The effects of DPP-4 inhibitors on vascular endothelial function in diabetes

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

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Salheen Madani T. Salheen
Date: 28.07.2015
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Summary

Diabetes mellitus is an independent risk factor for cardiovascular disease. It is well known that hyperglycaemia causes overproduction of reactive oxygen species that leads to vascular endothelial dysfunction. Endothelial cells, lining all blood vessels, modulate the tone of the underlying smooth muscle by responding to mechanical forces and neurohumoral mediators with the release of a variety of contracting and relaxing factors. Endothelium-dependent relaxing factors include nitric oxide (NO), prostacyclin and an unidentified cause of endothelium-dependent hyperpolarization (EDH). It is well established that endothelial dysfunction plays a critical role in the initiation and development of cardiovascular complications. The aim of this thesis was to investigate the effects of a high concentration of glucose, hyperglycaemia in diabetes, and consumption of a western, high fat, diet on the mechanism(s) of endothelium-dependent relaxation in rat mesenteric artery. Further, the effect of acute and chronic treatment with dipeptidyl peptidase-4 (DPP-4) inhibitor linagliptin was investigated on endothelium-dependent relaxation in normal and diabetic rat mesenteric artery. As oxidative stress plays a critical role in the initiation of endothelial dysfunction, it was hypothesised that drugs with antioxidant activity might be helpful in maintaining normal vascular function in diabetes.

In the first study, the aim was to investigate the effects of DPP-4 inhibitors and a glucagon-like peptide 1 receptor (GLP-1R) agonist, exendin-4, on the mechanism(s) of endothelium-dependent relaxation in rat mesenteric arteries exposed to a high concentration of glucose (40 mM). Organ bath techniques were employed to investigate vascular endothelial function in rat mesenteric arteries in the presence of normal (11 mM) or high (40 mM) glucose concentrations. Incubation of mesenteric artery rings with high
glucose for 2 h caused a significant increase in superoxide anion generation and a significant impairment of endothelium-dependent relaxation. Exendin-4 and the DPP-4 inhibitor linagliptin, but not sitagliptin or vildagliptin, significantly reduced vascular superoxide levels and improved endothelium-dependent relaxation in the presence of high glucose. The beneficial actions of exendin-4, but not linagliptin, were attenuated by the GLP-1R antagonist exendin fragment (9-39). Further experiments demonstrated that the presence of high glucose impaired the contribution of both nitric oxide and endothelium-dependent hyperpolarization to relaxation and that linagliptin improved both mechanisms involved in endothelium-dependent relaxation. These findings demonstrate that high glucose impaired endothelium-dependent relaxation can be improved by exendin-4 and linagliptin, likely due to their antioxidant activity and independently of any glucose lowering effect.

In the second study, the aim was to investigate the effect of the acute presence of linagliptin, a DPP-4 inhibitor, *in vitro* on the mechanism(s) of endothelium-dependent relaxation in rat mesenteric arteries isolated from streptozotocin (STZ)-induced diabetic rats after 10 weeks of diabetes. The study demonstrated that endothelial dysfunction was due to a decreased contribution of both NO- and EDH to endothelium-dependent relaxation. Furthermore, endothelial dysfunction was associated with elevated oxidative stress due the increased activity of NADPH-oxidase. This study demonstrated that acute treatment with linagliptin reduced the levels of oxidative stress and improved endothelial function by improving of the contribution of NO and EDH to endothelium-dependent relaxation in mesenteric artery from the diabetic rats.
A high-fat ‘western’ diet (WD), a trigger for the development of type 2 diabetes, may cause endothelial dysfunction as one of the earliest events in atherogenesis. In the third study, the aim was to examine whether consumption of a WD affected endothelium-dependent relaxation of rat mesenteric arteries and whether the DPP-4 inhibitor linagliptin improves endothelium-dependent relaxation. Wistar Hooded rats were fed a standard diet (SD, 7% total fat) or WD (21% total fat) for 17 weeks. Consumption of the WD significantly increased superoxide release from mesenteric arteries assayed by lucigenin chemiluminescence and linagliptin significantly reduced the vascular superoxide production. WD significantly reduced the sensitivity to acetylcholine (ACh) and acute treatment with linagliptin improved endothelial function. The contribution of EDH to ACh-mediated relaxation was determined in the presence of L-NNA and ODQ to block NOS and guanylate cyclase respectively. The study demonstrated that EDH-type relaxation was improved by linagliptin. Furthermore, acute treatment with linagliptin also significantly improved the contribution of NO (determined in the presence of TRAM-34 + apamin to block IKCa and SKCa) to relaxation. The results demonstrated that acute treatment with linagliptin in vitro significantly reduced vascular superoxide levels and improved the contribution of both NO and EDH to preserve endothelium-dependent relaxation in small mesenteric arteries isolated from rats fed a high fat diet.

In the fourth study, we hypothesized that long-term chronic treatment with DPP-4 inhibitor linagliptin in vivo (4 weeks, 2 mg/kg per day oral gavage), would improve relaxation in mesenteric arteries from STZ-induced diabetic rats. The lucigenin-enhanced chemiluminescence assay was used to measure superoxide production in mesenteric arteries from normal and diabetic rats. The study demonstrated that chronic treatment with linagliptin did not affect plasma glucose but markedly reduced the levels of superoxide
production and improved endothelium-dependent relaxation in mesenteric arteries from type1 STZ-induced diabetic rats. Treatment with linagliptin selectively improved NO-mediated relaxation in rats associated with an increased expression of eNOS, reduced eNOS uncoupling and down regulation of the NADPH-oxidase subunit Nox2 expression.

In conclusion, this thesis extends our understanding of the pathological process underlying endothelial dysfunction in the microvasculature caused by exposure to high glucose concentrations *in vitro*, the consumption of a high fat diet, and sustained hyperglycaemia. All of these factors caused oxidative stress via increased activation/expression of NADPH-oxidase and eNOS uncoupling and subsequently endothelial dysfunction. Long-term chronic treatment with linagliptin in STZ-induced diabetic rats had no effect on plasma glucose levels, indicating that linagliptin exerted its improvement in vascular function via a mechanism independent of glucose lowering. The beneficial protective actions of linagliptin likely occur via at least two mechanisms; a GLP-1 receptor independent pathway in which the linagliptin is able to rapidly scavenge ROS and inhibit NADPH oxidase as a source of superoxide production and reduced eNOS uncoupling to preserve NO bioavailability or through a GLP-1 receptor dependent pathway. These beneficial actions make linagliptin a potential adjunctive treatment for diabetic vascular complications in patients with both type 1 and type 2 diabetes importantly expanding the range of patients able to benefit from the use of this drug.
Chapter 1

Literature review

1.1 Introduction

Diabetes mellitus, a metabolic disorder characterized by hyperglycaemia, is a disease that has been rapidly increasing worldwide. According to the fact sheet of the World Health Organization (WHO), there are more than 346 million people worldwide suffering from diabetes which can lead to complications such as atherosclerosis, nephropathy, and retinopathy (WHO, 2011). In 2004, an estimated 3.4 million people died from the consequences of high blood glucose (WHO, 2011). It has been reported that by 2030, about 438 million people worldwide are expected to have diabetes (Donald et al., 2012). In 2010, the prevalence of diabetes mellitus in adults in the United Kingdom and the United States was 7% and 11% respectively (Noble et al., 2011). In Australia, more than 3.5 million Australians have diabetes. Diabetes mellitus in Australia is the cause of a considerable portion of the total burden of diseases, and significant costs. For example, Type 2 diabetes costs Australia $3 billion a year (Australia Diabetes, 2011).

Hyperglycaemia can result as a consequence of a deficiency in the release of insulin or result from insulin resistance. Patients with diabetes are classified into two groups; patients having type 1 (insulin-dependent) or type 2 (insulin-independent) diabetes mellitus. Prolonged periods of type 1 or type 2 diabetes mellitus, and thus an extended exposure to hyperglycaemia, can have severe effects on both the macro- and microvasculature. This is as an outcome of disturbances in several metabolic and
haemodynamic mechanisms that occur as a result of abnormal blood glucose homeostasis. Pathological conditions associated with these complications may develop in the nervous system (neuropathy), the eye (retinopathy), kidney (nephropathy), and cardiovascular system, all of which lead to the increased mortality and morbidity rates linked to advanced cases of diabetes mellitus (Australia Diabetes, 2011).

The principle of current medicines used in the treatment of diabetes is based on the idea of insulin replacement for type 1 diabetes, since insulin is not created by β-cells in the pancreas, or to enhance the insulin sensitivity or secretion for patients who are suffering from type 2 diabetes. These anti-hyperglycaemic treatments attempt to provide tight control of glucose levels and decrease detrimental macro- and microvascular risks and dysfunction caused by hyperglycaemia. But, large scale prospective studies for the two types of diabetes 1 and 2 such as the Diabetes Control and Complications Trial (DCCT/EDIC) and the UK Prospective Diabetes Study (UKPDS), demonstrated that tight control and adjustment of glucose level may slow, but cannot prevent the development of cardiovascular complications (David et al., 2005, Holman et al., 2008). Accumulating evidence suggests that reactive oxygen species (ROS), provoked by hyperglycaemia is the primary cause of the initiation and progression of diabetic cardiovascular complications including impairment of the modulatory role of the vascular endothelium that could initiate the development of these diabetic complications (Brownlee, 2001, De Vriese et al., 2000, Pieper, 1998). As a consequence, it is desirable to obtain alternative therapies that directly address the cardiovascular complications in order to minimize the high prevalence of morbidity and mortality related to diabetes.

Incretin based therapies are a pharmacological class of glucose lowering agents used as a new strategy in the treatment of patients with type 2 diabetes mellitus (Drucker, 2003). These compounds have less risk of hypoglycaemia and positively impact on the
obesity observed in this patient population (Amori et al., 2007). Dipeptidyl peptidase-4 (DPP-4) inhibitors, by inhibiting the action of the DPP-4 enzyme and increasing the availability of glucagon-like peptide-1 (GLP-1) and/or increasing its receptor (GLP-1R) stimulation, act to stimulate insulin secretion and inhibit glucagon secretion.

This review describes the critical role of high glucose, high fat diet and hyperglycaemia in diabetes-induced ROS production and their detrimental effects on the vascular endothelial cells. Furthermore, the evidence to suggest that DPP-4 inhibitors may be a promising therapy for treating cardiovascular complications in diabetes independently of their glucose lowering effect is described.

1.2 Biological action of vascular endothelium

The vascular endothelium, a simple monolayer of cells that covers the interior surface of all blood vessels, has been recognized to have several important physiological functions and is now considered as a purposefully placed, multifunctional organ (Epstein et al., 1990). The endothelium is located as a barrier between the circulating blood and the vascular smooth muscle and dynamically participates in the maintenance and control of vascular function (Behrendt and Ganz, 2002). The vascular endothelium play an important biological role in the regulation of vascular tone by producing endothelium-derived relaxing factors (EDRF) which include nitric oxide (NO), prostacyclin (PGI₂), and endothelium-dependent hyperpolarisation (EDH) (Figure 1.1).
Figure 1.1 Diagram representing the endothelium-derived vasoactive substances. Nitric oxide synthase (eNOS), stimulated by shear stress and/or acetylcholine, results in synthesis and release of nitric oxide (NO) which exerts its action via relaxation of vascular smooth muscle. The diagram also shows other endothelium-derived factors such as prostacyclin (PGI₂) and endothelium-dependent hyperpolarization (EDH). Adapted from (Vanhoutte et al., 2009).
1.2.1 Nitric oxide (NO) as EDRF

In 1980 Furchgott and Zawadzki, reported that the endothelium was necessary to mediate the ability of acetylcholine (ACh), and other agonists of muscarinic receptors, to induce relaxation of rabbit isolated aorta (Furchgott and Zawadzki, 1980). This effect of acetylcholine is mediated by an endothelium-derived relaxing factor that later was identified as nitric oxide (NO) (Palmer et al., 1987, Moncada et al., 1991). NO is a small molecule which readily diffuses to underlying smooth muscle and is generated, along with L-citrulline, from the cationic amino acid L-arginine by the enzyme nitric oxide synthase (eNOS) (Palmer et al., 1988). The activity of eNOS requires cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide, flavin dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH$_4$) and calmodulin (Bayraktutan, 2002, Hevel and Marletta, 1992) (Figure 1.2).

Besides the action of ACh, NO is synthesized and released from the endothelial cells in response to a large variety of chemical, physical and humoral stimuli including shear stress, platelet products (serotonin, adenosine diphosphate (ADP)), circulating hormones (catecholamines), receptor-independent agonists (e.g. calcium ionophores) and autocoids (histamine, bradykinin) which leads to stimulation of the eNOS enzyme and release of NO.

NO is a very unstable molecule with a half-life of 4-50 seconds before it is scavenged by oxygenated haemoglobin, superoxide anions or molecular oxygen (Rao, 1997, Nakagawa and Yokozawa, 2002). NO is recognized as a very strong vasodilator which, in addition, possesses antiatherogenic properties such as reducing the adhesion of platelets and leucocytes to the endothelium, and inhibition of VSMC migration (Radomski et al., 1987, Kubes et al., 1991). These actions are mainly mediated by the activation of soluble guanylate cyclase (sGC) which increases formation of cyclic
guanosine monophosphate (cGMP) inside smooth muscle cells or platelets causing relaxation and reduced aggregation respectively (Moncada et al., 1991, Ignarro et al., 1981).

1.2.2 Prostacyclin (PGI₂) as EDRF

Prostacyclin is thought to be one of the most important compounds in the prostanoids, a family of bioactive lipid mediators that are produced by cyclooxygenase (COX) from arachidonic acid (AA) present in the membrane of all cells of the body (McAdam et al., 1999). Under normal physiological conditions, prostacyclin acts as an effective vasodilator and contributes to the inhibition of platelet aggregation, adhesion of leukocytes, and proliferation of vascular smooth muscle cells (VSMC) (Gryglewski et al., 1976). These effects of PGI₂ are controlled by the stimulation and activation of PGI₂ receptors (IP) causing activation of adenylate cyclase and as a result, production of cyclic adenosine monophosphate (cAMP) is increased (Narumiya et al., 1999).
Figure 1.2 Shows the suggested mechanisms that regulate the creation of nitric oxide (NO) in endothelial cells. Activation of receptors leads to an increase in the concentration of Ca$^{2+}$. NO is synthesised by the conversion of L-arginine to L-citrulline through the action of eNOS; endothelial nitric oxide synthase. Some cofactors are required to synthesise NO such as BH$_4$; tetrahydrobiopterin, calmodulin, NADPH; nicotinamide adenine dinucleotide phosphate, FMN; flavin mononucleotide, and FAD; flavin adenine dinucleotide. EC; endothelial cell. Adapted from (Vanhoutte et al., 2009).
1.2.3 Endothelium-dependent hyperpolarization (EDH) as EDRF

EDH may be caused by an as yet chemically unidentified factor that is responsible for causing hyperpolarization of the VSMC that is not due to the action of NO or PGI₂ (Garland et al., 1995). EDH acts to regulate vasodilatation and mediates hyperpolarization of vascular smooth muscles (McGuire et al., 2001, Luksha et al., 2009, Chen et al., 1988, Edwards et al., 1998). The ability of EDH to contribute to endothelium-dependent relaxation is mainly noted in resistance vessels (Shimokawa et al., 1996, Busse et al., 2002) which are essential in maintaining blood pressure and regulating organ perfusion. EDH induced relaxation can be classified into two general classes, that is, a classical form and a non-classical form of EDH responses (Figure 1.3).
Figure 1.3 An illustration of the suggested EDH pathways associated with endothelial and smooth muscle ion channel opening. EETs; epoxyeicosatrienoic acids, HNO; nitroxyl, BK$_{Ca}$; large-conductance Ca$^{2+}$-activated K$^+$ channel, SK$_{Ca}$; small-conductance Ca$^{2+}$-activated K$^+$ channel subtype 3, IK$_{Ca}$; intermediate-conductance Ca$^{2+}$-activated K-channel, K$_{IR}$; inwardly-rectifying K-channels, [Ca$^{2+}$]; intracellular calcium concentration, EDH; endothelium-dependent hyperpolarization; MEGJ, myoendothelial gap-junction, EC; endothelial cell, VSMC; vascular smooth muscle cell. Adapted from (Edwards et al., 2010).
1.2.3.1 Classical form of EDH response

Endothelial cell activation by endothelium-dependent agonists such as ACh, leads to increased intracellular Ca$^{2+}$ which causes the beginning of the EDH response. Classical EDH responses basically involve the opening of Ca$^{2+}$-activated K$^{+}$-channels (K$_{Ca}$) both small conductance Ca$^{2+}$-activated K$^{+}$ channels (SK$_{Ca}$) and intermediate conductance Ca$^{2+}$-activated K$^{+}$ channels (IK$_{Ca}$) (Edwards et al., 1998, Komori and Vanhoutte, 1990). Application of a combination of apamin (selective SK$_{Ca}$ blocker) and charybdotoxin (non-selective inhibitor of large-conductance Ca$^{2+}$-activated K$^{+}$-channels (BK$_{Ca}$), and (IK$_{Ca}$) abolished EDH responses, indicating the vital role of these channels since iberiotoxin, a selective BK$_{Ca}$ blocker, was unable to substitute for charybdotoxin (Waldron, 1994). The SK$_{Ca}$ and IK$_{Ca}$ channels are located in the endothelium and are organized in endothelial microdomains, especially within projections close to the adjacent smooth muscle cells and nearby the inter-endothelial gap junctions. Hyperpolarization of the endothelial cells by the stimulation and activation of either SK$_{Ca}$ or IK$_{Ca}$ channels leads to K$^{+}$ efflux through them which can act as a diffusible EDH which stimulates vascular smooth muscle Na$^{+}$/K$^{+}$-ATPase and inwardly-rectifying K-channels (K$_{IR}$) respectively (Edwards et al., 1998, Sandow et al., 2006, Absi et al., 2007), resulting in smooth muscle hyperpolarization and relaxation.

The SK$_{Ca}$ and IK$_{Ca}$ channels are located in the endothelial cells in different endothelial microdomains and they facilitate the hyperpolarization process in the vascular endothelial cells through different downstream pathways (Sandow et al., 2006, Absi et al., 2007, Dora et al., 2008). SK$_{Ca}$ channels are located in caveolar partitions and preferentially stimulate vascular smooth muscle cell K$_{IR}$ channels (Absi et al., 2007). This has been supported by observations which demonstrated that the hyperpolarization of the VSMC, because of the opening of endothelial SK$_{Ca}$ channels by CyPPA, a selective opener of
SK_{Ca}, are specifically abolished not only by apamin, but also by a K_{IR} selective blocker, barium (Weston et al., 2010). In contrast, IK_{Ca} channels are non-caveolar (Weston et al., 2008) and largely exist within the endothelial cell projections that cross the internal elastic lamina. Hyperpolarization of VSMC, particularly by the activation of Na^+/K^+-ATPase, is due to the opening of IK_{Ca} channels (Sandow et al., 2009, Dora et al., 2008).

It is well recognized that K^+ efflux is a mediator of some EDH responses (Chrissobolis et al., 2000, Edwards et al., 1998, McNeish et al., 2005). The identification of specialized microdomains that are located on both endothelial cells and vascular cells has provided an enhanced understanding of how hyperpolarization can be enabled through electrical coupling via projections for myoendothelial gap junctions (MEGJs), with the probability of contribution from the changes in extracellular K^+ which works as a chemical mediator (Sandow et al., 2006). A number of experimental observations using rat mesenteric artery demonstrate that there is a coupling between adjacent endothelial cells by connexins such as CX37, CX40, and CX43 that are located in MEGJs which are spatially close to zones where there are clusters of SK_{Ca} channels (Sandow et al., 2006). On the other hand, densities of IK_{Ca} in the same blood vessels are established to be spatially connected with CX37 and CX40 MEGJs. This is consistent with supporting electrophysiological experiments which also suggested that IK_{Ca} were mainly expected to be linked with microdomains that are close to MEGJs in the endothelial cells (Crane et al., 2003). The significance of endothelial-VSMC communication and the presence of specialised microdomains is also supported by data which demonstrate the possibility to attenuate the electrical coupling of endothelial cells and VSMC through MEGJs by the application of inhibitors to block cell-cell coupling, resulting in impairment of the hyperpolarization and relaxation of VSMC by EDH (Edwards et al., 1999, Yamamoto et al., 1999, Little et al., 1995, Mather et al., 2005). In general, K^+ and myoendothelial
electrical coupling as possible candidates for EDH seem equally attractive interpretations of classical EDH responses, however perhaps not in all cases. Dora and colleagues, based on data from small mesenteric artery of rat, suggested a model of EDH responses wherein activation of endothelial cells by agonists such as ACh leads to increase Ca\(^{2+}\) in endothelial cells, consequently activating SK\(_{Ca}\) with subsequent spread via MEGJs, and the following activation of IK\(_{Ca}\) is dependent on the concentrations of Ca\(^{2+}\) in the area of endothelial cell projections (Dora, 2010, Dora et al., 2008). By the activation of IK\(_{Ca}\), which seems to be limited to the endothelial cell projections, leads to hyperpolarization of VSMC due to sufficient endothelial cell K\(^+\) efflux causing activation of VSMC Na\(^+\)/K\(^+\)-ATPase and possibly K\(_{IR}\) (Dora et al., 2008). Taken together, the mechanism of action of these two classical EDH pathways may act in a separate, parallel or even synergistic way. At any rate, activation of endothelial K\(_{Ca}\) channels as a starting point is essential in the action of both pathways.

1.2.3.2 Non-classical form of EDH response

Whereas the classical EDH response works via the opening of endothelial K\(_{Ca}\) channels, the non-classical EDH response causes hyperpolarization and relaxation of the VSMC independently of channels on the endothelium. It is suggested that when the endothelial cells are stimulated by shear stress or by local hormones such as bradykinin or substance P, elements such as nitrogen oxides (NO and nitroxy (HNO)), hydrogen peroxide (H\(_2\)O\(_2\)), and epoxyeicosatrienoic acids (EETs) are released from the endothelial cells and diffuse to VSMCs, causing hyperpolarization and relaxation (Bolotina et al., 1994, Edwards et al., 2000, Andrews et al., 2009).

It is widely documented that NO is an endothelium-derived relaxing factor, causing relaxation of the blood vessels through the activation of soluble guanylate cyclase (sGC)
(Palmer et al., 1987, Rees et al., 1989), but there is also evidence that NO causes VSMC hyperpolarization through the opening of BK$_{\text{Ca}}$ channels (Bolotina et al., 1994, Tare et al., 1990). In addition, the one electron reduced and protonated form of NO, HNO is synthesised endogenously and causes hyperpolarization and relaxation (Andrews et al., 2009, Ellis et al., 2000, Wanstall et al., 2001). The hyperpolarizing effect of HNO can be partially attenuated by iberiotoxin or 4-aminopyridine, demonstrating the involvement of both BK$_{\text{Ca}}$ and K$_{\text{v}}$ channels (voltage-gated channels) respectively (Andrews et al., 2009, Favaloro and Kemp-Harper, 2009, Yuill et al., 2011, Bullen et al., 2011).

Epoxyeicosatrienoic acids (EETs) are synthesised by the vascular endothelium as cytochrome P450 metabolites of AA. EET production occurs in response to agonists such as ACh and bradykinin or shear stress (Fisslthaler et al., 1999, Campbell et al., 1996) and there is evidence to suggest that K$_{\text{Ca}}$ channels on the VSMC and the endothelium are activated by EETs producing hyperpolarization and relaxation (Baron et al., 1997, Hoebel et al., 1997, Earley et al., 2005, Vriens et al., 2005, Edwards et al., 2000). Further observations indicate that EETs are able to activate BK$_{\text{Ca}}$ channels in the VSMC (Baron et al., 1997, Edwards et al., 2000). It has been reported that bradykinin stimulates the endothelial release of 11, 12 EET and 14, 15 EET which causes EDH responses in porcine coronary arteries via activation of endothelial SK$_{\text{Ca}}$ and IK$_{\text{Ca}}$, as well as maxi K$_{\text{Ca}}$-dependent VSMC hyperpolarization. This effect was blocked by the EET antagonist 14, 15-EEZE in addition to iberiotoxin (Edwards et al., 2000, Weston et al., 2005). Additionally, experimental results have shown the capacity of EETs to stimulate BK$_{\text{Ca}}$ channels in VSMC (Baron et al., 1997, Edwards et al., 2000). The mechanism of activation of BK$_{\text{Ca}}$ channels by EETs is complicated and isoform-dependent but the most physiologically applicable pathway is through the activation of transient receptor potential (TRP) channels in order to promote the influx of Ca$^{2+}$ into the VSMC which then stimulate
the release of Ca\(^{2+}\) from ryanodine sensitive Ca\(^{2+}\) stores and subsequently, the local increase of Ca\(^{2+}\) will activate \(\text{BK}_{\text{Ca}}\) which then leads to hyperpolarization and relaxation of the VSMC (Earley et al., 2005, Vriens et al., 2005, Watanabe et al., 2003, Grgic et al., 2009). In addition to activation of TRP channels in VSMC, TRP channels on the endothelium can be activated by EETs to generate endothelial hyperpolarization and consequently VSMC hyperpolarization (Earley et al., 2009, Fleming et al., 2007). Overall, these different actions indicate that EETs display both classical and non-classical EDH responses but, conclusions in regard to the mechanism of action of EETs differs depending on the type of arteries examined (Baron et al., 1997, Earley et al., 2009, Edwards et al., 2000, Fleming et al., 2007, Weston et al., 2005, Watanabe et al., 2003).

\(\text{H}_2\text{O}_2\) is synthesized through the reduction of superoxide anions that occur either spontaneously or by the enzyme Cu-Zn superoxide dismutase. Studies on the mice mesenteric artery have demonstrated that \(\text{H}_2\text{O}_2\) was able to cause relaxation and hyperpolarization of VSMC via the opening of \(\text{K}_{\text{Ca}}\) channels and production of \(\text{H}_2\text{O}_2\) is in response to ACh stimulation (Fujiki et al., 2005, Shimokawa, 2010). This EDH-mediated hyperpolarisation and relaxation in wild type and eNOS\(^{-/-}\) mice was inhibited by catalase, which metabolises \(\text{H}_2\text{O}_2\) (Fujiki et al., 2005, Shimokawa, 2010). In addition, there is evidence that \(\text{H}_2\text{O}_2\) -induced hyperpolarization is mediated, at least in part, by the opening of \(\text{BK}_{\text{Ca}}\) (Barlow and White, 1998) and \(\text{K}_{\text{ATP}}\) channels (Wei et al., 1996).

### 1.3 Generation of reactive oxygen and nitrogen species in diabetes

Diabetes-induced hyperglycaemia is now recognized to result in oxidative stress (Flammer and Luscher, 2010). “The term oxidative stress refers to a condition in which cells are subjected to excessive levels of molecular oxygen or its chemical derivatives
called reactive oxygen species (ROS)"(Bayraktutan, 2002). Reactive oxygen species are byproducts of normal cellular metabolism which can be valuable or detrimental to human health. Oxidative stress describes a condition in which intracellular production of ROS challenges the capability of cellular antioxidant systems to neutralise them, consequently causing serious cellular damage and complications such as endothelial dysfunction (Cai and Harrison, 2000). ROS can have either a useful or harmful effect to the body. The beneficial effects of ROS are exerted at low physiological concentrations and are used in host defence processes and also in many cellular signalling pathways such as responses to growth factors and stress (Yoon et al., 2002). The detrimental effect of ROS occurs when there is over production of ROS and/or impairment of endogenous antioxidant mechanisms (Flammer and Luscher, 2010). Overproduction of ROS in diabetes may cause damage to cellular DNA, lipids, and proteins through denaturation, thus impairing normal cellular function. For example, during diabetes, overproduction of ROS in the vascular endothelium may be the cause of endothelial dysfunction that could contribute to cardiovascular complications. Cellular enzyme systems that act as important sources of ROS include NADPH oxidase, endothelial nitric oxide synthase (eNOS), xanthine oxidase and the mitochondrial respiratory chain. These systems are discussed in detail below.

1.3.1 Categories of reactive oxygen species and nitrogen species

Reactive oxygen and nitrogen species may be present either in the form of free radicals or non-radicals. The free radicals are molecules that contain one or more unpaired electrons and have a high level of reactivity. In mammalian cells, molecular oxygen acts as a terminal electron acceptor in order to metabolize organic carbon and produce energy, therefore, free radicals obtained from molecular oxygen are the most important type of
radical species. The main types of ROS are superoxide anion \( (\text{O}_2^-) \), hydroxyl radicals \( (\cdot \text{OH}) \), peroxynitrite \( (\text{OONO}') \), and non-radicals such as \( \text{H}_2\text{O}_2 \).

### 1.3.1.1 Superoxide anion

Superoxide anions are a crucial source of ROS and have the ability to interact with other molecules either directly or indirectly via enzymatic reactions or metal catalyzed processes to increase the formation of secondary ROS. For example, in normal conditions, superoxide is short-lived and either rapidly metabolized to hydrogen peroxide \( (\text{H}_2\text{O}_2) \) through superoxide dismutase (SOD) or to peroxynitrite as a result of reaction with NO (Reiter et al., 2000, Beckman, 2003).

### 1.3.1.2 Hydroxyl radical

The hydroxyl radical is considered as one of the most reactive free radicals. Hydroxyl radicals have a very short half-life and have the ability to randomly oxidize many of the close targets within cells such as DNA (mutation), lipids (peroxidation) and proteins (oxidation) (Kehrer, 2000). The hydroxyl radical can be formed chemically either through the reaction between superoxide and hydrogen peroxide (Haber–Weiss reaction; \( \text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \text{O}_2 + \text{OH} + \cdot \text{OH} \)) (Kehrer, 2000), or as a result of the reaction of hydrogen peroxide in the presence of reduced metals (Fenton reaction; \( \text{H}_2\text{O}_2 + \text{Fe}^{2+}/\text{Cu}^{+} \rightarrow \text{Fe}^{3+}/\text{Cu}^{2+} + \text{OH} + \cdot \text{OH} \)) (Thomas et al., 2009).

