

Studies on Incretins and Cardiovascular Function

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TO MY FAMILY

Abstract

BACKGROUND

Type 2 diabetes (T2DM) is a strong risk factor for coronary heart disease (CHD). A great many diabetic subjects suffer from congestive heart failure (CHF), a condition with a high concomitant mortality. So far, attempts aimed at reducing macrovascular complications in T2DM have been essentially futile. Hence, there is a need for finding glucose-lowering agents that exert direct positive effects on vasculature and the heart. Glucagon-like peptide-1 (GLP-1) is a peptide secreted from the L-cells in the small intestine. GLP-1 decreases plasma glucose by increasing insulin secretion without an increased risk for hypoglycemia.

AIMS

The aim of this work was to evaluate the putative role and potential effects of GLP-1 and related peptides on the vasculature and heart and to elucidate the mechanisms behind these effects.

STUDY I AND II

These papers are based on studies performed within the ULSAM (Uppsala longitudinal study of adult men) cohort, a community-based prospective cohort study of elderly men started in 1970. The participants were examined at age 50 and 70 and the data was completed with annual updates on mortality and morbidity. The examination at age 70 forms the baseline of the present studies. A subsample from this cohort consisting of 509 men performed an oral glucose tolerance test (OGTT), plasma samples were stored frozen and GLP-1 concentrations were analyzed in 2007. At baseline, echocardiographic measurements of left ventricular function were done. Information concerning incident disease was collected from official Swedish registries. The studies did not reveal any longitudinal associations between plasma GLP-1 levels and the incidence of CHD or CHF. Cross-sectionally, however, we found correlative associations between plasma GLP-1 levels and impaired glucose tolerance and diastolic cardiac dysfunction.

STUDY III

This was a double-blinded randomized cross-over study. Twenty patients with T2DM hospitalized for decompensated congestive heart failure (CHF) were enrolled in the study. Primary outcome was the proportion of subjects achieving a 20% increase of cardiac index (CI) and a 20% decrease of pulmonary capillary wedge pressure (PCWP), i.v. infusions with exenatide or placebo, 18 hours apart. Hemodynamic variables were monitored by heart catheterization. Exenatide evoked a 21% increase in CI, a 29% increase of heart rate, a 15% decrease of PCWP and a 17% decrease of right atrial pressure.

STUDY IV

The aim of this study was to investigate whether exenatide could protect against endothelial dysfunction induced by lipotoxicity and if there were any differences in vasorelaxant capacity between GLP-1 (7-36), the degradation metabolite GLP-1 (9-36) and exenatide in femoral arterial rings from non-diabetic rats *ex vivo*. Exenatide did not protect against lipotoxicity, whereas GLP-1 (7-36) and GLP-1 (9-36) exerted vasorelaxation with 23% and 38%, respectively, vs. only 3% with exenatide.

STUDY V

We studied the effects of exenatide, GLP-1 (7-36) and GLP-1 (9-36) on human coronary artery endothelial cell (HCAEC) proliferation and potential underlying mechanisms. Exenatide, GLP-1 (7-36) and GLP-1 (9-36) elicited dose-dependent increases in DNA synthesis and increased cell numbers. This was associated with enhanced eNOS and Akt activity, which – along with the augmented cell proliferation – were inhibited by PKA-, PI3K-, Akt- and eNOS-inhibitors and by a GLP-1 receptor antagonist.

CONCLUSIONS

Altered plasma GLP-1 levels were not found to predict incident CHD or CHF, while significant cross-sectional correlations were found between GLP-1 impaired glucose tolerance (IGT) and diastolic cardiac function in elderly men. GLP-1 and related peptides stimulate proliferation of HCAEC cells, exert vasorelaxant effects on rat arterial rings *ex vivo* and evoke potent hemodynamic effects in T2DM patients with CHF. These effects seem to occur independent of changes in glucose concentrations. These findings prompt further efforts and mechanistic studies aimed at characterizing potential beneficial cardiovascular effects of incretin hormones in the clinical management of T2DM patients.

List of publications

- I. Nathanson D, Zethelius B, Berne C, Holst JJ, Sjöholm Å, Nyström T (2010). Reduced plasma levels of glucagon-like peptide-1 in elderly men are associated with impaired glucose tolerance but not with coronary heart disease. *Diabetologia* 53(2): 277-80.
- II. Nathanson D, Zethelius B, Lind L, Andrén B, Berne C, Ingelsson E, Holst JJ, Nyström T (2010). Plasma levels of glucagon like peptide-1 associate with diastolic function in elderly men. *Diab Med*, in press
- III. Nathanson D, Ullman B, Löfström U, Hedman A, Frick M, Sjöholm Å, Nyström T (2010). Acute hemodynamic effects of exenatide in type 2 diabetic patients with decompensated heart failure. *Manuscript*.
- IV. Nathanson D, Erdogdu Ö, Pernow J, Zhang Q, Nyström T (2009). Endothelial dysfunction induced by triglycerides is not restored by exenatide in rat conduit arteries ex vivo. *Regul Pept* 157(1-3): 8-13.
- V. 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. *Mol Cell Endocrinol* 325(1-2): 26-35.

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List of abbreviations

CHD	Coronary heart disease
CHF	Congestive heart failure
CI	Confidence interval
Clamp	Euglycemic insulin clamp
CVD	Cardiovascular disease
DPP-4	Dipeptidyl peptidase-4
FPG	Fasting plasma glucose
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
Δ GLP-1	Difference between 60 min OGTT-stimulated and fastingGLP-1
GLP-1R	GLP-1 receptor
HCAECs	Human coronary artery endothelial cells
HR	Hazard ratio
2hPG	2-hours post glucose challenge
IFG	Impaired fasting glucose
IHD	Ischemic heart disease
IGT	Impaired glucose tolerance
MI	Myocardial infarction
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
OR	Odds ratio
p-eNOS	Phospho-eNOS
PYAR	Person-years at risk
RAP	Right atrial pressure
T2DM	Type 2 diabetes mellitus
U-albumin	Urinary albumin

Introduction

Diabetes is considered by the WHO as one of the major threats to human health in the 21st century [1] and the latest estimate by the WHO (2000) gives that 171 million people worldwide currently suffer from diabetes and that this number will increase to 366 million in 2030 [2]. Cardiovascular disease (CVD) is by far the most common complication of diabetes (T2DM). Suffering from diabetes does not only significantly increase the risk of CVD but is also associated with poor survival, both acutely and in the long term, after a myocardial infarction (MI) [3]. In fact, total mortality from coronary heart disease (CHD) in subjects with diabetes without a previous MI is as high as that of non-diabetic individuals with a previous MI [4].

Congestive heart failure (CHF) is a clinical syndrome with high coexistent morbidity and mortality. In a population based study from Reykjavik, Thrainsdottir et al found a prevalence of 11.8% of heart failure in patients with diabetes, compared to 3.2% in control subjects [5]. The prevalence of diabetes in CHF amounts to ~ 20 - 35% [6].

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 [7-12].

Endothelial dysfunction is commonly observed in association with T2DM and other situations characterized by insulin resistance, and is related to a heightened risk for initial or recurrent cardiovascular malfunction [13-15]. Although the hypothesis that specifically reversing endothelial dysfunction can reduce the risk for CVD has not been tested directly, many

different alterations in lifestyle [16-18] and pharmacological interventions designed to improve endothelial function are known to lower this risk [19-21]. The traditional “gluco-centric” approach, i.e. to focus solely on the treatment of the hyperglycemia, has failed to reduce the incidence of CVD [22-24]. In fact, there is strong evidence for the efficacy of the multifactorial approach [20, 21]. So far, no particular antidiabetic treatment has proven superior to other hypoglycemic agents in reducing CVD risk so there is a clear need to identify new weapons of the pharmacologic armamentarium that positively impact the endothelium and cardiovascular system

There is today a growing body of evidence that Glucagon-Like-Peptide-1 (GLP-1) might have a beneficial role in cardiovascular function beside its well known antihyperglycemic action [25, 26].

To this end, the over-arching aim of this thesis was to explore the potential effects of GLP-1 and related peptides on the endothelium, vasculature and the heart and to reveal underlying mechanisms of these effects in a translational research setting.

Diabetes mellitus

The origin of the word “diabetes” is the Greek word for siphon. The Greek physician Aretus found that some patients had a marked polyuria - and that water in these patients - passed like in a siphon. He named the condition Diabetes.

Diabetes mellitus is a disease associated with hyperglycemia. Diabetes can be caused by two principal mechanisms:

1. Inadequate production of insulin.
2. Inadequate sensitivity of cells to the

action of insulin.

The two main types of diabetes that correspond to these two mechanisms are called type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM is characterized by a marked decrease in insulin production, a subacute onset of hyperglycemia and is ketosis prone. T2DM is characterized by an adult onset of hyperinsulinemia and decreasing insulin sensitivity, a slow progression of hyperglycemia and is associated with obesity. T2DM is accounting for approximately 90% of all cases of diabetes [1].

Both T1DM and T2DM are independent risk factors for CVD [4, 27, 28]. This thesis will only deal with T2DM and consequently, T2DM is herein referred to, as “diabetes”.

Definitions

The hyperglycemic conditions impaired glucose tolerance (IGT), impaired fasting glucose (IFG) and diabetes are simply defined according to the degree of hyperglycemia. IGT is defined as a moderate elevated postprandial glucose concentration determined by an oral glucose tolerance

test (OGTT). IFG is defined as isolated moderate elevated fasting glucose and diabetes is defined as elevated fasting glucose or elevated postprandial (OGTT). IGT and IFG are associated with an increased risk of developing diabetes and CVD [1, 29, 30].

The currently used criteria for glucometabolic classification according to the WHO [31] are presented in Table 1.

T2DM

Estimations by the WHO and the international diabetes federation (IDF) gives a prevalence of diabetes in 5.1% of the adult population. In addition, it is likely that these numbers will be matched or exceeded by subjects with IGT and IFG, which both are dysglycemic states that currently affect approximately 8% of adults worldwide [32]. In total approximately 13% of the global adult population therefore suffer from abnormalities in glucose metabolism. T2DM is characterized by an increasing insulin resistance and decreasing β -cell function and eventually also loss of β -cell mass [33-36].

Table 1. Definitions of normoglycemia and different states of glucose abnormalities according to the WHO in 2005 (venous plasma glucose).

Glucometabolic state	Classification criteria (mmol/l)
Normal glucose tolerance	FPG < 6.1 and 2hPG < 7.8
Impaired glucose tolerance	FPG < 7.0 and 2hPG \geq 7.8 and \leq 11.1
Impaired fasting glucose	FPG \geq 6.1 and < 7.0 and 2hPG < 7.8
Diabetes mellitus	FPG \geq 7.0 or 2hPG \geq 11.1

Insulin resistance

Insulin resistance is usually defined as an abnormal glucose turnover in the face of normal or raised glucose and insulin concentrations. Insulin sensitivity is determined not only by the number and affinity of the insulin receptors, but also by the functional state of the intracellular signaling pathways that transduce insulin binding to various downstream effectors e.g. glucose transport, phosphorylation and oxidation, glycogen synthesis, lipolysis and ion exchange [37]. Cellular resistance of the glucose metabolic pathway is caused by a dysfunction of the signal transduction machinery. The various insulin effectors are, at least in part, independent of one another. As a consequence, cellular insulin resistance can vary in degree and pathway specificity. To the extent that they have preserved their sensitivity, other pathways may be overly stimulated by the compensatory hyperinsulinemia. The pathophysiological implication of this phenomenon is that, in insulin resistant states, any abnormality that is found to be associated with altered glucose metabolism e.g. dyslipidemia, hypertension, platelet hypercoagulation, or prothrombotic changes theoretically can be the result of either the insulin resistance per se or the chronic effects of the attendant hyperinsulinemia [37]. Zethelius et al. found that proinsulin is a strong predictor of CHD independent of other major risk factors as smoking, elevated blood pressure and serum cholesterol concentrations whereas insulin does not seem to be a predictor of CHD. Although rather than being directly associated with its cause, proinsulin may be a marker of an underlying metabolic disturbance predisposing to atherosclerosis [38].

There is strong evidence for the association between inflammation and insulin resistance and links of these features to CVD

[12]. Epidemiologic studies show strong correlation between proinflammatory biomarkers, e.g. CRP, interleukin 6, and tumor necrosis factor and perturbations in glucose homeostasis and arteriosclerosis [39]. Moreover, alterations in CRP concentrations might be independently associated with insulin resistance [40, 41]. In recent years, numerous studies have been published that link subclinical chronic inflammation to the development of insulin resistance and T2DM [12].

The metabolic syndrome

The 'metabolic syndrome', as we currently know it, was born as 'the insulin resistance syndrome'. In 1988, Reaven [42] formalized the concept that insulin resistance clusters with glucose intolerance, dyslipidemia and hypertension to heighten cardiovascular risk. The definition by the WHO of the metabolic syndrome is based on any sign of insulin resistance, i.e. IGT, IFG or diabetes, together with two or more of the components hypertension, hypertriglyceridemia, low HDL cholesterol, central obesity and microalbuminuria. The syndrome is clearly overrepresented among T2DM subjects [43, 44].

T2DM and CVD

CHD is a dominant cause of mortality and morbidity in the industrialized world [45]. The underlying state, atherosclerosis, is a progressive process starting early in life and silently proceeding during many years. T2DM is an independent risk factor for developing CHD, and the risk of CHD or stroke is up to four times higher in patients with diabetes compared to non-diabetic subjects [4]. T2DM, hypertension and dyslipidemia are all states associated with insulin resistance and an increased risk for CHD (figure 1) [46, 47]. The preva-

lence of diabetes in patients with acute MI is high, approximately 30%, and is further increased in older populations [48, 49].

Results from a large Finnish database showed that for every 1% increase of HbA1c, CVD mortality increased by about 10% in T2DM [50]. The United Kingdom prospective diabetes study (UKPDS), a large cohort of newly diagnosed people with T2DM, indicated a 16% increased risk of MI for every unit (%) increase of HbA1c [51]. A global meta-analysis of all available epidemiological data for mortality from ischemic heart disease in relation to FPG has demonstrated an overall increase of 20 % for every 1 mmol/l increase of FPG above the optimum level [52]. In fact, the concentration of a single, random blood glucose measurement in the acute phase of macrovascular complications, for example, acute MI, was found highly

predictive whenever investigated, both for short-term prognosis during hospital stay and long term over several years [53].

The endothelium

The endothelium consists of a single layer of cells that line all the vessels in the body. These cells provide an interface between the blood and the tissues. The endothelium is known to work in an autocrine, paracrine and endocrine manner and is proposed to be involved in multiple functions, including maintenance of vascular tone, coagulation, fibrinolysis, and platelet and leukocyte adherence [11, 54, 55]. The interplay between endothelium and vessel is dependent on type and location of the vessel, i.e. in conduit arteries endothelial cells limit the activation of clotting and pro-inflammatory factors, in resistance

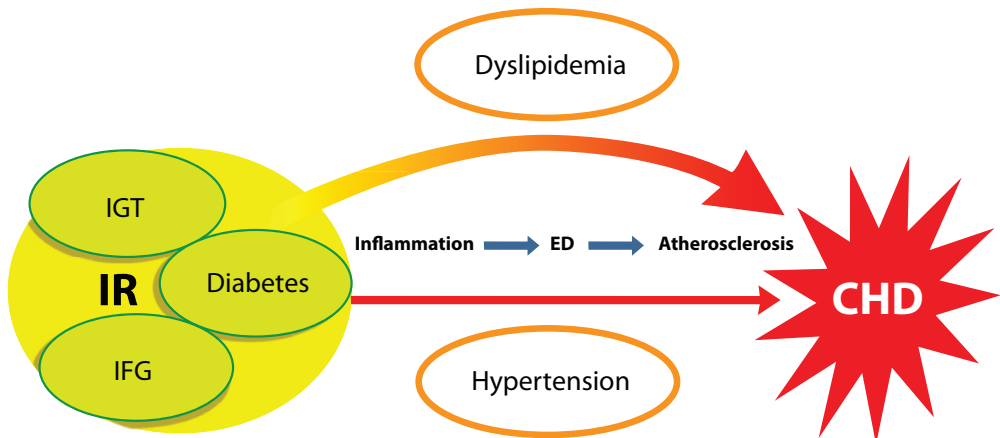


Figure 1. Contributing risk factors for coronary heart disease (CHD).

IGT: impaired glucose tolerance, IFG: impaired fasting glucose, IR: insulin resistance, ED: endothelial dysfunction, CHD: coronary heart disease.

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vessels they contribute to the regulation of blood flow, and in precapillary arterioles the endothelium transports and distributes nutrients and hormones. Almost three decades ago, Furchgott and Zawadzki demonstrated the important role of the endothelium in vasodilator activity [56]. This vasodilator effect was demonstrated to be mediated by the endothelium-derived relaxing factor (EDRF), subsequently identified as nitric oxide (NO), the key vasodilator factor in the endothelium [57]. Endothelial cells (EC) constitutively express NO synthase (eNOS) that generates NO using L-arginine as a substrate together with FAD, FMN, NADPH and tetrahydrobiopterin as cofactors (figure 2) [57].

NO rapidly diffuses into vascular smooth muscle cells and binds to soluble guanylate cyclase. This event results in formation of cyclic GMP (cGMP), activating a cGMP-dependent protein kinase. This leads to inhibition of the contractile machinery and thus vasodilatation [58]. Several different agonists releasing NO via receptor-operated mechanisms have been recognized, such as acetylcholine (ACh), bradykinin, serotonin and prostacyclin (PGI₂) [59]. Besides the potent vasodilator effect of NO, it mediates many other protective functions in the endothelium. NO inhibits the expression of pro-inflammatory cytokines, chemokines and leukocyte adhesion molecules, thereby limiting vascu-

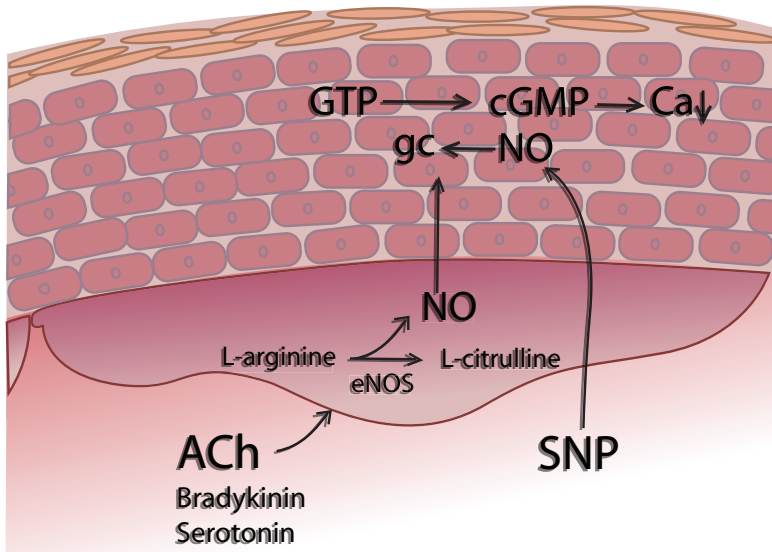


Figure 2. The nitric oxide pathway in the vasculature.

Endothelial cells constitutively expressing nitric oxide synthase (eNOS) generate NO using L-arginine as a substrate together with certain cofactors. NO then rapidly diffuses into vascular smooth muscle cells and binds to guanylate cyclase. This event results in formation of cyclic GMP (cGMP), activating a cGMP-dependent protein kinase, which leads to an increased extrusion of Ca²⁺ from the cytosol inhibiting the contractile machinery and thereby evoking vasodilation. Reprinted from *Molecular and Cellular Endocrinology*, 297, Nathanson D., Nyström, T., Hypoglycemic pharmacological treatment of type 2 diabetes: Targeting the endothelium, page no. 114, © (2009), with permission from Elsevier.

lar recruitment of leukocytes and platelets [60]. It also inhibits vascular smooth muscle cell (VSMC) proliferation, an early sign of atherosclerosis [60].

Diabetes and endothelial dysfunction

Endothelial dysfunction is a common feature in T2DM patients and in individuals with insulin resistant conditions [19]. Endothelial dysfunction can occur at any level in the arterial system and is a risk factor for the development of atherosclerosis [19]. In the healthy endothelium, there is an appropriate balance between vasodilatation and vasoconstriction. As mentioned above, NO is a key factor in regulating vascular tone, e.g., vasodilatation. There are also antagonizing factors promoting vasoconstrictor effects in the vasculature. The main vasoconstrictor factor is believed to be endothelin-1 (ET-1). The chain of processes that leads from endothelial dysfunction to atherosclerosis refers to an imbalance in the production between these mediators, which may disrupt endothelial homeostasis and predispose the endothelium to a pro-thrombotic and pro-atherogenic milieu [59]. Although the molecular basis of endothelial dysfunction is not completely understood, numerous studies point to loss of NO biological activity and/or biosynthesis as a culprit lesion [61]. In the presence of suboptimal concentrations of substrate or cofactors for the synthesis of NO, eNOS can become uncoupled. This results in the production of reactive oxygen species (ROS), e.g. superoxide anion and hydrogen peroxide, an event commonly referred to as oxidative stress [62].

Evidence for the hypothesis that endothelial dysfunction can precede diabetes is the finding of impaired vascular reactivity in subjects with normal glucose toler-

ance and with a family history of diabetes [63]. Endothelial dysfunction in subjects with T2DM seems to be multifactorial; hypertension, dyslipidemia, obesity, microalbuminuria, low grade inflammation, hyperglycemia and impaired insulin effects (insulin resistance) on the endothelium have all been associated with endothelial dysfunction [19].

The heart

Physiology

During the cardiac cycle, blood continuously flows from the inferior vena cava/superior vena cava and coronary sinus to the right atrium, and from the pulmonary veins into the left atrium [64]. As atrial pressure increases, blood is pushed into the ventricle through the open atrio-ventricular (AV) valve. As the ventricles contract, the pressure builds up rapidly and causes closure of the AV valves. The pressure continues to rise rapidly against closed valves. This is often referred to as isovolumetric ventricular contraction [64]. When ventricular pressure exceeds arterial pressure, the pulmonary and aortic valves open and blood is ejected into the arteries. Approximately 60% of the blood is ejected by a healthy left ventricle, i.e. the ejection fraction [64]. As the muscles relax, the pressure in the ventricles falls (known as the isovolumetric ventricular relaxation). When ventricular pressure is less than the atrial pressure, the AV valves open and the aortic and pulmonary valves close. Pressure is the product of flow and resistance. Cardiac output (CO) is the amount of blood ejected from the left ventricle into the aorta each minute and the rate of CO is determined both by stroke volume and heart rate [64].

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Ischemic heart disease

Ischemic heart disease (IHD), or myocardial ischemia, is a disease usually due to coronary artery disease (CAD). Traditional risk factors for IHD and MI include positive family history, increasing age, hypertension, dyslipidemia, cigarette smoking, marked obesity, physical inactivity and diabetes [45]. CAD is the leading cause of death in the Western world [45]. Manifestations of stable ischemic heart disease include angina and decreased exercise tolerance. Unstable IHD typically presents with central chest pain or other symptoms at rest, or rapidly worsening effort angina. In 2000, the European Society of Cardiology (ESC) and the American College of Cardiology (ACC) recommended a new definition for MI [65] that combines ischemic symptoms with changes in the ECG and in biochemical markers of myocardial necrosis and emphasizes the use of troponins.

Heart failure

Congestive heart failure (CHF) is a pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate corresponding with the requirements of the metabolizing tissues. CHF is commonly described as a syndrome caused by cardiac dysfunction, and recognized by a constellation of signs and symptoms. Estimates of the prevalence of symptomatic CHF in the general European population range from 0.4 to 2% [66]. The prevalence of heart failure increases rapidly with age, with the mean age of the heart failure population being 74 years [66]. As the proportion of the population that is elderly is increasing, this partly accounts for the rising prevalence of heart failure. In Europe, myocardial dysfunction secondary to CAD, usually as a consequence of MI, is the most

common cause of heart failure among patients under 75 years [67] of age and clear abnormalities in systolic function are usually present. Systolic hypertension and cardiac hypertrophy, cell loss and fibrosis may be more important causes of heart failure in the elderly and may be more likely to manifest predominantly as abnormalities of diastolic function [66]. Valvular abnormalities, diabetes and insulin resistance are also independent risk factors for CHF [66, 68, 69].

Cardiac metabolism

The capacity of the human heart to oxidize fat and carbohydrates is of great importance. In a series of enzyme-catalyzed reactions, the heart converts chemical energy into mechanical energy [70, 71]. The human heart uses several kilograms of ATP as an energy source every day [70, 71]. In a classic series of studies, Randle et al. demonstrated that fatty acids compete with glucose for substrate oxidation in isolated rat heart muscle and rat diaphragm muscle. They speculated that increased fat oxidation causes the insulin resistance associated with obesity, diabetes and starvation [72-74].

When the heart is acutely stressed, it is able to switch from fat to carbohydrate as fuel for oxidative energy production (figure 2) [75]. The advantage of increased carbohydrate oxidation relate to a higher effective value for the ratio of ATP synthesized/ O_2 consumed for carbohydrate vs. lipid [75]. Furthermore, if the heart is exposed to changes in ventricular pressures it reactivates its fetal gene program [71, 76]. Reactivation of these fetal genes includes a switch from fat to glucose oxidation [77], which though initially adaptive, ultimately results in a loss of insulin sensitivity and hence, a loss of metabolic

flexibility [71]. Change in workload of the heart may lead to insulin resistance [77]. However, maneuvers to sustain myocardial glucose oxidation and/or to prevent high fatty acid oxidation at least in obesity should be evaluated with great caution as a very recent published study on transgenic mice with cardiac-specific over-expression of the insulin-dependent glucose transporter GLUT-1 demonstrated that chronic increases in myocardial glucose uptake and oxidation reduce the metabolic flexibility and render the heart susceptible to contractile dysfunction [78].

Diabetes and ischemic heart disease

In diabetic patients over the age of 65 years, 68% of deaths are from CHD and 16% are from stroke [79]. Insulin resistance, a cardinal feature of T2DM, is associated with a cluster of metabolic and biochemical abnormalities, including hyperglycemia, hypertension, atherogenic dyslipidemia, inflammation, endothelial dysfunction, and impaired fibrinolysis [12, 63, 80-83]. Each of these abnormalities promotes the development of atherosclerosis and clinical CVD. Longitudinal epidemiologic studies report a similar incidence of cardiovascular and all-cause mortality between subjects who have diabetes without evidence of prior CHD and subjects who have CHD without prior diabetes [4, 84]. Notwithstanding the growing incidence of T2DM in young adults and adolescents requires that any claims about diabetes as a CHD risk equivalent should be qualified by the age of the affected individual [81].

Diabetes and congestive heart failure

Despite improvements of treating CHF,

mortality and hospitalization rates in diabetic patients with CHF remain high. The increased incidence of CHF in diabetic subjects remains high, despite controlling for confounders such as hypertension or CHD [85]. This might be caused by the direct effect of hyperglycemia on cardiac structure and function [86]. This feature is often called diabetic cardiomyopathy. Recently it has been confirmed that a large proportion of patients presenting with the clinical syndrome of heart failure have a normal EF [87]. Among these patients, diastolic dysfunction is believed to be common [88]. A large proportion of the diabetic patients suffers from diastolic dysfunction [89]. Approximately half of patients with overt CHF have diastolic dysfunction without reduced EF [89]. In a community based study of the prevalence of diastolic dysfunction Redfield et al. found that the frequency of CHF increased dramatically with increasing severity of diastolic dysfunction. However, even severe diastolic dysfunction is often subclinical with no recognized CHF diagnosis [89]. Moreover, there seems to be an inverse association between the degree of metabolic control and signs of early diastolic dysfunction [88].

In heart failure, myocardial glucose uptake is compromised independent of etiology. This phenomenon is associated with cellular deficits of insulin signaling. Insulin resistance in heart failure can be detrimental, because transcriptional shifts in metabolic gene expression favor glucose over fat as a substrate (figure 3) for high-energy phosphate production [90]. There are several lines of evidence that insulin resistance is associated with CHF [91, 92]. In a prospective community based study of elderly men, with a follow-up time of 8.9 years, Ingelsson et al. found that insulin resistance predicted CHF incidence

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independently of established risk factors such as diabetes [93].

The cardiotoxic triad of CHD, hypertension/ventricular hypertrophy, and diabetic cardiomyopathy also leads to decreased ventricular function in chronic and acute situations [68]. Diabetes changes the myocardial extracellular matrix, which is evident from elevated interstitial and perivascular fibrosis, increased expression of collagen type I, and down regulation of collagen-degrading matrix metalloproteases [94]. These pathologic mechanisms are mediated by hyperglycemia, oxidative stress, and elevated aldosterone and angiotensin II levels [94].

Endothelial dysfunction predicts cardiovascular mortality, correlates with the functional capacity of chronic CHF patients and plays a central role in the pathophysiology of CHF, T2DM, hypertension

and chronic renal failure [95, 96]. The important messenger molecule NO exerts favorable effects on left ventricular diastolic distensibility. Low myocardial NO bioavailability has been demonstrated to reduce LV preload reserve in CHF patients [97]. Hyperglycemia induces oxidative stress via several mechanisms, which include glucose auto-oxidation, formation of advanced glycation end products (AGE), activation of the polyol pathway, and increased levels of free fatty acids (FFA) [98]. eNOS is one prominent vascular source of NO. In oxidative environments, paucity of tetrahydrobiopterin induces eNOS uncoupling, which leads to production of superoxide instead of NO, thereby amplifying oxidative stress and endothelial dysfunction [86]. Evidence for uncoupling of eNOS has been obtained in patients with diabetes, hypertension, and hypercholes-

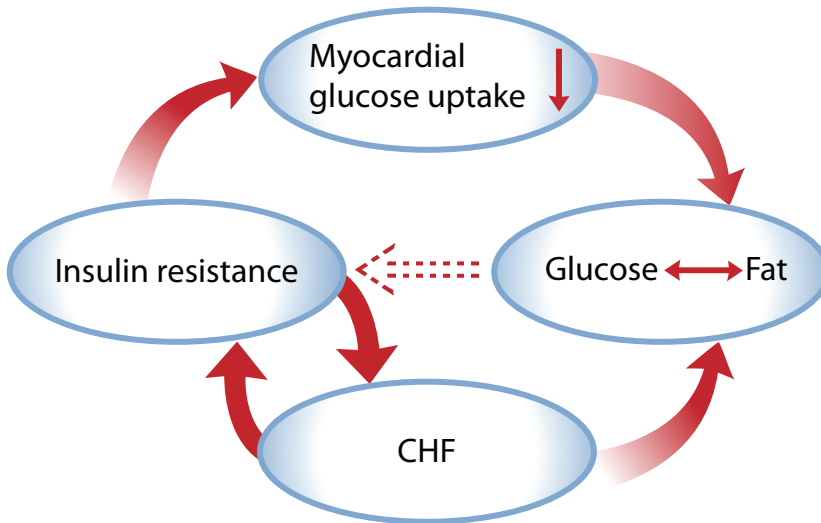


Figure 3. Metabolic changes in the decompensated heart, and associations between insulin resistance, congestive heart failure and glucose-fat oxidation.

When the heart is acutely stressed, it is able to switch from fat to carbohydrate as fuel for oxidative energy production. If the heart is exposed to changes in ventricular pressures it reactivates its fetal gene program. Reactivation of these fetal genes includes a switch from fat to glucose oxidation, which in the end might lead to increased insulin resistance. Change in workload of the heart results in a loss of insulin sensitivity.

terolemia [99].

Prevention of diabetic cardiovascular complications

A growing body of evidence suggests that diabetes can be prevented by lifestyle changes, such as an increase in physical activity and lowered consumption of fat, carbohydrates and an increase in fiber intake [18]. Increasing participation in vigorous and non-vigorous exercise has been correlated with improved insulin sensitivity in subjects with normal glucose tolerance, IGT, and early T2DM [100]. Likewise, exercise is associated with reduced total cholesterol levels and increased low-density lipoprotein cholesterol particle size, which are biomarkers for cardiovascular events [101]. Disappointingly, there is little evidence to support a clear benefit of lifestyle interventions alone on cardiovascular morbidity or mortality in patients with T2DM or prediabetes [102].

The landmark study exploring antihyperglycemic treatment effectiveness on CVD complications in (newly diagnosed) T2DM is still the UKPDS [22, 51, 103, 104]. In this study, intensive antihyperglycemic treatment with sulfonylureas and insulin did not significantly reduce the risk for CVD, whereas metformin – as monotherapy - did so in a subgroup analysis of obese subjects [103]. However, the most striking long-term effect of lowering blood glucose appeared during the open post-study observation period [51]. It is worth mentioning that the effectiveness of treating T2DM was more marked in reducing cerebrovascular and peripheral vascular disease but only of borderline significance for CHD.

The studies that so far report the most successful results of preventing CVD in T2DM adopted a multifactorial strategy;

in STENO 1 and 2, intensive intervention with multiple drug combinations and behavior modification had sustained beneficial effects with respect to vascular complications and on rates of death from any cause and from cardiovascular causes [20, 21]. In fact, the number needed to treat for preventing one cardiovascular event in this study was as low as 3.4!

In 2008, a triad of randomized studies, Action to Control Cardiovascular Risk in Diabetes (ACCORD), Veterans Affairs Diabetes Trial (VADT) and Action in Diabetes and Vascular Disease: Preterax and Diamicon Modified Release Controlled Evaluation (ADVANCE), in total some 23,000 T2DM patients, have reported conflicting results on macrovascular endpoints in the context of various blood glucose-lowering regimes [21, 24, 105]. In the ACCORD trial, the intensive treatment arm surprisingly had to be prematurely stopped after 3.5 years due to increased mortality [23]. In contrast, the ADVANCE-trial reported a 10% reduction in a combined micro- and macrovascular endpoint, primarily as a consequence of a 21% relative reduction in nephropathy, but intensive treatment did not yield any significant reduction of macrovascular events [24]. In the VADT trial, meticulous glucose control had no significant effect on incident major cardiovascular events, death, or microvascular complications with the exception of progression of albuminuria [105]. In summary, trials evaluating patients with shorter duration of T2DM and a milder disease, i.e. the UKPD [22, 51, 103,104] seem to show more benefit of intensive glucose control compared to patients with more severe disease and longer diabetes duration, i.e. ACCORD, ADVANCE and VADT [23, 24, 105]. Interestingly there is evidence that some antidiabetic drugs may exert beneficial cardiovascular effects independently

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of their antihyperglycemic action. I will briefly discuss evidence for such effects below.

Anti-diabetic drugs with cardiovascular effects

Metformin

The biguanide drug metformin has been used in the treatment of T2DM almost for half a century and is recommended as first line treatment for T2DM, along with diet and exercise. Metformin exerts its antihyperglycemic effects primarily by decreasing hepatic glucose output through activation of AMP-activated protein kinase (AMPK) [106, 107], and by increasing glucose disposal in skeletal muscle [108] with a minimal risk of hypoglycemia. Also, it is increasingly clear that metformin contributes to weight gain limiting effects [109]. Some investigators suggest that metformin ameliorates endothelial function independently of glycemia [110, 111].

In the UKPDS [103], metformin as monotherapy, but not in combination with sulfonylurea (SU), was associated with a decrease in macrovascular morbidity that appeared to be independent of glycemia. There is also some epidemiologic inference that metformin might be superior to sulfonylurea in terms of decreasing cardiovascular mortality, as the drug in a retrospective cohort study was associated with lower mortality and morbidity in patients with T2DM and heart failure compared to sulfonylurea [112, 113].

Sulfonylureas

Although there are studies indicating possible negative effects on vascular function by glibenclamide [114], there are also a few studies indicating that certain other

SUs may improve endothelial function. Gliclazide protects human umbilical vein endothelial cells (HUVECs) from glucapoptosis through anti-oxidative effects [115]. Also, glimepiride has been shown to influence endothelial cell function in a potentially positive way, inasmuch as it induces NO release from HCAECs by activating the PI3K/Akt pathway [116] and promoting eNOS phosphorylation [117]. A small prospective study, aimed to investigate the progression of carotis intima-media thickness (cIMT) in T2DM, showed that cIMT increased less in the gliclazide-treated group than in the glibenclamide group [118].

In the UKPDS, there were no differences in mortality and morbidity between groups treated with glibenclamide, insulin and chlorpropamide, respectively [22].

In a retrospective cohort study, consisting of 20,450 T2DM patients, McAlister and coworkers found that users of high-dose sulfonylureas were more prone to develop incident CHF than users of high-dose metformin [119].

Thiazolidinediones

TZDs bind to the γ subtype of the peroxisome proliferator-activated receptors (PPARs). This leads to modulation of insulin sensitivity and glucose homeostasis concomitant with a lowering of plasma FFAs [120, 121]. TZDs enhance NO bioavailability in endothelial cells, including activation of eNOS [122, 123]. Direct effects of TZDs on vascular smooth muscle cells have also been observed [124]. Recently, it was demonstrated that the TZD pioglitazone improves coronary endothelial function in non-diabetic patients with CHD [125], and their progression of coronary atherosclerosis compared with glimepiride [126]. These data support the

view that TZDs' beneficial effects on the endothelium might, at least in part, be independent of changes in glycemia. In the prospective pioglitazone clinical trial in macrovascular events (PROactive) study [127], there was a non-significant trend towards a relative risk reduction of the composite primary endpoint for pioglitazone-treated patients. More importantly, there was a significant reduction in the secondary endpoint of all cause mortality, MI or stroke. There was also a significantly increased number of edema and heart failure cases in the pioglitazone-treated group [127]. Peripheral edema is a well known side effect from TZD treatment; it is a dose-dependent phenomenon and occurs early, up to 6 months after commencement of a TZD [128, 129]. In addition, pulmonary edema and CHF can occur from weeks to years after initiation of TZDs [130]. The mechanism of TZD induced edema is not fully understood but the kidney has been shown to differentially express all three PPAR subtypes. Lower levels of lithium clearance, which indirectly measures renal proximal sodium absorption, has been shown during administration of pioglitazone. Taken together, this suggests an increased reabsorption of sodium in the proximal tubules due to PPAR stimulation [131].

In a meta-analysis, data from 42 trials were analyzed, concerning the cardiovascular safety of rosiglitazone [132]. In the rosiglitazone group, as compared to the control group, the odds ratio for MI was 1.43 and the odds ratio for death from cardiovascular causes was 1.64. The conclusion drawn was that rosiglitazone is associated with a significant increase in the risk of MI, with an increased the risk of death from CVD. As a consequence of this and of other recently published meta-analyses [133, 134] the European medicines agency

decided in September 2010 temporarily to recommend suspension of rosiglitazone-containing drugs.

Insulin

Human endothelial cells express the insulin receptor [135]. It has become increasingly clear that insulin may exert vasoactive effects, insofar as it can enhance blood flow and vasodilatation in a NO-dependent fashion upon i.v. administration [136-138]. Insulin enhances blood flow through different types of vessels, e.g. capillary and resistance vessels [139].

There is compelling evidence that insulin directly enhances the production of NO by the endothelium via signaling pathways involving PI3K activation, which in turn stimulates eNOS phosphorylation [140-142]. In addition to activation of the PI3K-eNOS pathways, insulin also appears to influence the endothelium via MAPK-dependent pathways that promote smooth muscle cell migration and ET-1 production [143].

In the UKPDS, intensive glucose control with insulin or SU significantly reduced microvascular complications [22]. For MI, the relative risk reduction was borderline significant, but neither insulin nor SU did, despite weight gain and hyperinsulinemia, increase the risk of CVD events. The recently published UKPDS 80 report demonstrated a continued reduction of microvascular risk and an emergent risk reduction for MI, despite an early loss of glycemic differences [51].

Interestingly there are data suggesting that infusion of glucose-insulin-potassium (GIK) might induce acute effects on the heart and left ventricular performance. Studies investigating hemodynamic effects of GIK shows that GIK infusion results in increased cardiac output, less need for ino-

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tropic agents, and reduction in duration of intensive care unit stay in patients directly after coronary artery bypass grafting, both in nondiabetic and diabetic patients [144-146]. Smaller experimental studies performing echocardiography during GIK infusion in several types of patients report an enhancement in segmental wall motion in dyssynergic segments. [147, 148]. Klein and coworkers demonstrated that GIK improves regional left ventricular function and allows the detection of myocardial viability to a similar extent as low-dose dobutamine in patients shortly after infarction [149]. In the DIGAMI 1 study, insulin-glucose infusion followed by intensive subcutaneous insulin therapy in diabetic patients with acute MI was found to improve long term survival; overall mortality was reduced by 11% (absolute risk reduction) [150]. These differences were sustained for more than three years. The DIGAMI 2 study was designed to investigate whether this substantial reduction in mortality was due to glycemia or insulin effects per se. However, the study failed to show any differences between the intervention groups, in terms of impact on long term and short term mortality [151]. Regrettably, this study must be considered inconclusive as the three groups (group 1, acute insulin-glucose infusion followed by insulin-based long-term glucose control; group 2, insulin-glucose infusion followed by standard glucose control; and group 3, routine metabolic management according to local practice) did not differ in HbA1c levels. The epidemiological analysis from the DIGAMI 2 study supports the concept that meticulous glucose control, rather than insulin treatment per se, is the major determinant of improved survival in subjects with hyperglycemia and MI [151]. However, it was recently suggested by the same investigators that insulin might be

harmful unless normoglycemia is achieved [152].

Despite a large body of data from clinical trials, the issue remains unsettled as to whether insulin treatment in patients with diabetes and recent MI can reduce the risk of recurrent CVD and death.

Incretins

After meal ingestion, multiple gastrointestinal hormones are secreted. These hormones are involved in the regulation of gut motility, secretion of pancreatic enzymes, gall bladder contraction and nutrient absorption. The observation that the insulin secretion after orally ingested glucose exceeded the insulin secretion levels elicited by an equal amount of i.v. administered glucose led to the conclusion that there must be endogenous factors in the gut besides the glucose load per se promoting this enhanced insulin response. These putative factors were named incretins and the effect was termed "the incretin effect" [153]. Two major mediators were subsequently identified: Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1. GLP-1 exists in two circulating equipotent forms: (GLP-1) (7-37) and GLP-1 (7-36) amide [154]. The first study of GLP-1 (7-36) amide (herein referred to as GLP-1) was published almost 3 decades ago with the cloning of a glucagon-related peptide from the preproglucagon gene in anglerfish [155]. GLP-1 is secreted mainly from the L-cells in the small bowel and colon and is a product of the partial proteolysis of preproglucagon. GLP-1 lowers blood glucose by augmentation of insulin secretion and inhibiting glucagon secretion [156-159], but it also diminishes bowel motility and promotes the feeling of satiety [160-163]. GLP-1 is rapidly degraded by the ubiquitous en-

zyme dipeptidyl peptidase 4 (DPP-4) [160-163]. The biological half-life of GLP-1 in plasma is therefore very short, i.e. 1-2 min. The degradation metabolite GLP-1 (9-36) amide lowers glucose to a much lesser extent compared to native GLP-1, effects that seem to be independent of insulin and glucagon secretion and the rate of gastric emptying [164]. The different GLP-1 isoforms are listed in table 2.

and subsequent characterization of the binding of radio-labeled GLP-1 to these cells [165]. Human pancreatic GLP-1R is approximately 90% homologous with the corresponding rat protein [166, 167].

Expression of GLP-1R has been detected in a wide range of tissues, including the pancreatic islets, lungs, kidneys, heart,

GLP-1 receptors

The actions induced by GIP and GLP-1 are believed to be mediated through G-protein coupled receptors. The GLP-1 receptor (GLP-1R) was first detected by transient transfection of a cDNA library prepared from rat pancreatic islets into COS cells

Table 2. GLP-1 isoforms

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

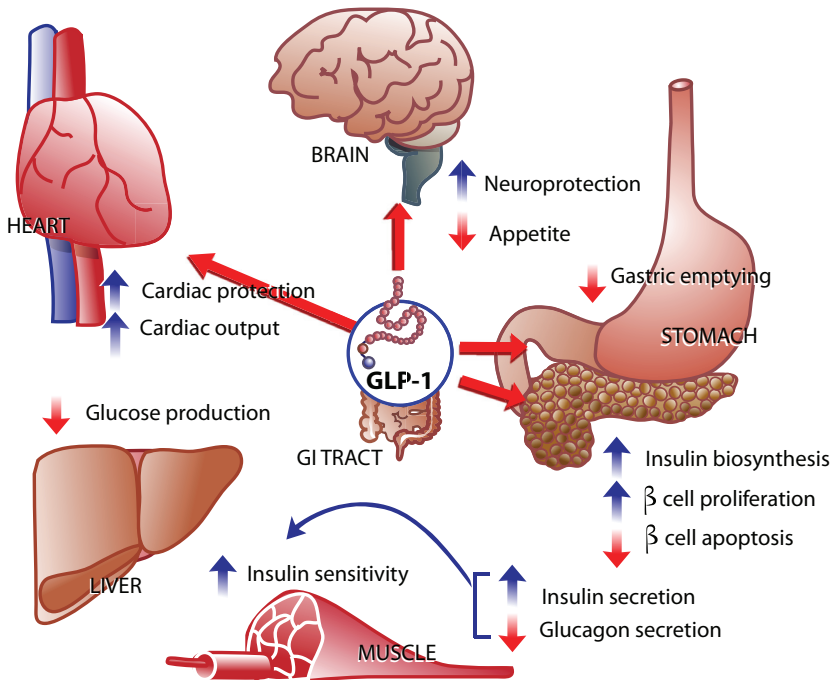


Figure 4. GLP-1 actions in peripheral tissues.

GLP-1 acts directly on the endocrine pancreas, heart, stomach, and brain, whereas actions on liver and muscle are indirect. Reprinted from Cell Metabolism, volume 3, issue 3, Drucker DJ, The biology of incretin hormones, page 154, © 2006, with permission from Elsevier.

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vessels, stomach, intestines, pituitary, skin and several regions of the CNS (figure 4) [168]. Although physiological actions of GLP-1 on the liver, skeletal muscle and fat cells have been reported, evidence for the expression of GLP-1R in these tissues is ambiguous. Ligand-binding analyses reveal that the K_d for binding of GLP-1 to its receptor is approximately 1 nM [169], and that exenatide displays a similar affinity [170, 171]. Exendin (9-39), an NH_2 -terminally truncated form of exenatide, also binds to the GLP-1R and is a strong antagonist of the receptor-dependent actions of GLP-1 and exenatide [171].

Stimulation of pancreatic β -cells by GLP-1 or exenatide, believed to be mediated via adenylate cyclase and the cAMP/PKA pathways, potentiates glucose-induced closure of ATP-sensitive K^+ channels, thereby generating cellular depolarization, activation of voltage-dependent Ca^{2+} channels (VDCCs) and a consequent influx of Ca^{2+} that sets secretion of insulin in motion [172]. Agonists of GLP-1R also evoke several other responses in the rodent pancreas, including enhanced proliferation and inhibition of apoptosis of β -cells [173, 174].

Antihyperglycemic effects of GLP-1

GLP-1 exerts a transient rise in insulin secretion and suppression of glucagon, both of which are glucose-dependent [156, 157, 160-163]. This dual mechanism of glucose dependent hormone secretion minimizes the risk of GLP-1-induced hypoglycemia, in contrast to other insulin secretagogues such as SU and glinides [175]. Several studies have shown that short-term infusions, i.e. (i.v. and s.c.) of GLP-1 lower blood glucose levels in both type 1 and type 2 diabetic subjects [156, 157, 176].

In a 6-week study in T2DM patients, GLP-1 infusion resulted in augmentation of insulin secretion, reduction of HbA1c (1.2%), a weight loss of 1.9 kg and improvements of insulin sensitivity [177]. In contrast to GLP-1, GIP seems to lose its insulinotropic effects in diabetic subjects possibly through receptor down-regulation or desensitization [158]. The ability of GIP to stimulate second phase insulin secretion is absent in T2DM, although a first phase response is present [178].

Incretin agonists

Due to the very short half-life of GLP-1, caused by DPP-4 degradation, GLP-1 agonists resistant to this degradation and DPP-4 inhibitors have been developed. The first known GLP-1R agonist, resistant to DPP-4 degradation, that came to be used for clinical purposes was originally found in the saliva of the lizard *Heloderma suspectum*. This peptide was named exendin-4 (exenatide is synthetic exendin-4). It shares a 53% amino acid sequence homology with that of native GLP-1 (figure 5) giving it a circulating plasma half-life of 60-90 min [179]. In clinical trials with T2DM patients, addition of exenatide on top of metformin, SU or both reduces HbA1c with 0.8-1.0% and gives a weight reduction of 0.9- 2.5 kg in overweight patients [180-182].

Liraglutide, almost a GLP-1 "twin" with ~ 97% homology to the native peptide, contains an arginine₃₄lysine substitution, a glutamate substitution and a FFA addition to lysine. It also binds to albumin, resulting in a plasma half-life of 10-14 hours after s.c. injection [154, 183]. Liraglutide can be administered once daily and reduces both fasting and postprandial glycemia. The Liraglutide Effect and Action in Diabetes (LEAD) program was

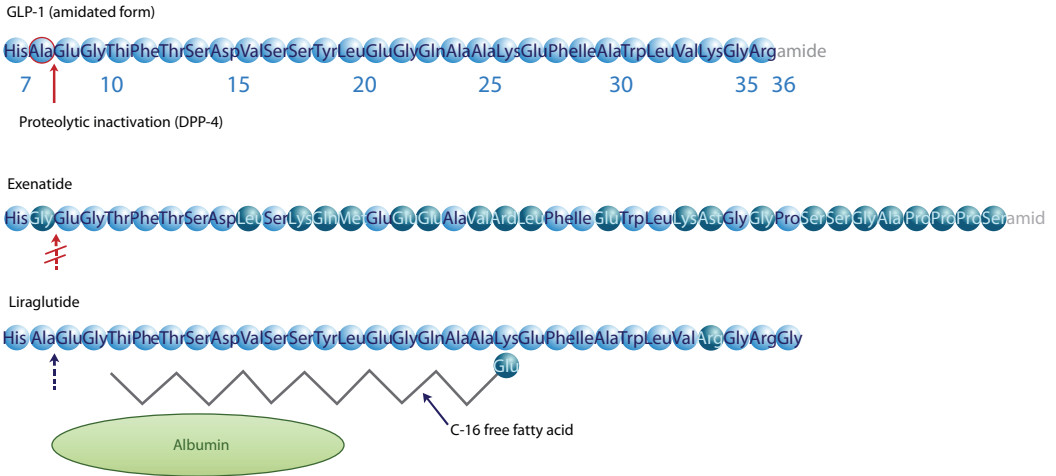


Figure 5. Structure of GLP-1 (7-36), GLP-1R agonists liraglutide and exenatide.

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.

designed to compare the efficacy and tolerability of once-daily liraglutide alone or in combination with other commonly used oral agents, for T2DM [184-188]. In all these studies (except for LEAD II), reductions of HbA1c of up to 1.6 % were observed with liraglutide as monotherapy or in combination with oral antidiabetes agents (OAD) [184-188]. Liraglutide also improved surrogate measures of β -cell function, reduced systolic blood pressure up to 6.7 mm Hg and was associated with weight loss up to 3.2 kg [184-189].

DPP-4 inhibitors

The inhibitors of DPP-4 in current clinical use are low-molecular weight compounds with good oral bioavailability, in contrast to peptides, i.e. GLP-1 analogues. DPP-4 knock-out mice have increased levels of

intact GLP-1 with concomitant increased insulin and decreased glucose levels after OGTT [190]. The first DPP-4 inhibitor approved for clinical purposes was sitagliptin. Vildagliptin and saxagliptin are other DPP-4 inhibitors in clinical use, and several others, e.g. linagliptin and alogliptin, are in phase II-III clinical trials. These agents are highly selective for DPP-4 inhibition, thus not appreciably affecting DPP-8 and DPP-9. In contrast to GLP-1 analogues, DPP-4 inhibitors are not associated with inhibited gastric emptying, nausea or weight loss, probably due to the relatively modest incretin effect (postprandial GLP-1 levels 15-25 pmol/l) [154]. The plasma half-life of sitagliptin is 8-14 h, resulting in an 80% inhibition of plasma DPP-4 activity 24 h after oral administration.

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GLP-1 secretion

The plasma levels of GLP-1 rise after ingestion of glucose, sucrose or fat [191]. Normally, basal levels of the peptide are approximately 5 pmol/l and they rise to 15- 40 pmol/l one hour after food ingestion. In a study by Laakso et al. differences in GLP-1 concentrations between NGT, IGT and IFG subjects could be seen from 60 to 120 min post-glucose challenge. In this study, NGT subjects had significantly higher post challenge GLP-1 levels compared to IGT and IFG subjects. However, a recent review has challenged the view that glucose intolerant individuals have GLP-1 deficiency [192].

GLP-1 and insulin resistance

After food intake, the rate of insulin secretion depends not only on the degree of glycemia, but also on the insulinotropic effects of the incretin hormones GLP-1 and GIP, and many other types of input [193]. There are to date conflicting data as to whether GLP-1 acutely affects insulin sensitivity or not. In one study on subjects with stable CHD and T2DM, infusion with GLP-1 did not affect whole body glucose uptake [194]. A study in non-diabetic men showed that the GLP-1 and GIP responses to a mixed meal correlated significantly with the degree of insulin resistance [195]. Recently, a study randomizing T2DM patients to either glibenclamide or exenatide (5 µg bid) showed - in contrast to the other studies reported in literature - positive effects of exenatide on insulin resistance and inflammatory state parameters like resistin, retinol binding protein-4, and hs-CRP [196]. In this study insulin resistance was measured by HOMA-IR, which decreased significantly in response to exenatide compared to glibenclamide treatment. However, the improvement of insulin sensitiv-

ity and inflammatory biomarkers in the exenatide arm might have been caused by the decrease in body weight (6.3 kg) or BMI (2.2 kg/m²) in these subjects.

Incretin effects on the vasculature and heart

In addition to the multitude of well-characterized actions of GLP-1 on glycemic endpoints, expression of GLP-1R has been detected in the vasculature [194, 197, 198] and a growing body of evidence has accumulated that implicate incretin action in the vasculature and the human heart.

Preclinical observations

Vasculature

Early experiments by Barragan showed that i.v. [199, 200] and intracerebroventricular administration of GLP-1 and exenatide to rodents elevates both blood pressure and heart rate [201-203]. In one study vagotomy was found to prevent the blood pressure and heart rate elevating effects of intracerebroventricularly administered GLP-1, as did intracerebroventricular administration of the GLP-1R antagonist exendin 9-39 [201]. These findings suggest that GLP-1 exerts cardiovascular effects through the central nervous system, initially by parasympathetic transmission. Furthermore, it has been shown that GLP-1 can pass through the blood-brain barrier by simple diffusion [204]. There are also studies that reveal dose-dependent relaxant effects of GLP-1 on vascular rings isolated from rodents ex vivo [205, 206]. The GLP-1 analogue liraglutide has recently been shown to exert an anti-inflammatory effect on vascular endothelial cells by increasing NO production and suppressing NF-κB activation, probably partly caused by AMPK activation [207]. Investigations

with chronic GLP-1 treatment of Dahl rats fed a high-salt diet partially 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 receiving vehicle alone [208].

The GLP-1 analogue exenatide also induces Fos-like immunoreactivity (Fos-IR) in neurons in the paraventricular nucleus of the hypothalamus [203, 209]. Interestingly it is proposed that increased activity of these neurons contributes to increases in blood pressure and heart rate [203]. These observations suggest that the cardiovascular actions of exenatide involve integration of both indirect neural and direct cardiac effects, thereby linking signaling through the GLP-1R to activation of the sympathetic nervous system.

It was recently demonstrated that brain GLP-1R signaling simultaneously controls arterial blood flow and glucose utilization, and hypothalamic NOS activity in a glucose-dependent manner [210]. However, findings concerning the possible non-adrenergic [199] vs. adrenergic effects [203] of GLP-1 appear to be ambiguous. Exenatide causes substantial β -adrenoceptor-mediated hindquarter vasodilatation and tachycardia through sympathoadrenal activation, and it was recently proposed that this substance may cause opposing hemodynamic effects, i.e. mesenteric vasoconstriction and hindquarter vasodilatation by activating both β - and α -adrenoceptors [211]. Furthermore, administration of exenatide or GLP-1 to rats with streptozotocin/nicotinamide-induced diabetes nearly normalized their vascular tone [212]. The restoration of endothelial function in these two models appeared to be secondary to the improvement in glycemic control, rather than direct endothelial effects of exenatide or GLP-1.

Heart

Interestingly, some recent papers have reported cardiac actions of the endogenous metabolite of GLP-1, i.e. GLP-1 (9-36) amide, independent of the known GLP-1R [197]. Furthermore several studies, in various animal models, have shown that GLP-1 or GLP-1 analogs may limit myocardial infarct size [197, 213-219]. Moreover exenatide has been shown to reduce intimal hyperplasia in Zucker fatty rats [220]. Very recently a group demonstrated that isolated mouse heart rapidly converts GLP-1 to GLP-1 (9-36) amide [221]. In this study it was shown that the metabolite GLP-1 (9-36) amide exerts cardioprotective actions in the mouse heart *ex vivo*, limiting infarct size after ischemia-reperfusion injury. Additionally, GLP-1 (9-36) amide, but not exenatide, also promoted cell survival in isolated cardiomyocytes from GLP-1R^{-/-} mice and in human coronary artery endothelial cells (HCAEC) [221]. The authors concluded that this supports the existence of an alternative receptor for GLP-1 (9-36) amide. Another study shed some light on the question whether increasing circulating GLP-1 levels by DPP-4 inhibition can exert effects direct on the heart. In this study Ye et al. showed that pretreatment with sitagliptin could limit myocardial infarct size, along with increased myocardial levels of cAMP and PKA activity in mice. The myocardial infarct size limiting effects of sitagliptin was completely blocked by a PKA inhibitor, suggesting that this effect is PKA dependent [222].

In animal *in vivo* studies, exploring the effects of GLP-1 on left ventricular function, GLP-1 has been shown to ameliorate left ventricular dysfunction in dogs with pacing-induced dilated cardiomyopathy [90] and to limit myocardial stunning in conscious canines [223]. A recently pub-

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lished study showed that GLP-1 infusion in conscious dogs with pacing-induced dilated cardiomyopathy stimulates myocardial glucose uptake through a p38 α MAP kinase-mediated and NO-dependent mechanism [224]. In this study, indications that the improvement in LV function was not mediated by β -adrenergic stimulation were found [224], in contrast to other studies with exenatide and GLP-1 [211]. Interestingly there are several studies demonstrating that central GLP-1R stimulation increases heart rate, in both anesthetized and freely moving rodents [199, 203, 210, 225]. Finally, there is some evidence that the the positive chronotropic effects of central GLP-1 receptor stimulation may result from changes in parasympathetic activity [199, 203, 210, 225, 226].

Clinical observations

Vasculature

In patients, both the systolic and diastolic blood pressure are reduced following 82 weeks of treatment with exenatide, probably as a consequence of improvements in blood levels of glucose and lipids and a reduction in body weight [228]. A recently published metaanalysis explored the effects of exenatide vs. placebo or insulin on blood pressure in pooled data from six trials including 2,171 subjects studied for at least 6 months. The authors in this analysis concluded that overall 6 months of exenatide treatment was associated with a significantly greater reduction in systolic BP (SBP) compared with placebo (difference of -2.8 mm Hg) or insulin (difference of -3.7 mm Hg). There was a weak, albeit statistically significant, correlation between weight loss and the reduction of SBP [229]. One study with shorter follow-up time failed to detect significant reductions of SBP in exenatide-treated patients

[230]. Vilsboll et al. reported that 14 weeks of treatment with the long-acting GLP-1 analog liraglutide improved glycemic control and lowered body weight, while significantly decreasing SBP [231]. Interestingly, the reduction in SBP occurred prior to the effect on body weight, indicating direct effects of liraglutide on SBP. Moreover, GLP-1 increases sympathetic vasoconstrictor neural activity but does not appear to affect cardiac sympathetic or parasympathetic activity in healthy humans [227].

Acute infusion of GLP-1 into T2DM patients with stable CHD ameliorates their endothelial dysfunction [194]. Short-time infusion of GLP-1 into healthy human subjects increased both normal and ACh-induced vasodilatation independent of alterations in glycemia and insulin levels and these effects were abolished by glibenclamide, an effect suggested to be mediated via K_{ATP} channels [232].

In a study investigating changes in endothelial function measured by peripheral arterial tonometry after a high-fat meal, Koska et al found that exenatide ameliorates postprandial endothelial dysfunction and that changes in triglycerides concentrations explained 64 % of this effect [233].

Heart

Nikolaidis et al. published the first study that showed that GLP-1 infusion can improve left ventricular function in patients after successful reperfusion following an MI [234]. In 2006, Sokos et al. found that a 5-week infusion with GLP-1, compared to standard therapy, improved left ventricular function and quality of life in patients with New York Heart Association class III/IV heart failure [235]. Interestingly, benefits were seen in both diabetic and non-diabetic patients in these two studies. Very recently it was demonstrated that inhibition of DPP-4 improved global and regional

wall LV function during dobutamine stress [236]. However, not all publications have shown cardiovascular beneficial effects of GLP-1 treatment; a study determining cardiac index in non-diabetic patients could not detect any improvement of left ventricular function after short-time infusion with GLP-1 [237].

Exenatide treatment has been associated with improved cardiovascular risk biomarkers, e.g. high-sensitive CRP, endothelin-1 levels and increased total adiponectin concentrations, independently of changes in body composition [238]. Treatment with liraglutide is known to associate with reduction of plasminogen activator -1 (PAI-1) and brain natriuretic peptide (BNP) but not significantly with high sensitivity CRP (hs-CRP) levels [239].

Epidemiological observations

There are to date very few epidemiological studies exploring potential associations between GLP-1 and CVD. In a Japanese study on individuals at high risk for CVD, a positive correlation between fasting GLP-1 levels and accumulation of components of the metabolic syndrome was found [240]. Very recently, a retrospective analysis of the LifeLink database demonstrated a lower risk of CVD events in 39,275 exenatide-treated subjects compared to 381,218 non-exenatide treated diabetic subjects [241].

Ongoing studies

The scientific field exploring cardiovascular effects of GLP-1 and its analogs is now expanding very fast. This is illustrated by a PubMed search on GLP-1 and heart that showed 116 published original papers in April 2010, twice as many as published in January 2007.

At present, several studies evaluating long-term cardiovascular effects of

incretins are ongoing: The Exenatide Study of Cardiovascular Event Lowering Trial (EXSCEL), Sitagliptin Cardiovascular Outcome Study (TECOS) and Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results - A Long Term Evaluation (LEADER) study are all randomized long - term studies that will investigate the impact of exenatide, sitagliptin and liraglutide on major cardiovascular outcomes.

Aims

The primary aim of this thesis was to investigate whether a group of peptides, incretins, with antihyperglycemic properties exert any physiologic or pharmacologic role in the endothelium, vasculature and human heart.

The specific aims were:

- I. To analyze whether GLP-1 plasma levels may predict CHD incidence, and to investigate potential cross-sectional associations between GLP-1 plasma levels, glucose abnormalities (i.e. diabetes and IGT) and insulin sensitivity.
- II. To analyze whether GLP-1 plasma levels may predict CHF incidence, and to investigate potential cross-sectional associations between GLP-1 plasma levels and echocardiographic measures of left ventricular function.
- III. To investigate therapeutic effects of the GLP-1 mimetic exenatide on left ventricular function and hemodynamic profile in T2DM patients with decompensated heart failure.
- IV. To investigate if exenatide 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 exenatide.
- V. To explore whether incretins influence proliferation of HCAECs *in vitro*, and to elucidate the molecular mechanisms behind such effects.

Material and methods

Translational research

Translational research by definition transforms scientific discoveries arising from laboratory, clinical, and population studies into clinical applications to reduce disease incidence, morbidity, and mortality. This thesis could be referred to as the product of a translational research effort as it is based on five studies using completely different methods, ranging from epidemiological analyses in cohort studies to molecular cell biological analyses of intracellular signal transduction pathways controlling the proliferation of human coronary artery endothelial cells to address a common issue: do incretins *per se* have an impact on the cardiovascular system?

Patients and study protocols

Study I and II

Study population

These studies are based on the ULSAM (Uppsala Longitudinal Study of Adult Men) cohort (<http://www.pubcare.uu.se/ULSAM/>). All men born in 1920 to 1924, who were residents of the municipality of Uppsala, Sweden, were invited to participate in a health examination survey carried out between April 1970 and October 1973, which aimed at identifying risk factors for CVD. Of the 2,841 men invited, 2,322 participants (82%) participated in the investigation at age 50 [242]. The men were invited for reexamination at age 60, 70, 77 and 82. The data has been completed with annual updates on mortality and in-hospital morbidity using national registers. The examination at age 70, which forms the

baseline of the present study, was carried out between August 1991 and May 1995. Of the 1,681 available 70-year-old men 1,221 (73%) attended. Out of these men, random plasma samples from 509 subjects were stored frozen at -70°C and analyzed for their GLP-1 concentrations in 2007. In study I (figure 6), 62 men were excluded due to prior MI or angina pectoris (ICD-9 codes: 410–414, ICD-10 codes: I20–25) in the prospective analyses. In study II (figure 6), seven participants were excluded due to previous diagnosis of CHF and four due to a diagnosis of valvular disease in the hospital discharge register at baseline. In the cross-sectional part of study II, associations between echocardiographic measurements of left ventricular systolic and diastolic (i.e. E/A ratio) function and GLP-1 concentrations were investigated. This cross-sectional study consisted of 377 out of 509 men, who had technically satisfactory echocardiographic evaluations, i.e. acoustic window limitations, atrial fibrillation/flutter and valvular disease ($n=132$) were all excluded. Fifteen participants with $\text{EF} < 50\%$ were excluded in an attempt to adjust for pseudo-normalization of the E/A ratio.

Outcome definitions

T2DM, IGT and NGT were defined according to the WHO criteria from 1999 [243], i.e. OGTT using 2-h post glucose load concentrations and/or use of OHAs.

Information concerning mortality and morbidity from incident disease was collected from the official Swedish registries: Cause of death (CDR), Swedish cancer (SCR) and in-patient (IPR) registries held by The Center for Epidemiology, National Board of Health and Welfare in Sweden. In

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study I the outcome CHD was defined using the registry data as death, as recorded in the CDR or for the first time hospitalized for CHD (ICD-9 codes 410-414, ICD-10 codes I20-I25), as recorded in the IPR, (censor date December 31, 2006). In study II, heart failure was chosen as outcome. Consequently, ICD heart failure codes 428 (ICD-9) and I50 (ICD-10) and hypertensive heart disease with heart failure, I11.0 (ICD-10) were eligible. The medical records from the hospitalization were reviewed for adjudication by two physicians blinded to the baseline data, who classified the cases as definite, questionable, or mis-coded [93]. After this validation, 40 definitive cases of heart failure were included in the cohort.

Study III

Study population

The study subjects were patients with T2DM hospitalized for heart failure at the Departments of Cardiology and Internal

Medicine at Södersjukhuset, Stockholm. Twenty male patients aged 57-79 years with T2DM and worsened heart failure (NYHA class III-IV) were recruited for the study.

Protocol

This was a randomized, cross-over, double-blinded study. The patients underwent i.v. infusions during two consecutive days with a) exenatide ($0.12 \text{ pmol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and b) saline for 6 hours separated by a washout period for 18 hours (figure 7). The order of the two sessions (a and b) was randomized. Hemodynamics; pulmonary capillary wedge pressure (PCWP), right atrial pressure (RAP), and pulmonary arterial mean pressure (PAP) were monitored with a pulmonary artery catheter (Swann-Ganz) and cardiac output (CO) was determined by thermodilution. Cardiac index (CI) was calculated from CO and body surface area (BSA). Mean arterial blood pressure (MAP) was determined by an intra-arterial catheter. Dur-

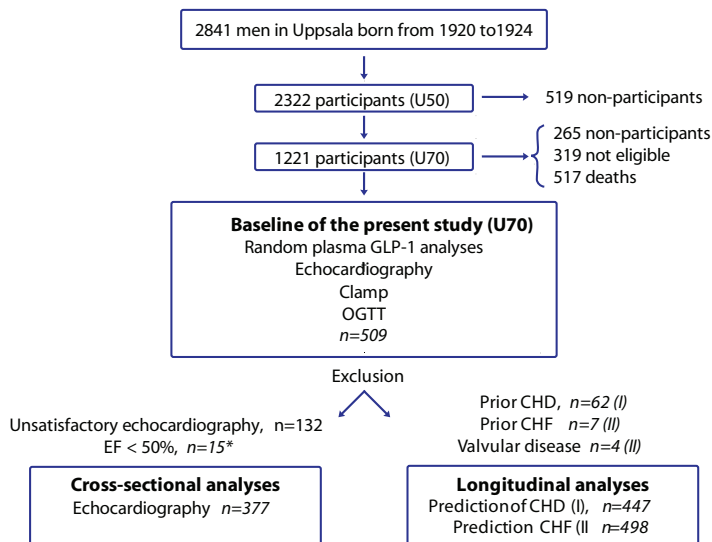


Figure 6. Schematic overview of the Uppsala Longitudinal Study of Adult Men. Study I and study II.

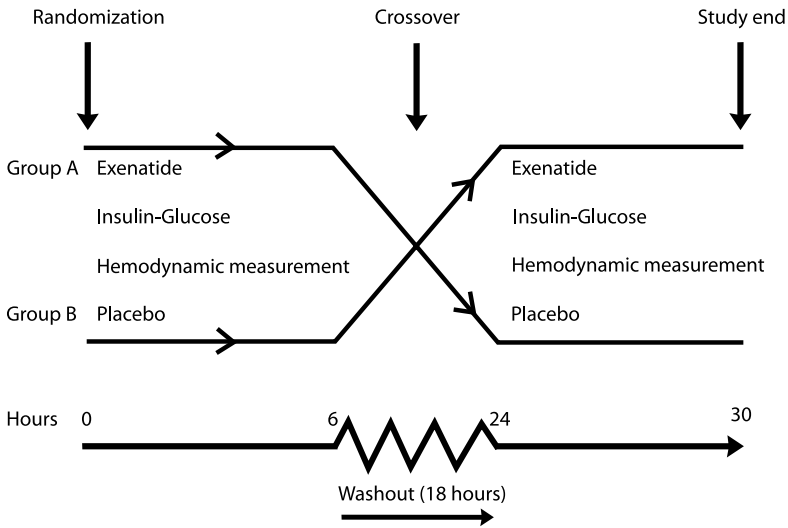


Figure 7. Schematic overview of the study III protocol

ing the 6-hour infusions of placebo/exenatide, all antihyperglycemic drugs were stopped and replaced with i.v. infusion of regular insulin (Actrapid®) to achieve normoglycemia (4–6 mmol/l). During the infusions, CO, PCWP, RAP, PAP and MAP were measured at baseline, 1 hour, 3 hours and 6 hours after start of infusions. Blood samples for analyses of insulin, C-peptide, glucagon, exenatide and NEFA concentrations were drawn simultaneously as the hemodynamic parameters were monitored.

Laboratory studies- protocols

Study IV

Sixty-nine non-diabetic male Sprague-Dawley rats (weight 250–350 g) were anesthetized with a mixture of fluanisone and fentanyl (Hypnorm®, Janssen, Beerse, Belgium) and midazolam (Dormicum®, Hoffman-LaRoche, Basel, Switzerland) (2.5, 0.08 and 1.25 mg/kg, respectively, i.m.). The rats were then killed by excision of the heart. The contractile func-

tion of the vascular segments was tested by administration of phenylephrine. Endothelium-dependent and -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 pending 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% CO_2 in O_2 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), the former serving as a positive control. Vessels were then frozen and stored at -80°C for further analysis, i.e. phosphorylation of eNOS and expression of GLP-1R.

Study V

Human coronary artery endothelial cells (HCAECs) (passage 4-13), isolated from normal human coronary arteries, purchased from Clonetics (Lonza, Walkersville, MD), were grown in complete 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 gentamicin/amphotericin-B at 37°C in a humidified (5% CO₂, 95% air) atmosphere as recommended by the supplier. Confluent cell 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 and Western blotting.

To examine the effects of exenatide on cell viability, DNA synthesis, eNOS- and Akt phosphorylation, HCAECs were grown to 90% confluence, followed by an

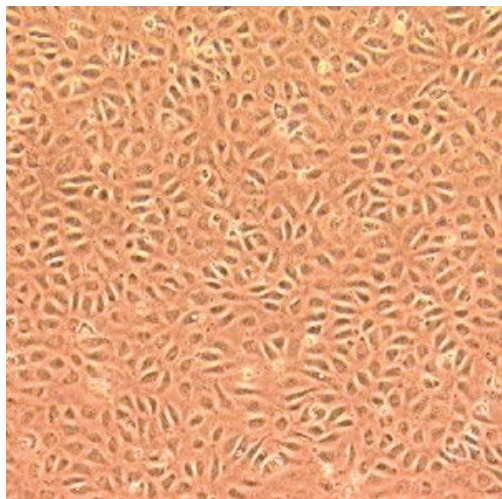


Figure 6. Typical light microscopical appearance of semi-confluent HCAEC cultures.

incubation overnight in serum-deficient EBM medium containing 0.5% FBS and 2 mM L-glutamine. The eNOS inhibitor L-NAME (1 mM) or the PI3K inhibitor LY294002 (2 μM) were added 30 min prior to exenatide stimulation and continuously present as the incubation was continued for 48 h.

Biochemical analyses, clinical investigations and laboratory methods

Plasma glucose

Concentrations of plasma glucose were analyzed by the glucose dehydrogenase method (Gluc-DH, Merck, Darmstadt, Germany).

GLP-1 (Study I and II)

The plasma concentrations of GLP-1 were measured by radio-immunoassays after extraction of plasma with 70% ethanol (vol/vol, final concentration). Carboxy-terminal GLP-1 immunoreactivity was determined using antiserum 89390. This antiserum has an absolute requirement for the intact amidated carboxy-terminus of GLP-1 (7-36) amide and cross-reacts less than 0.01% with carboxy-terminally truncated fragments and 89% with GLP-1 (9-36) amide, the primary metabolite of DPP-4-mediated degradation. Sensitivity was below 5 pmol/l and the intra-assay coefficient of variation below 10% [191].

Immunoreactive insulin (Study I)

Serum immunoreactive insulin (IRI) concentrations were determined with the enzymatic immunological assay Enzymmun®

(Boehringer Mannheim, Mannheim, Germany, [ELISA]) performed in an ES300 automatic analyzer (Boehringer Mannheim, Mannheim, Germany) [244].

OGTT (Study I and II)

A 75 gram OGTT was performed and blood samples for plasma glucose were drawn immediately before and 120 min after the ingestion of glucose. Samples drawn immediately before and 60 min after ingestion of glucose were available for analysis of GLP-1. We calculated the differences between plasma levels of GLP-1 at 60 min after the OGTT (60GLP-1) and fasting plasma levels of GLP-1 (fGLP-1), creating the dynamic predicted variable Δ GLP-1 (60GLP-1 - fGLP-1 = Δ GLP-1).

Euglycemic insulin clamp (Study I and II)

An euglycemic insulin clamp was performed to estimate in vivo sensitivity to insulin with the technique described by DeFronzo [245], with insulin (Actrapid® Human, Novo) infused i.v. at a constant rate of 56 mU/min per BSA (m²) calculated to achieve nearly complete suppression of hepatic glucose output [246]. The target plasma glucose concentration was 5.1 mmol/l. The glucose disposal rate (M) was calculated as the amount of glucose (mg) taken up per kilogram (kg) of body weight per min during the last 60 min of the clamp study and is given in mg · kg⁻¹ · min⁻¹ / (100mU/ml). M/I thus represent the amount of glucose metabolized per unit of plasma insulin. The CV was 13.9%. The OGTT and the clamp procedure were separated in time from each other by one week.

Blood pressure (Study I and II)

Blood pressure was measured once in the right arm to the nearest 5 mm Hg in the supine position after 10 min rest. Systolic and diastolic blood pressure was defined as Korotkoff phases I and V, respectively.

Serum lipids (Study I and II)

Analyses of cholesterol and triglyceride concentrations were performed on a Technicon Auto Analyzer type II. LDL cholesterol levels were calculated using Friedewalds formula: LDL = serum cholesterol - HDL - (0.42 x serum triglycerides) [247].

Cardiac output (Study III)

The thermodilution catheters were inserted via the external or internal jugular veins and the tip of the catheter was advanced 3-5 cm beyond the point where pressure tracings indicated the passage of the pulmonic valve. 7.5-F pulmonary artery thermodilution catheters (AH-05050, Arrow international, Inc., Bernville, PA) sensor and the Siemens sirecust SC 9000XL computer (Siemens, Denver, CO) were used to calculate CO from the modified Stewart-Hamilton equation [64]. The injections were made by hand and always completed within 3 s. The injections were started as soon as the CO computer indicated that the pulmonary artery temperature was stable, that is without taking into consideration the relationship between the injection and the respiratory cycle. The average result of five injections of 10 ml of iced 5% glucose solution was defined as the "true" CO. The computer integrates the temperature curve from the start of the fall in pulmonary artery temperature until the temperature has again risen to 60% above the nadir.

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Invasive arterial blood pressure (Study III)

A catheter with an arterial line primed with NaCl (0.9%) was positioned in the radial artery on the right wrist of all patients. A sterile line set with a disposable pressure biomedical sensor from BD critical care systems (Yishan ave. 7, Singapore) and a positive pressure flushing system was attached to a Siemens HemoPod with a 3-m wired connection to a Siemens sirecust SC 9000XL computer (Siemens, Denver, CO).

Glucagon (Study III)

Glucagon was assayed by a competitive radioimmunoassay (Euria-Glucagon®, Euro-Diagnostica) using a rabbit antiserum raised against a glucagon-albumin conjugate. Glucagon in standards and samples compete with ^{125}I -labeled glucagon in binding to the antibodies in a two-step incubation. ^{125}I -glucagon binds in a reverse proportion to the concentration of glucagon in standards and samples. Antibody-bound ^{125}I -glucagon was separated from the unbound fraction using double antibody solid phase. The radioactivity of the bound fraction was measured in a gamma counter [248].

Insulin and C-peptide (Study III)

Serum levels of insulin and C-peptide were measured by an immunometric method with monoclonal antibodies (Modular E 170, Roche Diagnostics Scandinavia AB).

NEFA (Study III)

Non-esterified fatty acid (NEFA), concentrations were determined colorimetrically with the NEFA-HR(2) kit (Wako Chemicals GmbH, Germany) on a Thermo T20x-

ti instrument (Kone Oy, Finland). In brief, NEFAs were enzymatically converted to acyl-CoA and formed a purple complex with 4-aminantipyrine. The shift in color was spectrophotometrically detected at 546 nm wavelength.

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 into 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 Instruments 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 CO_2 in O_2 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 -independent relaxations were determined by the administration of ACh and SNP, respectively. ACh and SNP were added to the organs baths at cumulatively increasing concentrations (10^{-9} – 10^{-5} mol/l), during a stable contractile tone induced by Phe (10^{-5} mol/l). The relaxatory response following preincubation with a studied substance was always compared to the preceding control response in the same vascular seg-

ment. Exenatide (Neosystems, 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 exenatide, GLP-1 (7-36) (Neosystems, Strasbourg, France) and GLP-1 (9-36) amide (Bachem, Bubendorf, Switzerland) were evaluated by adding cumulative 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 μ M), 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 or vasorelaxant effects per se. Finally, to investigate whether exenatide might co-activate the ACh relaxation, exenatide 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 exenatide against the triglyceride-induced endothelial dysfunction, exenatide (final concentration 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 and V)

Western blotting was applied to quantify the total and phosphorylated eNOS (Ser 1177) or Akt 1/2/3 (Ser 473) proteins in HCAECs (study V) and in aortas from

Sprague-Dawley rats (study IV).

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 \times protease inhibitor cocktail and 2% Triton X-100 in PBS (pH 7.5) for 30 min. The cell lysates were centrifuged (5,000 rpm, 5 min, 4° C) and the supernatant was collected. Equal amounts of protein (10-20 μ g) 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) with 5% non-fat dry milk, followed by an overnight incubation with phospho-eNOS (Ser 1177) or phospho-Akt (Ser 473) antibodies (1:500) in TBS-T/1% bovine serum albumin at 4° C. The membranes were extensively washed and subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG (1:10,000) 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 blots were stripped in Re-blot plus strong solution and probed with either anti-phospho-Akt 1/2/3 (Ser 473) (1:500), anti-eNOS (1:500) or anti- β -actin. The intensities of the bands thus obtained were quantified by densitometry (Gel Doc™, Bio-Rad laboratories, with software Quantity One).

In study IV, frozen artery rings were thawed and homogenized in 100 μ l 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 was examined by a phosphor-spe-

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cific antibody (at amino acid residue Ser 1177) as a measure of eNOS enzymatic activity (Santa Cruz) in a similar manner as described in study V.

NO (Study V)

Direct measurement of NO release from HCAECs was performed using the cell-impermeable fluorescence indicator DAF-2 as described [249]. Cells were incubated in 12-well plates in the presence or absence of exenatide-4 (10 nM) or the inhibitors (1 mM L-NAME, 2 μ M LY294002 or vehicle) in serum-deficient medium for 48 h. 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 NaH₂-PO₄, 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 a positive control. The same concentrations of exenatide or the inhibitors were present in the corresponding wells during the incubation. At the end of the incubation, the supernatants were transferred into black microplates and the fluorescence was measured with a microplate reader (Infinite M200, TECAN) at an 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 lysis buffer containing (in mM) 80 Na₂HPO₄, 20 NaH₂PO₄, 100 NaCl, 1% Triton X-100 (pH 7.5).

Akt activity (Study V)

Phospho-Akt was measured using Pathscan phospho-Akt1 sandwich ELISA kit, according to the manufacturer's instructions. The phospho-Akt specific ELISA detects Akt phosphorylated at serine 473.

Samples were prepared from cells after a 48 h incubation in the presence or absence of exendin-4. (7-36), and 100 μ l aliquots of samples containing equal amount of protein were applied to each well.

[³H]thymidine incorporation (Study V)

Rates of [³H]thymidine incorporation into DNA were analyzed as previously described [250] as a measure of DNA synthesis. In brief, HCAECs were grown in 60-mm Petri dishes and cultured 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 & luminescence counter (Wallac MicroBeta Trilux, PerkinElmer). Results were normalized to the protein concentrations of the samples, determined using BCA kits.

Cell counting and viability (Study V)

HCAECs were incubated in the presence or absence of exenatide 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.

Statistical analyses

All statistical analyses were performed using the statistical software package SPSS 17.0 for PC. Tests were two-tailed, and a P value of less than 0.05 was deemed statistically significant.

Distribution

The distribution of a continuous variable was tested using Shapiro Wilk's test. Skewed variables were log transformed to reach normal distribution (study I). Continuous variables are presented as means \pm standard error of mean (SEM) or \pm SD.

Correlation (study I and II)

Correlations between continuous baseline variables were analyzed using Pearson product correlations.

Group comparisons (Study I, II, IV and V)

Differences between groups were assessed by analysis of variance (ANOVA), one-way repeated measurement, followed by a Bonferroni post-hoc test (Study I, II, IV and V). In analyses using small sample sizes, results from ANOVA were checked with the Kruskal-Wallis and Mann-Whitney tests (study IV and V).

Logistic regression analysis (Study I)

Logistic regression analysis was used to assess associations between baseline GLP-1 variables and dichotomous-dependent variables. Potential associations were presented as odds ratio (OR) with corresponding 95% confidence intervals (CIs) and tested with the Wald test. All analyses were adjusted for age at baseline. In the multivariate models, adjustments were made for the possible confounding effects of M/I, plasma insulin and waist circumference regarding IGT and T2DM subjects. Goodness of fit was assessed by Hosmer-Lemeshow tests.

Survival analyses (Study I and II)

Cox's proportional hazard regression analyses were used in the prospective analyses. Hazard ratios (HRs) were obtained from the Cox's models and are presented with their 95 % CI. Subjects were divided into quartiles according to their Δ GLP-1 levels, and the time from baseline to event in the different groups of quartiles was compared with the use of the log-rank test and the results are presented as Kaplan-Meier curves. Log-minus-log plots were performed to confirm proportionality of the hazards.

Mixed models (Study III)

Linear mixed repeated models, with subjects as a random factor and with treatment and time as fixed and repeated factors, were used to test the effects of treatment on continuous parameters. The unstructured covariance structure was chosen after comparison with other covariance structures. If an overall significant difference was present, pair-wise comparisons with Tukey's least significant difference (LSD) were performed to determine differences between active treatment and placebo.

Ethical considerations

Study I was approved by the ethics committee of the Faculty of Medicine at Uppsala University. Study II was approved of the central ethical review board in Stockholm and the Medical Products Agency (MPA). All human studies conformed to good clinical practice guidelines and followed the recommendations of the Helsinki Declaration. All patients provided written and oral informed consent before enrolment.

RESULTS

Study IV was approved by the regional ethics committee for animal research and conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH publication # 85-23, revised 1985).

Results

Study I

In this study, we demonstrated a reduced glucose-stimulated GLP-1 plasma concentration (Δ GLP-1) in the IGT group compared with the NGT group (figure 9). IGT was associated with lowered Δ GLP-1, lowest vs. the highest quartile (OR 0.3, CI: 0.12-0.58), while no such association was discernable in the T2DM group (OR 1.0,

CI: 0.38-2.86). No association was demonstrated between IGT and fGLP-1 (OR 1.4, CI: 0.78-2.53) or 60GLP-1 (OR 0.82, CI: 0.42-1.6). There was a significant association between Δ GLP-1 and M/I in the T2DM group ($r=0.38$, $P<0.01$), with no such associations noticeable in the IGT ($r=0.11$, $P=0.28$) or NGT ($r=0.10$, $P=0.16$) groups (figure 10). During follow-up, 69 of 294 NGT subjects (rate 2.6/100 PYAR), 42 of 141 IGT subjects (rate 3.5/100 PYAR) and 32 of 74 T2DM subjects (rate 6.0/100 PYAR) developed a CHD event. The median follow-up time was 12.0 years (range, 0.2 to 13.8) with a total of 4,396 PYAR. No association was found between the lowest vs the highest quartile of Δ GLP-1 and CHD, based on 143 individuals that developed CHD (HR 1.0, CI: 0.52-2.28). Unad-

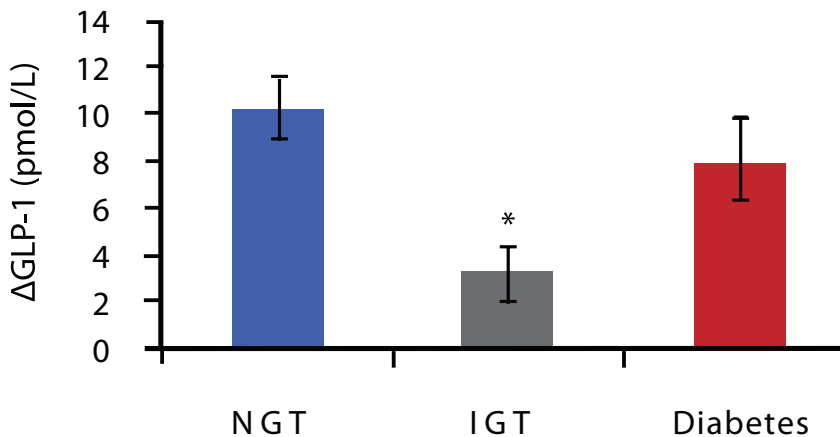


Figure 9. Δ GLP-1 levels among NGT, IGT and T2DM subjects (Study I).

IGT subjects demonstrate significant lower Δ GLP-1 levels compared to NGT subjects. * $P=0.005$. Bars indicate \pm SEM.

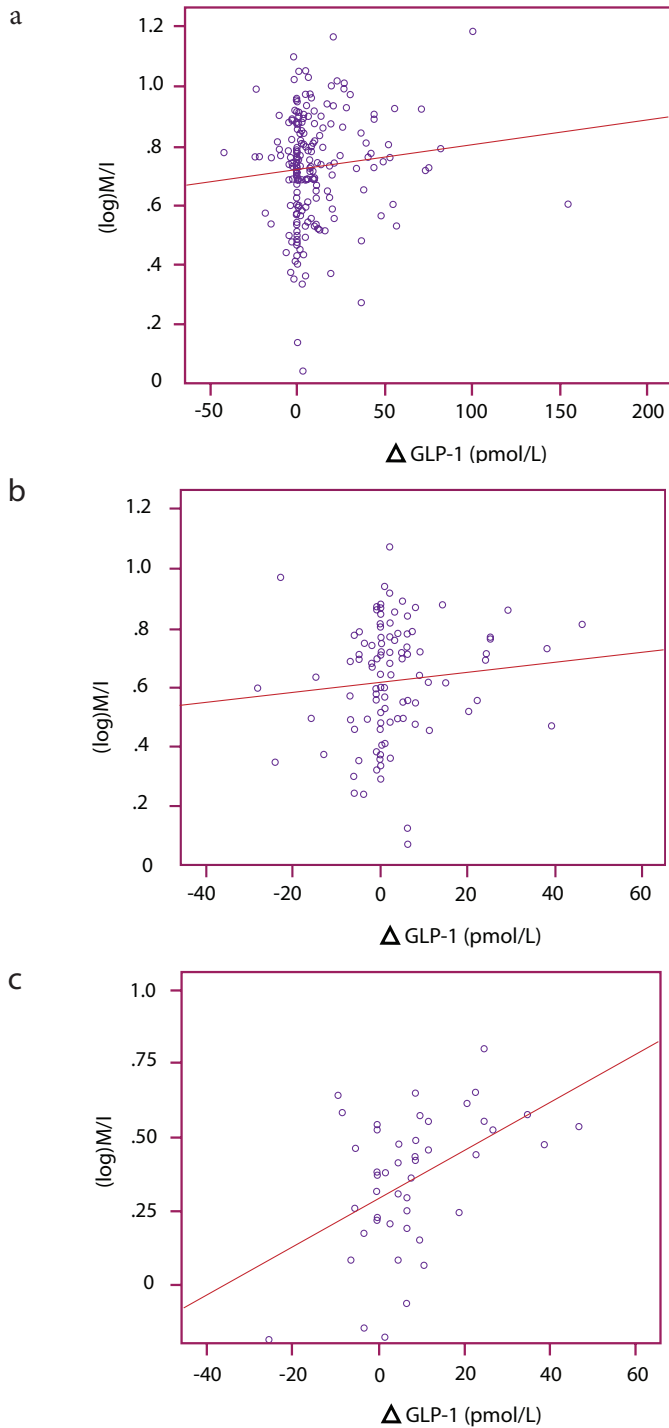


Figure 10. Scatter plots showing correlations between insulin sensitivity index (\log M/I) and Δ GLP-1 at baseline separated by groups (Study I).

a) NGT ($r=0.10$, $p=0.16$), b) IGT ($r=0.11$, $p=0.28$) and c) T2DM ($r=0.38$, $p<0.01$).

RESULTS

justed Kaplan-Meier survival curves could not reveal any significant difference between quartiles of Δ GLP-1 regarding the risk for CHD. Neither fGLP-1 (HR 0.94, CI: 0.45-0.98) nor 60GLP-1 (HR 0.79, CI: 0.37-1.6) correlated with CHD in the same follow-up period.

Study II

During the follow-up period, 16 of 290 participants with NGT experienced a CHF event (rate 0.7/100 PYAR), as did 8 of 136 (rate 0.8/100 PYAR) with IGT and 9 of 72 (rate 1.7/100 PYAR) with T2DM (Figure 11.). Although plasma GLP-1 concentrations did not predict CHF (fGLP-1: HR 0.98, 95% [CI 0.4-2.4], 60GLP-1: HR 1.1, 95% [CI 0.4- 2.6], Δ GLP-1: HR 0.9, 95 % [CI 0.3-2.3]), there was an association between left ventricular diastolic function

(E/A ratio) and fGLP-1 ($r=0.19$, $P=0.001$), 60GLP-1 ($r=0.20$, $P<0.001$), and Δ GLP-1 ($r=0.18$, $P=0.004$). When the associations between E/A ratio and GLP-1 were analyzed in the group that was investigated with echocardiography at baseline, these correlations remained significant. Interestingly, we now found a stronger correlation between glucose-stimulated GLP-1 levels (60 min post challenge) and E/A ratio in the T2DM group ($r=0.58$, $P=0.04$).

Study III

Twenty male T2DM patients with known CHF were enrolled and completed the protocol. Baseline hemodynamic variables were consistent with decompensated heart failure with depression of CI and elevation of PCWP (Table 3). Exenatide compared

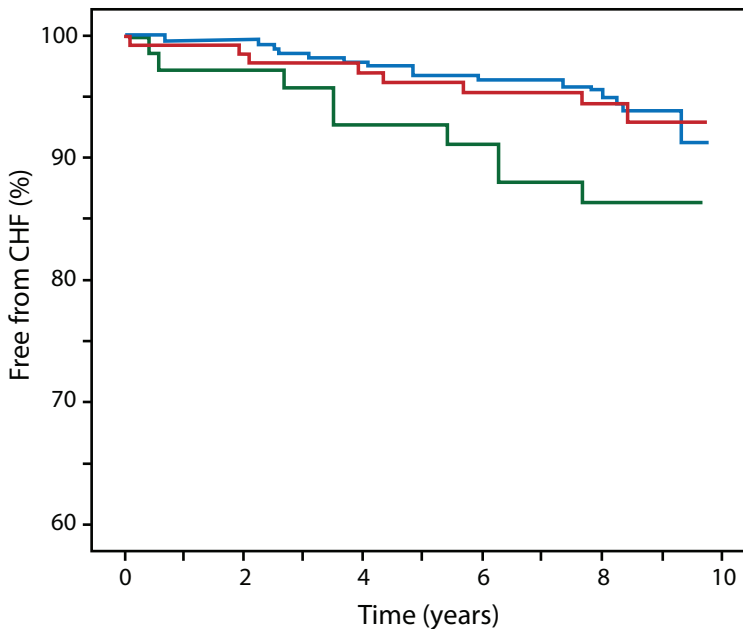


Figure 11. Kaplan-Meier survival curves for participants free from subsequent CHF events during 9.8 years of follow-up (Study II).

NGT (blue line), IGT (red line) and T2DM (green line). The risk of CHF was significantly higher in men with T2DM than for those with NGT (log rank test, $P=0.02$).

Table 3. Baseline hemodynamic and metabolic parameters.

Parameter	Value
Baseline hemodynamics	
HR, (bpm)	73±4
RAP (mmHg)	9±1
PAP (mmHg)	28±2
PCWP (mmHg)	17±2
MAP (mmHg)	92±4
CO (L · min ⁻¹)	4±0.2
CI (L · min ⁻¹ · m ⁻²)	1.8±0.1
SV (ml)	57±4
PVR (Wu)	3±0.4
SVR (dyne · s · cm ⁻⁵)	1750±120
Baseline metabolic parameters	
Plasma glucose (mmol/L)	6.6±0.3
Serum C-peptide (nmol/L)	1.2±0.2
Serum insulin (pmol/L)	440±120
Serum glucagon (pmol/L)	40±3
Plasma NEFA (mmol/L)	0.3±0.06
Plasma lactate (mmol/L)	1±0.09

Values are means ± SE. HR, heart rate; RAP, right atrial pressure; PAP, pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; MAP, mean arterial blood pressure; CO, cardiac output; CI, cardiac index; SV, stroke volume; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance NEFA, non-esterified fatty acids.

with placebo increased CI by a maximum of 21% concomitant with lowering of PCWP and right atrial pressure (RAP) by 15% and 17%, respectively (figure 12). The increase in CI was entirely contingent upon an increase in HR of 29% without any change in SV indicative of a positive chronotropic – but not inotropic – effect of exenatide in this acute setting. Glu-

cose concentrations were kept within the normoglycemic range during exenatide-placebo infusions by adjustment of the insulin-glucose infusion rate. There were no differences in glucagon and insulin concentrations during exenatide vs. placebo infusions. NEFA levels tended to rise during both infusions but were significantly

RESULTS

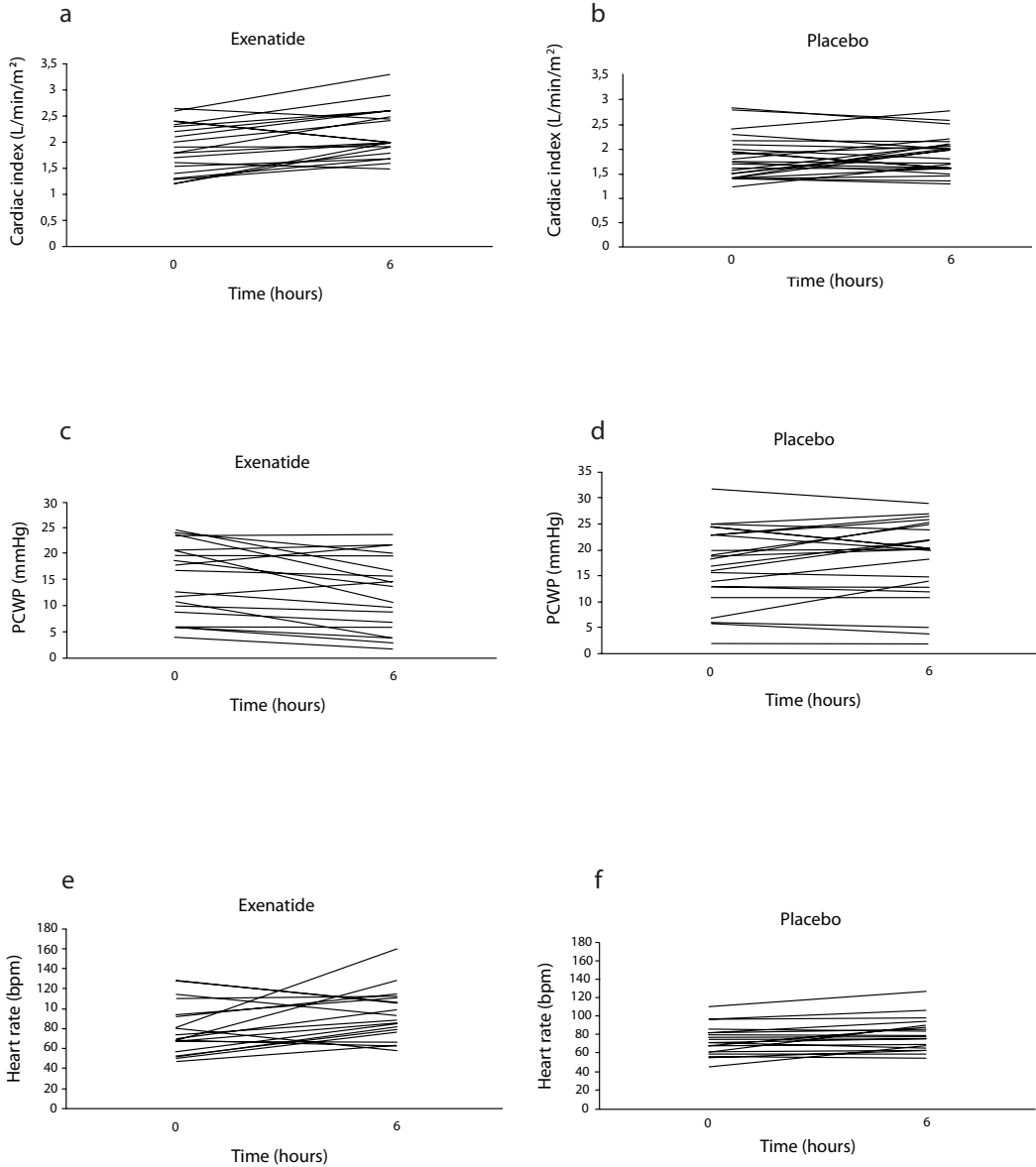


Figure 12. Changes in CI, PCWP and HR during 6 hours of infusion of exenatide and placebo.

Individual data. a, changes in CI during exenatide infusion. b, changes in CI during placebo infusion. c, changes in PCWP during exenatide infusion. d, changes in PCWP during placebo infusion. e, changes in HR during exenatide infusion. f, changes in HR during placebo infusion.

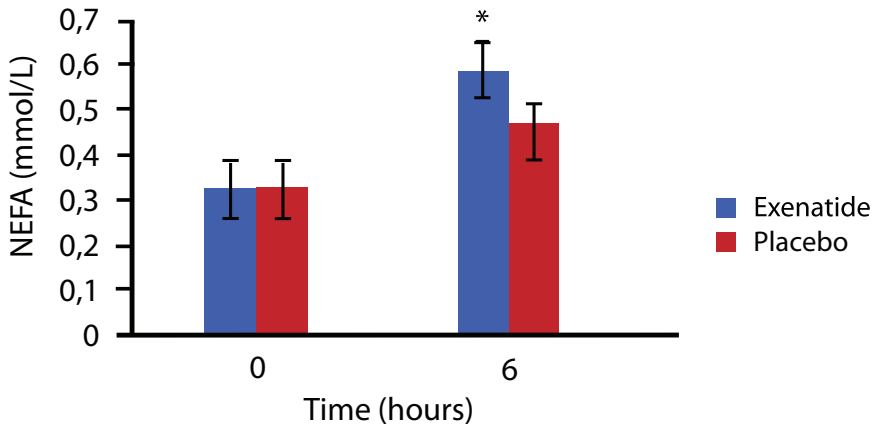


Figure 13 . Effects of infusion with exenatide on NEFA levels.

Fatty acid levels are shown for exenatide and placebo over 6-hour double-blind infusions. Values are means \pm SE.* $P=0.03$ (exenatide vs. placebo at 6 hours).

higher during exenatide infusion compared to placebo infusion ($P=0.03$; figure 13). All participants completed the protocols. However, we observed ten adverse events during the exenatide infusion. Six of these were related to nausea and vomiting, but two patients experienced a temporary increase in heart rate to such an extent that it required treatment with digoxin i.v. and in one patient an increase in systemic blood pressure necessitated a short term i.v. infusion with nitroglycerine.

Study IV

The main finding in this study is that both native GLP-1 (7-36) and its main metabolite GLP-1 (9-36), but not exenatide, exert acute vasorelaxant effects in non-diabetic rat arterial rings in an ex vivo model. The primary aim was to investigate whether exenatide may confer protection against endothelial dysfunction, induced by a triglyceride-rich fat emulsion (Intralipid[®]), in these vascular rings. However, this short-term exenatide exposure did not protect

against the triglyceride-induced endothelial dysfunction. Immunoblotting data revealed a single band with a molecular mass of 67 kDa, corresponding to the GLP-1 receptor, demonstrating expression of the GLP-1 receptor in the artery. Immunoblotting also revealed a significant increase in eNOS enzymatic activity by Intralipid[®] incubation at both 0.5 and 2% of the emulsion. There was a clear tendency towards decreased Intralipid[®]-induced eNOS activation by co-incubation with exenatide, although this effect did not attain statistical significance.

Study V

The main result of this study was that exenatide, GLP-1 and GLP-1 (7-36) dose-dependently enhanced HCAEC DNA synthesis, as reflected by an increased rate of ³H-thymidine incorporation. Consistent with the stimulatory effect of exenatide on DNA synthesis, the total cell number was significantly increased in the presence of exenatide, thus confirming that DNA

GENERAL DISCUSSION

synthesis was indeed followed by mitosis. Application of exenatide, GLP-1 (7-36) and GLP-1(9-36) to the HCAECs also elicited a dose-dependent phosphorylation of eNOS at Ser-1177, thereby enhancing its catalytic activity. Exenatide GLP-1 (7-36), GLP-1 and GLP-1 (9-36) also augmented Akt phosphorylation, indicating activation of the enzyme. In the presence of a specific PI3K inhibitor, the effect of exenatide, GLP-1 (7-36) and GLP-1 (9-36) on eNOS and Akt phosphorylation was abolished, indicating that eNOS phosphorylation induced by these peptides is mediated by PI3K-Akt-dependent signaling pathways. NO production stimulated by these peptides was completely abolished by either eNOS inhibitor (L-NAME) or PI3K inhibitor (LY294002) at the same concentrations that blocked phosphorylation of eNOS.

The stimulated DNA synthesis was further evaluated in the presence of the eNOS inhibitor L-NAME and the PI3K inhibitor LY294002. At the same concentrations that abolished phosphorylation of Akt and eNOS, the augmented DNA synthesis rate evoked by the peptides was completely abolished by L-NAME and suppressed by ~ 70 % by the PI3K inhibitor. Finally, the increases in DNA synthesis and eNOS and Akt activation evoked by the peptides, were inhibited by PKA inhibitors and blocked by a GLP-1 receptor antagonist. A cartoon schematically depicting the intracellular chain of events, occurring between GLP-1R occupancy to cell mitosis, is shown in figure 14.

General discussion

T2DM does not only increase the risk of incident CVD and associates with poor survival but is also an independent risk factor for CHF which is a malignant dis-

ease with a dire prognosis. To date it is, despite considerable efforts, difficult to point out which of the existing anti-hyperglycemic drugs that might be the treatment of choice targeting this increased risk of CVD and CHF. The most recent contribution to the armamentarium of antihyperglycemic treatment is incretin-based drugs. There is now a growing body of evidence that these

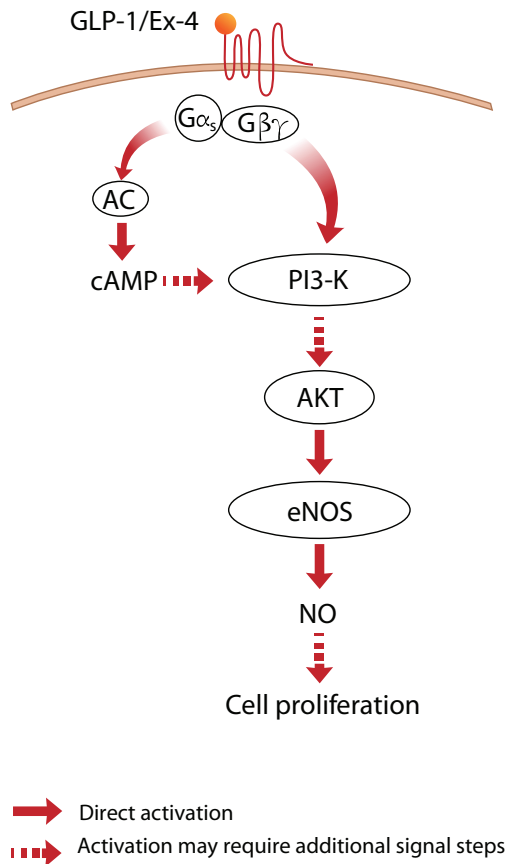


Figure 14. Schematic model of GLP-1-stimulated proliferation of HCAECs.

Stimulation of the endothelial cells with GLP-1/exenidin-4 through occupancy of their cell surface GTP-binding protein-coupled receptor 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.

agents exert, besides their well known anti-hyperglycemic and weight-reducing properties [176, 180-182, 185, 186], direct effects on the endothelium, vasculature and the failing heart [194, 197, 206, 211-214, 234-236, 251-256].

Our intention has been to investigate physiologic actions of incretins and pharmacologic effects on the human heart and the vasculature and to reveal the underlying mechanisms behind these putative effects. To this end, we have employed several widely different methodological approaches, such as epidemiologic analyses, a randomized clinical trial of intervention and finally laboratory experimental investigations on animals and cells. In the cellular perspective, we gathered evidence for proliferative effects of native GLP-1 (7-36), its degradation metabolite GLP-1 (9-36) and its stable analog exenatide in HCAECs. These effects might serve to limit the adverse consequences of the macrovascular complications of diabetes as proliferative dysfunction of endothelial cells is believed to contribute to premature development of atherosclerosis [257]. More specifically, it is our belief that GLP-1 might “patch up” an early endothelial lesion occurring in the highly pro-atherogenic diabetic milieu by rapidly covering it with endothelial cells. