent Form
Version 024/00 Version 2 Dated 03/03
Site St Vincent’s Hospital

Full Project Title: Glycooxidation, HDL and Diabetic Angiopathy

I have read, or have had read to me in my first language, and I understand the Participant Information version 2 dated 03/03.

I freely agree to participate in this project according to the conditions in the Participant Information.

Specifically I agree to:

☐ provide blood, urine and DNA
☐ provide blood and urine, but not DNA
☐ have a heart ultrasound

I will be given a copy of the Participant Information and Consent Form to keep.

The researcher has agreed not to reveal my identity and personal details if information about this project is published or presented in any public form.

Participant’s Name (printed) ..........................................................

Signature ................................................ Date

Name of Witness to Participant’s Signature (printed) ..........................................................

Signature ................................................ Date

Researcher’s Name (printed) ..........................................................

Signature ................................................ Date
I certify that I have translated the above explanation and declaration and assisted Dr. ................. With the oral translation to the person below in the ................. language which the person has indicated he/she understands.

Interpreter: ................................................................. Date:
Revocation of Consent Form

Full Project Title: Glycoxidation, HDL and Diabetic Angiopathy

I hereby wish to WITHDRAW my consent to participate in the research proposal described above and understand that such withdrawal WILL NOT jeopardise any treatment or my relationship with St Vincent’s Hospital.

Participant’s Name (printed) .................................................................

Signature  Date