### 1.3.1.3 Peroxynitrite

The reaction between NO and superoxide produces a reactive oxidant form peroxynitrite (Squadrito and Pryor, 1995, Szabo, 2003). Peroxynitrite is documented to act
as an influential oxidizing mediator causing nitration and hydroxylation of phenolic compounds, particularly tyrosine residues (Reiter et al., 2000, Jourd'heuil et al., 2001). In addition, hydroxyl radicals are generated by the protonation of peroxynitrite through the following chemical reactions (Hogg et al., 1992):

\[ \text{NO}^- + \text{O}_2^- \rightarrow \text{ONOO}^- \]

\[ \text{ONOO}^- + \text{H}^+ \rightarrow \cdot \text{OH} + \cdot \text{NO}_2 \]

1.3.1.4 Hydrogen peroxide

Hydrogen peroxide is a small, stable, non-radical ROS that has the ability to readily diffuse through both cellular and nuclear membranes. Physiologically, H_2O_2 is produced by several pathways. For example, H_2O_2 can be produced either by peroxisomes from molecular oxygen or as a consequence of superoxide anion dismutation by SOD. Within the cells there are a number of enzymes that act to reduce H_2O_2 to H_2O such as catalase, glutathione peroxidase (GPX), and thioredoxin (Yeldandi et al., 2000, Carter et al., 1994).

1.3.2 Diabetic sources of reactive oxygen and nitrogen species

Under diabetic conditions, it is thought that high glucose levels (hyperglycaemia) cause damage to tissues via four main pathways: (1) glucose and other sugars being highly metabolised through the polyol pathway; (2) overproduction of advanced glycation endproducts (AGEs) intracellularly and also increased coupling to its receptor; (3) increased activity of the hexosamine pathway; and (4) activation of protein kinase C (PKC) isoforms. The findings of clinical studies in which one of these pathways was inhibited are unsatisfactory, as hyperglycaemia-induced tissue injury was not significantly
changed (Geraldes and King, 2010, Ramasamy and Goldberg, 2010), however, there is evidence that all of these pathways are activated as a result of a single stimulus, that is overproduction of ROS from mitochondria. Furthermore, by controlling mitochondrial ROS, the tissue damage due to hyperglycaemia is minimized (Brownlee, 2001, Nishikawa et al., 2000).

1.3.2.1 Mitochondria as a source of superoxide

Mitochondria play an important role in oxidative phosphorylation via the electron-transport chain. In the mitochondria, the system involved in oxidative phosphorylation consists of five major multi-enzyme complexes, called, complexes I, II, III, and IV and ATP synthase and the mobile electron carrier ubiquinone (coenzyme Q), and cytochrome c (Figure 1.4). Under normal conditions, when a molecule of glucose is taken up into the cells, glycolysis causes production of pyruvate from glucose and the pyruvate then enters the mitochondria. The pyruvate is then oxidized by the action of tricarboxylic acid (TCA) cycle to produce nicotinamide adenine dinucleotide (NADH) together with flavin adenine dinucleotide (FADH2). Transformation of electrons derived from oxidative reaction of NADH and FADH2 activated through the electron transport chain involves complexes I-IV to finally reach the electron acceptor, molecular oxygen. Throughout the course of electron transfer through the chain, protons are driven across the mitochondrial membrane yielding a voltage gradient. This voltage is then collapsed and finally yields ATP through ATP synthase. During the electron transport procedure, and as a result of the escape of an electron before it is added to an oxygen molecule, the mitochondria generate superoxide radicals. The escape of electrons occurs at 2 prime locations ie complex I (NADH dehydrogenase) and the interface between complex II and coenzyme Q (Turrens, 1980, Turrens et al., 1985). Furthermore, the reduction of ROS occurs due to uncoupling protein-
UCP-1 (UCP-1) which leads to a collapse of the proton gradient across the mitochondrial membrane. Under normal physiological settings these processes are firmly controlled making this system an important source of ROS. It is estimated that around 1% of oxygen is condensed to superoxide instead of entirely to water under normal physiological conditions.

In diabetes, hyperglycaemia causes more pyruvate to be oxidized in the TCA cycle and this acts to drive more electron donors (NADH and FADH2) into the electron transport chain. This process increases the voltage gradient across the mitochondrial membrane until a critical threshold is reached as a result of the increase of electron efflux to the point at which the electron inside complex III is unsettled (Korshunov et al., 1997), leading to generation of superoxide as a result of accumulation of electrons at coenzyme Q which donates the electrons one at a time to molecular oxygen, thus producing superoxide (Nishikawa et al., 2000). Overproduction of ROS by the mitochondria is considered as a key initiating factor in the stimulation of four main fundamental pathways: (1) increased flux of glucose and other sugars into the cells via the polyol pathway (Lee et al., 1995); (2) increased production of intracellular glucose-derived advanced glycation endproducts (AGEs) and binding to its receptor for AGEs (Brownlee, 1995); (3) stimulation of protein kinase C (PKC) isoforms and (4) activation of hexosamine pathway (Koya and King, 1998) (Figure 1.5).
**Figure 1.4** The generation of superoxide through the electron-transport chain in mitochondria. Hyperglycaemia causes high mitochondrial membrane potential ($\Delta \mu_{H^+}$) by deriving electron donors from the TCA cycle (NADH and FADH$_2$) and pumping protons through the inner membrane of the mitochondria. Consequently this prevents electron transport at complex III leading to an increase in the half-life of free radical intermediates of coenzyme Q (ubiquinone) that produce superoxide from reduction of O$_2$. Figure obtained from (Brownlee, 2001).
**Figure 1.5** A summary of signalling pathways causing diabetic tissue impairment due to hyperglycaemia. Adapted from (Nishikawa et al., 2000).
1.3.2.2 Polyol pathway

The polyol pathway is essentially related to the family of aldo-keto enzymes that are utilized as carbonyl substrates. NADPH acts to reduce the carbonyl compounds to their particular sugar alcohols (polyol). The aldose-reductase enzyme acts to metabolise glucose to sorbitol, thus, in diabetic states there is a high concentration of intracellular glucose which is changed to sorbitol by aldose-reductase mediated by NADPH as a cofactor. It seems that during hyperglycaemia, utilization of NADPH by this reaction prevents the replacement of glutathione, which is needed to maintain glutathione peroxidase (GPx) activity. This would eventually reduce cellular antioxidant activity and also because NADPH is a cofactor required to reproduce reduced glutathione which is an important scavenger of ROS, this could initiate or intensify intracellular oxidative stress (Ii et al., 2004, Lee and Chung, 1999, Chung et al., 2003). It has been reported that in diabetic mice, atherosclerosis is increased due to the overexpression of human aldose-reductase and decreased gene expression that plays an important role in the regulation of glutathione regeneration (Vikramadithyan et al., 2005).

Once the glucose is transformed to sorbitol, then the sorbitol is oxidized to fructose in the presence of sorbitol dehydrogenase. This process is accompanied by reduction of NAD$^+$ to NADH, providing more substrate to complex I of the mitochondrial respiratory chain. As described above, in diabetes, the mitochondrial respiratory chain is recognized to be a major supplier of ROS.
Overproduction of Advanced glycation end-product (AGEs)

In normal physiological situations, AGEs are produced from the non-enzymatic reaction of glucose with other glycating compounds that are obtained from both glucose and over-oxidation of fatty acids with proteins. In diabetic conditions, the production of AGEs and its receptor is increased as a result of the constant action of hyperglycaemia and oxidative stress (Fu et al., 1994, Thornalley et al., 1999). The binding of AGEs to its receptors may lead to up-regulation of the transcription factor NF-κB via the activation of cytokines which eventually causes an increase in the production of ROS. Therefore, diabetes results in the damaging series of ROS formation, overproduction of AGE and its receptor, activation of NF-κB, and cytokine production and excessive free radical formation (Bayraktutan, 2002).

Activation of protein kinase C (PKC)

PKCs are a family of around eleven isoforms, with the ability to phosphorylate various protein targets. Both Ca\(^{2+}\) ions and phosphatidyl serine are recognized as activators of classical isoforms of PKC. The activity of these classic isoforms is also largely enhanced by diacylglycerol (DAG). In addition, hyperglycaemia causes de novo synthesis of DAG, leading to increased activity of mostly β- and δ- isoforms of PKC (Inoguchi et al., 1992). This results in amplifying stress signaling pathways such as Jak/STAT, p38MAPK, and NF-κB (Haneda et al., 1997). It is documented that activation of PKC has been involved when the production of NO is suppressed (Derubertis and Craven, 1994), and that the insulin-stimulated expression of eNOS in cultured endothelial cells has been inhibited due to the activation of PKC (Kim et al., 2006). Furthermore, PKC activation may enhance the activity of NADPH oxidase which acts as a ROS generating enzyme,
causing dysfunction of endothelial cells that can be ameliorated by using a PKC inhibitor (Hink et al., 2001).

1.3.2.5 Increased hexosamine pathway activity

The hexosamine pathway has been demonstrated to be involved in the pathogenesis of diabetic complications (Federici et al., 2002). In diabetes, hyperglycaemia has been reported to reduce activity of glyceraldehyde-3-phosphate dehydrogenase in bovine aortic endothelial cells through increased production of mitochondrial superoxide which in turn causes increased activity of the hexosamine pathway (Du et al., 2000). In diabetes there is high intracellular glucose and most of the glucose is metabolized firstly to glucose-6 phosphate via glycolysis and then subsequently to fructose-6 phosphate and then on through the rest of the glycolytic pathway. However, some of fructose-6 phosphate is diverted to act as a signalling pathway in which an enzyme GFAT (glutamine: fructose-6 phosphate amido-transferase) transforms fructose-6 phosphate into glucosamine-6 phosphate and finally forms UDP (uridine diphosphate) N-acetyl glucosamine. After that, N-acetyl glucosamine uses serine and threonine amino acids residues as transcription factors in a similar way to the normal phosphorylation process. The elevated modification by this glucose amine causes pathological changes in gene expression (Sayeski and Kudlow, 1996, Kolm-Litty et al., 1998, Wells and Hart, 2003). For example, elevated-modification of the transcription factor Sp1 causes increased expression of transforming growth factor-β1 and plasminogen activator inhibitor-1. Both have harmful effect on the vasculature in diabetes (Du et al., 2000). Overall, increased glucose flux through the hexosamine pathway has been documented as a factor in the pathogenesis of diabetic complications and plays an important role both in hyperglycaemia-induced abnormalities of glomerular cell gene expression (Kolm-Litty et al., 1998) and in cardiomyocyte
dysfunction in cell culture induced by hyperglycaemia (Clark et al., 2003). Furthermore, in diabetes associated carotid artery plaque, modification of proteins in endothelial cells via the hexosamine pathway is markedly increased (Federici et al., 2002).

1.3.3 Sources of reactive oxygen and nitrogen species in vascular endothelial cells

As discussed previously, vascular health is maintained by the appropriate function of the endothelial cells. Mitochondria have been recognized as a major source of ROS production in diabetes, but there are additional enzymes such as xanthine oxidase, NADPH oxidase, and eNOS in the vascular endothelial cells that are thought to have a significant role in increased ROS production.

1.3.3.1 Xanthine oxidase

In the endothelial cells, the enzyme xanthine oxidase acts to catalyze the oxidation of hypoxanthine to urate by utilizing oxygen molecules as electron acceptors and liberating ROS such as superoxide and hydrogen peroxide (Figure 1.6). Immunohistochemistry studies demonstrated that xanthine oxidase is situated in endothelial cells (Jarasch et al., 1981) and observations from experiments using electron spin resonance technologies confirmed that xanthine oxidase is a crucial source of formation of vascular superoxide in type I diabetes (Matsumoto et al., 2003).

1.3.3.2 NADPH oxidase

NADPH oxidase is a superoxide-producing enzyme primarily recognized as a cytosolic enzyme complex in neutrophils where it contributes to the nonspecific defense
mechanism against pathogens via the formation of low quantities of superoxide by the mechanism of electron transport (Babior et al., 2002). The NADPH oxidase complex consists of five subunits involving a membrane-associated p22phox and a gp91phox subunit and around four cytosolic subunits: p47phox, p67phox, p40phox, and GTPase rac1 or rac2 (Babior, 2004). NADPH oxidase is also present at physiological levels in vascular tissues (Valko et al., 2007). In addition to the control of vascular cell growth, proliferation, activation, and migration, the NADPH oxidase-derived superoxide is also involved in modulation of vascular tone (Feng et al., 2010). Conversely, in many of cardiovascular diseases, including diabetes, NADPH activity and expression is up-regulated to form large quantities of ROS causing oxidative stress (Figure 1.6) (Guzik et al., 2002, Takayama et al., 2004, Ray and Shah, 2005, Drummond et al., 2011, Paravicini et al., 2004).

1.3.3.3 Endothelial nitric oxide synthase

The isoforms of NOS can be classified into three categories, namely, inducible (iNOS), neural (nNOS), and endothelial (eNOS). The common action of all of these isoforms is to convert L-arginine to L-citrulline and thus to liberate NO. This reaction is catalyzed by a number of cofactors such as calmodulin, BH4, flavin mononucleotide, and adenine dinucleotide. The second two isoforms produce low quantities of NO in a short period of time and are mainly present in endothelial and neuronal tissue. In diabetic conditions, where there is an increase of oxidative stress an unbalanced production of nitric oxide and superoxide leads to further elevated formation of superoxide by the NADPH oxidase, xanthine oxidase, and the mitochondria. The reaction between superoxide and NO leads to the formation of a highly reactive intermediate called peroxynitrite and lower NO bioavailability in the vascular regions, causing vascular dysfunction (Figure 1.6). Peroxynitrite causes eNOS uncoupling, where the eNOS is
transformed from a dimer to a monomer to produce superoxide instead of NO. This also occurs under conditions of diminishing levels of L-arginine and/or BH₄ oxidation (Cai et al., 2002, Hink et al., 2001, Ryoo et al., 2008, Münzel et al., 2010, Cai et al., 2005).
Figure 1.6 Scheme shows the suggested mechanism, sources, and pathways of the production of ROS in endothelial cells. EC, endothelial cell; O$_2^-$, superoxide anion; O$_2$, oxygen molecule; H$_2$O$_2$, hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; OONO$^-$, peroxynitrite. Adapted from (Sugamura and Keaney, 2011).
1.3.4 Sources of endogenous antioxidants in diabetes

Under diabetic conditions, the phenomenon of oxidative stress arises when the cellular redox balance favours pro-oxidant production over endogenous antioxidant defences (Rains and Jain, 2011). This is usually observed when there is an overproduction of ROS (as described above) and/or in case of compromised function of endogenous antioxidant systems. There are several sources of antioxidant systems involved endogenously such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and thioredoxin.

1.3.4.1 Catalase

Catalase enhances the degradation of hydrogen peroxide to water and oxygen. In diabetes, although there is increased expression of catalase, the activity was reduced due to overproduction of hydroxyl radicals, thereby contributing to oxidative stress (Shi et al., 2007, Heales, 2001, Góth et al., 2001, Góth, 2008).

1.3.4.2 Superoxide dismutase enzyme (SOD)

SOD catalyses the dismutation of superoxide producing oxygen and hydrogen peroxide, having a vital role as an antioxidant agent to prevent cellular damage that may be induced by superoxide radicals (Scandalios, 1993). Within the cells there are isoforms of SOD situated in different compartments: (1) copper-zinc SOD (SOD 1) is located in the cytoplasm, (2) manganese SOD (SOD 2) is positioned in the mitochondria, and also there is extracellular copper-zinc SOD (SOD 3) (Faraci and Didion, 2004).
### 1.3.4.3 Glutathione peroxidase (GPx)

GPx reduces lipid hydroperoxides to lipid alcohol and reduces free hydrogen peroxide to water (McCay et al., 1976). GPx1 is present in the cytoplasm of almost all mammalian tissue and is the most abundant isoform of GPx. Under diabetic conditions, the level of GPx expression has been reported to be diminished (Sindhu et al., 2004). Additionally, studies show that GPx plays a role in the prevention of diabetes associated atherosclerosis. For example, a study using ApoE knockout mouse demonstrated that the extra deletion of GPx1 caused acceleration in the development of atherosclerosis by upregulation of profibrotic and proinflammatory pathways (Lewis et al., 2007, Torzewski et al., 2007). This accelerated progression of atherosclerosis was reduced by treatment of knockout mice with ebselen, a mimetic of GPx1 (Chew et al., 2010).

### 1.3.4.4 Thioredoxin

The thioredoxin system is a fundamental system for preserving the balance of cellular redox in endothelial cells and vascular smooth muscle (Yamawaki et al., 2003, Yamawaki and Berk, 2005). The thioredoxin system includes; thioredoxin, thioredoxin reductase, and NADPH that, through the interaction with redox-active centre of thioredoxin (Cys, Gly, Pro, Cys), reduces the oxidized cystein groups on protein. This forms a disulfide bond, which subsequently causes a reduction of thioredoxin reductase and NADPH (Yamawaki et al., 2003). Thioredoxin peroxidase plays a role in the antioxidant properties of thioredoxin by using SH groups as reducing equivalents. The reduction of the oxidized form of thioredoxin peroxidase by the action of thioredoxin, causes scavenging of ROS like hydrogen peroxide (Yamawaki et al., 2003). The thioredoxin-interacting protein, an endogenous inhibitor of thioredoxin, modulates the antioxidant activity of thioredoxin. Under diabetic conditions, it has been demonstrated
that reduced activity of thioredoxin contributes to oxidative stress, where diabetes increases the expression of thioredoxin-interacting protein (TXNIP) and reduces the antioxidant activity of thioredoxin (Qi et al., 2009, Tan et al., 2011).

1.4 Endothelial dysfunction in diabetes

Endothelial dysfunction is defined as an impairment of the balance between vasodilating and vasoconstricting substances that are produced by the endothelium (De Vriese et al., 2000). It is considered an important contributor to the pathogenesis of vascular diseases and the complications of diabetes mellitus (Hadi and Suwaidi, 2007). Indeed, under physiological conditions there is a delicate balance of the release of endothelium-derived relaxing factors and endothelium-derived contracting factors, however, this sensitive balance is altered adversely in cardiovascular diseases, thus, contributing to the detrimental effect on the vascular tissues and ending with damage to organs (Tan et al., 2002). Endothelial dysfunction is recognized as an early and independent predictor of the pathological cardiovascular outcomes of diabetes mellitus and plays a major role in the initiation of cellular events and complications associated with diabetic conditions (Hink et al., 2001). Endothelial dysfunction is associated with a reduction in the relaxation of blood vessels in response to endothelium-dependent vasodilators, such as ACh or bradykinin, or to flow-mediated vasodilatation. In humans, studies to assess endothelial function by both invasive and non-invasive techniques have been used in order to measure both agonist-induced and flow-mediated vasodilatation (Barac et al., 2007, Buscemi et al., 2009). In addition to reduced production of endothelium-derived relaxing factors such as NO, EDH, and PGI₂, there are also changes in the presence of proinflammatory molecules, the expression of adhesion molecules, and
enhanced production of vasoconstrictor molecules such as COX-derived metabolites of arachidonic acid (AA), ROS, and endothelin-1 (ET1) that all have pathological consequences. In diabetes, the impairment of endothelium-dependent relaxation is usually associated with the production of ROS and increased expression of proinflammatory molecules (Brownlee, 2001). Collectively, it is the combination of proinflammatory and proliferative conditions and the impairment of endothelium-dependent responses to stimuli that underlies endothelial dysfunction and offers the basis for the development of micro- and macro-vascular disease. Although the aetiology of vascular endothelial dysfunction under conditions of diabetes has been extensively examined in studies using humans or animal models, the pathway which could be the primary mechanism to explain the connection between diabetes and its associated vascular disease complications remains unclear. Therefore, in the next section the possible mechanisms that play a role in the initiation of endothelial dysfunction in diabetic vascular disease are described.

### 1.4.1 Reduction of NO bioavailability

Endothelium-derived NO bioavailability, as estimated by excess of NO over ROS levels, is a one of the most important indices for determining vascular endothelial function. Any condition in which there is reduced eNOS activity or where the generation of ROS is elevated would lead to a reduction of NO bioavailability and consequent impairment of endothelium-dependent relaxation. In diabetes there are many mechanisms involved in the reduction of NO-mediated relaxation include: (1) over-generation of superoxide anions leading to scavenging of NO and consequently increased levels of peroxynitrite; (2) reduced synthesis of NO by eNOS enzyme due to eNOS uncoupling; (3) increased activity of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS; (4) increased
uncoupling of eNOS and decreased bioavailability of BH4 which will be discussed in detail in the following sectors.

1.4.1.1 Expression and phosphorylation of eNOS

In addition to inhibition of NO by superoxide, NO synthesis can be inhibited at an enzymatic level. An alteration of the levels of eNOS expression may play a role in endothelial dysfunction associated with diabetes. Using cultured endothelial cells and/or isolated blood vessels from diabetic animals some studies have shown that the expression of eNOS may be decreased (Okon et al., 2005, Srinivasan et al., 2004, Salt et al., 2003), whereas there are other reports that the expression of eNOS is unchanged (Matsumoto et al., 2006, Pannirselvam et al., 2006) or increased (Ding et al., 2007). It has been reported that reduction of NO production may be attributed to the mechanism of phosphorylation of eNOS at Ser1177 which activates the enzyme (Michell et al., 1999). Studies demonstrated that in aortae from diabetic mice, phosphorylation of eNOS at Ser1177 was reduced (Zhang et al., 2009, Zhong et al., 2007b, Leo et al., 2011b), as well as in renal arteries (Zhong et al., 2007a), resulting in decreased synthesis of NO.

1.4.1.2 Direct reduction of NO bioactivity

In diabetes, the direct inactivation of NO has been attributed to the effect of overproduction of ROS in the vasculature (Bitar et al., 2005). Indeed, several reports show that diabetes causes imbalance of bioactive radicals by which the expression and/or activity of pro-oxidant enzymes such as lipoxygenases increased, therefore, the generation of superoxide anions increased and impairing endothelium-derived NO-mediated
relaxation (Woodman et al., 2008, Woodman, 2009, Malakul et al., 2008, Leo et al., 2010).

1.4.1.3 Augmented arginase activity

One of the main roles of arginase is to catalyze the hydrolysis of L-arginine to ornithine and urea, therefore, causing competitive inhibition of eNOS to utilize its common substrate, L-arginine to synthesize NO. In diabetes, it has been reported that there is an increase in the expression of arginase and as a consequent increase in its biological activity causing impairment of endothelium-dependent relaxation which could be ameliorated by acute application of an arginase inhibitor (Romero et al., 2008). Furthermore, arginase activity is increased and NO synthesis is significantly reduced by exposing endothelial cells to a medium containing a high glucose concentration (25 mmol/L, 24 hour) (Romero et al., 2008). The activity of arginase in cells exposed to high glucose can be prevented by transfection of endothelial cells with arginase I small interfering RNA which also normalizes NO production. Thus in diabetes, the activity of arginase may have a role in reducing NO production (Romero et al., 2008, White et al., 2006).

1.4.1.4 Amplified levels of ADMA

ADMA is an endogenous inhibitor of NOS. Under diabetic conditions, it has been reported that the levels of ADMA increased causing impairment of the response to ACh in endothelium-dependent relaxation in aortae (Xiong et al., 2003). In diabetes, the activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA, is reduced causing accumulation of ADMA resulting in impairment of NOS activity (Xiong
et al., 2003). The levels of ADMA are significantly reduced by restoration of DDAH activity through two mechanisms, either by drug intervention or gene transfer which increases the expression of DDAH and consequently increases NO synthesis and improves endothelium-dependent relaxation in diabetic aortae (Lu et al., 2010, Xiao et al., 2009, Yin and Xiong, 2005).

1.4.1.5 Uncoupling of eNOS and BH₄

BH₄ is a cofactor which plays an essential role in the modulation of eNOS activity. BH₄ is produced by guanosine 5-triphosphate cyclohydrolase I (GTPCH) (Li et al., 2010). More than 60% of BH₄ is expressed in the wall of blood vessels and associated with endothelium and modulated by shear stress (Harrison et al., 2010, Tsutsui et al., 1996, Widder et al., 2007, Li et al., 2010, De Bono and Channon, 2007). In order to be stabilized as a dimer, eNOS requires appropriate levels of BH₄, or the eNOS will act as an NADPH oxidase if it is in a monomeric state, where the molecular oxygen becomes an electron acceptor rather than L-arginine (Cai et al., 2005). In the monomeric state of eNOS, superoxide anions are produced rather than NO (Cai et al., 2005). More important than the level of BH₄, it seems that the maintenance of optimal endothelial function depends critically on the ratio of BH₄ to oxidized biopterins. Indeed, it has been reported that the levels of BH₄ in small mesenteric arteries from db/db diabetic mice were not significantly reduced in comparison to mesenteric arteries from normal animals, rather, the levels of oxidized biopterin products such as 7,8-dihydrobiopterin (BH₂) were increased, therefore, the BH₄/BH₂ ratio was reduced (Pannirselvam et al., 2003). Furthermore, it has also been reported that BH₂ has the same affinity as BH₄ to bind to eNOS, thus, BH₂ competitively displaces bound BH₄ from its site in eNOS to impair NO production by forming an uncoupled eNOS-BH₂ complex, indicating the importance of BH₄/BH₂ ratio in order to
maintain the function of endothelial cells (Crabtree et al., 2008, Crabtree et al., 2009a). Several studies showed that a reduced ratio of BH₄/BH₂ leads to a reduction of NO bioavailability via uncoupling of eNOS causing endothelial dysfunction. This reduction of ratio can be reversed by dietary provision of the BH₄ precursors such as sepiapterin or supplementation of BH₄ (Cai et al., 2002, Cai et al., 2005, Hink et al., 2001). In addition to overproduction of superoxide induced by hyperglycaemia and consequent decrease of the ratio of BH₄/BH₂, it has also been demonstrated that the reduction of the BH₄/ BH₂ ratio is modulated by the production of peroxynitrite. In diabetes which initiates the mechanism of ubiquitination and 26S proteasome-dependent degradation of GTPCH which causes further decrease in BH₄/BH₂ ratio (Wang et al., 2009, Wang et al., 2010, Xu et al., 2007). All these effects induced by hyperglycaemia are produced by increased expression of SOD or inhibition of eNOS or by the effects of inhibitors of 26S proteasome such as MG132 or PR-11 (Xu et al., 2007). Similarly, the reduction of GTPCH and BH₄ in STZ-induced diabetic mice can be reversed by the SOD mimetic, tempol (Xu et al., 2007). In addition, the BH₄ biosynthetic pathway plays a role in maintenance of eNOS by BH₄ recycling via dihydrofolate reductase (DHFR) which has been identified as a critical modulatory factor in the regeneration of BH₄ from BH₂ (Crabtree et al., 2011, Sugiyama et al., 2009, Crabtree et al., 2009b). In diabetes, expression levels of DHFR could also participate in the contribution to uncoupling of eNOS, but, the data in this regard are not consistent. Several studies showed increases in the expression of DHFR in diabetes (Oak and Cai, 2007, Ren et al., 2008, Yamamoto et al., 2010). On the other hand, it has been reported that in diabetic aorta the expression of DHFR was decreased (Wenzel et al., 2008). Despite these inconsistencies of the reported data in regards to DHFR expression, all studies have shown that in diabetes the ratio of BH₄/BH₂ was diminished and the endothelial
dysfunction was concomitant to uncoupling of eNOS (Oak and Cai, 2007, Ren et al., 2008, Wenzel et al., 2008, Yamamoto et al., 2010, Channon, 2004).

1.4.3 Diminished EDH responses

The reports about the relative contribution of NO and EDH to endothelium-dependent relaxation in mesenteric arteries from diabetic arteries are inconsistent. For instance, it has been reported that the contribution of EDH to endothelium-dependent relaxation is increased in diabetes to offer some compensation for the reduction of bioavailability of NO (Shi et al., 2006). In contrast, another study showed that the action of NO is not affected by diabetes and the contribution of EDH is selectively decreased (Wigg et al., 2001). Generally, the EDH is complex in its nature and its contribution depends on the vascular beds investigated. Several studies have endeavored to investigate the impairment of EDH responses in arteries under pathological conditions such as diabetes via investigating the underlying cellular mechanisms responsible for this impairment (Burnham et al., 2006b, Young et al., 2008, Leo et al., 2011b). There are probable pathways attributed to be responsible factors causes decrease in EDH responses in diabetes include either modification of the expression or activity of KCa channel or impairment of MEGJ expression or activity.

1.4.3.1 Alteration in the expression or activity of endothelial KCa channels

Changes in the expression or the activity of KCa channels could alter the contribution of EDH to endothelium-dependent relaxation. In diabetes, the impairment of EDH responses has been extensively investigated and its correlation to the expression level of KCa channels, investigated but there is a lack of consistent data in this regard (Brondum et al., 2010, Ding et al., 2005, Pannirselvam et al., 2006). For instance, it has been
reported that in STZ-induced diabetic ApoE-deficient mice, the expression of SK_{Ca}2 and SK_{Ca}3 channels was reduced causing an impairment of EDH-mediated relaxation (Ding et al., 2005). In contrast, another study showed that in insulin resistant Zucker fatty rats, even though there is an impairment in EDH responses, the expression of SK_{Ca}3 channels was augmented in small mesenteric arteries (Burnham et al., 2006a). Moreover, several other reports showed that even where there is impaired EDH-mediated relaxation there is no difference in the expression levels of K_{Ca} channels in diabetic arteries compared to normal arteries (Pannirselvam et al., 2006, Brondum et al., 2010). It is important to mention that the measurement of the expression levels of either protein or gene of the endothelial K_{Ca} channels has the common problem that its commonly suggested that the channel function reflected by the molecular weight of the native single channels reflects that of the functional channel (Brondum et al., 2010, Hilgers and Webb, 2007). To some extent this is not the case, as the functional channel in normal intact tissue has been reported to be constituted by high molecular weight complexes of these channels (Chadha et al., 2010).

The deficiency of EDH-mediated relaxation response in mesenteric arteries from obese animals is modulated by the decrease in the expression and/or activity of the K_{IR} channels, which is a downstream effect on SK_{Ca}-mediate the EDH responses (Haddock et al., 2011). Furthermore, it has also been reported that the activity of K_{IR} channel and/or the activity of Na^{+}-K^{+}-ATPase is diminished in diabetes (Makino et al., 2000). Overall, despite the lack of consistent data in regard to the expression levels of K_{Ca} channels in the endothelial cells, all the studies demonstrated that EDH-mediated relaxation is impaired in diabetic arteries, indicating that the functional/bioactivity of the endothelial K_{Ca} channels is a more essential indicator of EDH type responses than endothelial K_{Ca} channel expression level.
1.4.3.2 Transformed expression or activity of MEGJs

Under diabetic conditions, myoendothelial cell communication can be directly affected to reduce EDH-mediated relaxation via transforming the distribution and/or expression of key vascular connexins of MEGJs (De Wit and Griffith, 2010). It has been reported that the contribution of EDH to endothelium-dependent relaxation is reduced in mesenteric arteries from db/db type 2 diabetic mice, although there is no change in mRNA expression level for the key vascular connexins, Cx37, Cx40, Cx43, Cx45 (Pannirselvam et al., 2006). On the other hand, a study in STZ-induced type 1 diabetic ApoE-deficient mice showed that Cx37 expression was reduced in small mesenteric arteries (Ding et al., 2005). Likewise, in aortic arteries from STZ-induced diabetic animals, the protein expression of the connexins Cx37 and Cx40 was diminished (Hou et al., 2008) and also in coronary arteries (Makino et al., 2008), suggesting that there is a connection between the reduction in EDH-mediated relaxation in diabetes and the expression of connexins. Furthermore, it has been reported that in small mesenteric arteries there is a general reduction in mRNA and protein levels of Cx40 as well as EDH responses (Young et al., 2008). The myoendothelial cell communication could be disturbed by the alteration of the expression of the vascular connexin. A positive correlation has been demonstrated between the levels of vascular connexin expression of Cx40 and Cx37 in the cremaster arterioles from Cx40-deficient mice (De Wit, 2010). In Cx40-deficient mice, the conduction of activated endothelium-dependent vasodilatation in cremaster arterioles is impaired due to a decrease in the expression of Cx37 (Figueroa et al., 2003, De Wit, 2010). Therefore, in diabetes, the impairment of EDH-mediated relaxation may be a result of the reduction of Cx37 expression which also resulted from the reduction of the expression of Cx40. Furthermore, it has been reported that in diabetes, the expression of Cx37, Cx40, and Cx43 can also be affected when high glucose levels inhibit the intercellular communications.
through gap junction in VSMC in bovine aorta via PKC-mediated phosphorylation of Cx43 (Kuroki et al., 1998). Similarly, Sato et al. (2002) reported that the activity of gap junction communication in rat microvascular endothelial cells was reduced due to high glucose and this reduction was concomitant with a reduction in Cx43 mRNA and protein expression (Sato et al., 2002). Therefore, in diabetes, changes in the expression and/or regulation of the connexins that make up MEGJs could play a critical role in the endothelial dysfunction.

1.5 The incretins and the dipeptidylpeptidase-4 inhibitors

1.5.1 Historical overview of the incretin concept

The incretins are hormones that are secreted from enteroendocrine cells and released from the gut into the blood stream within minutes of the ingestion of food (Baggio and Drucker, 2007). The incretin hormones have a physiological role in the modulation of the insulin secretory response to food nutrients (Baggio and Drucker, 2007). The “incretin effect” is a term referring to the insulin secretory response due to the stimulation of pancreatic β-cells by incretin hormones which have insulinotropic effect and account for at least 50% of the total insulin secreted after oral glucose administration (Jose and Inzucchi, 2012). In 1902, Bayliss and Starling described the mechanism of pancreatic secretion, where they found that the pancreas secretes juices via the pancreatic duct after acid infusion into the digestive system, and this continued even when the innervation of the intestine was inhibited (Bayliss and Starling, 1902). They conclude that the nature of the signal that stimulates the pancreas was most likely a chemical, rather than nervous, stimulus. After the interstinal wall had been stimulated by acid, they removed the extracts and injected it into the blood stream and once again, they saw juices coming from pancreatic duct of the injected animal, and they proved that the stimulation of the flow of
pancreatic juice was due to the stimulation of the pancreas by the extracts that must contain a substance that was usually secreted from the intestinal wall into the blood stream to stimulate the pancreas, and they called this substance “secretin” (Bayliss and Starling, 1902). Moor (1906) reported achieving some success in some experiments he applied on young diabetic patients who received by mouth extracts of intestinal mucosa (Moore, 1906). This was the first trial based on incretin therapies in order to treat diabetes; however, these experiments were essentially doomed, because the chemical nature of peptides which would be degraded when given orally. In 1921, Banting and Best extracted insulin from the pancreas to further investigate the probability of food entering the gut causing release of an excitant into the blood stream to finally stimulate insulin secretion and lower blood glucose (Banting et al., 1922). La Barre (1932) used the term “incretin” to refer to an extract secreted in the upper part of the gut mucosa to cause hypoglycaemia (La Barre and Ledrut, 1934). The concept of the incretin system progressed when radioimmunoassays techniques for insulin become accessible. Between 1964 and 1976 there were at least three studies which independently showed that glucose administered orally causes a greater insulin response when compared with intravenous glucose injection although the glucose level obtained by intravenous injection was the same (Elrick et al., 1964, McIntyre et al., 1964, Perley and Kipnis, 1967). Indeed, the three groups knew that the oral intake of glucose leads to enhanced incretin release into the blood stream and subsequently causes an increase in insulin secretion rather than did plasma glucose itself. In 1971, Brown and Dryburgh isolated and identified the structure of amino acid from a peptide that was obtained from intestinal mucosa (Brown and Dryburgh, 1971). It has been reported that the exogenous administration of peptides causes inhibition of gastric acid secretion in dogs and this peptide was called gastric inhibitory polypeptide (GIP) (Brown and Dryburgh, 1971). Furthermore, Brown and colleagues identified that GIP had
insulinotropic properties (Dupre et al., 1973). GIP was deduced as the first incretin been isolated and its properties identified. By the end of 1985, glucagon-like peptide-1 (GLP-1) (7-36) was isolated as a peptide secreted in the gut and reported as a potent insulinotropic factor (Schmidt et al., 1985). Subsequently clinical investigations demonstrated that GLP-1 taken intravenously to increase plasma levels of GLP-1 to supraphysiological levels could have the ability to increase insulin secretion and reduce glucose levels in patients with type 2 diabetes mellitus. Thus incretin based therapy was proposed as a new approach to treat type 2 diabetes mellitus.

1.5.2 General description of incretin hormones and exendin-4

1.5.2.1 Glucose-dependent insulinotropic peptide (GIP)

GIP, the first incretin hormone to be identified, consists of a single 42-amino acid peptide chain derived from a 153-amino acid precursor (Takeda et al., 1987). It is a member of the structurally related family of hormones including secretin, glucagon, and vasoactive intestinal peptide. GIP was initially observed to inhibit gastric acid secretion as well as gastrointestinal motility in dogs receiving supraphysiological dosages (Brown et al., 1974). It has been reported that GIP at physiological plasma levels exerts glucose-dependent stimulatory effects on the process of insulin secretion and enhances glucose uptake by tissues via an insulin-mediated process (Dupre et al., 1973). Many reports demonstrated that GIP production occurred in enterendocrine cells (called K-cells) which are located in the proximal small intestine (duodenum and jejunum) and GIP is released as a response to nutrients containing glucose or fat, and then subsequently released into the blood-stream in an amount higher than the amount produced in the fasted state (Dupre et al., 1973, Pederson et al., 1975, Elliott et al., 1993). It has been found in a study carried out in fasting glucose concentration state that the effects of GIP strongly depends on the
glucose level in the blood, where administration of oral fat alone, such as oral corn oil, without carbohydrate leads to stimulation of GIP release, however, this is not sufficient to enhance insulin secretion, indicating that GIP-mediated insulin secretion is a glucose-dependent process; i.e. it is necessary for glucose to be high in plasma in order to enhance GIP to act on stimulation of insulin secretion (Ross and Dupre, 1978). The insulinotropic effects of GIP can be achieved through the binding to its specific receptor (GIPR) causing increased levels of intracellular cAMP and Ca$^{2+}$ in pancreatic β-cells, and promoting cell proliferation and cell survival in β-cells (Trümper et al., 2001, Trumper et al., 2002). The biological half-life of GIP is very short due to the rapid degradation of GIP by the action of dipeptidyl peptidase 4 (DPP4). It has been reported that plasma GIP concentrations are normal or increased in type 2 diabetes, however, the insulinotropic effects are lacking (Ross et al., 1977, Vilsbøll et al., 2001). Although the mechanisms by which β-cells respond to GIP remains unclear, some studies suggest that the decrease in physiological response of β-cell to GIP was due to the effects of hyperglycaemia which causes down regulation of GIPR expression/activity (Lynn et al., 2001, Zhou et al., 2007).

1.5.2.2 Glucagon-like peptide-1 (GLP-1)

GLP-1 was discovered through the cloning process and identification of the proglucagon gene (Bell et al., 1983). GLP-1 is a product of post-translational cleavage of the proglucagon gene via the prohormone convertase enzyme PC1/3 (Zhu et al., 2002, Ugleholdt et al., 2004). GLP-1 has been considered as the second peptide that has incretin activity and potentially stimulates insulin secretion from β-cells of the pancreas (Schmidt et al., 1985). GLP-1 is primarily produced in enteroendocrine cells (called L-cells) that are located within the enterocytes across the small bowel and ascending colon (Doyle and Egan, 2007, Jang et al., 2007). There are multiple forms of GLP-1 hormone that have been
identified in humans and the COOH-terminally amidated form, GLP-1 (7-36) amide represents the major circulating, biologically active form (at least 80%) with lesser amount of the minor glucine extended form GLP-1 (7-37) (Ørskov et al., 1986). Similar to GIP, GLP-1 is positively coupled to a specific receptor (GLP-1R) in order to achieve its insulinotrophic effects by increasing intracellular cAMP and Ca\(^{2+}\) levels in β-cells. Furthermore, beside its insulinotrophic effects, GLP-1 plays a role in inhibiting gastric emptying and decreasing food intake (Willms et al., 1996), prevents secretion of glucagon (Komatsu et al., 1989), thus minimizing the rate of the production of endogenous glucose (Prigeon et al., 2003). Collectively, all of these actions should lower blood glucose levels in type 2 diabetes. GLP-1 has also been reported to play a role in protecting β-cells from apoptosis (Farilla et al., 2002) and to enhance the proliferation of β-cells via upregulation of the transcription factor in β-cells, pancreatic duodenal homobox-1 protein (PDX-1) (Perfetti et al., 2000, Stoffers et al., 2000), that recognizes increased transcription of the insulin gene and up-regulates glucokinase and glucose transporter 2 (GLUT2) (Wang et al., 1999). Several studies reported that the continuous treatment of type 2 diabetic patients with GLP-1 can normalize blood glucose levels, enhance the function of β-cells, and restore the first-phase of insulin secretion in β-cells (Holz et al., 1993, Zander et al., 2002), hence, recently GLP-1/GLP-1R have become important therapeutic targets for treating type 2 diabetes. GLP-1 has a short half-life (1.5-2 min) due to its degradation by the DPP-4 enzyme.

1.5.2.3 The incretin mimetic exenatide (exendin-4)

Exenatide (exendin-4) is a 39–amino acid incretin mimetic, purified from the salivary secretions of the Gila monster (Heloderma suspectum), which exerts its glucoregulatory activities in a similar manner to mammalian incretin hormone glucagon
like peptide 1 (GLP-1) (Koltermann et al., 2003, Fineman et al., 2003, Nielsen and Baron, 2003, Nielsen et al., 2004). Exendin-4 causes glucose-dependent promotion of insulin secretion and inhibition of high secretion of glucagon, and slows gastric emptying. The process of glucose-dependent enhancement of insulin secretion by exendin-4 is mediated by binding to the pancreatic GLP-1 receptor (Göke et al., 1993). In animal experimental models of diabetes and in insulin secreting cell lines, both exendin-4 and GLP-1 improve pancreatic β-cell function by increasing the expression of key genes responsible for insulin biosynthesis and secretion (Nielsen et al., 2004). Several studies reported that exenatide and GLP-1 are able to reduce food intake, enhance weight loss, and have an insulin-sensitizing effect (Nielsen and Baron, 2003, Young et al., 1999, Nielsen et al., 2004, Szayna et al., 2000, Gedulin et al., 2005). Investigators conclude that exendin-4 markedly promotes the formation of new β-cells (Xu et al., 1999).

Besides the beneficial insulinotropic effects of exendin-4, it may have favorable direct effects on endothelial cell function. It has been reported that exendin-4 reduces macrophage adhesion to the endothelium, an early step in the formation of atherosclerotic plaques and subsequent endothelial dysfunction (Arakawa et al., 2010). Findings in human clinical trials showed that the acute administration of exendin-4 counteracts endothelial dysfunction induced by ischemia and reperfusion in the heart (Ha et al., 2012) or after a high fat meal (Koska et al., 2010). It has also been reported that the GLP-1 receptor agonist liraglutide has a potential role in the inhibition of vascular disease progression via the prevention of atherogenesis, stabilization of plaque and endothelial function together with potential protection against major cardiovascular events in an ApoE−/− mouse model of atherosclerosis (Gaspari et al., 2013).

Both GLP-1 and exendin-4 have been shown to reduce the vulnerability of cells to apoptotic signaling under conditions of oxidative stress (Chen et al., 2006, Tews et al.,
Several mechanisms have been proposed to interpret this cytoprotective effect including, counter-regulation in the reduction in proteins electron transport chain (Tews et al., 2009), decrease the expression of pro-apoptotic thioredoxin-interacting protein (TXNIP) implicated in cell glucose toxicity process (Chen et al., 2006), reduction of endoplasmic reticulum stress (Tsunekawa et al., 2007), and down-regulation of NF-κB which acts as a promoter to activate β-cells and stimulate NO synthase and NO pathway (Liu et al., 2008).

1.5.3 Dipeptidylpeptidase and dipeptidylpeptidase inhibitors

1.5.3.1 Dipeptidylpeptidase (DPP-4)

DPP-4 is a complex enzyme consisting of 766 amino acids, expressed on the surface of several cell types including vascular endothelial cells (Fadini and Avogaro, 2011, Augustyns et al., 1999). It is a serine aminopeptidase that cleaves GLP-1 and GIP via dipeptide cleavages with alanine, proline, or hydroxyproline in the N-terminal amino acids (Drucker, 2007). It has been reported that in vitro, DPP-4 cleaves many chemokines and peptide hormones, however, in vivo, there are comparatively fewer peptides have been identified as endogenous physiological substrates for DPP-4. Both GIP and GLP-1 are endogenous physiological substrates for DPP-4. DPP-4 knockout mice studies showed improved glucose tolerance concomitant with increased bioavailability of GIP and GLP-1 and enhanced secretion of insulin subsequent to oral glucose challenge. This was accompanied by decreased accumulation of fat and diminished intake of food as well as increased resistance to diet-induced obesity (Marguet et al., 2000, Conarello et al., 2003). In vivo studies on mice which had their incretin receptors genetically inactivated showed that the mechanisms transducing the glucoregulatory effects of DPP-4 inhibitors were dominated by GIP and GLP-1 receptor-dependent pathways (Marguet et al., 2000). DPP-4
is expressed in the endothelial cells, particularly in the microvascular circulation (Matheeussen et al., 2011), but it has been reported that the expression and the activity of DPP-4 is only increased in microvascular endothelial cells in the presence of high glucose (Pala et al., 2010). This suggests that DPP-4 inhibitors may reduce the detrimental effects of hyperglycaemia on endothelial cells.

1.5.3.2 Dipeptidylpeptidase inhibitors

DPP-4 inhibitors are pharmacological glucose lowering compounds that have been introduced as a new strategy for treatment of type 2 diabetes mellitus (Barnett, 2006, Richter et al., 2008, Palalau et al., 2009, Hollander and Kushner, 2010, Scheen, 2011, Jose and Inzucchi, 2012). In recent years, several structurally dissimilar DPP-4 inhibitors such as (1) sitagliptin, (2) vildagliptin, (3) saxagliptin, (4) alogliptin, and (5) linagliptin (Figure 1.7), have been developed and are now being used clinically. The structural dissimilarities of DPP-4 inhibitors could affect their beneficial pharmacological action. For example, Kröller-Schön and colleagues (2012) reported that the DPP-4 inhibitor linagliptin significantly inhibited the oxidative burst in human leucocytes but this was not shared with other DPP-4 inhibitors such as sitagliptin, vildagliptin, alogliptin and saxagliptin (Kroller-Schon et al., 2012). DPP-4 inhibitors act as incretin hormone enhancers which control postprandial elevated levels of blood glucose by increasing insulin secretion and decreasing glucagon secretion via glucose-dependent manner. Thus, in presence of high plasma glucose levels, the incretin hormones signal to the liver to reduce glucose production (Jose and Inzucchi, 2012). The DPP-4 inhibitors are able to reduce both fasting plasma glucose (FPG) and post prandial glucose (PPG) levels (Aschner et al., 2006). It has been reported that DPP-4 inhibitors do not reduce elevated blood glucose levels to a greater extent than existing therapies (Fakhoury et al., 2009, Monami et al., 2010), but
they provide many potential benefits including the ability to induce a sustainable decrease in glycated HbA1c that is accompanied by a low risk of hypoglycaemia and is not associated with weight gain (Barnett, 2006).
Figure 1.7 DPP-4 inhibitors show dissimilarities in their chemical structure. Obtained from (Lin et al., 2013).
1.5.4.2.1 The DPP-4 inhibitor linagliptin

Linagliptin (originally: BI 1356), developed by Boehringer Ingelheim Pharmaceuticals (Ingelheim, Germany), inhibits the enzyme DPP-4 in a competitive and reversible manner. It prevents the degradation of GLP-1 and GIP hormones and increases insulin secretion from pancreatic \( \beta \)-cells (Gallwitz, 2012). Linagliptin has a unique chemical structure based upon a xanthine scaffold structure (BI 1356; 8-(3-(R)-aminopiperidin-1-yl)-7-but-2-ynyl-3-methyl-1-(4-methyl-quinazolin-2-ylmethyl)-3,7 dihydropurine-2, 6-Dione) (Figure 1.7) (Deacon and Holst, 2010). Analysis of the X-ray crystal structure of linagliptin in complex with human DPP-4 showed that the butynyl substituent of linagliptin occupies the S1 hydrophilic pocket of the DPP-4 enzyme and the aminopiperidine substitute in the xanthine scaffold occupies the S2 substitute and the primary amine of linagliptin interacts with the key amino acid residues, (Deacon and Holst, 2010). Linagliptin is characterized by its high potency and excellent selectivity for the DPP-4 enzyme (Thomas et al., 2008), with a long duration of action which is advantageous for once-daily dosing (Thomas et al., 2008, Eckhardt et al., 2007).
1.5.5 Biological actions of the gliptins as potential treatment for diabetic vascular complications

1.5.5.1 Modulation of glucose homeostasis by the incretin system

In normal physiological conditions, the gut-derived incretin hormones GLP-1 and GIP are released from the large and small intestine in response to food ingestion. Both of these hormones stimulate insulin secretion and release from the pancreatic β-cells in proportion to blood glucose levels (Jose and Inzucchi, 2012). In addition, GLP-1 decreases glucagon secretion, slows gastric emptying, and increases satiety (Jose and Inzucchi, 2012, Scheen, 2011, Drucker and Nauck, 2006). The actions of GLP-1 and GIP are rapidly terminated by dipeptidyl peptidase-4 (DPP-4) (Figure 1.8). Thus, the compounds that inhibit the biological action of DPP-4 enzyme such as DPP-4 inhibitors will prolong the half-life and increase the bioavailability of the incretin hormones and consequently improve their biological effect to maintain glucose homeostasis in individuals with type 2 diabetes.
Figure 1.8 The physiological effect of the incretin system. The DPP-4 enzyme is activated after meal ingestion. As blood glucose levels increase in the blood, incretin hormones such as GLP-1 and GIP are released from neuroendocrine cells in the intestinal tract. These hormones cause insulin secretion from beta-cells in the pancreas. Additionally, in a glucose dependent manner, GLP-1 decreases glucagon secretion from pancreatic alpha-cells, increases the feeling of satiety, and slows gastric emptying. Through the action of DPP-4, the incretin hormones (GLP-1 and GIP) are degraded rapidly into their inactive forms. Blocking the action of DPP-4 increases the biological half-life of incretin hormones and improves their physiological actions. Obtained from (Jose and Inzucchi, 2012).
1.5.5.2 Effects of DPP-4 inhibitors on vascular endothelial cells and NO

In diabetes, endothelial dysfunction plays an important role in the initiation and development of vascular complications such as atherosclerosis, hypertension, and coronary artery disease (Liu et al., 2012, Matsubara et al., 2012a). The risk of developing coronary artery disease in type 2 diabetic patients is 2-4 fold higher compared to individuals without diabetes (Grundy et al., 1999). In type 2 diabetes mellitus, treatment with gliptins showed a potential vascular protective effect via GLP-1-dependent mechanisms (Hu et al., 2013). Gliptin stimulate endothelium-dependent GLP-1 receptor (GLP-1R)-induced vasodilation involving the cyclic adenosine monophosphate-dependent kinase A (PKA) pathway (Seino et al., 2010). Involvement of PKA pathway sequentially activates several downstream mediators which stimulate eNOS and increased production of NO (Liu et al., 2012). In addition to engagement of GLP-1R-mediated signalling pathway, there is some gliptin compounds that potentially exhibit improvement in endothelial function and acts against pathological vascular developments via GLP-1R-independent signalling pathways (Shah et al., 2011b, Kroller-Schon et al., 2012, Ding and Zhang, 2012). The gliptins vascular protective effects are well documented in animal models. In study using mouse model of hind limb ischemia, the DPP-4 inhibitor MK-0626 increased eNOS expression in vascular tissue and showed potential improvement in blood circulation (Shih et al., 2014). Also, the DPP-4 inhibitors sitagliptin and saxagliptin increased bioavailability of NO and improved the endothelial function in spontaneously hypertensive rat models (Aroor et al., 2013, Liu et al., 2012, Mason et al., 2012, Mason et al., 2011). The DPP-4 inhibitor linagliptin improved endothelial function and reduced oxidative stress and inflammation in rat model of lipopolysaccharide-induced septic shock (Kroller-Schon et al., 2012). It has also been reported that linagliptin significantly improved the function of endothelial cells in a rat model of renovascular hypertension (Chaykovska et al., 2013). Taken together, data from
these animal models under pathological challenges demonstrate that gliptins exhibit enhancement in vasorelaxation and facilitate the increase in blood flow. Additionally, gliptins also showed direct endothelium-dependent vasorelaxation in rat aortae ex vivo and the DPP-4 inhibitor linagliptin was the most potent vasorelaxant and has antioxidant effect which was not shared with other gliptins involved in the study such as alogliptin and vildagliptin (Kroller-Schon et al., 2012). In a study using human umbilical vein endothelial cell culture, the acute presence of either alogliptin or its mediator, GLP-1, caused eNOS upregulation and significant increases in activity and expression of eNOS causing an increased in bioavailability of NO (Zhong et al., 2013, Ding and Zhang, 2012, Shah et al., 2011b, Shah et al., 2011a). In several human clinical trials, gliptins exhibit improvement in endothelial function with vasoprotective effects (Matsubara et al., 2012a, Kubota et al., 2012, Van poppel et al., 2011, Noda et al., 2013, Suzuki et al., 2012, Fadini et al., 2010). Potential vascular benefits have also been identified with gliptins such as vildagliptin and alogliptin. These benefits may be due to different pathways eventually causing an upregulation of NO-mediated relaxation (Van poppel et al., 2011, Noda et al., 2013).

1.5.5.3 Effects of DPP-4 inhibitors on blood pressure

There is limited evidence that DPP-4 inhibitors affect blood pressure (BP). Recent trials show that BP was significantly reduced with DPP-4 inhibition (Liu et al., 2012, Lee et al., 2013, Chaykovska et al., 2013, Mistry et al., 2008, Ogawa et al., 2011). The BP in Zucker diabetic fatty (ZDF) rats and SHRs was significantly reduced after less than 2-4 weeks of treatment with sitagliptin, saxagliptin, and linagliptin (Liu et al., 2012, Kroller-Schon et al., 2012, Mason et al., 2012). Indeed, because GLP-1 is known to reduce salt intake and increase urinary salt excretion, thus gliptins may show a reduction in BP in salt
Sensitive hypertension (Ogawa et al., 2011). This beneficial effect was found in a trial in patients having uncontrolled type 2 diabetes mellitus currently treated with antihyperglycaemia and antihypertensive medications and the DPP-4 inhibitor sitagliptin was introduced to their treatment programme (Ogawa et al., 2011). In contrast, other studies report that the gliptins have little effect on BP (Jackson et al., 2008, Koren et al., 2012, Van poppel et al., 2011).

1.5.5.5 Effects of DPP-4 inhibitors on inflammation and atherosclerosis

Atherosclerosis is an outcome of complex pathologic proinflammatory events in the blood vessels and it is linked with activation of leukocytes with an increased production of inflammatory cytokines (Hansson, 2005). The formation of atherosclerosis plaques is due to the accumulation of inflammatory mediators in the blood vessels (Barbieri et al., 2013, Ervinna et al., 2013). Hyperglycaemia is a major risk action for atherosclerosis, due to increased oxidative stress and inflammation in addition to a negative action on function of platelets (Barbieri et al., 2013). Taken together, all of these risk factors promote the risk of atherosclerosis among type 2 diabetic patients.

Several animal studies demonstrated that gliptins have the capacity to delay the development of atherosclerosis both in diabetic and nondiabetic conditions (Shah et al., 2011a, Chaykovska et al., 2013, Ervinna et al., 2013, Terasaki et al., 2013, Ta et al., 2011). Vildagliptin normalized the oxidative stress and inflammatory markers in the aortae of Long-Evans Tokushima fatty rats (Matsui et al., 2011). Studies using apoE-null mouse models showed that gliptins, such as alogliptin, anagliptin, linagliptin, and des-fluoro-sitagliptin, markedly diminished the expression of proinflammatory cytokines and reduced inflammation by inhibiting monocyte/macrophage activation and chemotaxis (Zhong et al.,...
Furthermore, sitagliptin significantly decreased postprandial plasma LDL and vLDL cholesterols and delayed the progression of atherosclerosis in a hamster model of insulin-resistant fructose-induced hyperlipidaemia by increasing incretin action and this was consistent with other findings of sitagliptin-treated mice and also with apoE-deficient mice treated with alogliptin and anagliptin (Ervinna et al., 2013, Ta et al., 2011, Hsieh et al., 2010). The beneficial effects of gliptins on postprandial lipid profile may reduce the risk of cardiovascular pathology for individuals with type 2 diabetes (Eliasson et al., 2012). Moreover, it has been reported that the DPP-4 enzyme itself may be inherently atherogenic, thus there is some speculation that may act as a risk factor for atherosclerosis, because it stimulates cell proliferation in human coronary smooth muscle and enhances monocyte migration, inflammatory reactions mediated by macrophages, and tissue remodelling. Overall, it is still unclear whether the gliptins have the ability to reduce hyperlipidaemia and cardiovascular consequences. Therefore new investigations are needed to understand their potential to prevent the initiation and development of atherosclerosis associated with diabetes.

### 1.5.5.4 Cardiovascular effects of DPP-4 inhibitors

Studies assessing the beneficial effects of DPP-4 inhibitors on actual cardiovascular outcomes are limited, and their conclusions should be considered as highly preliminary. Accumulating evidence suggests that the incretin-based therapy has favourable cardiovascular outcomes, where the incretin has the potential to be used as cardiovascular protective therapy in experimental models of myocardial infarction (MI) and heart failure (Bose et al., 2005, Anagnostis et al., 2011, Jose and Inzucchi, 2012). In human clinical trial, it has been reported that the DPP-4 inhibition by sitagliptin by increasing the plasma concentrations of GLP-1 could protect the heart from ischaemic left
ventricular dysfunction during dobutamine stress echocardiography (DSE) in patients with ischaemic heart disease (Khan et al., 2010).

In addition to the cardiac beneficial effects of DPP-4 inhibition which are possibly mediated by GLP-1, DPP-4 inhibitors could also have direct effects on the risk factors affecting the heart or vasculature, independently of the incretin system pathway. For example, the mechanism which involves an enhanced signalling via the bone marrow chemokine identified as stromal cell-derived factor (SDF)-1alpha. Once there is damage to the blood vessels, local growth factors and cytokines are released and signal the bone marrow to release endothelial progenitor cells directed to the injured places of the blood vessels and differentiated into mature endothelial cells and contributes to the reconstruction of the vasculature. Interestingly, one of the regulators of the endothelial progenitor cells which enhance its mobilisation is SDF-1alpha which is known to be a substrate for the DPP-4 enzyme. Thus the inhibition of DPP-4 will increase SDF-1alpha concentrations to eventually promote the delivery of endothelial progenitor cells to the site of injured blood vessels (Heissig et al., 2002). This beneficial effect of DPP-4 inhibition on this chemokine was demonstrated by Fadini et al., who reported that after four weeks of treatment of type 2 diabetic patients with sitagliptin, there was an increase in endothelial progenitor cells, as well as an increase in SDF-1alpha levels, in addition to a reduction in the proinflammatory chemokine monocyte chemotactic protein (Fadini et al., 2010).

Mistry and colleagues reported that non-diabetic patients with mild to moderate hypertension treated with sitagliptin showed a small but significant reduction in their systolic blood pressure (Mistry et al., 2008). In a clinical trial, treatment of individuals with type 2 diabetes with sitagliptin, taken as a monotherapy was not associated with an increased risk of major adverse cardiovascular events and was generally well tolerated in clinical trials of up to 2 years in duration (Williams-Herman et al., 2010). Frederich et al.
evaluated the relative risk of cardiovascular events in trials assessing saxagliptin in patients with type 2 diabetes mellitus and conclude that saxagliptin had a potential to reduce cardiovascular events in diabetic patients (Frederich et al., 2010). A clinical trial examined the relationship between linagliptin and cardiovascular outcomes in type 2 diabetic patients compared to other diabetic patients receiving a placebo with treatment duration of at least 12 months. Linagliptin does not increase cardiovascular risk and, also, to exhibit a potential reduction of adverse cardiovascular diabetic events such as cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, or hospitalisation for unstable angina compared with placebo or other treatment (Johansen et al., 2011). White et al. in a randomized clinical trial examined the effects of alogliptin on cardiovascular end points such as cardiovascular death, non-fatal myocardial infarction and non-fatal stroke in individuals with type 2 diabetes. The findings demonstrated that the risk of cardiovascular events was lower in diabetic patients treated with alogliptin compared with diabetic individuals receiving a placebo (White et al., 2010). Indeed, several large-scale clinical trials planned specifically to evaluate the effects of DPP-4 inhibitors on cardiovascular events are currently proceeding including, TECOS (Randomised, Placebo Controlled Clinical Trial to Evaluate Cardiovascular Outcomes After Treatment With Sitagliptin in Patients With Type 2 Diabetes Mellitus and Inadequate Glycaemic Control), EXAMINE (Cardiovascular outcomes Study of Alogliptin in Subjects with Type 2 Diabetes and Acute Coronary Syndrome), SAVOR-TIMI 53 (Multicenter, Randomised, Doubleblinded, Placebo-Controlled Phase IV Trial to Evaluate the Effect of Saxagliptin on the Incidence of Cardiovascular Death, Myocardial Infarction or Ischaemic Stroke in Patients with Type 2 Diabetes) and CAROLINA (Cardiovascular Outcome Study of Linagliptin Versus Glimepiride in Patients with Type 2 Diabetes). Overall, small clinical studies suggesting that there are beneficial effects of DPP-4
inhibitors on diabetic cardiovascular risk. However, the relationship between the DPP-4 inhibitors and the actual cardiovascular outcomes remains poorly understood. To address this doubt, several large scale clinical trials using different DPP-4 inhibitors are now underway. Together, these large scale clinical trials could improve our understanding of the potential effects of these glucose-lowering drugs on cardiovascular events in diabetes.
1.6 Hypothesis

The broad aim of the thesis is to investigate the pathological changes associated with vascular dysfunction in the acute presence of high glucose or in small arteries from diabetic rats or from high fat western diet (WD) fed rats. Based on our understanding of the biological action and pharmacological activity of DPP-4 inhibitors, as discussed above, we hypothesize that DPP-4 inhibitors may improve vascular endothelial function in diabetes or western diet via GLP-1 dependent and/or independent pathways.

1.6.1 Specific aims of the project

(1) This project aims to investigate whether high glucose increases ROS production and causes endothelial dysfunction after incubation of 2 hours. Further to investigate whether the acute presence in vitro of DPP-4 inhibitors (linagliptin, sitagliptin, vildagliptin) or the GLP-1R agonist, exendin-4 prevents the impairment of endothelial function in small mesenteric arteries exposed to 40 mM glucose and to compare their antioxidant effects and also to investigate the effects of linagliptin on the relative contribution of NO-mediated and EDH-mediated relaxation to endothelium-dependent responses in vessels exposed to high glucose.

(2) It is well documented that STZ-induced hyperglycaemia causes increases in oxidative stress and impairment of endothelial function after a duration of 10 weeks. This project aimed to determine the acute effects in vitro of the DPP-4 inhibitor (linagliptin) on the mechanism(s) of endothelium-dependent relaxation in small mesenteric arteries from STZ-induced type 1 diabetic and normal rats, where any benefit will occur independently of any change in glucose levels where it is not possible for glucose concentration to influence the action of linagliptin.
(3) It is well established that small arteries and arterioles are primarily involved in determination of circulatory resistance and tissue perfusion. However, few if any studies have explored the effect of WD on the change of microvascular function. This project aimed to examine the effects of the acute presence of the DPP-4 inhibitor (linagliptin) *in vitro* on the mechanism(s) of endothelium-dependent relaxation in small mesenteric arteries from rats fed a high fat WD. Additionally, to explore the beneficial effects of linagliptin on the contribution of NO and/or EDH-mediated relaxation in small resistance arteries from WD fed rats.

(4) Based on the findings from the earlier studies, it was show that high glucose, STZ-induced diabetes and western high fat diet causes endothelial dysfunction due to impairment of the contribution of both NO-mediated and EDH-type relaxation, this was associated with increased activity of NADPH-oxidase-derived superoxide production and eNOS uncoupling in the mesenteric artery. Also based on the results from (1), (2), (3), it was demonstrated that acute treatment with linagliptin *in vitro* reduced oxidative stress and improved endothelial function in small mesenteric artery. The project aimed to investigate the effects of chronic treatment *in vivo* with the DPP-4 inhibitor (linagliptin) on the mechanism(s) of endothelium-dependent relaxation in small mesenteric arteries from STZ-induced diabetic and normal rats, and if so, whether it acts via direct scavenging of ROS and/or by inhibiting the sources of ROS production in the diabetic microvasculature, where any benefit will occur independently of changes in glucose levels in STZ-induced type 1 diabetes.
2.1 Animal experiments

Male Wistar rats were purchased from Animal Resource Centre (Perth, WA, Australia). All the experimental animals were kept in the Research Animal Facility at RMIT University. Animal welfare conditions were taken in account where all animal were kept under controlled environments of illumination (12 h light/12 h darkness) and temperature (20-25°C). All animals received free access to food (standard pellet diet) and water *ad libitum*. All the procedures were approved by the Animal Experimentation Ethics committee of RMIT University and complied with the Australian National Health and Medical Research Council code of practice for the care and use the animal for scientific purposes (AEC approval numbers 1121 or 1309).

2.1.1 Induction of diabetes

Streptozotocin (STZ) which causes pancreatic islet cell destruction was used to reduce insulin release thus generating an experimental model of type 1 diabetes. Sterile 0.1 M citrate buffer at pH 4.5 was used to dissolve STZ and prepared to a stock concentration of 100 mg/ml. A single-dose of STZ was used to induce type 1 diabetes at 6-8 weeks of age (~200 g) after overnight fasting. The rats were temporarily exposed to heat lamps (~3-5 min) to raise blood flow to the tail vein directly prior to the injection. To restrain the movement of the rats, they were wrapped in a towel with the tail exposed for injection. Ethanol (70%) was used to clean the tail to reduce the risk of infection and about 0.1 ml of STZ (50
mg/kg of body weight) was injected into the tail vein (26G/27G needle). Age-matched rats used as a control group were injected with the same volume of vehicle (0.1 M citrate buffer). After the injection of the STZ-treated and vehicle, the two groups were housed separately and received free access to food and water.

2.1.2 Blood glucose analysis

One week after STZ injection, rats blood glucose levels were measured using a glucometer (Accu-chek Advantage, Roche, U.S.A). Briefly, the rats were wrapped by towel allowing access to the tail which was pre-heated under a lamp. Ethanol (70%) was used to clean the tail to diminish the risk of infection. Applying a 26G needle, the tail vein was lanced to let bleeding occur and one drop of blood was collected onto a blood glucose strip fitted to the blood glucometer. The rats were considered diabetic if the blood concentration was higher than 20 mM. The rats received a low dose of insulin (4-5 IU, Protaphane, Novo Nordisk, NSW, Australia) to keep glucose <30 mM and to promote weight gain and reduce mortality, without achieving euglycaemia. All high glucose animals received insulin treatment on Mondays, Wednesday, and Fridays in the evening (4-6 pm). The blood glucose for these rats was monitored weekly on Friday mornings (10-11 am). At the time of tissue collection ie the end of experimental period, blood samples were collected from the left ventricle using a heparinized 18G needle and 3 ml syringes, and the glucose concentration was determined using the same procedure.

2.1.3 Estimation of glycated haemoglobin

Left ventricular blood was also transferred into vacuum blood collection tubes to be used for subsequent analysis of glycated haemoglobin (HbA1c). The blood was diluted
1:1 with milliQ water in an eppendorf tube. HbA1c was measured using a Micromat HbA1c analyser (Biorad, Sydney, NSW, Australia) following the manufacturer instruction.

2.2 Isolation of vascular tissue

At the time point of tissue collection with the end of experimental period, the rats were killed by asphyxiation with CO2 followed by exsanguination. The mesenteric arteries were immediately placed in ice-cold Krebs bicarbonate solution (4.7 mM KCl, 118 mM NaCl, 1.18 mM MgSO4, 25 mM NaHCO3, 11.1 mM D-glucose and 1.6 mM CaCl2), which were used for additional functional experiments and ROS measurement. The remaining arteries were snap frozen in liquid nitrogen and stored at -80°C for subsequent western blot analysis.

2.2.1 Preparation of mesenteric arteries

After the tissue collection, the entire tissue mass of mesenteric arteries was moved onto a large sylgard coated Petri dish, having ice-cold Krebs bicarbonate solution. The whole tissue of mesenteric arteries was pinned to the sylgard Petri dish using 26G needles in order to expose the branches of mesenteric arteries. Small mesenteric arteries (the third order branch of the superior mesenteric artery, internal diameter ~300 µm) were isolated, cleared of fat and connective tissue using a dissection microscope and cut into small rings of about 2 mm in length. Stainless steel wire (40-µm) in diameter was used and inserted through the lumen of the mesenteric artery in a gentle approach and great care was taken in inserting the wires to avoid any damage of the lumen of the artery (endothelium). The rings of the mesenteric arteries after inserting of the wires were then mounted on a Mulvany-Halpern myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) by fixing the wire onto the jaws of the myograph to the isometric force transducer side.
The first wire was used a guide to locate the lumen of the arteries, the second wire was inserted in the same manner with great care and fixed to the jaws of the myography to the micrometer side. The micrometer reading was increased thus that both the wires in the lumen of the artery were slightly separated and were in close contact with the wall of the artery (Figure 2.1). After the rings of the mesenteric arteries were mounted and fixed to the myography, the vessels were allowed to stabilize at zero tension for around 15 min before normalization. The standard optimization method was used to set the optimal value of resting tension for each ring of the mesenteric arteries and was measured by applying the resting tension-internal circumference relationship (Mulvany and Halpern, 1977; McPherson, 1992). In summary, the rings of mesenteric arteries were stretched in slight boosts (2 mN) with equilibration period was allowed between stretches. The passive tension in the rings was recorded and the vessels stretched till the passive tension was above 90 mm Hg (mean arterial blood pressure in rats). The rings of the mesenteric arteries were then fixed to an internal circumference equal to 90% of the calculated circumference at a passive transmural pressure of 13.3 kPa for the rest of experiment, the maximum active wall tension is achieved as at this level of stretching is reached. Therefore, the stretching of the vessels determine the passive tension-internal circumference and achieves an internal circumference equal to 90% of that of the entire blood vessels under transmural pressure of 100 mmHg. All the experiments were made in equal criteria and at 37°C and bubbled with carbogen (95% O2 and 5% CO2). After the normalization was performed, the mesenteric rings were fixed at the optimal tension for 30 minutes to stabilize and recorded on a chart recorder (model 3721; Yokogawa, Tokyo, Japan).
Figure 2.1 Diagrammatic representation of the Mulvany-Halpern myography chamber used to mount small mesenteric arteries. The ring segment of mesenteric artery was mounted on jaws, one wire fixed to the force transducer and the other wire to the micrometer in chamber of 5 ml volume containing Krebs buffer which continually aerated with 5% CO₂/95% O₂.
2.3 Vascular functional experiments

After the process of stabilization was finished, the vessels were subjected to a maximum contraction with an isotonic high K⁺-containing physiological saline solution (KPSS), in which the Na⁺ ions were replaced by K⁺ ions ([K⁺]KPSS=123 mM). The tension was resumed to basal tension, after a series of washouts with normal Krebs solution. In order to evaluate the integrity of the endothelium, the arteries were precontracted with phenylephrine (PE, 0.01-1 µM) to a level equal to (approximately 50%) of the KPSS response and then relaxed with a high concentration of ACh (10 µM). If the relaxation induced by ACh was larger than 80% of the precontraction, the endothelium was considered intact and functionally fit to run the experiments. Mesenteric arteries with endothelium that was not intact were excluded from the experimental studies.

2.3.1 Mesenteric arteries relaxation and contraction assays

After several washouts with normal Krebs, the mesenteric arteries were precontracted again with PE (0.01-1 µM) to 50% of the KPSS response. Relaxant responses to cumulative concentration additions of ACh (0.1 nM-10 µM) and sodium nitroprusside (SNP, 0.01 nM-10 µM) were determined. All experiments were conducted in the presence of indomethacin (10 µM) to inhibit the production of prostanoids and to block any contribution of prostanoids to the vascular relaxation. Furthermore, cumulative concentration-response curves to ACh and SNP were investigated subsequent to incubation with pharmacological inhibitors (Table 2.1). The different combination of the inhibitors is discussed in detail in each chapter. An example of a trace of the experimental protocol is illustrated in figure 2.2.
**Figure 2.2** A trace of the vascular relaxation protocol. Maximum contraction of the mesenteric arteries was achieved with 123 mM KPSS. PE was used (black dots) to precontract arteries and the endothelium was considered intact when the maximum relaxation exceeded 80% relaxation. The cumulative concentration response curve to ACh (10^{-10} - 10^{-5} M) or SNP (10^{-11} - 10^{-5} M) (red dots) were evaluated either in the absence or presence of pharmacological inhibitors (Table 2.1).
<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibitory activity</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>COX</td>
<td>10 µM</td>
</tr>
<tr>
<td>N-nitro-L-arginine (L-NNA)</td>
<td>NOS</td>
<td>100 µM</td>
</tr>
<tr>
<td>1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)</td>
<td>sGC</td>
<td>10 µM</td>
</tr>
<tr>
<td>1-[(2 chlorophenyl)(diphenyl)methyl]1H pyrazole (TRAM-34)</td>
<td>IK$_{Ca}$</td>
<td>1 µM</td>
</tr>
<tr>
<td>Apamin</td>
<td>SK$_{Ca}$</td>
<td>1 µM</td>
</tr>
<tr>
<td>Iberiotoxin (Ibtx)</td>
<td>BK$_{Ca}$</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>Tempol</td>
<td>SOD mimetic</td>
<td>100 µM</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>Superoxide generator</td>
<td>30 µM</td>
</tr>
<tr>
<td>Linagliptin</td>
<td>DPP-4 inhibitor</td>
<td>1 µM</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>DPP-4 inhibitor</td>
<td>1 µM</td>
</tr>
<tr>
<td>Vildagliptin</td>
<td>DPP-4 inhibitor</td>
<td>1 µM</td>
</tr>
<tr>
<td>Exendin-4</td>
<td>GLP-1R agonist</td>
<td>1nM-1µM</td>
</tr>
<tr>
<td>Exendin-Fragment (9-39)</td>
<td>GLP-1R antagonist</td>
<td>1 µM</td>
</tr>
</tbody>
</table>
2.3.2 Estimation of basal NO level assay

After the endothelium was investigated and considered to be intact, the vessels were precontracted again with PE (10-100 nM) to approximately 20% KPSS. The addition of the NOS inhibitor, L-NNA (100 µM), would induce additional contraction to the vessels. Due to removal of the basal level of NO that was acting against PE contraction. The basal level of NO was assessed by the extent of L-NNA-induced contraction (Figure 2.3).
Figure 2.3 Pattern of the protocol to assess basal NO activity. The mesenteric arteries reached maximum contraction induced by 123 mM K^+. ACh (10^{-5} M) was added to induce relaxation and if the relaxation was greater than 80%, the endothelium was considered intact. The addition of the NOS inhibitor, L-NNA (100 μM) to the arteries precontracted to approximately 20% of KPSS, produced further contraction of the vessels and was used as an indicator of the basal activity of NO.


2.4 Estimation of reactive oxygen species

The level of ROS was measured by the estimation of the superoxide production by L-012 or lucigenin-enhanced chemiluminescence assays.

2.4.1 L-012 assay

L-012 is a chemiluminescent probe used to measure superoxide levels. L-012 reacts with superoxide, to produce photons which could be quantified by a photon counter. The rings of rat mesenteric arteries were incubated in high glucose for 2 h and then incubated at 37°C for 30 minutes in Krebs-HEPES buffer (composition (mM): NaCl 99.90, KCl 4.7, KH₂PO₄ 1.0, MgSO₄·7H₂O 1.2, D-glucose 11.0, NaHCO₃ 25.0, CaCl₂·2H₂O 2.5, Na HEPES 20.0, pH 7.4) either alone, in the presence of tempol, a cell permeable SOD mimetic (100 µM), linagliptin, DPP-4 inhibitor (1 µM), sitagliptin, DPP-4 inhibitor (1 µM), vildagliptin, DPP-4 inhibitor (1 µM), exendin-4, GLP-1 agonist (1 µM), and exendin-fragment (9-39), (1 µM). 300 µL of Krebs-HEPES buffer, containing L-012 (100 µM, Wako Pure Chemicals, Osaka, Japan) and the appropriate treatments were placed into a 96-well Optiplate, which was loaded into a Polarstar Optima photon counter (BMG Labtech, Melbourne, VIC, Australia) to measure background photon emission at 37°C. After background counting was completed, a single ring segment of mesenteric artery was added to each well and photon emission was re-counted. Background counts were subtracted from superoxide counts and normalized with dry tissue weight.

2.4.2 Lucigenin assay

The superior mesenteric artery was isolated, cleared of fat and connective tissue and incubated in Krebs solution. The arteries were cut into small rings (2- to 3- mm-long
segments) in Krebs-HEPES buffer. The mesenteric arteries were incubated for 45 min at 37°C in Krebs-HEPES buffer either alone or in the presence of NADPH (100 μM) as a substrate for NADPH oxidase, diphenyliodonium (DPI, 5 μM), a flavoprotein inhibitor that inhibits NADPH oxidase, and linagliptin (1 μM). 300 μl of Krebs-HEPES buffer, containing lucigenin (5 μM) and the suitable treatment were placed into a 96-well Optiplate. The Optiplate was loaded into a Polastar Optima photon counter (BMG Labtech, Melbourne, VIC, Australia) to measure background photon emission at 37°C. After the background reading was recorded, a single ring section of mesenteric artery was placed into each well and photon emission was recounted to measure the production of NADPH oxidase-driven superoxide.

2.5 Western Blot

2.5.1 Extraction of protein from tissue samples

The mesenteric arteries from 2-3 rats of the same treatment groups were pooled, snap frozen and considered as n=1. Using a 1 ml glass homogenizer, the mesenteric arteries (~ 200 mg) were homogenized in lysis buffer solution consisting of (100 mM NaCl, 10 mM Tris, 2 mM EDTA, 0.5% w/v sodium deoxycholate, 1% vol/vol triton X-100, pH7.4, protease and phosphatase inhibitor cocktails (Roche, Sydney, NSW,Australia). After 20 min of incubation with lysis buffer at 4°C, the mesenteric arteries tissues were homogenized and tissue samples were centrifuged for 10 min at 4°C and 13,200 RPM. The supernatant of tissue samples were collected and kept at -80°C to be used when required.

2.5.2 Bradford protein assay

The Bradford assay was used to measure the protein concentration of the tissue samples. Phosphate buffered saline (PBS) (100 μL) in a test tube was used to dilute the
tissue samples 1:100 and 100 μL of 0.2 M NaOH was added to the test tube. After an incubation period of 15 minutes, milliQ water (600 μL) and 200 μL of red protein assay reagent dye (Biorad, Sydney, NSW, Australia), were added to the test tube. The solution which turned blue with the addition of protein was vortexed and 300 μL of the solution was loaded to a 96-well plate and in a plate reader the absorbance (590 nM) of the samples was measure. In addition, in the same 96-well plate, a bovine serum albumin (BSA) standard curve was generated (0-20 μg/mL). Each of the standard and the protein samples were applied in duplicate and if the absorbance of the tested samples were not compatible with the range of absorbance in the standard curve, the unknown samples were further diluted and the procedures were repeated as defined above. The standard curve was used to calculate the protein concentration of the protein in the unknown samples. The amount of protein in each sample was equal (30 μg) and the protein samples were aliquoted and PBS was used to top up the samples so they contained identical amount of protein (30 μg) in the same total volume (10 μL or 15 μL) of 2X Sample buffer (20% w/v glycerol, 2% SDS, 62.5 mM Tris, 0.05% bromophenol blue and 5% -mercaptoethanol, pH 6.8) and was added to each of the samples and stored at -80°C until required.

2.5.3 Gel preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

According to the manufacturers’ instructions, the plates were collected (Biorad, Sydney, NSW, Australia). The 7.5%, 10%, 12% and 15% resolving gel (30% acrylamide, distilled water, 1.5 M Tris, pH 8.8, 10% SDS, 10% ammonium persulfate and TEMED) was arranged and moved into the glass plates by pipette; isopropyl alcohol (~100 μL) was added to remove bubbles. Acrylamide-resolving gel was formed after 1-1.5 hours at room temperature by the polymerization of the solution. The alcohol was removed once the
resolving gel had set by dabbing using filter paper and 4% stacking solution (30% acrylamide, distilled water, 0.5 M Tris, pH 6.8, 10% SDS, 10% ammonium persulfate and TEMED) was added on to the top of the resolving gel, the 15-well comb was inserted, and the gel was left to polymerize at room temperature for 0.5-1 hour.

2.5.4 Preparation of SDS-PAGE

The method of SDS-PAGE was carried out by a mini-PROTEAN device (Biorad, Sydney, NSW, Australia). Denaturation of protein samples (30 µg) in sample buffer was completed by heating the samples at 95°C for 5 minutes. The prestained kaleidoscope protein ladder (7 µL, Biorad, Sydney, NSW, Australia) and the denatured protein were loaded into wells. The running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) was used to perform the electrophoresis at 100 V until the separation was complete (2 hours). Subsequent to the process of electrophoresis the gels were carefully detached from the gasket and equilibrated in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.037% SDS, pH 8.3) which works to eliminate additional salt and detergents from the running buffer and leads to increase the conductivity of the transfer buffer and reduce the amount of heat produced during the transfer. In the same transfer buffer, the Hybond nitrocellulose membranes, filter papers and sponges were also equilibrated. According to the manual for Biorad wet transfer cell, the sponges, filter papers, nitrocellulose membranes and gels were assembled, and gels were transferred at 350 mA for 2-3 hours. At the end of the transfer process, ponceau stain was used to confirm the transfer of protein was successful.
2.5.5 Performance of low temperature SDS-PAGE

To investigate the expression of eNOS monomers and dimers, we used a low temperature SDS-PAGE technique. As described in section 2.6.2, a 6% resolving gel was prepared. In low temperature SDS-PAGE, an ice cold resolving and stacking gel were used. Non-denatured protein sample (60 μg) and protein ladder (7 μL) were loaded into the wells of 6% resolving gel and ice cold running buffer used to perform electrophoresis at 30 V overnight at 4°C. After the end of the electrophoresis stage, the proteins were transferred from the gel to the nitrocellulose membrane at 30 V overnight at 4°C. Likewise, the membrane was stained with ponceau stain to confirm the transferred of protein was successfully performed.

2.5.6 Procedures of immunoblotting

To perform immunoblotting of eNOS, Nox2 and caveolin-1 protein levels (all BD Transduction Laboratories, Lexington, KY, USA), calmodulin (Millipore, Billerica, MA, USA), either by 5% w/v skim milk in Tris Buffered Saline plus 0.1% Tween-20 (TBST) for 1 hour at room temperature or 5% BSA/TBST (phosphorylated proteins) for 1 hour at 4°C were used in order to block the nitro-cellulose membranes before the addition of the primary antibodies (in 5 mL 3% BSA/TBST). Subsequently, the nitro-cellulose membranes were incubated with primary antibodies (1:1000) overnight at 4°C. After that the membranes were washed (3 x 10 minute washes with 10 mL TBST), and then incubated with secondary antibody (sheep anti-mouse) (Millipore, Billerica, MA, USA) (in 5 mL, 5% skim milk/TBST) (1:2000) at room temperature for 1 hour. After the incubation, the membranes were washed with 10 mL TBST (3 x 10 minute washes). The detection of the secondary antibody which conjugated with horseradish peroxidase was performed after the incubation of the membrane with either enhanced chemiluminescence reagents.
or Supersignal West Femto (Thermo Scientific, Rockford, IL, USA) for 1 minute, and the detection of the chemiluminescence signals on the membrane were achieved by digital image scanner (Biorad Chemidoc). Densitometry used to quantify the detected protein bands. After blocking non nonspecific binding with 5% w/v skim milk, nitro-cellulose membranes were re-investigated with a loading control antibody (actin), diluted 1:1000 in 5 mL 1% BSA/TBST. Followed by 3 x 10 minute washes of the membranes with 10 mL TBST TBST, and once more explored with secondary antibody (Millipore, Billerica, MA, USA) diluted 1:2000 in 5 mL 5% skim milk/TBST for 1 hour, and the bands were imagined and qualified as described previously.

2.6 Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except for linagliptin (Boehringer Ingelheim, Germany), acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, UK), and ODQ (Cayman Chemical, Ann Arbor, MI, USA). All chemicals were dissolved in distilled water, with the exception of L-NNA, which was dissolved in 0.1 M sodium bicarbonate, indomethacin, which was dissolved in 0.1 M sodium carbonate, ODQ, and TRAM-34 which was dissolved in dimethyl sulfoxide (DMSO). Linagliptin, vildagliptin, sitagliptin, exendin-4, and exendin-fragment (9-39) which were dissolved in distilled water.

2.7 Statistical analysis

All results are presented as the mean ± SEM, n indicates the number of experiments. The concentration response data from rat isolated mesenteric arteries were fitted to a sigmoidal curve using nonlinear regression (Prism version 6.0, GraphPad
Software, San Diego, CA, USA) to calculate the pEC$_{50}$. The maximum relaxation (R$_{\text{max}}$) to ACh or SNP was determined as a percentage of the phenylpherine pre-contraction. pEC$_{50}$ and R$_{\text{max}}$ values were compared and analysed using one way ANOVA or two way ANOVA with post hoc analysis using Bonferroni’s test or Student’s unpaired t-test as appropriate. p<0.05 was considered statistically significant.
Chapter 3

The DPP-4 inhibitor linagliptin and the GLP-1 receptor agonist exendin-4 improve endothelium-dependent relaxation of rat mesenteric arteries in the presence of high glucose

3.1 Introduction

Vascular disease is the main cause of morbidity and mortality in diabetes and impairment of endothelial function is considered to be a principle factor in the development of vascular disease (Grundy et al., 1999, De Vriese et al., 2000). Hyperglycaemia is thought to be a critical cause of endothelial dysfunction resulting in reduced NO release, generation of oxygen free radicals and impairment of endothelium-dependent relaxation (Calles-Escandon and Cipolla, 2001, Rodriguez-Manas et al., 2003). Acute exposure to high glucose reduces the sensitivity to acetylcholine-induced endothelium-dependent relaxation (Guo et al., 2000) with a reduced NO release (Reyes-Toso et al., 2002) associated with an increase in reactive oxygen species (Guo et al., 2000).

Incretin-based therapies, including inhibitors of dipeptidyl peptidase-4 (DPP-4) and glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists are effective in the treatment of type 2 diabetes (T2DM) by increasing insulin secretion (Ussher and Drucker, 2014, Drucker and Nauck, 2006). The incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), are released by intestinal endocrine cells in response to a
meal (Kim and Egan, 2008). These hormones have a key role in the modulation of pancreatic islet insulin release and, thus, of glucose homeostasis (Kim and Egan, 2008). Drugs that inhibit the effect of DPP-4, which cleaves both GLP-1 and GIP, extend the half-lives of these hormones and increase islet function and glycaemic regulation in T2DM (Pratley and Salsali, 2007, Ahren et al., 2004).

In addition to their ability to lower glucose there is increasing evidence that DPP-4 inhibitors or GLP-1R agonists improve endothelial function in T2DM patients (J Motta et al., 2012, Shah et al., 2011b) and in type 1 diabetic rats (Özyazgan et al., 2005). It has been reported that GLP-1 improves endothelial function and exerts protective actions in type-2 diabetic patients with coronary artery disease (Nystrom et al., 2004) and it has been reported that linagliptin and sitagliptin improve endothelium-dependent relaxation in type 2 diabetic rats (Takai et al., 2014).

Diabetes is associated with overproduction of reactive oxygen species (ROS) which is in turn associated with impaired NO release and/or activity causing endothelial dysfunction (De Vriese et al., 2000, Leo et al., 2011b). There is growing evidence that GLP-1R agonists and DPP-4 inhibitors may improve endothelial function. GLP-1R agonists are reported to increase eNOS activity in isolated endothelial cells (Hattori et al., 2010, Erdogdu et al., 2010) and to enhance endothelium-dependent dilatation in human subjects (Basu et al., 2007). Similarly DPP-4 inhibitors increase eNOS activity (Shah et al., 2011b) to increase eNOS expression in zucker obese rats (Aroor et al., 2013) and to improve endothelium-dependent relaxation in spontaneously hypertensive rats (Liu et al., 2012). By contrast, there is also a report that sitagliptin and alogliptin impair endothelium-dependent dilatation in patients with type 2 diabetes (Ayaori et al., 2013). A contributing factor to improved endothelial function may result from antioxidant effects of both GLP-1R agonists and DPP-4 inhibitors (Kroller-Schon et al., 2012, Ávila et al., 2013, Erdogdu...
et al., 2013, Shiraki et al., 2012, Ishibashi et al., 2010, Hendarto et al., 2012) which could decrease NO inactivation by ROS.

As high glucose induced impairment of endothelium-dependent relaxation in mesenteric arteries is associated with oxidative stress, thus, the aim of this study was to investigate whether the acute presence of DPP-4 inhibitors (linagliptin, sitagliptin, vildaglptin) or GLP-1R agonist, exendin-4 prevents the impairment of endothelial function in small mesenteric arteries exposed to 40 mM glucose and compare the antioxidant effects of linagliptin with those of sitagliptin, vildaglptin, and exendin-4 in vitro. Furthermore, we sought to investigate the effects of linagliptin on the relative contribution of NO-mediated and EDH-mediated relaxation to endothelium-dependent responses in vessels exposed to high glucose. The interest in linagliptin was due to results obtained in earlier part of study where linagliptin, but not the other DPP-4 inhibitors reduced superoxide production in mesenteric arteries exposed to high glucose solution.
3.2 Methods

All procedures were approved by the Animal Experimentation Ethics committee of RMIT University and complied with the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes (AEC approval number 1121).

3.2.1 Isolation of mesenteric arteries

Male Wistar rats (~8-weeks of age, ~250g) were used. The mesenteric arcade was isolated after rats were killed by asphyxiation with CO\textsubscript{2} followed by exsanguination. The mesenteric arteries were immediately placed in ice-cold Krebs bicarbonate solution (4.7 mM KCl, 118 mM NaCl, 1.18 mM MgSO\textsubscript{4}, 25 mM NaHCO\textsubscript{3}, 11.1 mM D-glucose and 1.6 mM CaCl\textsubscript{2}) containing the cyclooxygenase (COX) inhibitor indomethacin (10 µM), to inhibit the production of prostanoids. The third order branch of the superior mesenteric artery (internal diameter ~300 µm) was isolated, cleared of fat and connective tissue and cut into small rings (~2 mm). The arteries were mounted on a Mulvany-Halpern myograph (Danish Myo Technology, Aarhus, Denmark). The artery rings were stabilized at zero tension for 15 min in Krebs solution bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2} at 37ºC. Passive tension of the blood vessels was identified through stretching to obtain an internal circumference comparable to 90% of that in blood vessels under transmural pressure of 100 mmHg (Mulvany and Halpern, 1977).

3.2.2 Vascular experiments

The maximum contraction of the mesenteric arteries was assessed by addition of an isotonic physiological saline solution containing a high concentration of K\textsuperscript{+} (123 mM,
KPSS). The basal tension was restored after 3-4 washes using Krebs solution. The integrity of the endothelium was examined by precontraction of the mesenteric arteries with phenylephrine (0.1-3 µM) to ~50% of the KPSS response and relaxed with a high concentration of acetylcholine (ACh, 10 µM). ACh-induced relaxation of the precontracted rings was more than 80% in all cases indicating that the endothelium was functionally intact. The mesenteric arteries were incubated for 2 hours with 11 mM glucose or 40 mM glucose and again precontracted with phenylephrine (0.1-3 µM) to ~50% of the KPSS response. The high concentration of glucose (40 mM) was based on a previous reports demonstrating that short incubations (4-6 h) with a similar concentration (44 mM) impaired endothelial function in isolated blood vessels (Tesfamariam et al., 1991, Qian et al., 2006). Cumulative concentration-response curves to the endothelium-dependent relaxant, ACh (0.1 nM-10 µM) and the endothelium-independent relaxant, sodium nitroprusside (SNP, 0.01 nM-10 µm) were determined after 20 min incubation with linagliptin (1 µM), sitagliptin (1 µM), vildagliptin (1 µM) or exendin-4 (1 µM). The concentration of linagliptin was based on the observations of Kroller-Schon et al. (Kroller-Schon et al., 2012) that 1 µM was the lowest concentration to inhibit the respiratory burst in polymorphonuclear leucocytes and in preliminary experiments we found that this concentration had significant antioxidant activity and the same concentration was chosen for other compounds. Exendin-4 was found to be equally effective to improve endothelial function at a concentration of 1 µM (supplementary Figure 1). Relaxant responses were also assessed in the presence of different combinations of N-nitro-L-arginine (L-NNA, 100 µM), an inhibitor of nitric oxide synthase (NOS), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 µM), an inhibitor of soluble guanylate cyclase (sGC), apamin (1 µM), a selective blocker of the small conductance calcium-activated K⁺ channel (SKCa), 1-[(2-chlorophenyl) (diphenyl)methyl]-1H-pyrazole (TRAM-34, 1 µM), a blocker of
intermediate conductance calcium-activated K⁺ channel (IKCa), iberiotoxin (Ibtx, 100 nM), a blocker of the large conductance calcium-activated K⁺ channel (BKCa), superoxide dismutase (SOD, 50 U/mL) used to reduce superoxide, and pyrogallol (30 µM) used to generate superoxide.

3.2.3 Superoxide measurement in mesenteric artery

Superoxide production in the mesenteric artery was measured by using L-012 and lucigenin enhanced-chemiluminescence assays. Segments of mesenteric arteries were incubated in 40 mM glucose for 2 h and then incubated at 37°C for 30 minutes in Krebs-HEPES buffer (composition (mM): NaCl 99.90, KCl 4.7, KH₂PO₄ 1.0, MgSO₄•7H₂O 1.2, D-glucose 11.0, NaHCO₃ 25.0, CaCl₂•2H₂O 2.5, Na HEPES 20.0, pH 7.4) either alone or in the presence of tempol, a SOD mimetic (100 µM) or linagliptin (1 µM). 300 µL of Krebs-HEPES buffer, containing L-012 (100 µM, Wako Pure Chemicals, Osaka, Japan) and the appropriate treatments were placed into a 96-well Optiplate, which was loaded into a photon counter (Polarstar, BMG Labtech, Melbourne, VIC, Australia) to measure background photon emission at 37°C. After background counting was completed, a single ring segment of mesenteric artery was added to each well and photon emission was recounted. Background counts were subtracted from superoxide counts and normalized with dry tissue weight. Artery-derived superoxide was also measured using lucigenin as previously described (Leo et al., 2011b)

3.2.4 Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except for linagliptin (Boehringer Ingelheim, Germany), acetylcholine perchlorate (BDH
Chemicals, Poole, Dorset, UK), and ODQ (Cayman Chemical, Ann Arbor, MI, USA). All chemicals were dissolved in distilled water with the exception of L-NNA, which was dissolved in 0.1 M sodium bicarbonate, and indomethacin, which was dissolved in 0.1 M sodium carbonate.

### 3.2.5 Statistical analysis

Results are presented as the mean±SEM, $n$ indicates the number of experiments. The concentration response data from isolated mesenteric arteries were fitted to a sigmoidal curve using nonlinear regression (Prism version 6.0, GraphPad Software, San Diego, CA, USA) to calculate the $pEC_{50}$. The maximum relaxation ($R_{max}$) to ACh or SNP was determined as a percentage of the phenylephrine precontraction. $pEC_{50}$ and $R_{max}$ values were compared using one way ANOVA with post hoc analysis using Bonferroni’s test. $p<0.05$ was considered statistically significant.
3.3 Results

3.3.1 Effects of DPP-4 inhibitors and exendin-4 on vascular superoxide production

Using L-012 and lucigenin chemiluminescence assays, the level of superoxide in mesenteric arteries incubated in 40 mM glucose was demonstrated to be significantly higher than arteries incubated in 11 mM glucose (Figure 3.1). Linagliptin (1 µM) significantly reduced superoxide production in the presence of normal and high glucose whereas exendin-4 (1 µM) only reduced ROS levels in the presence of high glucose (Figure 3.1a, b). In contrast, sitagliptin (1 µM) and vildagliptin (1 µM) had no significant effect on the superoxide levels in the mesenteric rings under either condition (Figure 3.1a). The ability of linagliptin to reduce superoxide from arteries exposed to normal or high glucose was also confirmed using lucigenin enhanced chemiluminescence (Figure 3.1b).
Figure 3.1 ROS measurement in mesenteric arteries using L-012 (a) and lucigenin (b) enhanced-chemiluminescence assays. Superoxide levels were increased in mesenteric rings exposed to 40 mM glucose. Superoxide levels detected in the presence of normal (11 mM) glucose were significantly reduced by the SOD mimetic tempol (100 μM) or DPI (5 μM), a flavoprotein inhibitor that inhibits NADPH oxidase and linagliptin (1 μM) but not by any of the other treatments. The elevation of superoxide caused by high glucose was significantly attenuated by exendin-4 (1 μM) and linagliptin but not by the other DPP-4 inhibitors. Results are shown as mean±SEM. n=6 experiments. *p<0.05 vs 11 mM glucose untreated, #p<0.05 vs 40 mM glucose untreated, Bonferroni’s test.
3.3.2 Effect of high glucose, DPP-4 inhibitors, and exendin-4 on endothelium-dependent relaxation

High glucose (40 mM) significantly reduced the sensitivity and maximum relaxation to ACh, in the mesenteric arteries (Figure 3.2a, Table 3.3). The sensitivity and maximum relaxation to ACh in mesenteric arteries exposed to mannitol (29 mM mannitol+11 mM glucose) was similar to that determined in mesenteric arteries exposed to 11 mM glucose, indicating that the high glucose-induced impairment of the endothelium-dependent relaxation was not due to a hyperosmotic effect (Figure 3.3). SNP responses were unaffected by the high glucose (Figure 3.2b). Linagliptin (1 µM) significantly increased the sensitivity to ACh and the maximum relaxation in arteries exposed to 40 mM glucose (Figure 3.4, Table 3.1). The other DPP-4 inhibitors, sitagliptin (1 µM) and vildagliptin (1 µM), showed a significant increase in the maximum relaxation, but no change in the sensitivity to ACh-induced relaxation of mesenteric arteries exposed to 40 mM glucose whereas the GLP-1R agonist exendin-4 (1 µM), also caused a significant increase in the maximum relaxation and the sensitivity to ACh in the presence of high glucose (Figure 3.4, Table 3.1). To investigate the role of the GLP-1 receptor in the beneficial effects of linagliptin and exendin-4, responses to ACh were tested during co-incubation with the GLP-1 receptor antagonist exendin-fragment (9-39) (1 µM). The presence of exendin-fragment (9-39) had no effect on ACh-induced relaxation of mesenteric arteries exposed to 11 or 40 mM glucose (Table 3.2). In the presence of high glucose exendin-fragment (9-39) prevented the ability of exendin-4 to improve ACh-induced relaxation (Figure 3.5B) but did not affect the capacity of linagliptin to improve the endothelium-dependent relaxation (Figure 3.5A, Table 3.2).
Figure 3.2 Vascular function in mesenteric arteries. Cumulative concentration-response curves to ACh (a) and SNP (b) in endothelium-undamaged mesenteric arteries. In each group of experiments (a, b), mesenteric arteries were precontracted with PE to similar levels: (a) 11 mM glucose 65±3, 11 mM glucose+linagliptin 63±2, 40 mM glucose 61±3, 40 mM glucose+linagliptin 60±1, (b) 11 mM glucose 60±2, 11 mM glucose+linagliptin 61±3, 40 mM glucose 62±3, 40 mM glucose+linagliptin 60±4 %KPSS, n=5-11 experiments. Results are shown as mean±SEM. *pEC$_{50}$ significantly different from 40 mM glucose (Bonferroni’s test, p< 0.05).
Figure 3.3 The addition of mannitol (29 mM) to glucose (11 mM) had no effect on the endothelium-dependent relaxation indicating that the glucose-induced dysfunction was not due to hyperosmosis. *pEC₅₀ significantly different from 11 mM glucose and Mannitol (Bonferroni’s test, p<0.05), #pEC₅₀ significantly different from 40 mM glucose (Bonferroni’s test, p<0.05), n=6-9. Results are shown as mean±SEM.
Table 3.1 The effects of linagliptin, sitagliptin, vildagliptin and exendin-4, on endothelium-dependent relaxation to ACh in mesenteric arteries in the presence of normal (11 mM) and high (40 mM) glucose concentrations.

<table>
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<tr>
<th></th>
<th>Glucose (11 mM)</th>
<th>Glucose (40 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACh</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>pEC$_{50}$</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>6.96±0.10</td>
</tr>
<tr>
<td>Linagliptin</td>
<td>7</td>
<td>7.21±0.05</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>8</td>
<td>6.59±0.05</td>
</tr>
<tr>
<td>Vildagliptin</td>
<td>8</td>
<td>6.58±0.02</td>
</tr>
<tr>
<td>Exendin-4</td>
<td>8</td>
<td>6.61±0.04</td>
</tr>
</tbody>
</table>

All experiments were conducted in the presence of indomethacin (10 µM). n=the number of experiments. *Significantly different to 11 mM glucose (p<0.05), ANOVA plus Bonferroni’s multiple comparison test. #Significantly different to control within each group (p<0.05), ANOVA plus Bonferroni’s multiple comparison test.
**Figure 3.4** Cumulative concentration-response curves to ACh in endothelium-intact mesenteric arteries exposed to 11 and 40 mM glucose. In each group of experiments, mesenteric arteries were precontracted with phenylephrine to a similar level. All experiments were performed in the presence of indomethacin. Results are shown as mean±SEM of 6-11 experiments. *pEC$_{50}$ significantly different from 11 mM glucose (Bonferroni’s test, $p<0.05$). #pEC$_{50}$ significantly different from 40 mM glucose (Bonferroni’s test, $p<0.05$). See Table 3.1 for pEC$_{50}$ and R$_{max}$ values derived from these curves.
3.3.3 Effect of pyrogallol on endothelium-dependent relaxation

The effect of the superoxide generator, pyrogallol on ACh-induced relaxation is shown in (Figure 3.6). Pyrogallol (30 µM) significantly reduced the sensitivity to ACh (pEC\textsubscript{50} control 7.66±0.06, pyrogallol 7.07±0.06, p<0.05, Bonferroni’s multiple comparison test) but the maximum relaxation to ACh was not affected (R\textsubscript{max} control 96±1%, pyrogallol 95±3%). The addition of superoxide dismutase (SOD, 50 U/mL) as a scavenger of superoxide, improved the sensitivity to ACh in the presence of pyrogallol (7.64±0.07, p<0.05, Bonferroni’s multiple comparison test) (Figure 3.6). The sensitivity to ACh in the presence of pyrogallol was increased by linagliptin (7.64±0.14, p<0.05) or exendin-4 (7.17±0.12, p<0.05) demonstrating that linagliptin and exendin-4 effectively scavenged superoxide ions produced by pyrogallol.
Table 3.2 The effect of the GLP-1 receptor antagonist exendin-fragment (9-39) on the ability of linagliptin and exendin-4 to preserve ACh-induced endothelium-dependent relaxation in mesenteric arteries.

<table>
<thead>
<tr>
<th>ACh</th>
<th>Glucose (11 mM)</th>
<th>Glucose (40 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>7.03±0.18</td>
</tr>
<tr>
<td>Exendin-fragment (9-39)</td>
<td>5</td>
<td>6.51±0.06</td>
</tr>
<tr>
<td>Linagliptin+exendin-frag.</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Exendin-4+exendin-frag.</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

All experiments were conducted in the presence of indomethacin (10 µM). n = the number of experiments. Results are shown as mean±SEM of 5 experiments.

*Significantly different to 11 mM glucose (p<0.05), ANOVA plus Bonferroni’s multiple comparison test.

#Significantly different to 40 mM glucose (p<0.05), ANOVA plus Bonferroni’s multiple comparison test, ND= Not determined.
Figure 3.5 Demonstration of the beneficial effects of linagliptin (panel A) and exendin-4 (panel B), on responses to ACh in the presence of high glucose. The additional presence of the GLP-1 receptor antagonist exendin fragment (9-39) did not affect the protective actions of linagliptin but prevented the beneficial effect of exendin-4. In each group of experiments, mesenteric arteries were precontracted with phenylephrine to a similar level. All the experiments were conducted in the presence of indomethacin. Results are shown as mean±SEM of 5 experiments. *pEC$_{50}$ significantly different from 11 mM glucose (Bonferroni’s test, p<0.05). #pEC$_{50}$ significantly different from 40 mM glucose (Bonferroni’s test, p<0.05). See Table 3.2 for pEC$_{50}$ and R$_{max}$ values derived from these curves.
Figure 3.6 Cumulative concentration-response curves to ACh in the absence (control) or presence of pyrogallol (30 μM). Pyrogallol significantly decreased the sensitivity to ACh and this effect was prevented by the presence of linagliptin or exendin-4. Mesenteric arteries were precontracted with phenylephrine to a similar level. All the experiments were conducted in the presence of indomethacin. Results are shown as mean±SEM of 5 experiments. *pEC$_{50}$ significantly different from control (Bonferroni’s test, p<0.05). The values for the pEC$_{50}$ under different conditions are given in the text.
3.3.4 Effect of linagliptin on NO-mediated relaxation

To investigate the NO-mediated component of relaxation in mesenteric arteries responses to ACh were assessed in the presence of the combination of TRAM-34 and apamin. The addition of TRAM-34+apamin to vessel rings exposed to 11 mM glucose reduced the sensitivity, but not the maximum relaxation, to ACh in comparison to the control (Figure 3.7A, Table 3.3). By contrast, in arteries exposed to high glucose, the addition of IKCa and SKCa blockers did not change the sensitivity to ACh, however, the maximum relaxation was significantly diminished in comparison to the control (Figure 3.7B, Table 3.3). When responses to ACh were compared between arteries (Figure 3.5b) high glucose reduced the maximum relaxation to ACh in the presence of TRAM-34+apamin (Table 3.3), indicating that high glucose impaired the contribution of NO to endothelium-dependent relaxation. The additional presence of linagliptin had no effect on arteries exposed to 11 mM glucose but significantly increased the maximum relaxation induced by ACh in arteries exposed to 40 mM glucose in the presence of TRAM-34+apamin (Table 3.3) indicating that linagliptin enhanced the contribution of NO to relaxation when the glucose level was elevated.

3.3.5 Effect of high glucose on NOS and Nox2 related protein expression

Incubation of the rings of mesenteric arteries in 11 mM glucose concentration did not change the expression of total eNOS, whereas, 40 mM glucose concentration significantly decreased the expression of total eNOS (Figure 3.8a). The expression of Nox2 was significantly increased in the mesenteric arteries which incubated in 40 mM glucose compared to 11 mM glucose (Figure 3.8b).
Figure 3.7 Examination of the effect of high glucose on the contribution of NO to endothelium-dependent relaxation in mesenteric arteries. Cumulative concentration-response curves to ACh in the absence (control) or in the presence of TRAM-34+apamin in endothelium-intact mesenteric arteries exposed to 11 mM (A) or 40 mM glucose (B). In each group of experiments, mesenteric arteries were precontracted with phenylephrine to a similar level. All the experiments were conducted in the presence of indomethacin. Results are shown as mean±SEM of 5-9 experiments. *pEC$_{50}$ (A) or R$_{max}$ (B) significantly different from control (Bonferroni’s test, p<0.05). #pEC$_{50}$ significantly different from TRAM-34 + apamin (Bonferroni’s test, p<0.05). See Table 3.3 for pEC$_{50}$ and R$_{max}$ values.
Figure 3.8 Protein expression of eNOS (a) and Nox2 (b) in mesenteric arteries exposed to 11 mM and 40 mM glucose determined by western blot analysis. High glucose (40 mM) significantly reduced the expression of eNOS and increased the expression of Nox2. n=6 experiments. Representative blots are shown on each the corresponding graphs. Results are shown as mean±SEM. *Significantly different either from 11 mM glucose. (Student’s unpaired t-test, p<0.05).
3.3.6 Effects of linagliptin on EDH-mediated relaxation

To investigate the EDH-mediated relaxation, the contribution of NO was eliminated by the presence of L-NNA+ODQ to inhibit eNOS and soluble guanylate cyclase respectively. L-NNA+ODQ reduced the sensitivity to ACh in arteries exposed to 11 mM glucose (Figure 3.9A, Table 3.3). The sensitivity to ACh in arteries exposed to 40 mM glucose was also significantly reduced in comparison to arteries exposed to 11 mM glucose in the presence of L-NNA+ODQ (Table 3.3), suggesting that high glucose impaired the non-NO, non-prostanoid component of endothelium-dependent relaxation.

Linagliptin had no effect on EDH-mediated relaxation in mesenteric arteries exposed to 11 mM glucose (Figure 3.9A, Table 3.3) but improved the sensitivity to ACh-mediated EDH-mediated relaxation in arteries exposed to high glucose (Figure 3.9B, Table 3.3). When the contribution of NO was eliminated by L-NNA+ODQ, the maximum relaxation to ACh was unimpaired in mesenteric arteries exposed to either normal or high glucose concentration. The addition of TRAM-34 and apamin in the presence of L-NNA+ODQ significantly reduced ACh-induced relaxation in arteries exposed to 40 mM glucose (Table 3.3). When Ibtx was added to the combination of L-NNA+ODQ+TRAM-34+apamin, the relaxation to ACh in arteries exposed to 40 mM glucose was abolished (Table 3.3). In artery rings exposed to 11 mM glucose, ACh continued to cause a maximum relaxation of 15% in the presence of combination of L-NNA+ODQ+TRAM-34+apamin and the addition of Ibtx caused no further inhibition (Table 3.3). The presence of linagliptin had no effect on the maximum relaxation induced by ACh in the presence of L-NNA+ODQ+TRAM-34+apamin or with the addition of Ibtx in mesenteric arteries exposed to 11 mM glucose (Table 3.3), in contrast, acute treatment with linagliptin improved the ACh-induced relaxation in arteries exposed to 40 mM glucose in the presence of L-NNA+ODQ+TRAM-34+apamin (Table 3.3).
Table 3.3 Effect of L-NNA, ODQ and potassium channel blockers on ACh-induced relaxation of rat mesenteric arteries exposed to normal (11 mM) and high (40 mM) glucose in the absence or presence of linagliptin (1 µM).

<table>
<thead>
<tr>
<th>ACh</th>
<th>Glucose (11 mM)</th>
<th>Glucose (11 mM)+linagliptin</th>
<th>Glucose (40 mM)</th>
<th>Glucose (40 mM)+linagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>pEC50</td>
<td>Rmax</td>
<td>n</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>7.31±0.07</td>
<td>95±1</td>
<td>7</td>
</tr>
<tr>
<td>TRAM34+apamin</td>
<td>6</td>
<td>6.55±0.07</td>
<td>93±1</td>
<td>7</td>
</tr>
<tr>
<td>L-NNA+ODQ</td>
<td>9</td>
<td>6.64±0.12*</td>
<td>93±1</td>
<td>8</td>
</tr>
<tr>
<td>LNNA+ODQ+TRAM-34+apamin</td>
<td>7</td>
<td>5.71±0.39*</td>
<td>15±2*</td>
<td>5</td>
</tr>
<tr>
<td>LNNA+ODQ+TRAM-34+apamin+Ibtx</td>
<td>4</td>
<td>ND</td>
<td>5±1*</td>
<td>5</td>
</tr>
</tbody>
</table>

A comparison of the sensitivity (pEC50) and maximum relaxation (Rmax) to ACh in the absence (control), or the presence of TRAM-34 (1 µM)+apamin (1 µM), L-NNA (100 µM)+ODQ(10 µM), L-NNA (100 µM)+ODQ(10 µM)+TRAM-34 (1 µM)+apamin (1 µM) or L-NNA (100 µM)+ODQ(10 µM)+TRAM-34 (1 µM)+apamin (1 µM)+Ibtx (100 nM) in endothelium intact mesenteric arteries. All experiments were conducted in the presence of indomethacin (10 µM).
n=the number of experiments.
*Significantly different to control within each group (p<0.05), Dunnet’s test,
#Significantly different to 11 mM glucose within inhibitor group (p<0.05), Bonferroni’s test,
ΩSignificantly different to 40 mM glucose within inhibitor group (p<0.05), Bonferroni’s test,
ND, not determined.
Figure 3.9 Examination of the effect of high glucose on the contribution of EDH to endothelium-dependent relaxation in mesenteric arteries. Cumulative concentration-response curves to ACh in the absence (control) or in the presence of L-NNA+ODQ in endothelium-intact mesenteric arteries exposed to 11 mM (A) or 40 mM (B) glucose. In each group of experiments, mesenteric arteries were precontracted with phenylephrine to a similar level. All the experiments were conducted in the presence of indomethacin. Results are shown as mean±SEM of 7-9 experiments. *pEC$_{50}$ significantly different from control (Bonferroni’s test, p<0.05). #pEC$_{50}$ significantly different from L-NNA+ODQ (Bonferroni’s test, p<0.05). See Table 3.3 for pEC$_{50}$ and R$_{max}$ values.
3.4 Discussion

This study demonstrated that impairment of endothelial function caused by high (40 mM) glucose in rat mesenteric arteries was prevented by the presence of the DPP-4 inhibitor linagliptin or by exendin-4, a GLP-1R agonist. Both agents reduced the increase in vascular ROS caused by the high glucose concentration whereas the other DPP-4 inhibitors sitagliptin and vildagliptin failed to either reduce oxidative stress or to improve endothelial function. Importantly, whereas the protective action of exendin-4 involved activation of the GLP-1R the antagonist exendin fragment (9-39) had no effect on the beneficial action of linagliptin. Thus linagliptin acted independently of GLP-1 as expected in this isolated tissue and rather has direct antioxidant activity, perhaps by radical scavenging. High glucose impaired the contribution of both NO and EDH to endothelium-dependent relaxation and linagliptin improved the contribution of both of these relaxing factors.

3.4.1 Effects of DPP-4 inhibitors and exendin-4 on vascular superoxide generation

The findings in this study indicate that incubation of mesenteric arteries in high glucose for 2 hours significantly increased generation of superoxide. This increase in oxidative stress is consistent with other observations made in blood vessels isolated from rats with either type 1 or type 2 diabetes (Leo et al., 2011b), which is considered a key cause of endothelial dysfunction and consequent vascular disease (Fatehi-Hassanabad et al., 2010). We investigated the ability of the DPP-4 inhibitors, linagliptin, sitagliptin, and vildagliptin as well as the GLP-1R agonist, exendin-4 to reduce vascular superoxide levels and found that, whereas linagliptin and exendin-4 showed a significant reduction of superoxide production, the other DPP-4 inhibitors, sitagliptin and vildaglinptin, had no significant effect. Takai et al. (Takai et al., 2014) reported that linagliptin and sitagliptin improve endothelial function in type 2 diabetic rats but it is likely that effect is, at least in part, secondary to the lowering of
plasma glucose. Our observations are similar to that of Kroller-Schon et al. (Kroller-Schon et al., 2012) who reported that whereas linagliptin significantly inhibited the oxidative burst in human leucocytes sitagliptin, vildagliptin, alogliptin and saxagliptin were ineffective. The difference in the antioxidant activity of the DPP-4 inhibitors may be explained by their structural dissimilarities. Linagliptin has been reported to have antioxidant activity by inhibiting xanthine oxidase (Yamagishi et al., 2014) but this seems unlikely to explain the observations in isolated vascular tissue. Perhaps linagliptin acts as a radical scavenger in a manner similar to polyphenols such as flavonoids (Woodman and Chan, 2004), supported by its ability to prevent pyrogallol-induced impairment of endothelium-dependent relaxation. There are reports that vildagliptin can reduce oxidative stress after chronic treatment in rats with type 1 diabetes (Ávila et al., 2013) and after renal ischaemia and reperfusion (Glorie et al., 2012) but the mechanism of action has not been established. Reports that exendin-4 has antioxidant activity (Erdogdu et al., 2013, Padmasekar et al., 2013) and that GLP-1 decreased ROS production from cardiac microvascular endothelial cells cultured in a high glucose medium accompanied by a decreased expression of NADPH oxidase subunits (Wang et al., 2013) indicate a role for activation of GLP-1R in vivo. Thus linagliptin, when administered in vivo may have 2 mechanisms by which it acts as an antioxidant, direct radical scavenging and increased activation of GLP-1R secondary to DPP-4 inhibition.

3.4.2 Effects of DPP-4 inhibitors and exendin-4 on vascular endothelial function

In this study, 40 mM glucose significantly reduced the sensitivity and maximum response to the endothelium-dependent relaxant ACh without affecting endothelium-independent relaxation as previously reported (Ceriello et al., 1996, De Vriese et al., 2000, Cosentino et al., 1997, Cosentino et al., 2003). We confirmed that the impairment was not due to hyperosmosis as the presence of mannitol (29 mM) in addition to normal glucose
concentration had no effect on endothelium-dependent relaxation. To test whether DPP-4 inhibitors or activation of the GLP-1R improved endothelial function after exposure to high glucose ACh responses were assessed in the presence of linagliptin, sitagliptin, vildagliptin and exendin-4. Linagliptin and exendin-4 improved endothelium-dependent relaxation such that the sensitivity and maximum response to ACh in the presence of high glucose was restored to the control levels. By contrast sitagliptin and vildagliptin had no effect. The ability to improve endothelial function parallels the capacity of linagliptin and exendin-4 to reduce vascular superoxide in the presence of high glucose. To determine whether these beneficial effects involved activation of the GLP-1R we repeated the experiments in the presence of the antagonist exendin fragment (9-39) revealing that, whereas the protective actions of exendin-4 were prevented by the antagonist, the actions of linagliptin were maintained demonstrating that exendin-4 had GLP-1R-dependent actions but linagliptin did not. Data in this study is consistent with reports showing that exendin-4 inhibited ROS-induced injury to endothelial cells in a GLP-1R-dependent manner (Oeseburg et al., 2010, Erdogdu et al., 2013). Together these observations suggest that when administered in vivo linagliptin has the capacity to exert an antioxidant effect through 2 mechanisms, direct radical scavenging and by increasing the activity of GLP-1 at its receptor.

3.4.3 Effect of linagliptin on NO-mediated relaxation

To further examine the mechanism of the improved response to ACh in the presence of linagliptin we separately investigated the contribution of NO and EDH to relaxation. To examine the role of NO-mediated relaxation, EDH-mediated relaxation was abolished by the endothelial K_{Ca} channel blockers TRAM-34 and apamin. Under those conditions high glucose reduced the maximum response to ACh indicating an impaired contribution of NO to relaxation as has been reported previously (Cosentino et al., 1997) and as observed in animal
models of diabetes (Leo et al., 2011b). Interestingly, acute treatment with linagliptin in vitro significantly increased the maximum relaxation to ACh in high glucose exposed arteries in comparison to normal glucose. The restoration of NO activity by linagliptin treatment is consistent with its antioxidant effect reducing vascular superoxide and increasing the bioavailability of NO.

3.4.4 Effect of linagliptin on EDH-mediated relaxation

In order to evaluate the relative contribution of EDH to endothelium-dependent relaxation, we examined ACh-induced relaxation in the presence of L-NNA and ODQ to prevent the effect of NO synthesis and sGC activity respectively. The sensitivity to ACh in mesenteric arteries exposed to high glucose was significantly reduced in the presence of L-NNA and ODQ in comparison to normal glucose, indicating that high glucose impaired the contribution of EDH to endothelium-dependent relaxation, as previously reported (Ozkan and Uma, 2005, Liu and Gutterman, 2002, Clark and Fuchs, 1997). Acute treatment with linagliptin of the mesenteric arteries exposed to high glucose showed enhanced EDH-mediated relaxation which might be due to the reduction of superoxide levels by acute linagliptin treatment.

3.4.5 Conclusion

Acute treatment of rat mesenteric arteries exposed to high glucose in vitro with the DPP-4 inhibitor linagliptin or the GLP-1R agonist exendin-4 reduces superoxide generation and preserves the contribution of both NO and EDH to endothelium-dependent relaxation. Interestingly, whilst the protective actions of exendin-4 were GLP-1R dependent, linagliptin-induced improvement of endothelium-dependent relaxation was not affected by the GLP-1R
antagonist consistent with a capacity to directly scavenge ROS. Linagliptin enhanced both NO and EDH activity to improve endothelial function in the mesenteric arteries. Importantly, under the conditions of these experiments, linagliptin and exendin-4 improved endothelial function in the absence of any change in glucose levels. This suggests that it may be worthwhile to explore the potential for these agents to improve vascular function in pathologies beyond their current use in type 2 diabetes.
Chapter 4

Acute treatment with the DPP-4 inhibitor linaglaptan improves endothelium-dependent relaxation of rat mesenteric arteries from STZ-induced type 1 diabetic rats

4.1 Introduction

The impairment of endothelium-dependent relaxation has been identified as a critical factor in the initiation and development of diabetes-induced vascular complications (De Vriese et al., 2000, Fatehi-Hassanabad et al., 2010). Diabetes mellitus type 2 is characterised by insulin resistance and beta-cell dysfunction, causing hyperglycaemia and secondary micro-macrovascular complications (Bailey, 2008, Kimura et al., 2003). Hypoglycaemic agents and insulin when used in the management of diabetes do not prevent the development of parallel vascular complications (Brown et al., 2010, Holman et al., 2008). Under diabetic conditions, generation of oxygen radicals, primarily superoxide anion as a consequence of hyperglycaemia, has a key role in the pathogenesis of vascular complications (Brownlee, 2001, Forstermann, 2008, Li et al., 2013, Nishikawa et al., 2000). Potential targets for pharmacological therapies include eNOS, NADPH-oxidase and mitochondria which all have been reported as sources of ROS generation in diabetes (Fatehi-Hassanabad et al., 2010, Hink et al., 2001).

Dipeptidyl peptidase-4 (DPP-4) inhibitors are compounds used in the management of type 2 diabetes mellitus (Drucker and Nauck, 2006). We (Chapter 3) and other have
demonstrated that in addition to their glucose lowering action, the DPP-4 inhibitors might exhibit a variety of other biological actions such as antioxidant and vasorelaxant effects. (Kroller-Schon et al., 2012). Furthermore, a number of studies have revealed that DPP-4 inhibitors improve the activity of vascular eNOS (Shah et al., 2011b) or increased the expression of eNOS, for example in Zucker obese rats (Aroor et al., 2013). In the previous chapter, the acute antioxidant effect of the DPP-4 inhibitor linagliptin was demonstrated to restore endothelial function in small mesenteric arteries exposed to a high glucose concentration (Chapter 3). It was therefore hypothesised that acute treatment with linagliptin would have the ability to reduce the oxidative stress and inhibit the source of ROS generation in diabetic vasculature and consequently to improve endothelial function.

Endothelial dysfunction induced by hyperglycaemia in diabetes arises due to impairment of both NO and/or EDH-mediated relaxation and vascular oxidative stress (Heitzer et al., 2000, Mather et al., 2001, Antoniades et al., 2004, Leo et al., 2011b). Thus, the aim of the present study was to explore whether acute treatment of small mesenteric arteries from type 1 diabetic rats in vitro with the DPP-4 inhibitor linagliptin restores microvascular endothelial function, and if so, whether the effects are through direct scavenging of ROS. Additionally, we investigated whether linagliptin improved contribution of NO and/or EDH to endothelium-dependent relaxation in diabetic arteries.

4.2 Materials and Methods

The experiments were approved by the Animal Experimentation Ethics Committee of RMIT University (AEC approval number 1121) and complied with the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes.
4.2.1 Experimental animals and induction of type 1 diabetes

Diabetes was induced according to the methods described in Chapter 2.1. Briefly, male ~8 week old Wistar rats, weighing approximately 200 g, were randomly divided into two groups: normal and diabetic. Type-1 diabetes was induced by a single injection of streptozotocin (STZ, 50 mg/kg) into the tail vein after overnight fasting. The normal group received an equivalent volume of the vehicle (0.1 M citrate buffer, pH 4.5). Blood glucose was measured at the time of experiment for all groups of rats using ACCU-CHECK Advantage II monitor (Roche, Mannheim, Germany). Rats with a blood glucose of >20 mmol/L were considered to be diabetic.

4.2.2 Vascular function assay

The vascular function experiments were performed according to methods described in Chapter 2.3. Briefly, mesenteric arteries were precontracted to a similar level using PE (0.1-3 µM), and cumulative concentration-response curves to ACh (0.1 nM/L-10 µM) and SNP (0.01 nM-10 µM) were investigated. In addition, responses to ACh and SNP were examined after 20 minutes incubation with different combinations of L-NNA (100 µM), a non-selective eNOS inhibitor, ODQ (10 µM), a sGC inhibitor, TRAM-34 (1 µM), a selective blocker of IKCa, Ibtx (100 nM), a selective blocker of BKCa and apamin (1 µM), a SKCa inhibitor.

4.2.3 Assessment of ROS in mesenteric artery

ROS in the mesenteric artery was measured using either L-012 or lucigenin as described in Chapter 2.4.1.
4.2.4 Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except for ODQ (Cayman Chemical, Ann Arbor, MI, USA), and acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, UK). Linagliptin was a gift from (Boehringer Ingelheim, Germany). All drugs were dissolved in distilled water with the exception of indomethacin, which was dissolved in 0.1 M sodium carbonate, and L-NNA, which was dissolved in 0.1 M sodium bicarbonate.

4.2.5 Statistical analysis

Data were expressed as mean ± SEM, n indicates the number of experiments. The concentration response data from rat isolated mesenteric arteries were fitted to a sigmoidal curve using nonlinear regression (Prism version 6.0, GraphPad Software, San Diego, CA, USA) to calculate the pEC$_{50}$. The maximum relaxation ($R_{\text{max}}$) to ACh or SNP was determined as a percentage of the phenylephrine precontraction. Differences between groups of pEC$_{50}$ and $R_{\text{max}}$ values were compared and analysed using one way ANOVA with post hoc analysis using Bonferroni’s test or Student’s unpaired t-test as appropriate. p<0.05 was considered statistically significant.

4.3 Results

4.3.1 Body weights and blood glucose

Table 4.1 shows the levels of body weight gained, blood glucose, and HbA1$_c$. Ten weeks after treatment with STZ, the body weight gained was significantly higher in the control than in diabetic group (Table 4.1). In diabetic rats, the blood glucose and HbA1$_c$ levels were significantly greater compared to normal rats (Table 4.1).
4.3.2 Effect of diabetes and linagliptin on superoxide production

The superoxide level in mesenteric arteries was measured by L-012 and lucigenin chemiluminescence assays. The level of superoxide generation in intact mesenteric arteries from diabetic rats was significantly greater than in normal rats. Treatment with tempol, a cell permeable SOD mimetic or DPI, a flavoprotein inhibitor that inhibits NADPH-oxidase, reduced the production of superoxide anions to equivalent levels in normal and diabetic rats (Figure 4.1a, b). The acute presence of linagliptin (1 µM) attenuated the generation of superoxide anions in mesenteric arteries from normal and diabetic rats (Figure 4.1a, b).
Table 4.1 Mean body weight gained, blood glucose, and HbA1c levels at the end of experiment of normal and diabetic rats.

10 weeks after vehicle or STZ treatment

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Normal</th>
<th>n</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>10</td>
<td>524±17</td>
<td>9</td>
<td>372±16</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>8</td>
<td>5.3±0.2</td>
<td>9</td>
<td>28.5±1.0*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9</td>
<td>5.2±0.1</td>
<td>9</td>
<td>13.0±0.2*</td>
</tr>
</tbody>
</table>

n= the number of rats.

*Significantly different from normal group (Student’s unpaired t-test, p<0.05).

Results are shown as mean±SEM.
Figure 4.1 Measurement of ROS in intact mesenteric arteries. In diabetic arteries, superoxide (a), NADPH-oxidase activity (b) was increased and were decreased by acute presence of linagliptin or by the presence of DPI, a flavoprotein inhibitor that inhibits NADPH oxidase, or tempol, a cell permeable SOD mimetic. Results are shown as mean±SEM. n=5-9 experiments. *p<0.0001 vs normal, †p<0.0001 vs diabetic.
4.3.3 Effect of diabetes and linagliptin on vascular function

The mesenteric arteries from diabetic rats showed a significant impairment of their relaxation response to ACh with a reduction in sensitivity, but not the maximum relaxation (Figure 4.2a, Table 4.2). In contrast, the sensitivity (diabetic, 7.56±0 vs. normal, 7.47±0, n=4-5, p<0.05) and maximum relaxation (diabetic, 97±16% vs. normal, 97±7%, n=4-5, p<0.05) to SNP were not affected (Figure 4.2b). The acute presence of linagliptin in arteries from normal rats did not affect the sensitivity and response to ACh, but, in mesenteric arteries from diabetic rats linagliptin, caused a significant increase in the sensitivity (Figure 4.2a, Table 4.2). The presence of linagliptin did not affect the sensitivity or maximum response to SNP in normal or diabetic rats (Figure 4.2).
Figure 4.2 Cumulative concentration-response curves to ACh (a), SNP (b) in endothelium-intact mesenteric arteries. In each group of experiments (a, b), mesenteric arteries were precontracted with PE to similar levels: (a) normal 60±1, normal+linagliptin 62±3, diabetic 59±1, diabetic+linagliptin 63±1, (b) normal 58±2, normal+linagliptin 57±3, diabetic 61±3, diabetic+linagliptin 59±4 %KPSS, n=5-12 experiments. Results are shown as mean±s.e.m. *p<0.05 vs. diabetic. See Table 4.2 for pEC$_{50}$ and $R_{max}$ values for ACh.
4.3.4 Role of NO in normal and diabetic mesenteric arteries

The response to ACh-induced relaxation in mesenteric arteries from normal rats was reduced by the presence of TRAM-34 and apamin to block the IKCa and SKCa channels (Figure 4.3a, Table 4.2). In contrast, in mesenteric arteries from diabetic rats, the sensitivity to ACh was unaffected by the presence of a combination of TRAM-34 and apamin (Figure 4.3b, Table 4.2), but the maximum relaxation was significantly reduced (Figure 4.3b, Table 4.2). The presence of a combination of TRAM-34, apamin and Ibtx inhibited the maximum relaxation of vessels from both normal and diabetic rats (Table 4.2). This is indicating that NO contributes to endothelium-dependent relaxation which is impaired due to diabetes.

4.3.5 Role of EDH-type relaxation in normal and diabetic mesenteric arteries

The sensitivity to ACh in both normal and diabetic mesenteric arteries were significantly reduced by the presence of NO synthase inhibitor, L-NNA and a sGC inhibitor, ODQ (Figure 4.4a, b, Table 4.2). When the contribution of NO was eliminated by L-NNA+ODQ, the sensitivity to ACh was significantly lower in the diabetic compared to the normal arteries (Table 4.2), indicating that diabetes impairs the contribution of a non-NO and non-prostanoid factor to endothelium-dependent relaxation. The maximum relaxation to ACh was unaffected by the presence of L-NNA+ODQ in normal or diabetic arteries.
**Table 4.2** Effect of L-NNA, ODQ and potassium channel blockers on ACh-induced relaxation of mesenteric arteries from normal and diabetic rats in the absence or presence of linagliptin (1 µM).

<table>
<thead>
<tr>
<th>ACh</th>
<th>Normal</th>
<th>Normal+linagliptin</th>
<th>Diabetic</th>
<th>Diabetic+linagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>R&lt;sub&gt;max&lt;/sub&gt; (%)</td>
<td>n</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>7.20±0.11</td>
<td>98±0</td>
<td>6</td>
</tr>
<tr>
<td>TRAM34+apamin</td>
<td>6</td>
<td>6.62±0.12&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>93±1</td>
<td>7</td>
</tr>
<tr>
<td>L-NNA+ODQ</td>
<td>10</td>
<td>6.65±0.12&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>92±2</td>
<td>10</td>
</tr>
<tr>
<td>LNNA+ODQ+TRAM-34+apa+Ibtx</td>
<td>4</td>
<td>ND</td>
<td>6±1&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>6</td>
</tr>
</tbody>
</table>

A comparison of the sensitivity (pEC<sub>50</sub>) and maximum relaxation (R<sub>max</sub>) to ACh in the absence (control), or the presence of TRAM-34 (1 µM) +apamin (1 µM), L-NNA (100 µM)+ODQ (10 µM), L-NNA (100 µM)+ODQ (10 µM)+TRAM-34 (1 µM)+apamin (1 µM) or L-NNA (100 µM) +ODQ (10 µM)+TRAM-34 (1 µM)+apamin (1 µM)+Ibtx (100 nM) in endothelium intact mesenteric arteries. All experiments were performed in the presence of indomethacin (10 µM). n = the number of experiments.

*Significantly different to control within each group, p<0.05, Bonferroni’s test,

<sup>φ</sup>Significantly different to normal within inhibitor group, p<0.05, Bonferroni’s test,

<sup>¥</sup>Significantly different to diabetic within inhibitor group, p<0.05, Bonferroni’s test. Results are shown as mean±SEM, ND= not determined.
4.3.5 Effect of linagliptin on NO-mediated relaxation

In order to investigate the role of the NO-mediated component of the relaxation, responses to ACh were assessed in the presence of the KCa blockers, TRAM-34, apamin and Iberio-toxin. When the responses were compared between mesenteric arteries from normal and diabetic rats, it is apparent that diabetes significantly reduced the maximum relaxation induced by ACh in the presence of TRAM-34+apamin, indicating that NO-mediated relaxation was impaired by diabetes. Acute treatment with linagliptin had no effect in normal mesenteric arteries (Figure 4.3c, Table 4.2), but significantly increased the maximum relaxation in mesenteric arteries from diabetic rats (Figure 4.3d, Table 4.2). A similar finding was observed in linagliptin treated diabetic mesenteric arteries in the presence of combination of TRAM-34+apamin+Ibtx, indicating that linagliptin was able to improve the contribution of NO to endothelium-dependent relaxation in mesenteric arteries from diabetic rats (Table 4.2).
Figure 4.3 Contribution of NO to endothelium-dependent relaxation in mesenteric arteries. Investigation of NO-mediated relaxation in mesenteric arteries isolated from normal (a), diabetic (b) in the absence or presence of linagliptin, normal+linagliptin (c), diabetic+linagliptin (d) rats. In each group of experiments, arteries were precontracted with PE to similar level. n=6-7 experiments. Results are shown as mean±s.e.m. *p<0.05 vs diabetic. See Table 4.2 for values.
4.3.6 Effect of linagliptin on EDH-type relaxation

To identify the role of EDH-mediated relaxation, the action of NO-mediated relaxation was abolished via the addition of L-NNA and ODQ. In the presence of L-NNA+ODQ, the sensitivity but not the maximum response to ACh was significantly reduced in mesenteric arteries from diabetic rats compared to normal rats, suggesting that diabetes impaired the EDH type response. The presence of linagliptin in vitro had no significant effect on ACh-induced EDH-mediated relaxation in mesenteric arteries from normal rats (Figure 4.4a, Table 4.2), but significantly increased the sensitivity to ACh in mesenteric arteries from diabetic rats (Figure 4.4b, Table 4.2), indicating that linagliptin improved the contribution of EDH-type relaxation in diabetic rats. The addition of the combination of L-NNA+ODQ+TRAM-34+apamin+Ibtx, abolished ACh-induced relaxation in either untreated diabetic or linagliptin-treated diabetic as well as untreated normal or linagliptin-treated normal mesenteric arteries (Table 4.2).
Figure 4.4 Contribution of EDH to endothelium-dependent relaxation in mesenteric arteries. Examination of EDH-type relaxation in mesenteric arteries isolated from normal and diabetic rats in the absence or presence of linagliptin, (a), normal+linagliptin, (b) diabetic+linagliptin. In each group of experiments, arteries were precontracted with PE to similar level. n=8-10 experiments. Results are shown as mean±s.e.m. See Table 4.2 for values of pEC_{50} and R_{max} derived from these data.
4.4 Discussion

This study demonstrated that, after 10 weeks of diabetes, endothelial dysfunction was evident in the rat small mesenteric artery. This was due to a decreased contribution of both NO-mediated and EDH-type relaxation to endothelium-dependent relaxation which associated with increase in superoxide production. This study also demonstrated that acute treatment of mesenteric arteries from type 1 STZ-induced diabetic rats attenuates the high levels of oxidative stress caused by hyperglycaemia and ameliorates endothelial dysfunction in mesenteric arteries from diabetic rats. Interestingly, linagliptin acted independently of glucose lowering action and rather has direct antioxidant activity, possibly via radical scavenging as demonstrated in (Chapter 3). Hyperglycaemia in type 1 STZ-induced diabetic rats impaired the contribution of NO and EDH to endothelium-dependent relaxation and acute treatment in vitro with linagliptin enhanced the contribution of both these mediated-relaxation factors.

4.4.1 Effect of diabetes on superoxide and endothelium-dependent relaxation

In this study, the results indicate that type 1 STZ-induced diabetes augmented the level of ROS production in mesenteric arteries which is consistent with previous reports (Leo et al., 2010, Leo et al., 2011b, Inoguchi et al., 2000, Guzik et al., 2002). The increase in superoxide was concomitant with a selective impairment of endothelium-dependent relaxation in the mesenteric arteries. The level of superoxide production and the degree of impairment of endothelial function is consistent with the opportunity that hyperglycaemia-induced oxidative stress was responsible for the impairment of endothelium-dependent relaxation in the mesenteric arteries. The overproduction of superoxide by the mechanism of mitochondrial electron transport chain due to hyperglycaemia-induced oxidative stress
becomes an important interpretation for the pathogenesis of diabetic complications (Brownlee, 2001). This mechanism is followed by the activation of four downstream pathways: increased polyol pathway flux, increased formation of advanced glycosylation end products, activation of protein kinase C, and increased hexosamine pathway flux (Nishikawa et al., 2000).

### 4.4.2 Effect of diabetes on NO and EDH-type response

In the resistance vessels, such as the rat mesenteric artery, endothelium-dependent relaxation is mediated by multiple factors including NO and EDH (Edwards et al., 2010). High glucose concentration-induced oxidative stress has been associated with the impairment of both NO- and EDH-mediated relaxation (Chapter 3) (De Vriese et al., 2000). In diabetes, the increase in superoxide anions production can cause impairment in endothelium-dependent relaxation via a number of mechanisms. Superoxide reacts with NO immediately, to form peroxynitrite which simultaneously causes reduction in the availability of NO for relaxation and increases peroxynitrite-induced toxicity. For example, peroxynitrite has been demonstrated to uncouple endothelial NO synthase due to tetrahydrobiopterin (BH4) oxidation, therefore additional stimulating the synthesis of superoxide (Münzel et al., 2010), and leads to protein nitration of the calcium-activated potassium channels which are responsible for EDH-type responses (Liu and Gutterman, 2002). Furthermore, it has been reported that the peroxynitrite alone can cause impairment to EDH-type responses in the mesenteric arteries, which might be improved by the existence of antioxidant treatment (Li et al., 2008).
4.4.1 Effect of linagliptin on vascular superoxide and relaxation

As we described early, in diabetes, increases in the generation of superoxide caused an impairment in eNOS activity, increased NO inactivation, increased eNOS uncoupling via oxidation of BH4, further increased activity of NADPH oxidase, and increased formation of peroxynitrate which may promote the initiation of endothelial dysfunction. The presence of linagliptin in vitro significantly reduced increased level of superoxide generation detected, indicating that the linagliptin may reduce the oxidative stress, possibly by direct scavenging of superoxide and improved endothelium-dependent relaxation in mesenteric arteries from type 1 diabetic rats without affecting arteries from normal rats. Our findings in this study are consistent with our findings in (Chapter 3) and other observations demonstrated that treatment with linagliptin improves injury to cardiovascular tissue induced by salt-sensitive hypertension which seem to be attributed to the reduction of oxidative (Koibuchi et al., 2014) or enhancement of endothelial function and reduced vascular stress in aortic artery challenged to experimental sepsis (Kroller-Schon et al., 2012).

4.4.2 Effect of linagliptin on NO-mediated relaxation

To investigate NO-mediated relaxation, ACh-induced relaxation was assessed in the presence of endothelial KCa blockers to inhibit the EDH-mediated relaxation. This inhibition of the EDH-type response, revealed significant decrease in the sensitivity to ACh and the maximum relaxation in diabetic arteries compared to normal arteries. Acute treatment with linagliptin significantly increased the maximum relaxation mediated by ACh in mesenteric arteries from diabetic rats in comparison to untreated mesenteric arteries from diabetic rats, indicating that linagliptin improved the contribution of NO to endothelium-dependent relaxation in diabetic mesenteric arteries. The prevention of peroxynitrite formation and the reduction of ROS activity in diabetic vasculature in rats might be at least in one part via rapid
free radical scavenging effect of linagliptin as we demonstrated in (Chapter 3), in order to preserve the bioavailability of NO in vascular endothelial cells as we also observed in this study. The response to SNP-induced endothelium-independent relaxation in this study was comparable in both normal and diabetic rats, demonstrating that in both normal and diabetic rats, the responses to exogenous NO-induced relaxation in smooth muscle cells was not impaired.

4.4.3 Effect of linagliptin on EDH-mediated relaxation

In order to investigate the contribution of EDH-type relaxation to endothelium-dependent relaxation, the NO-mediated relaxation was inhibited by the application of L-NNA and ODQ to inhibit NO synthesis and sGC activity respectively. In the presence of L-NNA+ODQ, the sensitivity to ACh was decreased in diabetic arteries when compared to normal arteries, showing that diabetes impaired the contribution of EDH to endothelium-dependent relaxation as previously reported (Leo et al., 2011b, Makino et al., 2000, Wigg et al., 2001). It has been reported that superoxide generation by auto-oxidation of pyrogallol might impair EDH type responses in rat mesenteric arteries (Ma et al., 2008). Furthermore, it has been suggested that K⁺ as an one of EDH factor and the vasodilatation response to K⁺ is impaired in the mesenteric arteries in diabetic rats (Makino et al., 2000). Acute treatment with linagliptin of mesenteric arteries from diabetic arteries improved EDH-mediated relaxation which likely due to the antioxidant effect of linagliptin (Chapter 3).

4.4.4 Conclusion.

In the present study we have demonstrated that acute treatment of mesenteric arteries from diabetic rats in vitro with the DPP-4 inhibitor linagliptin significantly reduces the
generation of superoxide and preserves the contribution of both NO and EDH to endothelium-dependent relaxation. As we demonstrated in (Chapter 3), the attributed beneficial protective effect of linagliptin in this study, at least in part, by rapidly scavenge ROS and reduce oxidative stress to increase NO bioavailability and improve EDH-type response in vascular endothelial cells. Interestingly, the beneficial effect of linagliptin to protect NO and EDH activity observed in this study shows that it has potential as therapeutic agent for use in the reduction and/or prevention of microvascular complications associated with diabetes beyond its current use in type 2 diabetes.
Chapter 5

The DPP-4 inhibitor linagliptan restores endothelial function of mesenteric arteries from rats fed a western diet

5.1 introduction

Cardiovascular morbidity and mortality have been reported to be associated with metabolic syndrome (Isomaa et al., 2001). Endothelial dysfunction is known to be linked to obesity, atherogenesis, hypertension and insulin resistance that enhances the risk of cardiovascular diseases (Caballero, 2003). It has been observed that obesity, particularly in viscera, is correlated with the early stages of development of endothelial dysfunction (Arcaro et al., 1999, Caballero, 2003). Excessive considerations have been devoted to high-fat content of diet which is proposed to be a key event and responsible for the development of obesity and subsequent vascular dysfunction, even before progress to type 2 diabetes mellitus (Cuevas et al., 2000, Woo et al., 2004, Hajer et al., 2008) These observations demonstrate that the consumption of high-fat diet is associated with the development of the impairment of endothelium-dependent relaxation of large arteries due to transformed NO signaling mechanisms (Molnar et al., 2005, Roberts et al., 2000).

It is well established that small arteries and arterioles are primarily involved in determination of circulatory resistance and tissue perfusion. Nevertheless, few if any studies have explored the effect of western diet (WD) on the change of microvascular function. Also, it is of specific interest to investigate whether the DPP-4 inhibitor linagliptin which recently we (Chapter 3 and 4) demonstrated was able to act as an antioxidant compound that can
ameliorate endothelial dysfunction in small mesenteric arteries isolated from STZ-treated rats. Therefore, the aim of the studies presented in this chapter was to investigate whether acute *in vitro* treatment with linagliptin reverses endothelial dysfunction in small resistance mesenteric arteries from WD fed rats and, if so, whether it acts via direct scavenging of superoxide anions and/or prevention of the sources of ROS generation in endothelial cells in mesenteric arteries. Additionally, we sought to explore the beneficial effects of linagliptin on the contribution of NO and/or EDH to endothelium-dependent relaxation in small resistant arteries from WD fed rats.

### 5.2 Materials and Methods

All procedures were approved by the Animal Experimentation Ethics committee of RMIT University and complied with the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes (AEC approval number 1245).

#### 5.2.1 Experimental animals

Male Wistar Hooded rats (Monash University, Australia) weighing 180–200 g at the beginning of the study were used. At least one week acclimation was allowed prior to any testing and during this period rats were handled daily to reduce stress. At the start of the feeding regime, rats were housed in groups of four under a light/dark cycle (12 h/12 h), room temperature (21±1°C) and humidity (30–70%) kept constant with water available *ad libitum* in the home cage. Animals were randomly assigned to either a control (SD, Standard AIN93G rodent diet, 7% total fat including 1.05% total saturated fatty acids; Specialty Feeds, Perth, Australia) or western diet (WD SF00-219, 21% total fat including 1.80% total...
saturated fats and 0.15% cholesterol; Specialty Feeds, Perth, Australia). Animals were allowed ad lib access to diets for 7 weeks after which they were placed on a food restricted diet where animals received 70% of their normal daily food consumption for a period of 10 weeks. Food intake and body weight were measured twice weekly. The rats were killed 10 weeks after being placed on their restricted diet by asphyxiation by CO₂ inhalation, followed by decapitation, and their abdomen opened to isolate the mesenteric arteries. Blood samples were obtained from the carotid arteries and the blood glucose levels were measured using a one-touch glucometer (Roche, Sydney, New South Wales). Glycated haemoglobin (HbA₁c) was measured using the in2it™ (II) analyser (Bio-Rad, Hercules, CA, USA).

5.2.2 Isolation of mesenteric arteries

After rats were killed, the mesenteric arteries were isolated and immediately kept in ice cold Krebs bicarbonate solution. The third-order branch mesenteric arteries (internal diameter ~200-300 µm) were isolated and prepared as described in Chapter 2.2.

5.2.3 Vascular function experiments

Vascular reactivity was evaluated as previously described (Chapter 2.3). Briefly, after maximum contraction of the vessels and the assessment of the integrity of the endothelium, the mesenteric arteries were precontracted with PE (0.1-3 µM) to attain comparable active tension and cumulative concentration-response curves to ACh (0.1 nM – 10 µM) and SNP (0.1 nM–10 µM) were obtained. In some experiments, cumulative concentration- responses to ACh were assessed after 20 minutes incubation with different combinations of L-NNA (100 µM), a non-selective NOS inhibitor, ODQ (10 µM), a sGC inhibitor, TRAM-34 (1 µM), a selective blocker of IK<sub>Ca</sub> and apamin (1 µM), a SK<sub>Ca</sub> inhibitor.
5.2.4 Measurement of superoxide generation in mesenteric arteries

Superoxide anion generation in the mesenteric arteries was measured using the lucigenin-enhanced chemiluminescence assay as described in Chapter 2.5.1.

5.2.5 Western Blot

Tissue samples of first order, second order, and third order mesenteric arteries from 2 rats from the same treatment group were pooled and considered as n=1. Western blots were accomplished as described in Chapter 2.7. To explore eNOS mono/dimer development in tissue sample, a non-boiled sample was resolved by 6% SDS-PAGE at 4°C overnight (Chapter 2.7.5).

5.2.6 Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except for linagliptin (Boehringer Ingelheim, Germany), acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, UK), and ODQ (Cayman Chemical, Ann Arbor, MI, USA). All chemicals were dissolved in distilled water with the exception of L-NNA, which was dissolved in 0.1 M sodium bicarbonate, and indomethacin, which was dissolved in 0.1 M sodium carbonate.

5.2.7 Statistical analysis

Results are presented as the mean±SEM, n indicates the number of experiments. The concentration response data from isolated mesenteric arteries were fitted to a sigmoidal curve using nonlinear regression (Prism version 6.0, GraphPad Software, San Diego, CA, USA) to calculate the pEC$_{50}$. The maximum relaxation ($R_{max}$) to ACh or SNP was determined as a percentage of the phenylephrine precontraction. pEC$_{50}$ and $R_{max}$ values were compared using
one way ANOVA with *post hoc* analysis using Bonferroni’s test or Student’s unpaired t-test as appropriate. p<0.05 was considered statistically significant.

5.3 Results

5.3.1 Effect of WD on body weights

The body weights of SD and WD fed rats is shown in Figure 5.1. The final body weight in WD fed rats was significantly higher than in SD fed rats.

5.3.2 Effect of WD and linagliptin on oxidative stress in mesenteric arteries

The level of superoxide generation in mesenteric arteries from SD or WD fed rats was measured by using lucigenin-enhanced chemiluminescence. In WD fed rats, the level of superoxide induced fluorescence was significantly higher than that in SD fed rats (Figure 5.2). Acute treatment with linagliptin (1 µM) significantly reduced the level of superoxide generation detected in mesenteric arteries from WD fed rats (Figure 5.2).

5.3.3 Effect of linagliptin on endothelial function in mesenteric arteries from WD fed rats

The level of contraction to the high K⁺ physiological saline solution (KPSS, 123 mmol/L) was similar in arteries from SD and WD rats (Figure 5.3). The endothelium-dependent relaxation agonist ACh and the endothelium-independent relaxation agonist SNP were used to assess the effect of linagliptin on the vascular function in mesenteric arteries from SD and WD fed rats. In the mesenteric arteries from WD fed rats, the sensitivity to ACh, but not the maximum relaxation was significantly decreased compared with SD fed rats.
(Figure 5.4a), whereas, the sensitivity and the maximum relaxation to SNP were not altered (Figure 5.4b). Acute treatment with linagliptin had no effect on the response of the mesenteric arteries to ACh in SD fed rats, but in WD fed rats, linagliptin significantly increased the sensitivity to ACh (Figure 5.4a).
**Figure 5.1** Body weight gain over 10 wk of rats receiving a WD and SD. n=8-15. Results are shown as mean±SEM. n=8-15 experiments. *p<0.0001 vs SD.
Figure 5.2 Superoxide levels in intact mesenteric arteries isolated from SD and WD fed rats. NADPH activity was raised in WD mesenteric arteries which were attenuated with linagliptin treatment. Results are demonstrated as mean±SEM. n=6-8 experiments. *p<0.05 vs SD #p<0.05 vs WD.
Figure 5.3 Mesenteric arteries isolated from SD and WD fed rats induced to maximum contraction by KPSS (123 mM). KPSS caused a similar level of contraction in arteries from both groups of rats.
Figure 5.4 Vascular function in mesenteric arteries. Cumulative concentration-response curves to ACh (a), SNP (b) in endothelium-intact mesenteric arteries. In each group (a, b), mesenteric arteries were precontracted with PE to a similar level: (a) SD 66±2, SD+linagliptin 64±1, WD 65±1, WD+linagliptin 66±1, (b) SD 64±1, SD+linagliptin 64±1, WD 61±6, WD+linagliptin 66±1 %KPSS, n=7-10 experiments. Results are shown as mean±SEM. *p<0.05 vs SD.
5.3.4 Investigation of the contribution of NO and EDH-mediated relaxation in SD and WD

In mesenteric arteries from SD fed rats, the response to ACh was partially decreased by either the combination of N-nitro-L-arginine (L-NNA), a NO synthase inhibitor and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylate cyclase inhibitor or inhibitors of intermediate-conductance calcium-activated K⁺ channel (IK⁺Ca), small-conductance calcium-activated K⁺ channel (SK⁺Ca) with 1-[(2 chlorophenyl)(diphenyl)methyl]1H pyrazole (TRAM-34), apamin and iberiotoxin (Ibtx) respectively, indicating that both NO and EDH contribute to endothelium-dependent relaxation. Moreover, the response of mesenteric arteries from WD fed rats to ACh-induced relaxation was decreased in the presence of either NO inhibitors or EDH inhibitors compared with SD fed rats suggesting that both NO and EDH-mediated relaxation were impaired by consumption of WD by the rats.

5.3.5 Effect of linagliptin on NO-mediated relaxation in WD

In order to investigate the contribution of NO to endothelium-dependent relaxation, a combination of IK⁺Ca and SK⁺Ca channel inhibitors (TRAM-34+apamin) were used. The addition of TRAM-34+apamin to the mesenteric arteries from SD fed rats reduced the sensitivity but not the maximum relaxation to ACh, compared to the control (Figure 5.5a, Table 5.1). On the other hand, in mesenteric arteries from WD fed rats, the addition of IK⁺Ca and SK⁺Ca inhibitors significantly reduced both the sensitivity and the maximum relaxation in comparison to the control (Figure 5.5b, Table 5.1). In mesenteric arteries from WD fed rats, the reduction in the responses to ACh (the sensitivity and the maximum relaxation) in the presence of TRAM-34+apamin indicate that WD consumption impaired the contribution of NO to endothelium-dependent relaxation. Acute treatment with linagliptin significantly
increased the response to ACh in the presence of TRAM-34+apamin by increasing the sensitivity and the maximum relaxation in the arteries from WD fed rats (Table 5.1) indicating that linagliptin has the ability to enhance the contribution of NO to endothelium-dependent relaxation.

5.3.6 Effect of WD on the expression of NOS and Nox2

In mesenteric arteries from WD fed rats, the expression of eNOS significantly decreased in comparison to mesenteric arteries from SD fed rats (Figure 5.6a). In WD fed rats, the proportion of eNOS expressed as monomer was significantly higher than that in SD fed rats (Figure 5.6b). Nox2 expression was significantly increased in mesenteric arteries isolated from WD fed rats (Figure 5.6c).
Table 5.1 Effect of L-NNA, ODQ and potassium channel blockers on ACh-induced relaxation of rat mesenteric arteries isolated from SD and WD fed rats in the absence or presence of linaglaptin (1 µM).

<table>
<thead>
<tr>
<th></th>
<th>SD</th>
<th>SD+linagliptin</th>
<th>WD</th>
<th>WD+linagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACh</strong></td>
<td><strong>n</strong></td>
<td><strong>pEC&lt;sub&gt;50&lt;/sub&gt;</strong></td>
<td><strong>R&lt;sub&gt;max&lt;/sub&gt;</strong></td>
<td><strong>n</strong></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>7.56±0.08&lt;sup&gt;Ω&lt;/sup&gt;</td>
<td>94±2</td>
<td>8</td>
</tr>
<tr>
<td>TRAM34+apamin</td>
<td>6</td>
<td>6.97±0.0&lt;sup&gt;•Ω&lt;/sup&gt;</td>
<td>90±4</td>
<td>6</td>
</tr>
<tr>
<td>L-NNA+ODQ</td>
<td>5</td>
<td>6.71±0.10&lt;sup&gt;•Ω&lt;/sup&gt;</td>
<td>97±1</td>
<td>5</td>
</tr>
<tr>
<td>LNNA+ODQ+TRAM34+apamin+Ibtx</td>
<td>5</td>
<td>ND</td>
<td>10±1&lt;sup&gt;•Ω&lt;/sup&gt;</td>
<td>5</td>
</tr>
</tbody>
</table>

A comparison of the sensitivity (pEC<sub>50</sub>) and maximum relaxation (R<sub>max</sub>) to ACh in the absence (control), or the presence of TRAM-34 (1 µM)+apamin (1 µM), L-NNA (100 µM)+ODQ(10 µM), L-NNA (100 µM)+ODQ(10 µM)+TRAM-34 (1 µM)+apamin (1 µM) or L-NNA (100 µM)+ODQ(10 µM)+TRAM-34 (1 µM)+apamin (1 µM)+Ibtx (100 nM) in endothelium intact mesenteric arteries. All experiments were conducted in the presence of indomethacin (10 µM).

n=the number of experiments.

*Significantly different to control within each group (p<0.05), Bonferroni’s test,

#Significantly different to SD within inhibitor group (p<0.05), Bonferroni’s test,

ΩSignificantly different to WD within inhibitor group (p<0.05), Bonferroni’s test,

ND, not determined.
Figure 5.5 Contribution of NO to endothelium-dependent relaxation. Mesenteric arteries isolated from both SD and WD fed rats to examine NO-mediated relaxation in the presence of TRAM-34+apamin were isolated from SD (a), WD (b). In each group of experiments, arteries were precontracted with PE to similar levels: 63±2 (a), 65±0.6 (b) %KPSS, n=8-9 experiments. Results are shown as mean±SEM. *p<0.05 vs SD (control), *p<0.05 vs WD (control). See Table 5.1 for values.
Figure 5.6 Analysis of protein expression of eNOS (a, 130 kDa), eNOS dimer and monomer measured in on representative WB (b, 260 kDa) and Nox2 (c, 58 kDa) in SD and WD mesenteric arteries. In WD mesenteric arteries, the expression of eNOS significantly reduced and the proportion of eNOS was expressed as the dimer decreased, and the expression of Nox2 increased. n=6 experiments. Results are shown as mean±SEM. *p<0.05 compared to SD.
5.3.7 Effect of linagliptin on EDH-mediated relaxation in WD

To investigate the contribution of EDH to endothelium-dependent relaxation, NO-mediated relaxation was abolished by the addition of L-NNA+ODQ to block eNOS and soluble guanylate cycalse respectively. The sensitivity to ACh-enhanced relaxation reduced in the presence of L-NNA+ODQ in mesenteric arteries from SD fed rats (Figure 5.7a, Table 5.1). Also, the sensitivity to ACh, but not the maximum relaxation, in mesenteric arteries from WD fed rats was significantly reduced in the presence of L-NNA+ODQ (Figure 5.7b, Table 5.1), indicating that WD impaired the non-NO, non-prostanoid component in endothelium-dependent relaxation. Acute treatment with linagliptin did not increase the sensitivity to ACh and had no effect on EDH-mediated relaxation in arteries from SD fed rats (Figure 5.7a, Table 5.1), however, linagliptin increased the sensitivity to ACh-induced EDH-mediated relaxation in mesenteric arteries from WD fed rats (Figure 5.7b, Table 5.1). The addition of TRAM-34, apamin, and Ibtx in the presence of L-NNA+ODQ significantly reduced ACh-enhanced relaxation in mesenteric arteries from both SD and WD fed rats (Table 5.1). Acute treatment with linagliptin did not improve the relaxation induced by ACh in mesenteric arteries from SD or WD fed rats under those conditions.
Figure 5.7 Contribution of EDH to endothelium-dependent relaxation. Mesenteric arteries isolated from both SD (a) and WD (b) fed rats to investigate EDH-mediated relaxation in the presence of L-NNA+ODQ. In each group of experiments, arteries were precontracted with PE to similar levels: 62±0.7 (a), 65±0.8 (b) %KPSS, n=8-9 experiments. Results are shown as mean±SEM. See Table 1 for values of pEC₅₀ and Rₘₐₓ.
5.4 Discussion

This study showed that endothelial dysfunction in mesenteric arteries caused by consumption of WD (high fat diet) was prevented by acute treatment with the DPP-4 inhibitor linagliptin. Linagliptin reduced the increased production in vascular ROS caused by WD consumption. Consumption of WD impaired the action of both NO and EDH and acute treatment with linagliptin improved the contribution of both of these relaxing factors.

5.4.1 Effects of WD and linagliptin on oxidative stress

To investigate the effect of WD on oxidative stress, we measured the levels of superoxide generation in mesenteric arteries from SD and WD fed rats. The findings in this study indicate that the level of superoxide generation significantly increased in WD mesenteric arteries compared to SD fed rats. These findings suggest that WD promotes the development of oxidative stress and endothelial dysfunction primarily because of an increased NADPH oxidase-derived superoxide anion production which may react with NO, thereby enhancing the generation of the NO/superoxide reaction endproduct peroxynitrite which in turn causes uncoupling of eNOS and inactivation of NO as noted in this study. Furthermore, the findings also showed that the expression of Nox2 was increased in the mesenteric arteries of WD fed rats, indicating that the impairment of endothelial-dependent relaxation may be in part due to the overproduction of superoxide anions caused by increased expression of the enzymatic source of superoxide generation. We inspected the ability of the DPP-4 inhibitor linagliptin to decrease the levels of vascular superoxide and found that linagliptin expressed a significant decline in superoxide generation in WD mesenteric arteries by its ability to act as antioxidant compound as we have demonstrated in chapter 3.
5.4.2 Effect of WD diet on endothelial function

ACh was used to evaluate the effect of diet (SD and WD) on endothelium-dependent relaxation. The findings of this study indicate that the consumption of WD causes impairment of endothelial function in small resistant mesenteric arteries from these rats, and this is in agreement with some reports which demonstrated that consumption of high fat diet induced endothelial cell dysfunction in male and female rat aorta (Roberts et al., 2005, Reil et al., 1999) or obesity-induced insulin resistance, hyperinsulinemia, and hypercholesterolemia that have been anticipated to be involved in the development of endothelial dysfunction (Ohara et al., 1993, Arcaro et al., 1999, Caballero, 2003, Busija et al., 2005). Thompson and colleagues using coronary arteries and Woodman and colleagues using brachial arteries demonstrated that the consumption of high fat diet caused impairment of endothelial cell function in male and female pigs, respectively (Thompson et al., 2004, Woodman et al., 2003). The results in this study demonstrated that the impairment of endothelial function may be attributed, in part, to a reduced expression of eNOS, since WD consumption reduced the total protein content of eNOS and reduced dimerization of eNOS in mesenteric arteries and this is consistent with the report by Woodman et al, who noted that the consumption of high-fat diet induce a reduction in brachial artery eNOS using immunohistochemistry (Woodman et al., 2003). Additionally, SNP was used in order to investigate the effect of WD on vascular smooth muscle relaxation, SNP-induced relaxation in this study was similar in both SD and WD fed rats, indicating that the relaxation of smooth muscle in response to exogenous NO was not impaired in WD fed rats.

5.4.3 Effect of linagliptin on endothelial function in WD

The findings in this study showed that rats fed a WD had a significant reduction in the response to ACh-induced endothelium-dependent relaxation in comparison to SD fed rats.
Acute treatment with linagliptin in vitro improved endothelial function and increased endothelium-dependent relaxation of mesenteric arteries isolated from WD fed rats. Therefore we investigated whether linagliptin improved the endothelial function by increasing the contribution of NO and/or EDH to the endothelium-dependent relaxation.

5.4.4 Effect of linagliptin on NO-mediated relaxation in WD fed rats

ACh-induced relaxation was used in order to investigate the contribution of NO to endothelium-dependent relaxation in the presence of the combination of TRAM-34 and apamin as IKCa and SKCa inhibitors respectively, in mesenteric arteries isolated from SD and WD fed rats. Under the conditions of blocking the action of EDH-mediated relaxation, the maximum relaxation response to ACh was significantly reduced in mesenteric arteries from WD fed rats. Acute treatment with linagliptin in vitro, significantly improved the response to ACh-induced relaxation and increased the maximum relaxation in WD mesenteric arteries in comparison to the control. This is likely due to lingliptin reducing the oxidative stress by the antioxidant effect which in turn increased the bioavailability of NO in mesenteric endothelial cells by preventing the formation of peroxynitrite as a product of the reaction of superoxide and NO via rapid free radical scavenging action of linagliptin (Chapter 3, 4). Overall, acute treatment with linagliptin preserved the bioavailability of NO in mesenteric arteries of WD fed rats.

5.4.5 Effect of linagliptin on EDH-mediated relaxation in WD fed rats

In addition to NO-mediated relaxation, the endothelium-dependent relaxation in rat mesenteric arteries is mediated by both classical and non-classical EDH type responses (Edwards et al., 2010, Leo et al., 2011b, Garland et al., 2011). To investigate the contribution
of EDH to endothelium-dependent relaxation in mesenteric arteries from SD and WD fed rats, L-NNA+ODQ were added to prevent NO formation and sGC activity respectively. The sensitivity to ACh-induced relaxation in WD mesenteric arteries was significantly decreased in comparison to SD mesenteric arteries in the presence of L-NNA+ODQ, indicating that feeding rats with WD for 10 weeks impaired the contribution of EDH to endothelium-dependent relaxation. Acute treatment with linagliptin in vitro significantly improved the sensitivity to ACh-induced relaxation in mesenteric arteries from WD fed rats, indicating that linagliptin preserved the contribution of EDH to endothelium-dependent relaxation by reducing the oxidative stress under the conditions of feeding animals with WD.

5.5.5 Conclusion

The findings in this study demonstrated that consumption of WD induced oxidative stress, downregulation of vascular NOS, and reduced NO production to promote endothelial dysfunction. The acute treatment in vitro with the DPP-4 inhibitor linagliptin improves the endothelial function in mesenteric arteries developed in WD fed rats. This is probably via conserving the beneficial effects of both NO and EDH-mediated relaxation. In this study we showed that the DPP-4 inhibitor linagliptin has the ability to decrease the production of superoxide anions which is perhaps, at least in part, via direct radical scavenging action. The beneficial effect of DPP-4 inhibitor linagliptin by improving the vascular endothelial function in small resistance mesenteric arteries in WD fed rats demonstrated in this study shows it possesses the capability to act as a pharmacological intervention that may be anticipated to improve the NO and EDH-mediated relaxation and could be valuable in the prevention of the pathological role of WD in the development of endothelial dysfunction in the vasculature.
Chapter 6

The DPP-4 inhibitor linagliptin preserves endothelial cell function in mesenteric arteries from type-1 diabetic rats without decreasing plasma glucose

6.1 introduction

Cardiovascular disease starts with risk factors such as hyperglycaemia in diabetes, hypertension, and dyslipidaemia (Dzau et al., 2006). Endothelial dysfunction is considered a critical factor in the initiation and development of vascular complications induced by diabetes (De Vriese et al., 2000, Fatehi-Hassanabad et al., 2010). Macro- and microvascular complications are presently the main causes of morbidity and mortality amongst diabetic patients in both type 1 and type 2 diabetes mellitus (De Vriese et al., 2000). Endothelial cells play an active role to regulate the basal vascular tone and reactivity of blood vessels in both physiological and pathological conditions, by releasing a diversity of contracting and relaxing factors in responding to stimulating factors such as mechanical forces and neurohumoral mediators (Furchgott and Vanhoutte, 1989, Ferrara, 2002). The most important endothelium-derived relaxing factors (EDRFs) are nitric oxide (NO), prostacyclin and endothelium-dependent hyperpolarization (EDH) (Feletou and Vanhoutte, 1999, Woodman et al., 2000). It has been demonstrated that the endothelial dysfunction caused by the impairment of eNOS expression/activity and the reduction of NO production could be promoted by some risk factors such as hypercholesterolemia and diabetes, thus, the endothelium-dependent vasodilatation used as a parameter to investigate endothelial function in both physiological and pathological circumstances (Arnal et al., 1999, Leo et al., 2011b).
Dipeptidyl peptidase-4 (DPP-4) is a glycoprotein peptidase broadly expressed in various cell types which displays complex biological actions and has multifunctional properties (Drucker, 2006, Drucker, 2007, Cordero et al., 2009, Yazbeck et al., 2009). Inhibition of the DPP-4 system by DPP-4 inhibitors, which are class of blood glucose-lowering drug, signifies a new approach in order to treat type 2 diabetes and take advantage of their neutral effect on body weight and low risk of the occurrence of hypoglycaemia (Panchapakesan et al., 2013, Zhong et al., 2013). DPP-4 inhibitors prolong the half-life of incretins such as glucagon-like-peptide-1 (GLP-1) and glucagon induced peptide (GIP) by inhibiting the degradation of these incretins and thus consequently lower blood glucose via improved insulin secretion (Drucker, 2006). Interestingly, previous studies show beneficial effects of GLP-1 in situations such as in the regulation of endothelial function and cardiac remodeling (Zhao et al., 2006, Nikolaidis et al., 2004, Basu et al., 2007, Green et al., 2008). The DPP-4 inhibitors have the ability to prevent the impairment of cardiac diastolic function (Aroor et al., 2013), improve glomerulopathy (Nistala et al., 2014), ameliorate dysfunction in rat aortic artery in experimental sepsis (Kroller-Schon et al., 2012) and reduce oxidative stress in vascular endothelial cells (Ishibashi et al., 2013). We have recently found that acute treatment with linagliptin ameliorate vascular dysfunction in mesenteric arteries exposed to high concentration of glucose (40 mM) independently of glucose lowering effect.

We have previously demonstrated that in small arteries from STZ-treated rats with diabetes-induced endothelial dysfunction results from the impairment of both NO-mediated and EDH-mediated relaxation, associated with eNOS uncoupling and an increase in Nox2-derived superoxide generation (Leo et al., 2011b). We have also shown that treatment with 3’,4’-dihydroxy flavonol reduces oxidant stress and improves endothelium-dependent relaxation in type 1 diabetic rats (Leo et al., 2011a). Therefore, it is of particular interest to examine whether linagliptin, a DPP-4 inhibitor which we have recently demonstrated is able
to also act as an antioxidant, can alleviate endothelial dysfunction in diabetic vasculature independently of the glucose lowering properties. Thus, the aim of the present study was to examine whether chronic *in vivo* treatment with linagliptin, a DPP-4 inhibitor with unique xanthine-based structure, preserves endothelial function in small resistance mesenteric arteries from type 1 STZ-induced diabetic rats and, if so, whether it acts via the mechanism of direct scavenging of ROS and/or through the inhibition of the sources of ROS generation in microvascular endothelial cells in diabetic mesenteric arteries independently of reducing blood glucose effects.
6.2 Materials and Methods

Ethics statement: All the procedures were approved by the Animal Experimentation Ethics committee of RMIT University (AEC approval number 1309) and complied with the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes.

6.2.1 Induction of type 1 diabetes

Male Wistar rats (~ 8-weeks old and weighing approximately 200g) were randomly divided into two groups: normal and diabetic. A single injection of streptozotocin (STZ, 50 mg/kg IV) after the rats were fasted overnight was used to induce type 1 diabetes, whereas, rats in the control group received vehicle of a comparable volume (0.1 mol/L citrate buffer, pH 4.5). Blood glucose was monitored and when greater than 25 mmol/L, rats were treated with insulin (3-4 IU, s.c., 3 injections/ week, Protaphane, Novo Nordisk, NSW, Australia). Blood glucose concentration was measured by a glucometer (Roche, Sydney, NSW, Australia) and glycated haemoglobin (HbA1c) was measured using a Micromat HbA1c analyser (Biorad, Sydney, NSW, Australia). Blood samples were collected from the left ventricle at the end of the experimental period.

6.2.2 Linagliptin treatment

Six weeks after induction of diabetes, the two groups of rats were further divided into 4 subgroups (normal, normal+linagliptin, diabetic, diabetic+linagliptin) administering either vehicle (1% carboxy methylcellulose, CMC) or linagliptin (2 mg/kg) by daily oral gavage for a period of 4 weeks. The rats were administered the last dose of linagliptin at least 24 hours prior to the collection of tissue for experimentation.
6.2.3 Isolation of mesenteric arteries

After ten weeks of STZ treatment, the rats were killed by asphyxiation with CO\textsubscript{2} followed by exsanguination. The mesenteric arcade was isolated and collected and immediately placed in ice-cold Krebs bicarbonate solution (4.7 mmol/L KCl, 118 mmol/L NaCl, 1.18 mmol/L MgSO\textsubscript{4}, 25 mmol/L NaHCO\textsubscript{3}, 11.1 mmol/L D-glucose and 1.6 mmol/L CaCl\textsubscript{2}) containing the non-selective cyclooxygenase (COX) inhibitor indomethacin (10 µmol/L), to inhibit the production of prostanoids. Small mesenteric arteries (third order branch of the superior mesenteric artery, internal diameter ~300 µm) were isolated, cleared of fat and connective tissue and dissected into rings of about 2 mm in length and then mounted on a Mulvany-Halpern myograph (model 610M, Danish Myo Technology, Aarhus, Denmark). After the rings were mounted, they were allowed to stabilize at zero tension for 15 min before normalization. The blood vessels were stretched to attain an internal pressure comparable to 90\% of that of the blood vessel under a transmural pressure of 100 mmHg (McPherson, 1992, Mulvany and Halpern, 1977) to determine the passive tension-internal circumference. All experiments were conducted at 37\(^{\circ}\)C in the Krebs solution bubbled with carbogen (95\% O\textsubscript{2} and 5\% CO\textsubscript{2}).

6.2.4 Investigation of vascular function and reactivity

After equilibration for 30 minutes, blood vessels were subjected to maximum contraction by the addition of an isotonic physiological saline solution containing a high concentration of K\textsuperscript{+} (123 mmol/L, KPSS). Basal tension of vessels was restored after several washouts using Krebs solution. In order to investigate the integrity of the endothelium, the vessels were precontracted with phenylephrine (0.1-3 µmol/L) to ~50\% of the KPSS response and relaxed with a high single dose of acetylcholine (ACh, 10 µmol/L). ACh-induced relaxation of the precontracted rings was more than 80\% in all cases indicating that the
endothelium was functionally intact. After several washouts, the mesenteric arteries were precontracted again with phenylephrine (0.1-3 µmol/L) and cumulative concentration-response curves to the endothelium-dependent relaxant agonist, ACh (0.1 nmol/L-10 µmol/L) and the endothelium-independent relaxant agonist, sodium nitroprusside (SNP, 0.01 nmol/L-10 µmol/L) were examined. Furthermore, after 20 minutes, responses to ACh and SNP were investigated with different combinations of N-nitro-L-arginine (L-NNA, 100 µmol/L), an inhibitor of nitric oxide synthase (NOS), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 µmol/L), an inhibitor of soluble guanylate cyclase (sGC), apamin (1 µmol/L), a selective blocker of the small calcium-activated K+ channel (SKCa), 1-[(2-chlorophenyl)(diphenyl)methyl]-1H-pyrazole (TRAM-34, 1 µmol/L), a blocker of the intermediate calcium-activated K+ channel (IKCa), and iberiotoxin (Ibtx, 100 nmol/L), a blocker of the large calcium-activated K+ channel (BKCa).

6.2.5 Estimation of basal release of NO in mesenteric arteries

In order to investigate the effect of diabetes on the basal level of NO release, endothelium-intact blood vessels were exposed to L-NNA (100 µmol/L) after precontraction with phenylephrine (10-100 nmol/L) to ~ 20% KPSS. A contractile response to L-NNA was considered to indicate inhibition of basal release of NO (Leo et al., 2011b).

6.2.6 Western Blot

Western blots were performed by collecting the mesenteric arteries from normal and diabetic rats. From the same treatment group, the endothelium-intact small mesenteric arteries from 2 animals were pooled and considered as n =1. The same amount of protein homogenate of the treatment animal groups were exposed to SDS-PAGE and analyzed
through western blot processes with mouse/rabbit primary antibodies (all 1:1000, overnight, 4°C) includes endothelial NO synthase (eNOS), Nox2 (all BD Transduction Laboratories, Lexington, KY, USA). The membranes were reinvestigated with a loading control antibody (actin), in order to normalize for the amount of protein. After 1 hour incubation at room temperature with anti-mouse/rabbit secondary antibody (1:2000) (Millipore, Billerica, MA, USA), all proteins were identified by enhanced chemiluminescence reagents (Amersham, GE Healthcare, Sydney, NSW, Australia). Densitometry (Biorad Chemidoc, Sydney, NSW, Australia) was used to quantify the protein bands expressed as a ratio of the loading control. To examine tissue eNOS ratio of monomer/dimer formation, 6% SDS-PAGE at 4°C used to resolve a non-boiled sample and the membranes were examined and imaged as described above.

6.2.7 Measurement of superoxide release by mesenteric arteries

To measure superoxide generation in mesenteric arteries, lucigenin-enhanced chemiluminescence was used. The superior mesenteric artery was isolated, cleared of fat and connective tissue and incubated for 45 minutes at 37°C in Krebs-HEPES buffer solution containing NADPH (100 µmol/L) as a substrate for NADPH-oxidase in the presence or absence of diphenyliodonium (DPI, 5 µmol/L), a flavoprotein inhibitor that inhibits NADPH oxidase. 300 µL of Krebs-HEPES buffer, containing lucigenin (5 µM) and the suitable treatment were placed into a 96-well Optiplate. The Optiplate was loaded into a Polarstar Optima photon counter (BMG Labtech, Melbourne, VIC, Australia) to measure background photon emission at 37°C. After the background reading was recorded, a single ring section of mesenteric artery was placed into each well and photon emission was recounted. The mesenteric arteries were placed in Krebs-HEPES buffer that contain either lucigenin (5 µM) alone or with the presence of NADPH (100 µM) to measure the production of NADPH
oxidase-driven superoxide. The signal stimulated by NADPH was measured in the presence or absence of DPI (5 µM). The background counts were subtracted from superoxide counts and normalized with dry tissue weight.

6.2.8 Materials

Linagliptin was a gift from (Boehringer Ingelheim, Germany). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except for ODQ (Cayman Chemical, Ann Arbor, MI, USA), and acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, UK). All drugs were dissolved in distilled water with the exception of indomethacin, which was dissolved in 0.1 M sodium carbonate, and L-NNA, which was dissolved in 0.1 M sodium bicarbonate.

6.2.9 Statistical analysis

Data were expressed as mean ± SEM, n indicates the number of experiments. The concentration response data from rat isolated mesenteric arteries were fitted to a sigmoidal curve using nonlinear regression (Prism version 6.0, GraphPad Software, San Diego, CA, USA) to calculate the pEC\textsubscript{50}. The maximum relaxation (R\textsubscript{max}) to ACh or SNP was determined as a percentage of the phenylephrine precontraction. Differences between groups of pEC\textsubscript{50} and R\textsubscript{max} values were compared and analysed using two way ANOVA with post hoc analysis using Bonferroni’s test. p<0.05 was considered statistically significant.
6.3 Results

6.3.1 Body weights and blood glucose

The body weight, blood glucose and HbA1c levels of rats in the 4 groups are shown in Table 1. Ten weeks after treatment with streptozotocin or vehicle, the final body weight in normal rats was significantly higher than in diabetic rats (Table 6.1). Furthermore, in diabetic rats, the blood glucose and HbA1c levels were significantly greater than in normal rats. Four weeks treatment with linagliptin had no significant effect on body weight, HbA1c or blood glucose levels in either normal or diabetic rats.

6.3.2 Effect of linagliptin on vascular superoxide production

The superoxide level in mesenteric arteries was measured by lucigenin-enhanced chemiluminescence. In diabetic rats, the level of superoxide-induced fluorescence was significantly higher than that in normal rats (Figure 6.1). After 4 weeks of treatment with linagliptin, the level of NADPH oxidase-driven superoxide was significantly attenuated in diabetic rats, but there was no effect in normal rats (Figure 6.1). In all groups, diphenyliodonium was able to inhibit NADPH oxidase-driven superoxide production in the mesenteric arteries (Figure 6.1).
Table 6.1 Mean body weight gained, blood glucose and HbA1c levels at the end of the experiment of normal and diabetic rats with or without linagliptin (2 mg/kg oral gavage daily for 4 weeks) treatment.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Normal</th>
<th>n</th>
<th>Normal+linagliptin</th>
<th>n</th>
<th>Diabetic</th>
<th>n</th>
<th>Diabetic+linagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>8</td>
<td>498±18Φ</td>
<td>9</td>
<td>538±21Φ</td>
<td>11</td>
<td>346±11Ψ</td>
<td>11</td>
<td>379±25Ψ</td>
</tr>
<tr>
<td><strong>Blood glucose (mM)</strong></td>
<td>8</td>
<td>5.1±0.3Φ</td>
<td>8</td>
<td>4.5±0.2Φ</td>
<td>11</td>
<td>26.4±2Ψ</td>
<td>12</td>
<td>27.8±1.7Ψ</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>7</td>
<td>6.2±0.2Φ</td>
<td>8</td>
<td>5.8±0.2Φ</td>
<td>10</td>
<td>14.8±0.6Ψ</td>
<td>12</td>
<td>13.9±0.4Ψ</td>
</tr>
</tbody>
</table>

n= the number of rats. *Significantly different from normal group (Bonferroni’s test, p<0.05), Φ Significantly different to diabetic group, p<0.05, Bonferroni’s test. Ψ Significantly different to Normal+linagliptin group, p<0.05, Bonferroni’s test. Results are shown as mean±SEM.
6.3.3 Effect of diabetes and linagliptin on vascular function

Both diabetes and linagliptin treatment had no effect on the level of contraction to the high K\(^+\) physiological saline solution (KPSS, 123 mmol/L) (Figure 6.2). The sensitivity to ACh but not the maximum relaxation was significantly reduced in mesenteric arteries from diabetic rats (Figure 6.3a, Table 6.2), whereas the sensitivity and the maximum relaxation to sodium nitroprusside (SNP) were not affected (Figure 6.3b). 4 weeks of linagliptin treatment (2 mg/kg per daily, oral gavage) did not affect the response to ACh in mesenteric arteries from normal rats, but in diabetic rats treated with linagliptin, the sensitivity to ACh was significantly increased in comparison to the sensitivity and response to ACh in mesenteric arteries from diabetic untreated rats (Figure 6.3a, Table 6.2). The treatment with linagliptin had no effect on the response to SNP in normal rats or diabetic rats (Figure 6.3b).

The basal level of NO release was investigated by assessing the level of contraction induced by L-NNA in mesenteric arteries precontracted with PE to 20% of the KPSS response. In normal mesenteric arteries the L-NNA-induced contraction was significantly higher than that with diabetic mesenteric arteries (Figure 6.3c) showing that diabetes reduced the basal release of NO. Treatment with linagliptin significantly increased the L-NNA-induced contraction in diabetic mesenteric arteries compared to untreated diabetic arteries but had no effect on L-NNA-induced contraction in normal mesenteric arteries (Figure 6.3c).
Figure 6.1 ROS measurement in intact mesenteric arteries. NADPH activity was raised in diabetic mesenteric arteries which were reduced with linagliptin treatment. Results are shown as mean±SEM. n=7-10 experiments. *p<0.05 vs normal, #p<0.05 vs diabetic.
Figure 6.2 Mesenteric arteries were induced to maximum contraction by KPSS. Exposure of mesenteric arteries from both normal and diabetic rats with or without linagliptin (2 mg/kg per day orally for 4 weeks) treatment to KPSS (123 mM). Diabetes or linagliptin treatment did not affect the level of contraction of the mesenteric arteries.
Figure 6.3 Vascular function in mesenteric arteries. Cumulative concentration-response curves to ACh (a), SNP (b), and basal NO release (c) in endothelium-intact mesenteric arteries. In each group (a, b), mesenteric arteries were pre-contracted with PE to a similar level: (a) normal 66±2, normal+linagliptin 64±1, diabetic 65±1, diabetic+linagliptin 66±1, (b) normal 64±1, normal+linagliptin 64±1, diabetic 61±6, diabetic+linagliptin 66±1 %KPSS, n=7-10 experiments. Results are shown as mean±SEM. **p<0.01, ***p<0.001. See Table 2 or results section for pEC$_{50}$ and Rmax values.
6.3.4 Determination of the relative contribution of NO and EDH to endothelium-dependent relaxation

In mesenteric arteries from normal rats, the ACh-induced relaxation was partially reduced by either the combination of N-nitro-L-arginine (L-NNA), a NO synthase inhibitor and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylate cyclase inhibitor or inhibitors of intermediate-conductance calcium-activated K+ channel (IKCa), small-conductance calcium-activated K+ channel (SKCa) with 1-[(2 chlorophenyl)(diphenyl)methyl]1H pyrazole (TRAM-34), apamin and iberiotoxin (Ibtx) respectively, confirming that both NO and EDH were involved in endothelium-dependent relaxation (Figure 6.4, Table 6.2). Further, the response to ACh-induced relaxation in mesenteric arteries from diabetic rats was significantly further reduced in the presence of either NO inhibitors or EDH inhibitors in comparison to normal rats, indicating that the contribution of both NO and EDH to endothelium-dependent relaxation were impaired by diabetes.

6.3.5 Effect of linagliptin on NO- and EDH-mediated relaxation in the mesenteric arteries

The response to ACh was assessed in the presence of TRAM-34+apamin in order to investigate the contribution of NO to endothelium-dependent relaxation. Treatment with linagliptin for 4 weeks (2 mg/kg, daily oral gavage), significantly increased the maximum relaxation to ACh in mesenteric arteries from diabetic rats but had no effect on responses in normal rat arteries (Figure 6.4, Table 6.2) indicating that linagliptin could restore the contribution of NO to endothelium-dependent relaxation in diabetes.
To investigate the contribution of EDH-mediated relaxation, the actions of NO were inhibited by the addition of L-NNA and ODQ. Treatment of normal and diabetic rats for 4 weeks with linagliptin (2 mg/kg, daily oral gavage) significantly increased on ACh-induced EDH mediated relaxation diabetic arteries but not in arteries from normal rats (Figure 6.5, Table 6.2).
Table 6.2 Effect of L-NNA, ODQ and potassium channel blockers on ACh-induced relaxation of mesenteric arteries from normal and diabetic rats with or without linagliptin (2 mg/kg oral gavage daily for 4 weeks) treatment in the presence of indomethacin.

<table>
<thead>
<tr>
<th>ACh</th>
<th>Normal</th>
<th>Normal+linagliptin</th>
<th>Diabetic</th>
<th>Diabetic+linagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>R&lt;sub&gt;max&lt;/sub&gt; (%)</td>
<td>n</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>7.26±0.13</td>
<td>99±0</td>
<td>9</td>
</tr>
<tr>
<td>TRAM34+apamin</td>
<td>7</td>
<td>6.52±0.06*</td>
<td>91±2</td>
<td>7</td>
</tr>
<tr>
<td>L-NNA+ODQ</td>
<td>7</td>
<td>6.31±0.09*</td>
<td>95±0</td>
<td>7</td>
</tr>
<tr>
<td>LNNA+ODQ+TRAM34+apa</td>
<td>7</td>
<td>5.62±0.29*</td>
<td>27±8*</td>
<td>4</td>
</tr>
<tr>
<td>LNNA+ODQ+TRAM34+apa+Ibtx</td>
<td>4</td>
<td>5.27±0.17*</td>
<td>14±2*</td>
<td>4</td>
</tr>
</tbody>
</table>

A comparison of the sensitivity (pEC<sub>50</sub>) and maximum relaxation (R<sub>max</sub>) to ACh in the absence (control), or the presence of TRAM-34 (1 µM)+apamin (1 µM), L-NNA (100 µM)+ODQ(10 µM), L-NNA (100 µM)+ODQ(10 µM)+ TRAM-34 (1 µM) +apamin (1 µM) or L-NNA (100 µM)+ODQ(10 µM)+ TRAM-34 (1 µM) +apamin (1 µM)+Ibtx (100 nM) in endothelium intact mesenteric arteries. All experiments were performed in the presence of indomethacin (10 µM). n = the number of experiments.

*Significantly different to control within each group (p<0.05), Bonferroni’s test,
<sup>Φ</sup>Significantly different to normal within inhibitor group, p<0.05, Bonferroni’s test.
<sup>¥</sup>Significantly different to normal+linagliptin within inhibitor group, p<0.05, Bonferroni’s test.
<sup>£</sup>Significantly different to diabetic within inhibitor group, p<0.05, Bonferroni’s test.
Results are shown as mean±SEM, ND= Not determined.
Figure 6.4 Relative contribution of NO to endothelium-dependent relaxation. Mesenteric arteries to examine NO-mediated relaxation in the presence of TRAM-34+apamin were isolated from normal (a), diabetic (b), normal+linagliptin (c), diabetic+linagliptin (d) rats. In each group of experiments, arteries were precontracted with PE to similar levels: 63±2 (a), 65±0.6 (b), 65±1 (c), 63±1 (d) %KPSS, n=8-9 experiments. Results are shown as mean±SEM. See Table 6.2 for values.
Figure 6.5 Contribution of EDH to endothelium-dependent relaxation. Mesenteric arteries to examine EDH-mediated relaxation in the presence of L-NNA+ODQ were isolated from normal (a), diabetic (b), normal+linagliptin (c), diabetic+linagliptin (d) rats. In each group of experiments, arteries were precontracted with PE to similar levels: 62±0.7 (a), 65±0.8 (b), 62±2 (c), 64±0.8 (d) %KPSS, n=8-9 experiments. Results are shown as mean±SEM. See Table 2 for values.
6.3.6 Effect of linagliptin on Nox2, total-eNOS and eNOS monomer/dimer

In diabetic rats, the expression of total eNOS in the mesenteric arteries was significantly reduced as was the proportion of eNOS expressed as the dimer and the expression of Nox2 was significantly increased (Figure 6.6). Treatment with linagliptin did not affect the expression or dimerization of eNOS or the expression of Nox2 in normal rats. By contrast, in diabetic mesenteric arteries after linagliptin treatment there was a significant increase in eNOS, both in total and as a dimer, and a decrease in Nox2 expression
Figure 6.6 Western blot analysis of protein expression of eNOS (a, 130 kDa), eNOS dimers and monomers (b, 260 kDa) and Nox2 (c, 58 kDa) in normal and diabetic mesenteric arteries with or without linagliptin treatment. In diabetic mesenteric arteries, the expression of eNOS significantly reduced and the proportion of eNOS expressed as the dimer reduced, and the expression of Nox2 increased. Treatment with linagliptin increased the expression of eNOS significantly and reduced Nox2 expression and increased the proportion of eNOS expressed as the dimer. Representative blots are shown on each of the corresponding graphs. n=6 experiments. Results are shown as mean±s.e.m. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
6.4 Discussion

This study showed that treatment of type 1 STZ-induced diabetic rats with the DPP-4 inhibitor linagliptin (2 mg/Kg, daily oral gavage) for 4 weeks decreases the levels of vascular oxidative stress and improves endothelium-dependent relaxation in mesenteric arteries. Importantly in this type 1 diabetes model the beneficial vascular effects of linagliptin were achieved without affecting plasma concentrations of glucose or HbA1c demonstrating that linagliptin has actions in addition to the capacity to lower plasma glucose. A similar dose of linagliptin (1 mg/kg po) to that used in this study (2 mg/kg po) has been demonstrated to effectively inhibit DPP-4 activity and to increase GLP-1 in Zucker diabetic fatty rats (Jones et al., 2014) but in this type 1 model of diabetes where the beta pancreatic cells are destroyed by necrosis it is not possible to elevate insulin secretion to decrease glucose. The dysfunction of endothelial cells in the mesenteric arteries of diabetic rats is accompanied with an impairment of the contribution of both NO and EDH-mediated relaxation to endothelium-dependent relaxation. Treatment of diabetic rats with linagliptin improves the contribution of NO-mediated relaxation to endothelium-dependent relaxation which associated with an increased expression of total eNOS, decreased eNOS uncoupling and downregulation of Nox2 expression. In addition, the contribution of EDH to endothelium-dependent relaxation in diabetic mesenteric arteries was improved by the treatment with linagliptin.

6.4.1. Effect of linagliptin on vascular endothelial function

In the present study, Type 1 STZ-induced diabetes increased the level of vascular ROS generation concomitant with a selective impairment of endothelium-dependent relaxation and an increase in the expression of Nox2 in the mesenteric arteries consistent with previous reports (De
Vriese et al., 2000, Leo et al., 2011b, Ding et al., 2005). In diabetic rats, the in vivo treatment with the DPP-4 inhibitor linagliptin (2 mg/kg, per day, oral gavage) for 4 weeks improved endothelium-dependent relaxation in mesenteric arteries and decreased the generation levels of oxidative stress associated with decreased Nox2 and improved coupling of eNOS in the mesenteric arteries of diabetic rats. Our observations are similar to that of other observations that have shown that treatment with the DPP-4 inhibitor linagliptin protects against cardiovascular injury induced by salt-sensitive hypertension via a reduction of oxidative stress (Koibuchi et al., 2014) or improves endothelial function and reduces vascular stress in experimental sepsis in large conduit aortic artery (Kroller-Schon et al., 2012). Whilst there are various reports that the DPP-4 inhibitor, linagliptin could ameliorate endothelial dysfunction induced by diabetes, however, the mechanism of the vasoprotective effect of linagliptin remains ambiguous particularly on the contribution of NO and EDH to endothelium-dependent relaxation in diabetes.

6.4.2. Effects of linagliptin on NO-mediated relaxation in rats mesenteric arteries

In order to investigate NO-mediated relaxation, the EDH-mediated relaxation was blocked by endothelial K_{Ca} channel blockers. This clearly showed impaired release of NO. Under those conditions of blocking NO-mediate relaxation, the response to ACh and the maximum relaxation was significantly reduced in the mesenteric arteries from diabetic rats in comparison to mesenteric arteries from normal rats. Chronic treatment with linagliptin in vivo for 4 weeks significantly increased the maximum relaxation to ACh in diabetic mesenteric arteries in comparison to untreated diabetic mesenteric arteries. Furthermore, beside stimulated NO release, basal NO release was also reduced in mesenteric arteries of diabetic rats, in response to NOS
inhibition the contraction was impaired and this was reversed in arteries from linagliptin treated diabetic rats. Therefore, treatment with linagliptin was able to preserve the contribution of NO-mediated relaxation to the endothelium-dependent relaxation in diabetic mesenteric arteries. In our results, consistent with the impairment of NO mediated-relaxation we found that in the diabetic mesenteric arteries the expression of eNOS was reduced and the proportion of eNOS expressed as a dimer was decreased which indicated uncoupling of the total eNOS in the diabetic mesenteric arteries. In addition, in linagliptin treated diabetic rats, the expression of eNOS was comparable to the normal rats. Moreover, treatment with linagliptin stimulated the re-coupling of eNOS, which was expressed as a dimer more than monomer compared to untreated mesenteric arteries in diabetic rats and this normalization of eNOS expression and re-coupling of eNOS by chronic treatment with linagliptin would elucidate the improvement of the endothelium-dependent relaxation in mesenteric arteries of diabetic rats. A further factor to the repair of the activity of NO by chronic treatment with liangliptin could be the decrease of vascular superoxide generation in diabetic mesenteric arteries, and this could then increase the bioactivity of NO through avoiding the forming of peroxynitrite (ONOO−) via the degradation of NO by superoxide action. The reduction of ROS activity and prevention of peroxynitrite formation in diabetic vasculature could be either due to the rapid free radical scavenging effect of linagliptin in order to preserve the bioavailability of NO linagliptin through the inhibition of xanthine oxidase (Yamagishi et al., 2014) or by reducing the expression and/or activity of the enzymatic source of superoxide generation in the diabetic mesenteric arteries such as NAPDH oxidase subunit Nox2. Taken together, chronic treatment with liangliptin reserved the beneficial activity of NO via improving of eNOS expression, re-coupling of eNOS and reducing the expression of Nox2 which mediate superoxide generation in the mesenteric arteries of diabetic rats.
6.4.3 Effect of linagliptin on EDH-mediated relaxation in rat mesenteric arteries

The endothelium-dependent relaxation in rat mesenteric arteries is mediated by NO, classical EDH and non-classical EDH pathways (Edwards et al., 2010). In order to evaluate the contribution of EDH to endothelium-dependent relaxation in diabetes, we investigated the endothelium-dependent relaxation in the presence of L-NNA and ODQ inhibitions to block NO formation and sGC activity respectively. In addition to inhibit NOS, the guanylate cyclase inhibitor was also used to confirm the inhibition of the action of NO derived from nitrosothiols, a non-NOS sources of NO, which was earlier described to act as a source of NO in diabetic conditions (Leo et al., 2010). The sensitivity to ACh in diabetic mesenteric arteries was decreased significantly in comparison to normal mesenteric arteries in the presence of L-NNA+ODQ, indicating that diabetes impaired the contribution of EDH to endothelium-dependent relaxation, which is consistent with other reports (Leo et al., 2011b, Fukao et al., 1997, Matsumoto et al., 2003, Matsumoto et al., 2005). Chronic in vivo treatment with linagliptin improved EDH-mediated relaxation in mesenteric arteries of diabetic rats, indicating that treatment with linagliptin preserved the contribution of EDH to endothelium-dependent relaxation in diabetes. Although the main cause of the impairment of EDH in diabetic conditions remains uncertain (Leo et al., 2011b, Matsumoto et al., 2003, Matsumoto et al., 2006, Makino et al., 2000, Burnham et al., 2006a, Weston et al., 2008), however, is partly associated with over generation of superoxide and oxidative stress. It is reported that auto-oxidation of pyrogallol causes an increase in superoxide anions which could impair EDH-mediated relaxation in rat mesenteric arteries (Ma et al., 2008). Our results in this study demonstrated that the impairment of EDH-mediated relaxation in diabetes may be in part due to the overproduction of superoxide...
and treatment of diabetic rats with linagliptin improved the contribution of EDH to endothelium-dependent relaxation in mesenteric arteries which could be as a result of the reduction of superoxide levels by linagliptin treatment.

6.4.4 Conclusion

In this study we have demonstrated that the DPP-4 inhibitor linagliptin improves endothelial function in mesenteric arteries in diabetic rats by preserving the activities of both NO and EDH-mediated relaxation. In order to reduce inactivation of NO, linagliptin has protective actions via at least two mechanisms. The DPP-4 inhibitor linagliptin was able to reduce the generation of superoxide which may be due to direct radical scavenging action and/or attenuating the enzymatic source for superoxide generation where the chronic treatment of diabetic rats with linagliptin in vivo for 4 weeks prevents the uncoupling of eNOS, increases the expression of total eNOS, and decreases Nox2, hence facilitates maintenance of endothelium-dependent relaxation. Interestingly, the beneficial effects of linagliptin to preserve NO activity and improve EDH role in relaxation demonstrated in this study displays that linagliptin has the ability to act as a therapeutic compound independent of its glucose lowering action for use in the prevention of the microvasculature complications of diabetes.
Chapter 7

General Discussion

7.1 Effects of diabetes and WD on vascular function

Diabetes mellitus is one of the fast growing diseases all over the world both in developed and developing countries (Dunstan et al., 2002, Wild et al., 2004). Diabetes affects all the organs in the body, however cardiovascular complications are a major cause of high prevalence of morbidity and mortality in individuals with diabetes (Boyle et al., 2010). Vascular endothelial function is identified as a vital independent risk factor for cardiovascular disease. In diabetes, the endothelial dysfunction plays a critical role in the initiation and development of cardiovascular complications (De Vriese et al., 2000, Halcox et al., 2002, Al Suwaidi et al., 2000). In this thesis, we investigated the mechanisms underlying endothelial dysfunction over the course of exposure to high glucose concentration, STZ-induced type-1 diabetes, and western diet feeding regime.

It is widely acknowledged that hyperglycaemia causes an increase in oxidative stress which is known to cause endothelial dysfunction and is unfavourably related to an imbalance between NO and superoxide production in vascular endothelial cells. In investigations of small blood vessels (mesenteric arteries) exposed to high glucose, our findings (Chapter 3) indicate that there was an endothelial dysfunction related to an increase in ROS production and impairment in NO and EDH-mediated relaxation. Further investigation showed that the vascular endothelial function improved by increased bioavailability of NO derived from eNOS pathway
and improved EDH-type response which leads to increasing the contribution of these mediated relaxation factors to the endothelium-dependent relaxation in microvasculature. The results also suggested that both NO and EDH-mediated relaxation are vital modulators of vascular health and pharmacological compounds targeted to increase the bioavailability of NO and improve EDH-type response could be used to maintain vascular endothelial function under the pathological condition of diabetes.

In diabetes, prolonged hyperglycaemia causes overgeneration of ROS causing oxidative stress which leads to an impairment of the action of NO and EDH-mediated relaxation and subsequent endothelial dysfunction (De Vriese et al., 2000, Fatehi-Hassanabad et al., 2010) (Chapters 4 and 6). During diabetes, hyperglycaemia results in oxidative stress via the increase in NADPH oxidase expression/activity and uncoupling of eNOS that causes impairment of vascular endothelial function. This impairment in endothelial function was concomitant with a reduction in the contribution of both NO and EDH-mediated relaxation to the endothelium-dependent relaxation. This indicates that the increase in the expression/activity of NADPH oxidase and eNOS uncoupling are early contributors to hyperglycaemia-induced endothelial dysfunction. The increase in expression/activity of endothelial NADPH-oxidase, suggests that the observed impairment of EDH-type response may also be due to oxidative stress as a result of overgeneration of superoxide in the microvasculature which cause K_{Ca}-channels (IK_{Ca} and SK_{Ca}) alteration and interruption of downstream reactions of these channels (Leo et al., 2011b, Burnham et al., 2006a) (Chapters 4 and 6). Thus, therapies targeting ROS derived from NADPH-oxidase and eNOS uncoupling could have the potential to prevent microvascular complications and endothelial dysfunction concomitant with diabetes.
Metabolic syndrome is generally defined as a combination of risk factors or abnormalities strongly associated with obesity and insulin resistance which are noticeably associated with the increase in the risk of development of cardiovascular disease and diabetes mellitus (Stein and Colditz, 2004). ROS production is proposed to have an important role in the development of insulin resistance (Furukawa et al., 2004, Lin et al., 2005, Urakawa et al., 2003, Diniz et al., 2006). This study (Chapter 5) showed that the consumption of high fat diet (western diet) caused endothelial dysfunction in the mesenteric microvasculature. The findings showed that the ROS production in small blood vessels was significantly increased due to the consumption of western diet. This increase in oxidative stress was through the increase in the expression/activity of NADPH-oxidase and eNOS uncoupling that leading to vascular endothelial dysfunction. These findings suggest that ROS production may be the early event promoting western diet-induced pathologies and therefore may be an attractive therapeutic targeted for inhibiting insulin resistance and obesity caused by consumption of high fat diet or western diet.

7.2 Protection of diabetes or WD-induced endothelial dysfunction by the DPP-4 inhibitors

Exposure of blood vessels to high glucose induced oxidative stress, that has been demonstrated as a primary cause of premature vascular disease (Cosentino et al., 1997, Cosentino et al., 2003, Tesfamariam and Cohen, 1992). A recent study demonstrated that the DPP-4 inhibitor linagliptin, currently used as an antihyperglycaemic agent, was markedly more potent than other DPP-4 inhibitors in its anitoxidant capacity (Kroller-Schon et al., 2012). In this study (Chapter 3), the acute effects of the DPP-4 inhibitors linagliptin, sitagliptin and vildagliptin and the GLP-1 receptor agonist exendin-4 were examined on the endothelial function
of blood vessels exposed to high glucose. The findings demonstrated that high glucose impaired endothelium-dependent, but not endothelium-independent, relaxation in rat mesenteric arteries, due to an increased production of superoxide and the acute treatment with linagliptin and exendin-4 reduced vascular superoxide and improved endothelium-dependent relaxation and the beneficial effect on endothelium-dependent relaxation of exendin-4, but not linagliptin, was mediated by the GLP-1 receptor. Also, the NO and EDH-mediated relaxation were both impaired in the presence of a high glucose concentration and the actions of both mediators were improved by linagliptin. Taken together, the findings suggested that linagliptin and exendin-4 have antioxidant effects that may not be shared with other DPP-4 inhibitors. This antioxidant action may have additional benefit to the glucose lowering effects of linagliptin in the treatment of diabetes.

In Chapter 4, the acute effect of the DPP-4 inhibitor linagliptin in vitro on microvascular function from STZ-induced type 1 diabetic rats was investigated, demonstrating that acute treatment with linagliptin markedly reduce the high level of superoxide and improved endothelial function in diabetes. Endothelial dysfunction in the diabetic rats was associated with impairment in the modulatory role of NO and EDH-mediated relaxation. Acute treatment with linagliptin in vitro improved the contribution of both NO and EDH to the endothelium-dependent relaxation in small blood vessels from the diabetic rats, indicating that the DPP-4 inhibitor has potential as therapeutic agent for use in the reduction and/or prevention of microvascular complications associated with diabetes beyond its current use in type 2 diabetes. In Chapter 5, the acute treatment with DPP-4 inhibitor linagliptin in vitro on the endothelial function of small blood vessels from western diet fed rats was investigated. The observed findings indicated that superoxide production is increased in mesenteric arteries from western diet fed rats and in the
acute presence of linagliptin, the vascular superoxide production was significantly reduced. Western diet caused endothelial dysfunction and both the NO and the EDH-mediated relaxation was impaired in the microvasculature. The results showed that the acute presence of linagliptin improved endothelium-dependent relaxation in the mesenteric arteries from western high fat diet fed rats by selectively preserving the contribution of both NO and EDHmediate relaxation. This indicates that linagliptin could be in favour of the prevention of the pathological events caused by western diet including the development of endothelial dysfunction in small arteries.

In Chapter 6, treatment with linagliptin in vivo in STZ-induced type 1 diabetic rats had no effect on the plasma glucose level, indicating that the DPP-4 inhibitor improves the function in blood vessels independently of a glucose lowering pathway. Furthermore, in normal animals, treatment with linagliptin had no effect on vascular function. The beneficial action of linagliptin, when administered in vivo may have at least two mechanisms of action. The linagliptin may exhibiting its protective action via either as an antioxidant by rapidly directly scavenging ROS and/or inhibit the enzymatic source for superoxide production (i.e. NADPH oxidase and eNOS uncoupling) to preserve NO bioavailability or through a GLP-1 receptor dependent pathway through increased activation of GLP-1R secondary to DPP-4 inhibition. The beneficial action of linagliptin via its ability to reduce the oxidative stress in diabetic vasculature by inhibiting superoxide-derived from NADPH oxidase and eNOS uncoupling makes the DPP-4 inhibitor linagliptin a potential adjunctive treatment for diabetic vascular complications.
7.3 Future directions

Whilst the acute relaxant effects of the GLP-1 agonist exendin-4 are of interest, of great significance is the investigation of the capacity of long-term chronic treatment with GLP-1 agonist exendin-4 to improve endothelial function in conditions of cardiovascular risk including diabetes and western, high fat diet. This would help in better understanding of the effects of their effect on endothelial function.

Acute treatment of small blood vessels from western high fat diet fed rats with the DPP-4 inhibitor linagliptin markedly improved vascular function may have been by inhibiting the activity of NADPH oxidase derived ROS. Hence, future experiments would be useful to investigate the effects of long-term chronic treatment with linagliptin on endothelial function in conditions of western high fat diet.

The observations of preclinical studies have indicated a possible beneficial cardiovascular action of DPP-4 inhibitors through both glucagon-like peptide 1 (GLP-1)-dependent and GLP-1-independent pathways. Clinically, DPP-4 inhibitors improve several risk factors such as improvement of blood glucose homeostasis, reduced blood pressure, improve postprandial lipaemia, reduced inflammatory markers, reduced oxidative stress, and improved endothelial function in individuals with type 2 diabetes. Some beneficial effect was also observed in patients with ischaemic heart disease or congestive heart failure (Bose et al., 2005, Anagnostis et al., 2011, Jose and Inzucchi, 2012). However, the actual relationship between the beneficial effects of DPP-4 inhibitors such as linagliptin and cardiovascular events remains to be demonstrated, thus investigation of the effect of DPP-4 inhibitor linagliptin on the ischemic/reperfusion cardiac injury would help insight into the effects of gliptins such as linagliptin on cardiovascular outcomes.
7.4 Conclusion

Taken together, the set of studies conducted in the present thesis have contributed to better understanding of the beneficial action of the DPP-4 inhibitor linagliptin on endothelial function independent of decreasing blood glucose levels. The data presented in the thesis provides an insight into the effects of diabetic conditions and western high fat diet on the mechanism(s) of endothelium-dependent relaxation. Also, data obtained in this thesis would help in understanding the mechanism of action of the DPP-4 inhibitor linagliptin used in the treatment of endothelial dysfunction in disorders such as diabetes and metabolic syndrome. Endothelial dysfunction is the initial step of the commencement of cardiovascular complication such as atherosclerosis. Conservation of endothelial function would delay and/or avoid complications of cardiovascular risk factors and reduce the prevalence of the mortality and morbidity among diabetic patients. As the DPP-4 inhibitor linagliptin treatment has demonstrated valuable effects in improving endothelial function, it may be a useful adjunct therapy in the treatment of endothelial dysfunction in diabetes independent of lowering blood glucose. This would make the range of patients able to benefit from the use of this drug to be greatly expanded.
Chapter 8

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