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ROLE OF GLP-1 RECEPTOR AGONISTS IN ENDOTHELIAL FUNCTION

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TO MY FAMILY

ABSTRACT

BACKGROUND

The leading cause of death for patients suffering from type 2 diabetes is macrovascular disease. Endothelial dysfunction is one of the earliest events identified in the pathogenesis of atherosclerosis. Hence, there is a need for finding glucose-lowering agents that cause direct positive effects on vasculature in diabetic patients. The aim of this work was to evaluate the putative role and potential effects of incretins *i.e.* GLP-1 and exendin-4 on the vasculature and to elucidate the mechanisms behind these effects.

STUDY I

We investigated whether incretins influence proliferation of human coronary artery endothelial cells (HCAECs) *in vitro* and studied the molecular mechanisms behind such effects. Exendin-4, GLP-1 (7-36) and GLP-1 (9-36) elicited dose-dependent increases in DNA synthesis and increased cell number. This mitogenic effect was associated with increased eNOS and Akt activity, which along with the augmented cell proliferation were blocked by PKA-, PI3K-, Akt- and eNOS-inhibitors and by a GLP-1 receptor antagonist, exendin (9-39).

STUDY II

We studied the role of exendin-4 on apoptosis of HCAECs under lipotoxic conditions *in vitro*. Palmitate provoked apoptosis, an effect that was inhibited by exendin-4 or GLP-1 (7-36). In contrast, palmitate-induced apoptosis was not affected by GLP-1 (9-36). Palmitate alone resulted in increased eNOS, p-38 MAPK and JNK phosphorylation, which were neutralized by exendin-4. The protective effect of exendin-4 on apoptosis was prevented after treatment of the cells with specific inhibitors for PKA, PI3K, eNOS, p38 MAPK or for JNK. The effect of exendin-4 on lipopapoptosis was blocked by the GLP-1 receptor antagonist, exendin (9-39).

STUDY III

In this study, we investigated the long-term *in vitro* effect of palmitate or high glucose, and the role of exendin-4, on gene expression in HCAECs. Our data show that the expression of eNOS was up-regulated by exendin-4 in the presence of either palmitate or high glucose, as demonstrated by both microarray and Western blotting analyses. However, microarray analysis showed a suppressed eNOS expression by palmitate, which was not observed in Western blot.

The expression of tyrosine kinase receptor Tie-2 and its ligand Ang-1 was up-regulated in the presence of exendin-4. Moreover, exendin-4 increased the expression of tissue plasminogen activator (TPA) and cell adhesion molecules involved in angiogenesis, such as platelet endothelial cell adhesion molecule (PECAM), cadherin-5 and extracellular matrix protein fibronectin. Angiotensin I-converting enzyme (ACE) expression was up-regulated by high glucose, whereas exendin-4 inhibited expression of this gene at high glucose.

STUDY IV

The aim of this study was to investigate whether exendin-4 could protect against endothelial dysfunction induced by a triglyceride-rich fat emulsion, and if there were any differences in vasorelaxant capacity between GLP-1 (7-36), GLP-1 (9-36) and exendin-4 in rat femoral arterial rings from non-diabetic rats *ex vivo*. Exendin-4 did not protect against lipotoxicity, whereas GLP-1 (7-36) and GLP-1 (9-36) exerted vasorelaxation.

CONCLUSIONS

GLP-1 receptor agonists stimulate the proliferation of HCAECs, protect them from lipopapoptosis and improve endothelial function in part through regulating expression of genes involved in angiogenesis, inflammation and thrombogenesis by reversing glucolipotoxic gene regulation. Improvement of endothelial dysfunction may translate into beneficial effects on many cardiovascular risk factors and may thus have important clinical implications in preventing and treating macroangiopathy in type 2 diabetes.

Key words: Type 2 diabetes, endothelial dysfunction, glucagon-like peptide 1 (GLP-1), exendin-4, endothelial nitric oxide synthase (eNOS)

LIST OF PUBLICATIONS

- I. Erdogdu Ö, Nathanson D, Sjöholm Å, Nyström T, Zhang Q (2010). Exendin-4 stimulates proliferation of human coronary artery endothelial cells through eNOS-, PKA- and PI3K/Akt-dependent pathways and requires GLP-1 receptor. *Molecular and Cellular Endocrinology* 325(1-2):26-35.
- II. Erdogdu Ö, Eriksson L, Xu H, Sjöholm Å, Zhang Q, Nyström T. GLP-1 receptor agonist exendin-4 protects human coronary artery endothelial cells from lipoapoptosis through the GLP-1 receptor and by PKA, PI3K, eNOS, p38 MAPK and JNK dependent pathways (*Submitted*).
- III. Erdogdu Ö, Eriksson L, Nyström T, Sjöholm Å, Zhang Q (2012). Exendin-4 restores glucolipotoxicity-induced gene expression in human coronary artery endothelial cells. *Biochemical and Biophysical Research Communications* 419(4):790-79.
- IV. Nathanson D, Erdogdu Ö, Pernow J, Zhang Q, Nyström T (2009). Endothelial dysfunction induced by triglycerides is not restored by exenatide in conduit arteries *ex vivo*. *Regulatory Peptides* 157(1-3):8-13.

Other publications not included in the thesis:

Eriksson L, Erdogdu Ö, Nyström T, Zhang Q, Sjöholm Å (2012). Effects of some anti-diabetic and cardioprotective agents on proliferation and apoptosis of human coronary artery endothelial cells. *Cardiovascular Diabetology* 11:27.

LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
ACE	Angiotensin I-converting enzyme
ACh	Acetylcholine
AGE	Advanced glycation end products
BH ₄	Tetrahydrobiopterin
CAD	Coronary artery disease
CHD	Coronary heart disease
CHF	Congestive heart failure
CVD	Cardiovascular disease
DPP-4	Dipeptidyl peptidase-4
EC	Endothelial cells
ECG	Electrocardiography
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FFA	Free fatty acid
FMD	Flow-mediated vasodilation
GIP	Glucose-dependent insulintropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1R	GLP-1 receptor
HCAECs	Human coronary artery endothelial cells
H ₂ O ₂	Hydrogen peroxide
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IHD	Ischemic heart disease
IL-1	Interleukin 1
IR	Ischemia-reperfusion
K _{ATP}	ATP- dependent potassium channel
MI	Myocardial infarction
NO	Nitric oxide
O ₂ ⁻	Superoxide anion
ONOO ⁻	Peroxynitrite
OxLDL	Oxidized low-density lipoprotein
PAI-1	Plasminogen activator inhibitor 1
PCWF	Pulmonary capillary wedge pressure
PECAM-1	Platelet endothelial cell adhesion molecule 1
PGH ₂	Prostaglandin H2
PGL ₂	Prostacyclin
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
RAP	Right arterial pressure
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
TNF- α	Tumor necrosis factor alpha
TPA	Tissue plasminogen activator

TZD
VCAM-1
VSMC

Thiazolidinedione
Vascular cell adhesion molecule 1
Vascular smooth muscle cell

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INTRODUCTION

The rapidly increasing prevalence of diabetes mellitus worldwide is one of the most challenging health problems in the 21st century [1]. The number of people with diabetes grows faster than expected. By the year 2025, it is estimated that 333 million people worldwide will suffer from the disease [2].

Cardiovascular disease (CVD) is the major complication of type 2 diabetes. The devastating complications of type 2 diabetes are mostly macrovascular and microvascular diseases as a consequence of atherosclerosis. In fact, total morbidity from cardiovascular disease in patients with type 2 diabetes is two to four times greater than that of non-diabetic individuals [1].

Regardless of the risk factors involved, atherosclerosis is perceived as much of an inflammatory disease in which endothelial dysfunction plays an essential role at all stages of the atherosclerotic process [3-6].

Endothelial dysfunction is regarded as an important factor in the pathogenesis of vascular disease in type 2 diabetes [7]. Although the hypothesis that specifically improving endothelial dysfunction can reduce the risk for CVD has not been tested directly, many different changes in lifestyle [8] and pharmacological interventions designed to ameliorate endothelial function are known to reduce this risk [9]. To focus only on the treatment of hyperglycemia has failed to reduce the incidence of CVD [10,11]. Therefore, there is a clear need to identify new effective therapies of the pharmacologic armamentarium that positively impact the endothelium and cardiovascular system.

Glucagon-like peptide-1 (GLP-1) is a gut hormone, released from the intestine following a meal, with potent insulin releasing and glucose lowering actions that result in improved glycemic control. Besides its well known anti-diabetic and metabolic effects [12], preclinical and clinical studies clearly demonstrate additional effects on the cardiovascular system by GLP-1 and its analogues [12,13]. The aim of this thesis was to study the potential effects of GLP-1 and exendin-4 on the vasculature and to pin down the underlying mechanisms of these effects.

TYPE 2 DIABETES

Type 2 diabetes is a multifactorial disorder in which the interaction between environmental and genetic factors contribute to the development of insulin resistance, a state in which a given concentration of insulin produces a less than expected biological effect, and β -cell dysfunction [14]. Although insulin resistance is a contributing factor, β -cell dysfunction is the critical step in the development of type 2 diabetes. The pathophysiology of type 2 diabetes involves defects in several organs, *i.e.* liver, pancreas, adipose and skeletal muscle tissue that conspire together to produce abnormal glucose and lipid metabolism. In contrast, type 1 diabetes is caused by cell-mediated autoimmune destruction of β -cells leading to rapid insulin deficiency. Diabetes is diagnosed by fasting plasma glucose levels > 7 mmol/l, or by a plasma glucose concentration > 11.1 mmol/l 2 hours following an oral glucose tolerance test according to the criteria by the World Health Organization, WHO. This thesis will only focus on type 2 diabetes.

THE METABOLIC SYNDROME

The metabolic syndrome is a name for a group of risk factors that can lead to the development of CVD. The condition is also known as syndrome X or the insulin resistance syndrome. Reaven *et al* [15] developed the concept that insulin resistance and a cluster of other risk factors including impaired glucose regulation, hypertriglyceridemia, decreased HDL-cholesterol, hypertension and obesity lead to the development of CVD. In 1998, the WHO defined these risk factors collectively as the metabolic syndrome [16]. The WHO definition of the metabolic syndrome is based on any sign of insulin resistance, *i.e.* impaired glucose tolerance (IGT), impaired fasting glucose (IFG) or diabetes, together with two or more of the components: hypertension,

hypertriglyceridemia, low HDL-cholesterol, central obesity and microalbuminuria [17]. Shortly thereafter, the European Group for the study of Insulin Resistance (EGIR), omitted microalbuminuria from the definition and introduced waist circumference as the main indicator of obesity [18]. In 2001, the National Cholesterol Education Program adult Treatment panel III (NCEP:ATPIII) reported five components of the metabolic syndrome related to CVD, namely waist circumference, hypertriglyceridemia, low HDL-cholesterol, hypertension and IFG [19]. Based on these five components NCEP:ATPIII proposed in 2001 [19] that when three out of five factors are present, diagnosis of metabolic syndrome can be made. The main differences between the three definitions are that the NCEP:ATPIII focused less on type 2 diabetes or insulin resistance and more on CVD risk, while the WHO and EGIR definitions emphasize the role of insulin resistance.

CARDIOVASCULAR DISEASE AND TYPE 2 DIABETES

CVD is currently the leading cause of death globally, accounting for 21.9% of total deaths and are estimated to increase to 26.3% by 2030 according to the WHO [20]. Ischemic heart disease (IHD) and myocardial ischemia are usually due to coronary artery disease (CAD). Myocardial ischemia is defined as myocardial cell death due to a prolonged ischemia, and occurs when a fibrous cap in coronary artery vessel is ruptured followed by local thrombosis formation [21]. The definitions of an acute MI are based primarily on rapid rise and fall of biochemical markers of myocardial necrosis, such as CKMB and troponins, together with at least one of following: ischemic symptoms and development of pathological Q waves on the electrocardiography (ECG) [22]. Unstable IHD is characterized by chest pain or other symptoms at rest, or rapidly worsening effort angina. Symptoms of stable ischemic heart disease include effort angina and reduced exercise tolerance.

ATHEROSCLEROSIS

Atherosclerosis is regarded as a multifactorial disease associated with a wide range of risk factors *e.g.* hypertension, hypercholesterolemia, diabetes and obesity [23]. The atherosclerotic process can be divided into four different phases.

Atherosclerotic plaque formation

Increased endothelial permeability to lipoproteins and other plasma constituents results in up-regulation of endothelial adhesion molecules, such as E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule 1 (VCAM)-1 [24]. This activation mediates rolling of leukocytes -- mainly T-cells and monocytes -- to the intima, the innermost layer of the artery [25].

Fatty streak

Oxidized low-density lipoproteins (oxLDL) are considered to be the crucial pro-inflammatory stimulus that damages the artery wall and initiates the development of atherosclerosis [26, 27]. Once penetrated into the intima, oxLDL activates the endothelial cells to express adhesion molecules that contribute to the recruitment of circulating leukocytes, mainly monocytes and T-cells [28]. Within the intima, monocytes are converted into macrophages that engulf oxLDL by endocytosis through scavenger receptors and form foam cells. These play a central role in minimizing the endothelial damage, not only by functioning as lipid scavenger cells but also as immune-competent cells secreting pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin 1 (IL-1) triggering the ongoing process leukocyte adherence. Vascular smooth muscle cells (VSMC) start to migrate from the media to the intima, divide themselves during this process and produce collagen and other extracellular matrix components [25].

Advanced fatty streak formation

Advanced atherosclerotic lesions start to form a fibrous cap that covers a mixture of leukocytes, lipid, and debris, forming a necrotic core [29]. This process represents a type of healing or fibrous response to the injury. Release of cytokines not only exacerbates inflammation and lipid accumulation but also influences VSMC activity. Continued expansion of lesions may cause obstruction of coronary blood flow and ischemia.

Rupture of the fibrous cap

Another factor that exacerbates the development of atherosclerotic plaque is the increased digestion of collagen and other connective tissue components. Macrophages covered by fibrous caps release proteolytic enzymes, such as proteases, which digest the collagen produced from VSMCs, thereby leading to thinning of the fibrous cap that prevents the necrotic center from spilling into the lumen of the artery. Increased apoptosis of VSMCs may also prevent the production of collagen, resulting in increased arterial instability. Rupture of the fibrous cap can result in thrombus formation and occlusion of the artery, contributing to MI [25].

THE ENDOTHELIUM

The arterial wall consists of three separate layers: the intima, a layer of endothelial cells that line the internal lumen of blood vessels; the media, which consists mainly of smooth muscle cells; and the adventitia that harbors nutrient vessels, nerves and dense fibroelastic tissue. Before discovering the complexity of the endothelium, it was known to be an inert transporting tube. Today the endothelium is known to be the main regulator of vascular wall homeostasis [28]. It is therefore not only a barrier between the circulating blood and the tissue but also an important regulator of vascular tone and permeability, the balance between coagulation and fibrinolysis, the adhesion and extravasation of leukocytes and inflammatory activity in the vessel wall.

Nitric oxide production

There are three distinct nitric oxide synthase (NOS) isoforms, encoded by three distinct NOS genes: neuronal nNOS (also known as NOS-1) isolated from the brain, inducible iNOS (also known as NOS-2) produced by macrophages, and endothelial eNOS (also known as NOS-3) [30]. Interestingly, an alternative pathway for NO formation in mammals was described where inorganic nitrate, an inert NO oxidation product and undesired dietary constituent, is reduced to nitrite and then to NO and other bioactive nitrogen oxides [31]. Endothelial cells (EC) constitutively express eNOS that converts the amino acid L-arginine to L-citrulline and uses cofactors such as FAD, FMN, NADPH and (tetrahydrobiopterin) BH₄ to produce picomolar to nanomolar concentrations of NO [32]. The release of NO occurs either through receptor-operated mechanisms induced by acetylcholine (ACh), bradykinin, ATP, ADP, serotonin or prostacyclin (PGI₂), or through receptor-independent activation mechanisms such as physical stimuli. Shear stress or receptor activation of vascular endothelium will increase the intracellular concentration of Ca²⁺. These ions bind to calmodulin and form a Ca²⁺-calmodulin complex that activates eNOS [33, 34]. NO diffuses out of the ECs and into the neighboring VSMCs where it binds and activates soluble guanylate cyclase (sGC). This event results in formation of cyclic GMP, which triggers a response that causes the VSMCs to relax and thereby allows the vessel to dilate (Figure 1).

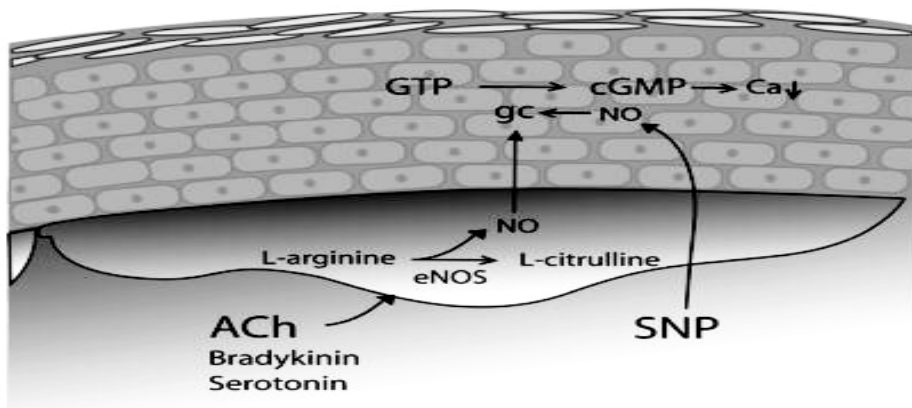


Figure 1. The nitric oxide pathway in the vasculature

Endothelial cells express eNOS that converts the amino acid L-arginine to L-citrulline and uses cofactors to produce NO. The release of NO is either through receptor-operated mechanisms induced by acetylcholine (ACh), bradykinin, ATP, ADP, serotonin or prostacyclin (PGI₂), or through receptor-independent activation mechanisms such as physical stimuli. Shear stress or receptor activation of vascular endothelium will increase the intracellular concentration of Ca²⁺. These ions bind to calmodulin and form a Ca²⁺-calmodulin complex that stimulates eNOS. NO diffuses out of the EC cells and into adjacent VSMCs where it binds and activates soluble guanylate cyclase (sGC), resulting in enhanced synthesis of cyclic GMP. Enhanced levels of cyclic GMP trigger a response that causes the VSMCs to relax, thereby allowing the vessel to dilate. Reprinted from *Molecular and Cellular Endocrinology*, volume 197, Nathanson D, Nyström T., *Hypoglycemic pharmacological treatment of type 2 diabetes: Targeting the endothelium*, page no. 114, © (2009), with permission from Elsevier.

Endothelium-dependent relaxation occurs in resistance vessels as well as in larger vessels. However, relaxation may be limited to certain blood vessels and therefore it might be more pronounced in arteries than in veins [35]. Apart from the potent vasodilator effect of NO, it regulates many other vasoprotective functions in the endothelium [36]. It decreases endothelial permeability; *i.e.* prevents lipoproteins from entering the vessel wall. Also, NO protects against vascular injury, inflammation and thrombosis by inhibiting leukocyte migration and adhesion to the endothelium, maintaining the vascular smooth muscle in a non-proliferative state [37-39].

ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction can be broadly defined as an imbalance in the production of vasodilating factors, *e.g.* NO, initially identified as endothelium-derived relaxing factor (EDRF) [35], PGI₂ and vasoconstricting factors, *e.g.* endothelin-1 (ET-1), angiotensin II and prostaglandin (PGH₂). When this balance is disrupted, the release of vasoconstriction factors may trigger the vasculature towards a pro-atherogenic milieu and initiate a number of processes that promote the development of atherosclerosis. These processes subsequently contribute to VSMC proliferation, platelet and leukocyte adhesion and production of cytokines [40-42]. Impaired endothelium-dependent vasodilatation due to an impaired NO synthesis, as a result of diminished levels of the cofactor BH₄ or augmented inactivation by superoxide, has been proposed as a major mechanism of endothelial dysfunction and contributor to atherosclerosis [42].

Although the molecular basis of endothelial dysfunction is not well understood, several studies suggest that NO may play a central role in this mechanism. A common feature of endothelial dysfunction is a reduced level of NO due to increased production of reactive oxygen species (ROS), resulting in an intima that is characterized by increased thrombus formation and dysregulated VSMC growth. Diminished NO bioactivity is considered a main contributor to vasoconstrictive remodeling and oxidative stress [32].

eNOS uncoupling

Pathological conditions, *e.g.* hyperglycemia, hyperlipidemia, inflammation and insulin resistance, may trigger superoxide anion (O_2^-) production through activation of NADPH oxidase and result in oxidative stress (Figure 2). O_2^- is known to react with NO to form the most potent oxidant peroxynitrite ($ONOO^-$), which oxidizes BH_4 and exerts oxidative damage to eNOS. In such a situation -- with excessive oxidation and depletion of BH_4 -- eNOS may function as an NADPH oxidase, thereby producing O_2^- instead of NO [34, 37, 43-45]. It has been suggested that not only NADPH oxidase but also eNOS has the potential to generate O_2^- [46] in rats with streptozotocin-induced diabetes, in which vascular O_2^- production was decreased by an eNOS inhibitor [47]. Moreover, a time-dependent decrease in myocardial BH_4 levels has been observed during ischemia in rats [48]. Administration of BH_4 has been shown to restore coronary flow and eNOS activity, an effect that resulted in reduced O_2^- production.

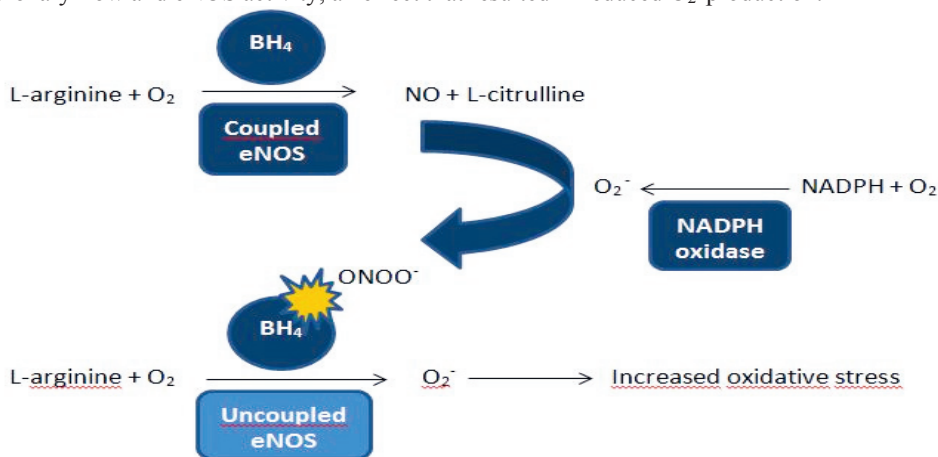


Figure 2. Coupled and uncoupled eNOS

NADPH oxidases are up-regulated in diabetes and the product superoxide anion (O_2^-) reacts with NO to form peroxynitrite ($ONOO^-$). This oxidizes BH_4 , the co-factor of eNOS, and eNOS may become uncoupled. This results in the production of reactive oxygen species (ROS), *e.g.* O_2^- and hydrogen peroxide (H_2O_2). A functional eNOS is now converted into a dysfunctional O_2^- -generating enzyme that contributes to vascular oxidative stress and endothelial dysfunction.

These results provide evidence that optimal concentrations of BH_4 are fundamentally important for the normal function of eNOS in ECs. Elevated levels of ROS or oxidative stress are scavenged by superoxide dismutases (SOD). Reddy *et al* [49] have demonstrated that over-expression of SOD inhibits H_2O_2 -induced apoptosis. However, once the potent oxidant $ONOO^-$ is formed, this scavenger system becomes incapacitated thereby exaggerating the oxidative stress further [50].

ENDOTHELIAL DYSFUNCTION AND TYPE 2 DIABETES

The role of endothelial dysfunction in type 2 diabetes is complicated due to the many independent factors involved, including hyperglycemia, dyslipidemia, insulin resistance, hypertension, abdominal obesity and low-grade inflammation [51]. All of these factors are associated with endothelial dysfunction [52].

Hyperglycemia

Several mechanisms have been proposed that can explain how hyperglycemia induces vascular complications. All these mechanisms are activated by mitochondrial overproduction of ROS. Hyperglycemia-induced ROS production plays an important role in the activation of several pathways such as the aldose reductase, hexosamine pathways, DAG/PKC activation and advanced glycation end products (AGE) formation [52]. All these pathways can overlap each other and produce oxidative stress [53, 54]. Also, hyperglycemia can induce endothelial dysfunction in non-diabetic individuals [53]. Diminished vascular response to methacholine chloride, but not to a calcium channel blocker, suggests involvement of NO in hyperglycemia-induced endothelial dysfunction [55]. These findings raised the possibility that L-arginine and/or BH₄ may be deficient in various conditions associated with impaired endothelial dysfunction [56]. There is a close correlation between eNOS synthesis and the concentrations of BH₄. In the settings of oxidative stress by hyperglycemia, BH₄ depletion is seen and this may result in reduced bioavailability of NO due to decreased eNOS synthesis [30]. Treatment with BH₄ has shown to increase endothelium-dependent vasodilatation in humans with hypercholesterolemia [57]. Also, administration of L-arginine and BH₄ has shown to ameliorate endothelial dysfunction in the forearm vasculature in patients with type 2 diabetes and CAD [58]. Notwithstanding these pathogenic mechanisms, improving hyperglycemia *per se* has shown only modest effects on diabetic macroangiopathy [9], indicating a clear need for agents that effectively address this.

Dyslipidemia

Increased plasma levels of oxLDL and cholesterol are risk factors for atherosclerosis and macrovascular disease. OxLDL has been shown to suppress eNOS and subsequently inhibit the release of NO. This pathological process may activate vasoconstrictor mediators, such as angiotensin II, ET-1, prostaglandins and ROS, thereby leading to reduced production of NO that causes endothelial dysfunction and increased apoptosis [59].

Insulin resistance

Insulin resistance is a condition where insulin becomes less effective in promoting glucose uptake from the bloodstream into skeletal muscle and adipose tissue [60]. Initially, the insulin-producing β -cells manage to compensate for insulin resistance by an increase in insulin secretion [61, 62]. In the presence of insulin resistance, adipocytes release excess levels of circulating free fatty acids (FFAs), which results in dyslipidemia including VLDL-hypertriglyceridemia, high plasma FFA and low HDL-cholesterol levels. This alteration has a negative impact on glucose and lipid metabolism. FFAs also diminish insulin sensitivity in muscle by inhibiting insulin-mediated glucose uptake. Both hyperglycemia and hyperlipidemia cause an increase in oxidative stress due to increased production of ROS and increased formation of AGEs [63]. Finally, in insulin resistance and type 2 diabetes a state of pro-coagulability and low-grade inflammation occurs, both of which have been linked to endothelial dysfunction [63]. At physiological concentrations insulin exerts anti-inflammatory effects, whereas hyperinsulinemia increases levels of oxidative stress. A previous study reported that insulin, at pathophysiological concentrations alone or in combination with low concentrations of TNF- α , promotes VCAM-1 expression [64].

Insulin resistance is characterized by specific impairment in PI3K-dependent pathways, whereas other insulin signaling branches -- such as MAPK-dependent pathways -- remain unaffected [63].

Inflammation

There is increasing evidence that adipose tissue is a highly active endocrine organ, releasing a variety of secretory products, *e.g.* cytokines, hormones and enzymes with the propensity to impair insulin sensitivity [65]. Altered plasma concentrations of adipokines (adiponectin and leptin) and cytokines (TNF- α , IL-6, PAI-1) have been suggested to play a role in inflammation, insulin resistance and CVD [65].

Adiponectin plays an important role in the regulation of insulin function and energy homeostasis; it is known to reduce insulin resistance and the development of atherosclerosis in an anti-inflammatory way [66]. It stimulates production of NO in endothelial cells [67]. Moreover, elevated levels of adiponectin are associated with a lowered risk of myocardial infarction [68].

Receptors for leptin are present on endothelial cells and in atherosclerotic plaques, suggesting that this peptide may contribute to the development of atherosclerosis [69].

Also, the recently described protein resistin that is expressed and secreted from adipocytes in mice [70], but not from mature adipocytes in humans [71], has been shown to impair glucose tolerance and insulin secretion [72]. In further support of its inflammatory profile, resistin has been reported to increase expression of pro-inflammatory cytokines -- such as TNF- α and IL-6 -- through activation of NF- κ B. Moreover, increased levels of TNF- α and IL-6 up-regulate NADPH oxidase, resulting in higher oxidative stress [73, 74]. Resistin also up-regulates endothelial expression of vascular cell adhesion molecules, such as ICAM-1 and VCAM-1. This indicates that resistin may promote the onset of cardiovascular complications [75].

IMPROVEMENT OF ENDOTHELIAL FUNCTION

Studies have shown that endothelial dysfunction is an independent risk factor for cardiovascular events in patients with peripheral vascular [76] and coronary artery disease [77]. This indicates that endothelial dysfunction contributes to the pathogenesis of CVD. Endothelium-mediated vasodilation is impaired in patients with chronic heart failure due to decreased NO levels [78]. One way to minimize the risk and improve the long-term prognosis of patients with CVD would be to improve endothelial function by increasing NO bioavailability. Endothelial dysfunction may therefore represent a therapeutic target. Although this has not been tested directly, numerous studies have evaluated lifestyle and pharmacologic interventions to improve endothelial function, and many of these interventions are known to reduce cardiovascular risk [79].

Lifestyle intervention

It is well known that insulin resistance coexists with obesity. Obesity, defined as body mass index (BMI) > 30, has increased dramatically worldwide and is considered a global epidemic by the WHO. In the U.S., the prevalence of obesity has doubled in the last 30 years [80]. Changes in lifestyle, such as regular exercise and/or dietary modification, can improve both insulin sensitivity and endothelial function [81, 82]. Body weight loss and modifications in food intake result in decreased lipid levels and inflammatory activity [83, 84]. Dietary caloric restriction alone normalizes β -cell function and hepatic insulin sensitivity, two major culprits underlying type 2 diabetes [85]. Also, the progression into overt diabetes can be prevented by moderate lifestyle changes in individuals with IGT [8, 86].

Pharmacological agents

Insulin

Insulin stimulates vasorelaxation through a mechanism that appears to be NO-dependent [87]. Previous publications have shown that HCAECs express the insulin receptor [88]. There is

evidence that insulin increases endothelial NO production through activation of the PI3K/Akt signaling pathway, which in turn stimulates eNOS phosphorylation [89, 90].

Hyperinsulinemia, which is a key feature in insulin resistance, over-activates MAPK-dependent pathways, thereby creating an imbalance between PI3K and MAPK-dependent functions of insulin [91, 92]. Extracellular regulated kinases 1 and 2 (Erk1/2), known to mediate vasoconstrictive effects of insulin, are responsible for the increased production of (plasminogen activator inhibitor 1) PAI-1, ET-1 and various proliferative events in VSMCs [93, 94]. This indicates that insulin resistance blocks potential antiatherosclerotic mechanisms but leaves certain proatherosclerotic mechanisms intact.

Metformin

Short-term treatment with metformin has been shown to improve markers of endothelial dysfunction and inflammatory activity, such as soluble vascular adhesion molecule 1, E-selectin, TPA and PAI-1 [95]. Also, it is increasingly clear that administration of metformin decreases hepatic glucose production through activation of AMP-activated kinase, with a minimal risk of hypoglycemia [96]. Some investigators have demonstrated that metformin may exert its vascular protective actions independent of glycemia [97].

The United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that metformin in monotherapy, but not in combination with sulfonylureas, may moderately reduce cardiovascular morbidity and mortality independent of glycemia in a subgroup analysis of obese subjects [98].

Sulfonylureas

The main target of action of sulfonylureas is to bind to the SUR/Kir 6.2 subunit of the ATP-dependent potassium channel (K_{ATP}) found in most cell types, including β -cells, skeletal muscle, VSMCs, kidney, the central nervous system and endothelium.

The cardiovascular effects of sulfonylureas have been widely debated. Glibenclamide, a conventional sulfonylurea, has been shown to exert negative cardiovascular effects, resulting in high incidence of cardiovascular death [99]. In contrast, gliclazide, a second generation sulfonylurea, not only lowers glycemia but also works as a free radical scavenger that improves endothelial function, reduces platelet reactivity [100] and decreases apoptosis and fibronectin expression [101]. Nevertheless, Jiang *et al* [102] have demonstrated that both glibenclamide and gliclazide relax mouse conduit arteries, an effect that seems to be NO-dependent.

Also, glimepiride has been reported to exert its cardiovascular effect through eNOS- and PI3K/Akt-dependent pathways [103, 104].

Thiazolidinediones (TZDs)

TZDs bind to the γ subtype of the peroxisome proliferator-activated receptor (PPAR). Activation of this receptor leads to increased insulin sensitivity concomitant with reduced plasma FFA levels [105]. The TZDs are also called insulin sensitizers that enhance whole body glucose uptake [105]. Administration of TZDs to endothelial cells increased eNOS activity and NO production [106]. In addition to lowering blood glucose, pioglitazone exerts a number of cardiovascular effects that are considered to be beneficial in atherosclerotic disease. Some studies have shown that pioglitazone decreases intima-media thickness of the carotid artery [107], whereas others have shown that pioglitazone directly influences endothelium-dependent vasodilatation independent of its glycemic effects in non-diabetic patients with CAD [108]. Furthermore, in high risk patients with type 2 diabetes and previous MI, pioglitazone reduced the recurrence of fatal and nonfatal MI [109].

Rosiglitazone is another anti-diabetic drug previously used in the treatment of type 2 diabetes. However, it has been withdrawn recently due to high incidence of cardiovascular events in patients with type 2 diabetes [110]. The mechanism by which rosiglitazone increases cardiovascular mortality is unclear.

Incretins

Multiple gastrointestinal hormones are secreted in response to nutrient ingestion. These hormones are capable of stimulating the pancreas to release substances that can reduce glycemia. Elrick *et al* [111] demonstrated that oral glucose elicits a higher insulin response when compared with the same amount of glucose given intravenously. This phenomenon is called “the incretin effect” and is caused by the two incretins, namely GIP and GLP-1. GLP-1 is secreted from L-cells in the distal small bowel and colon, whereas GIP is released from K cells of the proximal small intestine [112]. Both GLP-1 and GIP are insulinotropic in healthy humans [12]. However, glucagon secretion is inhibited only by GLP-1, not by GIP [113]. In contrast to GLP-1, GIP seems to lose its insulinotropic effects in diabetic patients, [114] probably through receptor down-regulation [115]. In addition to regulating glucose and insulin levels, GLP-1 slows down gastric emptying, reduces body weight and promotes satiety [112, 116-117].

GLP-1 is a product of the partial proteolysis of pre-proglucagon and released from the intestinal L-cells after food intake. The proglucagon gene is expressed in many cell types, including α -cells, intestinal L-cells and neurons. A single copy of this proglucagon gene leads to an mRNA transcript that after posttranslational processing becomes tissue specific. In the intestine, proglucagon is cleaved to glicentin, oxyntomodulin intervening peptide-2 (IP-2), GLP-1 and GLP-2 by pro-hormone convertase PC1/3 and PC2 [12] (Figure 3).

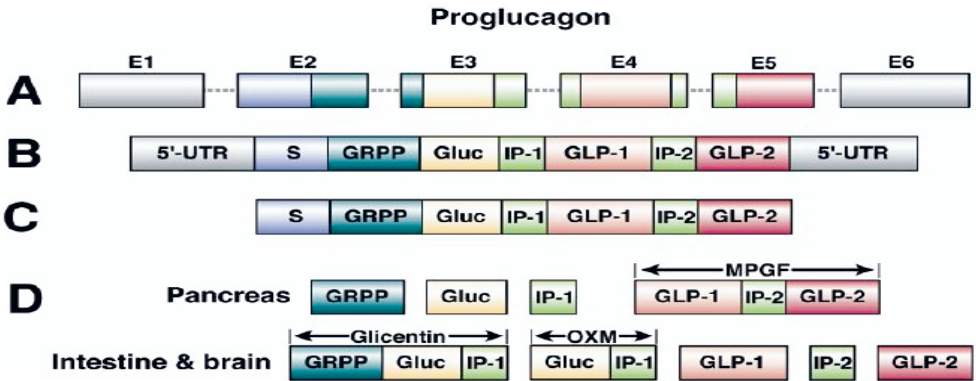


Figure 3. Structures of (A) the proglucagon gene, (B) mRNA, and (C) protein, (D) tissue specific posttranslational processing of proglucagon in the intestine liberates glicentin, oxyntomodulin, GLP-1, intervening peptide-2 (IP-2) and GLP-2. Reprinted from *Gastroenterology*, volume, 132, Baggio, LL, Drucker DJ., *Biology of Incretins: GLP-1 and GIP*, page no 2132, © (2007), with permission from Elsevier.

In terms of glucose-lowering, the biologically active forms of GLP-1 are GLP-1 (7-37) and GLP-1 (7-36) amide. The main circulating form of GLP-1 is the GLP-1(7-36) amide [12] (Table 1).

Table 1. GLP-1 isoforms

(7-36)amide
(7-37)
(1-36)amide
(1-37)
(9-36)amide
(9-37)

GLP-1 (7-37) and GLP-1 (7-36) amide are rapidly degraded by DPP-4 with a half-life of 1-2 min [118]. Interestingly, *in vitro* studies have demonstrated that GLP-1 (7-36) amide is

progressively degraded to GLP-1 (9-36) by DPP-4 present in serum [119]. DPP-4 cleavage generates the *N*-terminally truncated metabolites GLP-1 (9-37) and GLP-1 (9-36) amide, which are unable to stimulate the GLP-1R [120]. Due to its intact *C*-terminus, GLP-1 (9-36) amide can still bind to the receptor, albeit with an affinity of only < 1% compared to GLP-1 (7-36) amide [121]. Studies indicate that receptor binding in the absence of receptor activation results in a peptide antagonist [120, 122]. On the other hand, a 100-fold excess of GLP-1 (9-36) amide is needed to induce antagonism [120]. High levels (1 μ M) of GLP-1 (9-36) also exert a weak agonist activity on the GLP-1R, indicating that GLP-1 (9-36) amide may be a partial agonist of the GLP-1R [122].

It is well known that GLP-1 (9-36) amide and the truncated form of GIP are unable to stimulate insulin secretion [123]. *In vivo* studies have reported that in anesthetized pigs GLP-1 (9-36) amide in very high concentrations, compared to GLP-1 (7-36) amide, does not antagonize insulin secretion but exerts a glucose-lowering effect independent of insulin [124]. In contrast, others have demonstrated that GLP-1 (9-36) amide has no effect on insulin secretion, glucose elimination or insulin-independent glucose disposal in healthy humans [125] and in mice [126]. These results suggest that the effect of GLP-1 (9-36) amide on glucose disposal may become apparent only at pharmacological concentrations and in stressed metabolic states such as insulin resistance [127].

GLP-1 receptors

Both GLP-1 (7-36) amide and GIP exert their actions through structurally distinct G protein-coupled receptors. The GLP-1R was first cloned from rat pancreatic islets [128]. The GLP-1R is widely distributed and expressed in peripheral organs (pancreatic islets, stomach, heart, intestine, lung and kidney) and in the brain (Figure 4) [112, 129-131].

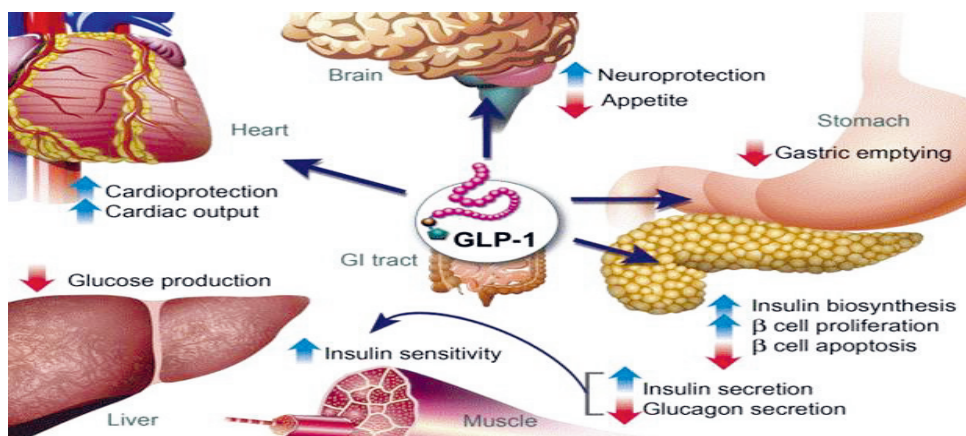


Figure 4. GLP-1 actions in peripheral tissues

GLP-1 acts directly on the endocrine pancreas, heart, stomach, and brain, whereas actions on liver and muscle are direct and/or indirect. Reprinted from *Cell Metabolism*, volume 3, issue 3, Drucker DJ, *The biology of incretin hormones*, page no 154, © (2006), with permission from Elsevier.

Previous publications have shown that GLP-1 promotes glycogen accumulation in rat hepatocytes [132, 133], independent of changes in insulin [134]. Recent studies further demonstrated that GLP-1 (9-36) inhibits hepatic glucose production, an effect that was particularly apparent during GLP-1R blockade [135]. Moreover, in isolated muscle and adipocytes, GLP-1 and exendin-4 increase insulin-stimulated glucose uptake through a receptor distinct from the classic GLP-1R [136, 137]. Activation of GLP-1R causes stimulation of adenylate cyclase through the G_{α_s} -coupled GLP-1 receptor, thereby resulting in the formation of

cAMP and activation of PKA [118]. Stimulation the pancreatic β -cell by GLP-1, in the presence of glucose, results in closure of ATP-sensitive K^+ channels and depolarization of the plasma membrane and opening of voltage-sensitive Ca^{2+} channels and a consequent influx of Ca^{2+} that sets secretion of insulin in motion [138, 139]. GLP-1-induced insulin secretion is thought to be mediated by this pathway. However, contribution of other signal transducing pathways, including PKC through activation of phospholipase C (PLC) [117, 138] and PI3-K γ [140], has been reported. Previous studies have shown that activation of PI3-K γ , through the $G_{\beta\gamma}$ subunit, plays an essential role in the regulation of GLP-1-stimulated insulin secretion. These findings demonstrate that PI3-K γ deficiency may result in dissociation between cAMP levels and glucose-dependent insulin secretion [140]. Nevertheless, increased cAMP levels are thought to be required for all of these pathways. Although GLP-1-induced insulin secretion is mainly through activation of cAMP and PKA, a study has proposed that physiological concentrations of GLP-1 may act through a cAMP-PKA independent pathway [141], possibly through activation of mitogen-activated protein kinase signaling [142, 143]. Nevertheless, GLP-1-stimulated insulin secretion is dependent on intracellular Ca^{2+} levels [141]. Activation of GLP-1R also elicits other responses, including stimulation of β -cell proliferation and protection against apoptosis in rodents [144] and human islets *ex vivo* [145, 146].

Antihyperglycemic effects of GLP-1

GLP-1 stimulates insulin release and suppresses glucagon release only in the presence of hyperglycemia, which ceases at glucose levels <4 mM, meaning that there is a low risk to produce hypoglycemic side effects [147]. GLP-1 is an important contributor in the regulation of insulin and glucagon secretion and these glucoregulatory effects play a crucial role in the maintenance of glucose homeostasis. Zander *et al* [148] demonstrated that in type 2 diabetic patients, infusion of GLP-1 increases insulin secretion, reduces HbA_{1c} levels and gives a weight loss of 1.9 kg, thereby leading to increased insulin sensitivity. Other studies have demonstrated that short-term intravenous infusions of GLP-1 lower blood glucose levels not only in patients with type 2 diabetes at early stages, but also at more advanced stages of the disease [149] as well as in type 1 diabetes of different duration [150, 151].

Incretin agonists

GLP-1 (7-36) amide is rapidly degraded by the ubiquitous DPP-4; therefore, GLP-1 analogs, resistant to this degradation and DPP-4 inhibitors, have been developed. Exendin-4, a 39-amino acid peptide hormone, was found in the venom of the Gila Monster (*Heloderma suspectum*) and it is a high-affinity agonist of mammalian GLP-1 receptors. It shares 53% of its amino acid sequence with the native GLP-1 [152]. Exendin-4 displays biological properties similar to GLP-1, as a regulator of glucose metabolism and insulin secretion. Unlike GLP-1, exendin-4 has much longer half-life (60-90 min) [153], and therefore is a viable candidate for the treatment of type 2 diabetes [152].

Exendin-4 can be truncated by 8 amino acid residues at its *N*-terminus without losing receptor affinity. However, truncation of the first 2-8 amino acid residues leads to the generation of antagonists.



Figure 5. Structure of GLP-1 (7-36) and its mimetic exendin-4

Reprinted from *The Lancet*: Drucker DJ, *The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 Diabetes*, page 1697, © 2006, with permission from Elsevier.

A significant difference between the *N*-terminal region of GLP-1 and exendin-4 is the second amino acid residue, alanine in GLP-1 and glycine in exendin-4, which gives exendin-4 its resistance to DPP-4 digestion (Figure 5). Truncation of the *N*-terminal region of GLP-1 (7-36) to GLP-1 (9-36) results in a large loss in affinity, whereas similar truncation of exendin-4 to exendin-4 (9-39) contributes less to affinity. This indicates that exendin (9-39) is a specific and competitive antagonist of GLP-1R that can block both GLP-1 and exendin-4 from receptor binding due to its high affinity interactions with the *N*-terminal extracellular domain (nGLP-1R) [154]. Studies have shown that the *N*-terminal domain of the GLP-1R binds exendin-4 with much higher affinity than GLP-1 [154]. A divergent residue in the central region of exendin-4 explains its higher affinity for the nGLP-1R [155].

Liraglutide is a GLP-1 analog with 97% homology to the native peptide, which contains an arginine 35 lysine substitution, a glutamate substitution and a FFA addition to lysine. The FFA addition increases liraglutide binding to albumin, resulting in a plasma half-life of 10-14 hours after s.c. injection [118]. Liraglutide can be administered once daily and reduces both fasting and postprandial glycemia. Liraglutide also has been shown to reduce systolic blood pressure and decrease weight up to 3.2 kg [156].

Lixisenatide is another GLP-1R agonist in late-stage development. The half-life of lixisenatide is 2-4 hours. Despite its short half-life, lixisenatide is intended for once daily dosing due to its strong binding affinity to the GLP-1 receptor. Lixisenatide has been shown to have beneficial effects on HbA_{1c} in combination with other commonly used oral agents for type 2 diabetes, with no increased risk of hypoglycemia [157].

DPP-4 inhibitors

GLP-1 (7-36) amide and GIP are rapidly degraded by DPP-4, a serine protease, which cleaves proteins that have alanine or proline in the second position. DPP-4 is widely distributed and expressed in both soluble and cell surface forms in many cell types including endothelial cells and intestinal cells [158]. Thus, the majority of GLP-1 passing the portal circulation has already been degraded by DPP-4 [159]. The first DPP-4 inhibitor approved for clinical purposes was sitagliptin, which inhibits plasma DPP-4 activity after oral administration [160]. Vildagliptin, saxagliptin and linagliptin are other highly selective DPP-4 inhibitors in clinical use in Europe [161-164]. Inhibition of DPP-4 results in increased levels of endogenous uncleaved, biologically active incretins [165]. In contrast to GLP-1 analogues, DPP-4 inhibitors do not cause major decreases in body weight [2, 166], probably due to the relatively modest incretin effect (raising [GLP-1] to 15-25 pmol/l) [118].

GLP-1 secretion

The plasma levels of GLP-1 rise after ingestion of glucose, fatty acids, proteins and dietary fiber [167]. In addition, ACh has been shown to stimulate GLP-1 release [168]. Basal levels of total GLP-1, including GLP-1 (7-36) amide, are between 5 to 10 pmol/l and rise to between 20 and 60 pmol/l after oral glucose or meals. In individuals with IGT, GLP-1 secretion is slightly reduced [169, 170]. Insulin resistance has also been described as a factor impeding GLP-1 release [171].

GLP-1 and insulin resistance

In type 2 diabetic subjects, GLP-1 concentrations have been reported to be reduced in some [172], but not all [169, 173], studies. Thus, the rate of insulin secretion depends not only on the degree of glycemia but also on the insulinotropic effects of GLP-1 [174]. There are to date conflicting results as to whether GLP-1 increases insulin sensitivity or not [175]. A previous publication has shown that infusion of GLP-1 did not affect whole body glucose uptake in subjects with type 2 diabetes and stable coronary artery disease [175]. In contrast, exendin-4 therapy for 3 years produced sustained improvements not only in glycemic control but also in other cardiovascular risk factors such as total cholesterol and body weight [176]. In *ob/ob* mice,

exendin-4 treatment reduced plasma glucose compared to placebo-treated mice while concomitantly improving insulin sensitivity [177]. Furthermore, a recent study reported that long-term treatment with GLP-1 analog increased insulin sensitivity in pre-diabetic UCD-T2DM rats, a model of polygenic obese type 2 diabetes, [178] and in *db/db* mice [179], as a consequence of reduced body weight.

Incretin effects on the vasculature

Previous studies have shown that i.v. and central (intracerebroventricular) administration of GLP-1 (7-36) amide and exendin-4 dose-dependently increase the blood pressure and heart rate in rodents [180-181], effects that were blocked by i.v. infusion of exendin (9-39) [181]. In addition, bilateral vagotomy was found to inhibit the blood pressure and heart rate elevating effects of intracerebroventricularly administered GLP-1 [182]. This indicates that GLP-1-stimulated increase in heart rate and blood pressure involves dual pathways originating from both the CNS and periphery. Long-term treatment of Dahl rats fed with a high-salt diet containing GLP-1 protected these animals from endothelial dysfunction, the vasodilator response of the GLP-1- treated rats to ACh was almost twice as great as that seen in the animals getting vehicle alone [183].

The GLP-1 mimetic exendin-4 also induces Fos-like immunoreactivity in neurons in the paraventricular hypothalamus [184]. It is proposed that increased activity of these neurons contributes to increases in both blood pressure and heart rate [184]. These findings indicate that the cardiovascular actions of exendin-4 involve integration of both indirect neural and direct cardiac effects, thereby linking signaling through the GLP-1R to activation of the sympathetic nervous system. In addition, others have shown that exendin-4 triggers tachycardia, vasoconstriction in mesenteric arteries and vasodilatation in hindquarters [185, 186]. In the presence of β -adrenoreceptor antagonist, the tachycardic effect of exendin-4 was suppressed [186]. These results suggest that exendin-4 exerts cardiovascular effects, some of which involve sympathoadrenal activation.

Moreover, administration of GLP-1 or exendin-4 to rats with streptozotocin/nicotinamide-induced diabetes restored their normal vascular tone [187]. The normalization of endothelial function in these two models appears to be a consequence of the improvement in metabolic control, rather than direct endothelial effects of GLP-1 and exendin-4. Furthermore, long-term treatment of patients with exendin-4 reduced the systolic and diastolic blood pressure, probably secondarily to improvements in glycemic control and weight loss [188]. Similarly, 14 weeks of treatment with a long-acting GLP-1 analog reduced systolic blood pressure and body weight and improved glycemic control [189]. However, infusion of GLP-1 to healthy human subjects had no effect on systolic or diastolic blood pressure [190].

Richter *et al* [191] published the first study that showed dose-dependent vasorelaxant effects of GLP-1 on arterial rings isolated from rats. Mechanical removal of the endothelium suppressed the vasorelaxant effect of GLP-1, suggesting that this effect is eNOS-dependent. Moreover, inhibition of eNOS with a specific inhibitor, L-NNA, abolished the vasorelaxant effect of GLP-1 in mesenteric arteries [192], which further confirms that the vasorelaxant effect of GLP-1 in the mesenteric artery is eNOS-dependent. Others have shown that GLP-1 increases ACh-mediated vasodilation in healthy non-diabetic individuals through K_{ATP} channels [193]. Studies by Green *et al* [194] show that not only GLP-1 (7-36) but also GLP-1 (9-36) and exendin-4 have vasorelaxant properties in the isolated rat aorta, an effect that appears to be mediated through cAMP and opening of K_{ATP} channels [194]. In addition, exendin-4 protects against ischemia-reperfusion (IR)-induced endothelial dysfunction through opening of K_{ATP} channels [195]. Previously, Nyström *et al* [175] demonstrated that GLP-1 ameliorates endothelial dysfunction in type 2 diabetic patients with CAD. A single injection of exendin-4 has been reported to improve postprandial endothelial dysfunction after a high-fat meal in individuals with IGT or recent-onset type 2 diabetes [196]. In contrast, others have shown that the vasorelaxant effect of GLP-1 in rat aorta does not seem to be mediated by the endothelium [197]. Studies carried out in rat

mesenteric arteries show that both GLP-1 (7-36) and its metabolite GLP-1 (9-36) exert a clear vasorelaxant response, whereas exendin-4 fail to exert vasorelaxation [192]. The reasons for these differences remain elusive.

Short-time treatment of human umbilical endothelial cells (HUVECs) with GLP-1 had no effect on Akt activation but enhanced PKA activity and cAMP response element binding protein (CREB) phosphorylation [198]. The GLP-1 analog liraglutide has recently been shown to exert anti-inflammatory effects on human vascular endothelial cells by inhibiting TNF- α and hyperglycemia-induced expression of VCAM-1 and PAI-1 [199, 200]. Others have shown that exendin-4 treatment decreases accumulation of monocytes or macrophages in the vascular wall by decreasing the expression levels of TNF- α through activation of the cAMP/PKA pathway [201]. Furthermore, in HUVECs, GLP-1 decreases ROS and VCAM-1 mRNA expression after treatment with AGEs [202].

GLP-1 and the heart

Several studies have demonstrated that GLP-1 or GLP-1 analogs may protect the myocardium by reducing infarct size through cAMP/PKA-, PI3K- and p44/42-dependent pathways [203, 204], an effect that requires GLP-1 receptor [205-210]. Several independent lines of evidence support multiple biological roles for GLP-1 (9-36), likely acting via a structurally and functionally distinct receptor [204]. Interestingly, Ban *et al* [192] previously reported that GLP-1 (9-36) protects against I/R injury and exerts vasodilatation through a NO/cGMP-associated mechanism independent of the known GLP-1R. Taken together, these findings suggest that GLP-1 (9-36) --which is known not to bind to the classical GLP-1R -- exerts cardioprotective effects through GLP-1R-dependent and -independent actions. Administration of GLP-1 (9-36) amide after global ischemia in rats improved left ventricular pressure without reducing infarct size [206]. Ye *et al* [211] attempted to find out whether increasing circulating GLP-1 levels, by DPP-4 inhibition, can elicit direct effects on the heart. Pre-treatment with oral sitagliptin limited myocardial infarct size through activation of PKA [211]. Furthermore, treatment with GLP-1 has been shown to attenuate myocardial stunning after ischemia-reperfusion in conscious canines [204]. A similar study by Read *et al* [212] showed that i.v. infusion of GLP-1 (7-36) protects the heart against ischemic LV dysfunction and reduces stunning after balloon occlusion in humans.

However, not all publications have shown beneficial effects of GLP-1 treatment. In one study in non-diabetic patients with stable chronic heart failure no improvement of left ventricular function after 48 hours infusion with GLP-1 could be detected [213]. In addition, Nathanson *et al* [214] reported that 6 hours of i.v. infusion of exendin-4 in male type 2 diabetic patients with congestive heart failure (CHF) decreased pulmonary capillary wedge pressure (PCWP) and right arterial pressure (RAP), but increased heart rate and FFA levels. The clinical impact of these findings is difficult to interpret since both positive (*i.e.* decreases of PCWF and RAP) and potentially detrimental (*i.e.* increases in heart rate and FFA levels) effects were observed. The increase in heart rate induced by exendin-4 is of interest, since increased heart rate is a risk factor for patients with CAD; therefore, further studies with hard clinical end-points, are very much needed.

AIMS

The over-arching aim of this work was to investigate whether incretin hormones exert any physiologic or pharmacologic role in the endothelium and vasculature. More specifically, the aims were:

- I. To explore whether incretins influence proliferation of HCAECs *in vitro*, and to elucidate the molecular mechanisms behind such effects.
- II. To investigate the role of the stable GLP-1R agonist exendin-4 on apoptosis of HCAECs under hyperlipidemic conditions *in vitro*.
- III. To investigate the long-term *in vitro* effect of palmitate or high glucose, and the role of exendin-4, on gene expression in HCAECs.
- IV. To investigate if exendin-4 can protect against lipid-induced endothelial dysfunction in rat vascular tissue *ex vivo* and to explore potential differences in vasoactivity between GLP-1 (7-36), GLP-1 (9-36) and exendin-4.

MATERIALS AND METHODS

Study protocols

In vitro experiments (study I-III)

Cell culture and incubation (study I-III)

HCAECs, isolated from normal human coronary arteries (passage 5-13), purchased from Clonetics (Lonza, Walkersville, MD), were grown in EGM-2 MV medium supplemented with hydrocortisone, human epidermal growth factor (hEGF), 5% fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), human fibroblast growth factor (hFGF)-B, R³-insulin-like growth factor (IGF)-1, ascorbic acid and gentamycin/amphotericin-B at 37°C in a humidified (5% CO₂, 95% air) atmosphere as recommended by the supplier. Confluent cultures were detached by trypsin-2-[2- (Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetic acid (EDTA) and seeded onto tissue culture dishes for evaluation of [³H]thymidine incorporation rates, cell counting, protein expression and apoptosis.

To examine the effects of exendin-4, GLP-1 (7-36) and GLP-1 (9-36) on cell viability, DNA synthesis, eNOS and Akt phosphorylation, HCAECs were grown to 90% confluence, followed by an incubation overnight in serum-deficient EGM medium containing 0.5% FBS and 2 mM L-glutamine. The eNOS inhibitor L-NAME (1 mM), the PI3-kinase inhibitor LY294002 (2 μM), the PKA inhibitor Rp-cAMP[S] (10 μM) or vehicle were added 30 min, and Akt inhibitor IV or U0126 were added 1 h, prior to exendin-4, GLP-1 (7-36) and GLP-1 (9-36) stimulation and continuously present as the incubation was continued for 48 h.

To examine the effects of exendin-4, GLP-1 (7-36) and GLP-1 (9-36) on apoptosis HCAECs were also grown to 90% confluence as mentioned previously. L-NAME (1 mM), LY294002 (1 μM), Rp-cAMP[S] (10 μM), p-38 MAPK inhibitor SB203580 (10 μM), JNK inhibitor SP600125 (5 μM), Akt inhibitor IV (0.5 μM) or vehicle were added 1 h prior to palmitate (0.125 mM) and exendin-4 stimulation and continuously present during the 24 h incubation.

To examine the effects of *in vitro* diabetic glucolipotoxicity on gene expression, and the influence of exendin-4 thereupon, HCAECs were grown to 80% confluence, followed by incubation overnight in EGM medium containing 2% FBS and 2 mM L-glutamine. Exendin-4 (10 nM) was added 1 h prior to high glucose (30 mM) or palmitate (0.125 mM palmitate/0.25%BSA or vehicle) and the incubation was continued for 48 h.

Western blot (study I-III)

Western blotting was applied to quantify the total and phosphorylated eNOS (Ser¹¹⁷⁷) (study I-III), Akt 1/2/3 (Ser⁴⁷³) (study I), p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) (study II), JNK (Thr¹⁸³/Tyr¹⁸⁵) (study II), angiopoietin 1 (study III), Tie-2 (study III), TPA (study III) or fibronectin (study III) proteins in HCAECs. HCAECs were grown and incubated in 100 mm Petri dishes. Cells were washed twice with PBS and lysed on ice in ice-cold lysis buffer containing 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 x protease inhibitor cocktail and 2% Triton X-100 in PBS, pH 7.5 for 30min. The cell lysates were centrifuged (5,000 rpm, 5 min, 4°C) and the supernatant was collected. Equal amounts of protein (10-30 μg) were subjected to SDS-PAGE under reducing conditions. The separated proteins were electrotransferred onto nitrocellulose membranes. The membranes were blocked in TBS-T (20 mM Tris-base, 137 mM NaCl (pH 7.6) with 0.05% Tween 20 and 5% non-fat dry milk, followed by an overnight incubation with anti-phospho-eNOS (Ser¹¹⁷⁷), phospho-Akt (Ser⁴⁷³) (1:500), phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) (1:2,000), phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵) (1:1,000), (angiopoietin 1 (1:200), Tie-2 (1:500), TPA (1:1,000) or fibronectin antibody (1:500) in TBS-T/1% BSA at 4°C. The membranes were extensively washed and subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG (1:10,000) or goat anti-mouse IgG in TBS-T with 1% BSA for 1 h at room temperature. The membranes were extensively washed and the immunostained proteins were visualized by ECL.

The intensities of the bands were quantified by densitometry (Gel DocTM, Bio-Rad laboratories) with software Quantity One.

NO (study I-II)

Direct measurement of NO release from HCAECs was performed using the cell-impermeable fluorescent indicator DAF-2 as described [215]. Cells were incubated in 12-well plates in the presence or absence of exendin-4 (10 nM) or the inhibitors (1 mM L-NAME, 2 μ M LY294002 or vehicle) in serum-deficient medium for 48 h (study I). Cells were incubated in the presence or absence of palmitate with/without exendin-4 or vehicle in serum-deficient medium for 24 h (study II). The cells were subsequently washed twice in Krebs-Ringer bicarbonate Hepes buffer (KRBH) buffer containing (in mM) 135 NaCl, 3.6 KCl, 5 NaHCO₃, 0.5 NaH₂PO₄, 0.5 MgCl₂, 1.5 CaCl₂ and 10 Hepes (pH 7.4), followed by an incubation with 5 μ M DAF-2 in 0.5 ml KRBH buffer for 2 h, at 37°C, using the eNOS substrate L-arginine (100 μ M) as positive control. At the end of the incubation, the supernatants were transferred into black microplates and the fluorescence was measured with a fluorescence microplate reader (Infinite M200, TECAN) at excitation wavelength of 488 nm and emission 515 nm. Results were normalized to the protein concentrations, determined using BCA kits, after the cells in each well were lysed in a lyses buffer containing (in mM) 80 Na₂HPO₄, 20 NaH₂PO₄, 100 NaCl, 1% Triton X-100 (pH 7.5).

Akt activity (study I)

Phospho-Akt was measured using Pathscan phospho-Akt1 sandwich ELISA kit, according to the manufacturer's instructions. This phospho-Akt-specific ELISA detects Akt phosphorylated at ser⁴⁷³. Samples were prepared from cells after a 48h incubation in the presence or absence of GLP-1 (7-36) and 100 μ l aliquots of samples containing equal amount of protein were applied to each well.

[³H]thymidine incorporation (study I)

Rates of [³H]thymidine incorporation into DNA were analyzed as previously described [216], as a measure of DNA synthesis. In brief, HCAECs were grown in 60-mm Petri dishes until 90% confluence. After serum starvation, cells were incubated in the presence or absence of kinase inhibitors (or vehicle) or exendin-4 for 48 h. Cells were pulsed with [³H]thymidine (1 μ Ci/ml) 6 h prior to the end of the incubation. [³H]thymidine incorporation into DNA was measured using a microplate scintillation and luminescence counter (Wallac MicroBeta Trilux, Perkin Elmer). Results were normalized to the protein concentrations of the samples, determined using BCA kits.

Cell counting and viability (study I)

HCAECs were incubated in the presence or absence of exendin-4 or inhibitors for 48 h in serum-deficient medium as mentioned above. Cell number was manually counted in a hemocytometer and cell viability was assessed by Trypan blue exclusion.

Analysis of ROS levels (study II)

ROS levels were measured using Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Life Technologies Europe BV) as previously described [217]. Briefly, the assay is based on a 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), a fluorogenic marker, which will be cleaved upon the presence of ROS. Cells were seeded into 6-well plates. When reaching 80% confluence, cells were first kept overnight in serum-deficient EGM medium containing 0.5% FBS and 2 mM L-glutamine followed by a 24 h incubation in the presence or absence of palmitate or vehicle, with or without exendin-4. Cells were then washed with Hank's balanced salt solution (HBSS) before adding 50 μ M carboxy-H2DCFDA to each well. After 30 minutes of incubation at 37°C, excess probe was removed by

washing the cells again with HBSS. Cells were then lysed in PBS containing 1% Triton X-100. Carboxy-DCF fluorescence in cell lysates was detected at an excitation/emission wavelength of 495/529 nm using a microplate reader (Tecan Group Ltd., Männerdorf, Switzerland). The fluorescence intensity was normalized against the protein concentration of each individual well.

Determination of caspase 3 activity (study II)

Caspase 3 activity was determined as a measure of apoptosis using a fluorometric substrate, Z-DEVD-AMC (EnzChek® Caspase-3 assay kit, Molecular probes, Life Technologies Europe BV) as previously described [218]. 24 h after stimulation with palmitate or vehicle with/without exendin-4, cells were incubated in 50 μ l Z-DEVD-AMC substrate at room temperature for 30 min. Substrate cleavage was quantified fluorometrically at 342 nm excitation and 441 nm emission with a fluorescent plate reader (Tecan Group Ltd., Männerdorf, Switzerland).

DNA fragmentation (study II)

DNA fragmentation, another marker of apoptosis, in HCAECs was assayed by the cell death detection kit ELISA plus (Roche Diagnostics Scandinavia AB, Stockholm, Sweden), according to the manufacturer's instructions. This ELISA measures cytoplasmic DNA-histone complexes that increase after apoptosis-associated DNA fragmentation.

Gene silencing (study II)

HCAECs were seeded into a 100-mm dish at a density of 2.5×10^5 cells per well and incubated for 24 h at 37°C in complete medium. The cells were washed twice with culture medium without serum and supplement. Control siRNA/eNOS siRNA (10 nM) was mixed and incubated with SilenceMag according to the standard protocol (Oz Biosciences, Marseille, France). After incubation with a magnetic field for 15 min, the magnet was removed from the culture plate. 8-24 h post transfection, the media in the cell culture plate were replaced with complete medium containing 5% FBS and then further incubated for 24 h. The cells were harvested and centrifuged at 14,000 rpm for 5 min to remove the supernatant.

Isolation of total RNA and real-time PCR (study II)

Total RNA was extracted from cells treated with eNOS siRNA and control siRNA, using Aurum Total RNA Mini kit (BioRad), according to the manufacturer's instructions. cDNA was prepared using Script cDNA Synthesis kit, BioRad (Life Science Research, CA). The PCR reaction mixture contained, in a final volume of 20 μ l, 4 μ l of cDNA, 10 μ l of KAPA SYBR FAST qPCR master mix (Kapa Biosystems, MA) and corresponding primers [219]. The gene expression level was normalized to the housekeeping gene, β -actin.

RNA extraction and sample preparation (study III)

High quality RNA was extracted from the cell lysate samples using Aurum total RNA mini kit (Bio-Rad, Stockholm, Sweden) according to the manufacturer's instruction. The quality and purity of the extracted RNA was confirmed by agarose gel electrophoresis and analysis using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Nano Technologies, Wilmington, DE) [220].

Gene expression profiling (study III)

Gene expression was examined using the Human Endothelial Cell Biology RT² Profiler™ PCR Array (SABiosciences, Qiagen) to detect the expression of 84 genes involved in permeability and vascular tone, angiogenesis, endothelial cell activation and endothelial cell injury by real-time PCR with a set of optimized real-time PCR primers. The PCR array performs gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray. The cDNA template prepared from highly purified RNA (10 μ g) was mixed with

the ready-to-use PCR master mix, aliquoted in equal volumes to each well of the same plate, and then run in the real-time PCR cycling program.

The RT² Profiler™ PCR Arrays include built-in positive control elements for proper normalization of the data (using data from species-specific housekeeping genes included in the pre-set wells), detection of genomic DNA contamination (using genomic DNA control primer set present in the wells to detect non-transcribed genomic contaminations), quality of the RNA samples (using reverse transcription controls), and general PCR performance (using positive PCR controls).

Ex vivo experiments (study IV)

Sixty-nine male Sprague-Dawley rats (weight 250-350 g) were anesthetized with a mixture of fluanisone and fentanyl (Hyp-norm®, Janssen, Beerse, Belgium) and midazolam (Dormicum®, Hoffman-LaRoche, Basel, Switzerland) (2.5, 0.08 and 1.25 mg/kg b.w., respectively, i.m.). The rats were then killed by excision of the heart. The contractile function of the vascular segments was tested by administration of phenylephrine (Phe). Endothelium-dependent and endothelium-independent relaxations were determined by administration of acetylcholine (ACh) and sodium nitroprusside (SNP), respectively. After the end of the functional tests, artery rings were frozen and stored at -80 °C for subsequent immunoblotting analysis. For determination of cAMP contents, the thoracic aorta was removed, cleaned and cut into two sections. Each vessel segment was equilibrated for 20 min in Krebs- Henseleit (KH) solution at 37 °C and bubbled with 5% CO₂ in O₂ to maintain a pH of 7.4, before incubation. One vessel segment was then incubated for 15 min with GLP-1 (100 nM) or exenatide (2.5 nM), respectively, and the other with an equivalent volume of the adenylyl cyclase activator forskolin (10 µM) or its solvent DMSO (10 µM), serving as positive control. Vessels were then frozen and stored at - 80 °C for further analysis, *i.e.* phosphorylation of eNOS and expression of GLP-1R.

Arterial rings in organ baths (Study IV)

Femoral arteries from non-diabetic male Sprague-Dawley rats were carefully dissected free from surrounding tissue, removed and put in organ baths with KH solution. Circular segments (1-2 mm in length) of the artery were mounted on two thin metal holders, one of which was connected to a force displacement transducer (model FT03, Grass Instrument Co, Quincy, MA) and the other to a movable device that allowed the application of a passive tension of 5 mN. The tension was recorded on a polygraph (model 7B, Grass). The mounted vascular segments were kept in 2 ml organ baths containing KH solution at 37 °C and continuously bubbled with CO₂ in O₂ to maintain a pH of 7.4. After preparation, the vascular segments were allowed to equilibrate for 60 min. The contractile function of the vascular segments was first tested by administration of K⁺-rich solution (127 mmol/l), prepared by replacing NaCl with equimolar amounts of KCl, and thereafter with phenylephrine (Phe; 10⁻⁵ mol/l). Endothelium- dependent and endothelium-independent relaxations were determined by administration of acetylcholine (ACh) and the NO donor sodium nitroprusside (SNP), respectively. ACh and SNP were added to the organ baths at cumulatively increasing concentrations (10⁻⁹-10⁻⁵ mol/l), during a stable contractile tone induced by Phe (10⁻⁵ mol/l). The relaxant response following pre-incubation with a studied substance was always compared to the preceding control response in the same vascular segment. Exendin-4 (Neosystem, Strasbourg, France) was added to the organ baths at cumulative increasing concentrations (10⁻¹³-10⁻⁸ mol/l) during baseline tension to evaluate contractile effects *per se*. The relaxant effects of exendin-4, GLP-1 (7- 36) amide (Neosystem, Strasbourg, France) and GLP-1 (9-36) (Bachem, Bubendorf, Switzerland) were evaluated by adding cumulatively increasing concentrations (10⁻¹³-10⁻⁸ mol/l) of these peptides to artery segments precontracted with Phe (10⁻⁵ mol/l). The relaxant effect of GLP-1 was also tested in separate experiments together with the DPP-4 inhibitor sitagliptin (5 µM), which was added 5 min before Phe-induced contraction. Sitagliptin was also added during baseline tension and during Phe-induced contraction to evaluate any contractile and vasorelaxant effects of the DPP-4 inhibitor *per se*.

Finally, to investigate whether exendin-4 might co-activate the ACh relaxation, exendin-4 was added in separate experiments to the organ baths 10 min before contraction with Phe. Thereafter the vascular segments were preincubated for 20 min with the triglyceride-rich emulsion Intralipid® (100 mg/ml) (Pharmacia-Upjohn, Uppsala, Sweden) diluted in KH solution to final concentrations of 0.5 and 1 %, corresponding to an approximate triglyceride level of 5 and 10 mmol/l, respectively. To examine any protective effect of exendin-4 against the triglyceride-induced endothelial dysfunction, exendin-4 (final concentration of 2.5 nM) was administered to the organ baths 20 min before the test of vascular response to ACh and SNP.

Western blot (study IV)

Western blotting was applied to quantify the total and phosphorylated eNOS (Ser¹¹⁷⁷) or Akt 1/2/3 (Ser⁴⁷³) proteins in aortas from Sprague-Dawley rats.

Frozen artery rings were thawed and homogenized in 100 µl of buffer. The tissue was minced, homogenized and incubated on ice for 30 min and centrifuged (5,000 g, 5 min). Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL). Phosphorylation of eNOS at Ser¹¹⁷⁷ was examined by a phospho-specific antibody (Santa Cruz) as a measure of eNOS enzymatic activity in similar manner as described in study I-III.

Statistical analyses (study I-IV)

Each assay was repeated a minimum of three times. All data are presented as mean ± SEM. Student's *t*-test was used to determine the statistical significance of differences between two groups. Differences between several groups were assessed by analysis of variance (ANOVA), one-way repeated measurement, followed by Student-Newman Keuls and Dunns-Bonferoni *post hoc* tests (study I-IV).

RESULTS

In vitro experiments (study I-III)

Study I

In this study, we demonstrated that exendin-4, GLP-1 (7-36) and GLP-1 (9-36) enhanced DNA synthesis, as reflected by an increased rate of [³H]thymidine incorporation. Consistent with the stimulatory effect of exendin-4 on DNA synthesis, the total cell number was significantly increased in the presence of exendin-4, confirming that DNA synthesis was indeed followed by mitosis. Combination of exendin-4 and GLP-1 (7-36) did not produce additive changes in cell proliferation. It is possible that exendin-4-stimulated proliferation is already maximized and cannot be further increased by GLP-1 (7-36). Administration of exendin-4 to the HCAECs exerted a sustained phosphorylation of eNOS and Akt. Administration of GLP-1 (7-36) or GLP-1 (9-36) also increased eNOS and Akt phosphorylation. Co-incubation with the PKA antagonist Rp-cAMP[S], the eNOS inhibitor L-NAME, the PI3K inhibitor LY294002 or Akt inhibitor IV, abolished the effect of exendin-4 on eNOS and Akt phosphorylation. This indicates that exendin-4-induced eNOS and Akt phosphorylation are mediated by PKA-PI3K/Akt-dependent signaling pathways. Exendin-4-stimulated NO production was completely inhibited by either L-NAME or LY294002.

The exendin-4-stimulated DNA synthesis was further evaluated in the presence of Rp-cAMP[S], L-NAME, LY294002 or Akt inhibitor IV. At the same concentrations that inhibited phosphorylation of Akt and eNOS, the augmented DNA synthesis rate evoked by exendin-4 was abolished by these inhibitors. Finally, the increases in DNA synthesis and eNOS and Akt activation evoked by the peptides, were blocked by the GLP-1R antagonist exendin (9-39).

Study II

The main result of this study was that 0.125 mM palmitate provoked apoptosis, and this effect was significantly inhibited by exendin-4 or GLP-1 (7-36). Incubating the cells with palmitate and exendin-4 in the presence of the GLP-1 receptor antagonist exendin (9-39) led to loss of exendin-4 protection against lipoapoptosis. In contrast, palmitate-induced apoptosis was not affected by the metabolite GLP-1 (9-36). When cells were incubated with palmitate and exendin-4 in the presence of Rp-cAMP[S], LY294002, Akt inhibitor IV or L-NAME, the anti-apoptotic action of exendin-4 was completely lost. This indicates that the protective effect of exendin-4 against lipoapoptosis requires cAMP/PKA, PI3K/Akt and eNOS activation. To determine whether palmitate influences eNOS activity, we examined activation of the enzyme by measuring its phosphorylation at Ser¹¹⁷⁷. Interestingly, treatment of the cells with palmitate significantly enhanced eNOS activity, an effect that was blocked by exendin-4. Treatment of HCAECs with palmitate also significantly enhanced NO production, an effect that was further augmented by co-incubation of the cells with palmitate and exendin-4. To further corroborate the role of eNOS in apoptosis, eNOS in HCAECs was silenced by siRNA. Knockdown of eNOS to 45% of controls was observed by real-time PCR and resulted in the protective effect of exendin-4 on palmitate-induced apoptosis being completely abolished. Taken together, these findings indicate that the protective effect of exendin-4 against lipoapoptosis in HCAECs is mediated by eNOS activation. In addition, palmitate was found to increase ROS, an effect that was decreased by co-incubation with exendin-4. Incubation of HCAECs with palmitate alone for 24 h resulted in increased p38 MAP kinase and JNK phosphorylation, an effect that was neutralized by exendin-4. The protective effect of exendin-4 on lipoapoptosis was prevented by specific inhibitors of p38 MAP kinase or JNK, indicating that these pathways are required for exendin-4 to provide lipoprotection in HCAECs

Study III

The main finding of this study was that at high glucose or palmitate, exendin-4 significantly enhanced expression of eNOS. In contrast, incubation of the cells with palmitate silenced the expression of the enzyme. Our *in vitro* studies further demonstrated that Tie-2 expression was down-regulated, whereas Ang-1 expression was increased, under hyperglycemic conditions. Importantly, both genes were up-regulated by exendin-4 in the presence of high glucose. Exposure of HCAECs to high glucose resulted in a significant decrease in the expression of TPA, whereas exendin-4 enhanced the expression of this gene.

The expression of cell adhesion molecules involved in angiogenesis, such as PECAM, cadherin-5 and the extracellular matrix protein fibronectin, was inhibited by high glucose. In contrast, incubation of the cells with exendin-4 increased expression of PECAM-1 and cadherin-5. Similarly, in the presence of high glucose, exendin-4 increased expression of fibronectin. Moreover, ACE expression was up-regulated by high glucose, an effect countered by exendin-4.

Consistent with the gene profiling studies, our Western blot data showed that the expression of eNOS was up-regulated by exendin-4 in the presence of either palmitate or high glucose, whereas expression of eNOS tended to be inhibited by palmitate but not significantly affected by high glucose. Our *in vitro* studies further demonstrated that high glucose led to a significant increase in Ang-1 expression; this was accompanied by a decrease in Tie-2 expression, although statistical significance was not reached. Incubation of the cells with exendin-4 further increased the expression of Ang-1 and reversed the suppressed expression of Tie-2 by high glucose. Furthermore, exposure of the cells to exendin-4 for 48 h significantly increased the expression of TPA and fibronectin, but the expression levels of these proteins did not reveal any significant changes at high glucose.

Ex vivo experiments

Study IV

The main result of this study was that both GLP-1 (7-36) and its metabolite GLP-1 (9-36), but not exendin-4, exert acute vasorelaxant effects in non-diabetic rat conduit artery rings *ex vivo*. The main aim of this study was to investigate whether exendin-4 restores endothelial dysfunction induced by the triglyceride-rich fat emulsion, Intralipid. However, short-time exposure to exendin-4 did not protect against the triglyceride-induced endothelial dysfunction *ex vivo*. In other words, this study shows that exendin-4 does not induce vasorelaxation or protects against the triglyceride-induced endothelial dysfunction. This could not be explained by a loss of exendin-4 bioactivity, as it significantly increased cAMP levels and insulin secretion. Immunoblotting data showed the expression of the GLP-1R in the femoral artery. Immunoblotting also showed an increased eNOS activity in the presence of Intralipid. In addition, we observed a clear tendency towards decreased Intralipid-induced eNOS activation in the presence of exendin-4, although this effect did not attain statistical significance.

GENERAL DISCUSSION

Impaired endothelial function is an early indicator of atherosclerosis and predicts CVD outcome in man [221]. Endothelial dysfunction in type 2 diabetes is associated with many factors, such as obesity and insulin resistance, and may explain the poor outcome in CVD in these patients [51]. Improved endothelial function or insulin sensitivity may confer treatment benefits and perhaps improve survival in patients with type 2 diabetes. The most recent contribution to the armamentarium of antihyperglycemic treatment is incretin-based drugs. There is now evidence that these agents cause, besides and independent of their known anti-hyperglycemic and weight-reducing properties [222, 223] direct effects on the endothelium and vasculature [187, 192, 197, 205, 224, 225].

The aim of this thesis has been to study the effects of GLP-1R-activating agents, such as exendin-4, on the vascular endothelium and to investigate the underlying molecular mechanisms behind these effects. In study I, we demonstrated that exendin-4 stimulates the proliferation of HCAECs *in vitro*, an effect shared by GLP-1 (7-36) and its major metabolite GLP-1 (9-36). These mitogenic effects of exendin-4 in HCAECs may prove beneficial in dampening or delaying coronary atherosclerosis, hypothetically by rapidly covering a vascular wall wound with endothelium and thus protecting it from further atherothrombotic events. In addition, angiogenesis can be of potential clinical benefit in patients with IHD [226]. Also, diabetic patients are at high risk of coronary re-stenosis and late stent thrombosis formation after angioplasty, such as percutaneous coronary intervention (PCI). This is believed to be due to vascular remodeling and neointima formation (decreased endothelial cell and increased smooth muscle cell formation) after PCI and incomplete or delayed stent endothelialization. Thus, our findings may be harnessed to advantage clinically in therapeutic efforts to minimize vascular remodeling and neointima formation after PCI and to accelerate stent endothelialization. Such effects may decrease the high risk of coronary re-stenosis and late stent thrombosis formation after PCI that diabetic patients endure to such a disproportionate extent. However, neovascularisation and vasoproliferation also contribute to the pathological processes leading to diabetes retinopathy [227].

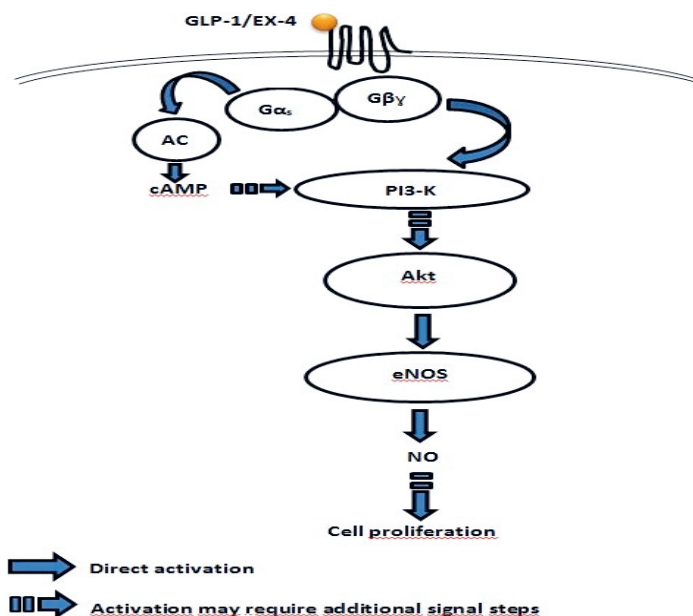


Figure 6. Mechanisms by which GLP-1- stimulated proliferation of HCAECs

Stimulation of HCAECs with GLP-1/exendin-4 through their G-protein coupled receptors results in activation of adenylyl cyclase (AC), generating cAMP and PI3K. The downstream PI3K effector, Akt, subsequently activates eNOS through phosphorylation, leading to NO-dependent proliferation of the cells.

Study II demonstrated that exendin-4 and GLP-1 (7-36), but not GLP-1 (9-36), protect HCAECs from lipoapoptosis, an effect that is mediated through the GLP-1R and involves PKA, PI3K, eNOS, p38 MAPK and JNK dependent pathways. Our findings indicate that activation of p38 MAPK and JNK not only contributes to lipoapoptosis, but are also involved in the anti-apoptotic effects of exendin-4. In contradiction to our results, Chai *et al* [228] demonstrated that p38 MAPK, but not JNK, mediates palmitate-induced apoptosis. This apparent discrepancy may occur partly because only SB203580, a specific inhibitor of p38 MAPK, was used to study the involvement of both p38 MAPK and JNK in palmitate-induced apoptosis and/or due to differences in the experimental conditions. Taken together, these results suggest that p38 MAPK not only plays a major role in apoptosis, but also in the regulation of cell survival.

Our microarray analyses demonstrated an inhibited eNOS expression by palmitate (study III). It is known that reduced availability of NO is associated with endothelial dysfunction and insulin resistance [32, 229-231]. Therefore, we would have expected palmitate to diminish eNOS activity [232- 234]. However, exposure of HCAECs to palmitate paradoxically increased eNOS activity, NO release, and production of ROS (study II). This finding is consistent with a prior study in human aortic endothelial cells in which high concentrations of glucose was found to increase the eNOS activity [235], probably due to increased eNOS expression. In addition, short-term exposure to a triglyceride-rich fat emulsion impairs endothelium-dependent vasorelaxation -- with a concomitant increase in eNOS activity -- in rat conduit artery rings *ex vivo* (study IV). The contribution of ROS production might explain the different results regarding eNOS expression in our studies. Our findings suggest that palmitate increased the activity of a dysfunctional, uncoupled eNOS, resulting in increased O_2^- production in our *in vitro* and *ex vivo* model of type 2 diabetes. Several studies have shown that excessive production of ROS results in oxidative stress leading to endothelial dysfunction and apoptosis [34, 37, 45]. To

further address a potential role of exendin-4 in the regulation of eNOS activity and O_2^- production, we treated HCAECs with both palmitate and exendin-4. Co-incubation with exendin-4 reduced eNOS activity and O_2^- production in palmitate-treated cells. This in turn resulted in reduced eNOS uncoupling and attendant O_2^- production, probably through inhibition of ONOO⁻ formation and thus less oxidation of the active form of eNOS cofactor BH₄. Taken together, these findings indicate that exendin-4-reduced eNOS activity markedly improved endothelial dysfunction, decreased ROS levels and significantly increased NO.

In study III, the microarray and Western blot analyses demonstrated that the expression of eNOS was up-regulated by exendin-4 in the presence of either palmitate or high glucose. However, microarray analysis showed an inhibited eNOS expression by palmitate but this was not confirmed by Western blot analysis. This discrepancy might be explained by post-transcriptional regulation of eNOS mRNA levels. Many factors can destabilize and shorten the half-life of eNOS mRNA, leading to decreased expression of eNOS protein. Importantly, it has been shown that eNOS mRNA levels do not correlate with gene transcription rates [236, 237]. Whether exposure to palmitate increases the half-life of eNOS mRNA, and therefore influences the level of eNOS protein, warrants further investigation. Incubation of the cells with high glucose up-regulated the expression of ACE, while exendin-4 reversed this effect. Reduction of ACE activity is a target for treatment of hypertension and its related cardiovascular diseases [238].

TPA is dynamically released by the endothelium to stimulate breakdown of fibrin clots and to maintain fibrinolysis [239]. Impaired fibrinolytic function, due to reduced TPA activity and increased PAI-1, has been found in patients with hypertension [238] and type 2 diabetes [240]. Furthermore, type 2 diabetic patients often show hypercoagulability due to increased numbers of pro-coagulant factors, such as fibrinogen [241]. As reported previously [240], an imbalance between coagulation and fibrinolysis may contribute to the premature development of atherothrombosis in obese patients with type 2 diabetes. Exendin-4 up-regulated the expression of TPA, suggesting that increased activity of TPA might be an important defense against atherothrombotic events.

Bcl-2 associated Bax protein is classified as a pro-apoptotic protein that regulates the release of cytochrome c through alteration of mitochondrial membrane permeability [242]. Exposure of HCAECs to high glucose increased the expression of Bax, an effect that again was reversed by exendin-4, which suggests that Bax down-regulation may be involved in the anti-apoptotic effects of exendin-4 in these cells. In their entirety, these findings suggest that exendin-4 not only protects HCAECs from lipoapoptosis, but also improves endothelial function in part through regulating expression of genes related to inflammation by reversing glucolipotoxic gene dysregulation.

It is well known that migration and proliferation of endothelial cells are important steps in angiogenesis. Our findings (study III) show that exendin-4 increases the expression of genes promoting angiogenesis at high glucose. These include Ang1 and its receptor Tie-2, a transmembrane tyrosine kinase essential to angiogenesis and endothelial cell survival. This suggests that exendin-4 induced over-expression of Tie-2 may be involved in rescuing the hyperglycemia-induced impairment of HCAECs. PECAM/CD31 [243] and cadherin-5 [244] are adhesion molecules expressed on the surface of endothelial cells that mediate cell-cell interactions. Exendin-4 was found to up-regulate PECAM-1 and cadherin-5, both of which play a role in many physiological events such as transendothelial migration of leukocytes during an inflammatory response and angiogenesis [245, 246]. Therefore, expression of PECAM/CD31 in endothelial cells is important during angiogenesis and inflammation. Fibronectin also promotes angiogenesis, as angiogenic processes entail interactions between endothelial cells and the extracellular matrix [247]. Collectively, these findings indicate that exendin-4 increases the expression of genes related to angiogenesis, which may be part of the mitogenic stimulation of HCAECs evoked by exendin-4.

In study IV, it was found that administration of GLP-1 (7-36) and its metabolite GLP-1 (9-36) produced vasodilatation *ex vivo* in rat conduit artery rings. In contrast to GLP-1 (7-36) and its metabolite, exendin-4 did not exert any vasorelaxant effects in this system. These findings indicate that there are differences in vasoactive potency between GLP-1 (7-36), GLP-1 (9-36) and the stable GLP-1 analogue exendin-4.

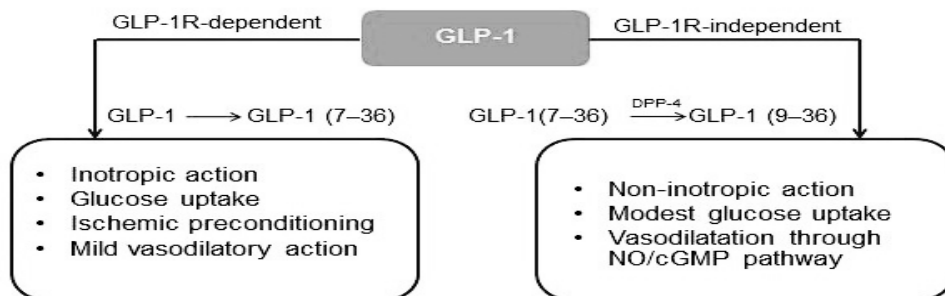


Figure 7. GLP-1 receptor-dependent and -independent effects

Adapted from *Circulation*: Ban, K, Cardioprotective and Vasodilatory actions of Glucagon-like Peptide 1 Receptor-dependent and -independent Pathways, *Circulation*, page 2340-2350, © (2008).

Our findings are consistent with a prior study in which GLP-1 (7-36) and GLP-1 (9-36), but not exendin-4, were found to exert vasodilatation in mesenteric arteries in both wild type and in GLP-1R^{-/-} mice [192] (Figure 7). In contrast, studies by Green *et al* [194] demonstrate that not only GLP-1 (7-36) and GLP-1 (9-36) but also exendin-4 produce vasorelaxant effects in isolated rat aorta. The reason for these differences in producing vasorelaxation remains elusive. Furthermore, in study IV exendin-4 failed to restore endothelial dysfunction induced by a triglyceride-rich emulsion. This might be due to differences in affinity to the endothelial GLP-1R receptors, but this was not experimentally addressed in this study. One possible explanation is that GLP-1 (9-36) mediates vasorelaxation by a GLP-1R- independent mechanism and that vasorelaxation observed with GLP-1 (7-36) is a result from its cleavage to GLP-1 (9-36) by ambient DPP-4 activity present in the preparations.

In summary, we have in this thesis focused on potential effects of GLP-1 and exendin-4 in the vasculature. In study I, we found that exendin-4, GLP-1 (7-36) and GLP-1 (9-36) stimulate endothelial cell proliferation through activation of eNOS in HCAECs. In contrast, short-term administration of exendin-4 failed to activate eNOS in rat conduit arteries *ex vivo* (study IV). The reason for the apparent discrepancy between study I and IV is not clear, although differences in species and treatment duration may be involved. In contrast to GLP-1 (7-36) and its metabolite GLP-1 (9-36), exendin-4 did not exert any vasorelaxant effects in our *ex vivo* study on rat femoral arteries (study IV). These differences might be due to the existence of another GLP-1 receptor.

In study II and III, we found that exendin-4 not only protects HCAECs against lipoapoptosis but also may improve endothelial function in part through regulating expression of genes related to angiogenesis, and inflammation by reversing glucolipotoxic gene dysregulation.

Limitations of the studies

I, II & III: We have only studied the effects of GLP-1 (7-36), GLP-1 (9-36) and exendin-4 on HCAECs *in vitro* from healthy individuals. Further studies on GLP-1R^{-/-} mice, and in HCAECs in which GLP-1R is either over-expressed or silenced, under varying glycemic conditions would have extended and fortified the conclusions of these studies. In studies II and III, we tested only palmitate because it is the most abundant fatty acid *in vivo*; however, many different fatty acids

are present in plasma and may have different effects. Also, various fatty acids may interact with each other to coordinate different pathological responses.

IV: It would have been useful to include resistance vessels from diabetic models and to include studies from both genders.

CONCLUSIONS

- Exendin-4, GLP-1 (7-36) and GLP-1 (9-36) stimulate proliferation and increase eNOS and Akt in HCAECs *in vitro*. The mitogenic effect of exendin-4 likely occurs through PKA-, PI3K/Akt- and eNOS-dependent pathways conveyed by a GLP-1R-mediated mechanism (summarized in Figure 6).
- Exendin-4 and GLP-1 (7-36), but not the GLP-1 (9-36) metabolite, protect HCAECs against lipoapoptosis, an effect that is mediated through the classical GLP-1R and involves PKA-, eNOS-, p38 MAPK- and JNK dependent pathways.
- Exendin-4 improves endothelial function in part through regulating expression of genes involved in angiogenesis, inflammation and thrombogenesis by reversing glucolipotoxic gene regulation.
- GLP-1 (7-36) and GLP-1 (9-36) exert vasorelaxant effects in femoral arteries *ex vivo* in non-diabetic rats. In contrast, exendin-4 does not and also does not protect against triglyceride-induced endothelial dysfunction.
- These direct actions of GLP-1R-agonistic agents on the endothelium may be of relevance for the cardiovascular effects of incretin-based therapy in diabetic patients.
- It is suggested that GLP-1R activation may be beneficial in repairing endothelial lesions, caused by the pro-atherogenic milieu in diabetes that may otherwise precipitate atherothrombotic events.

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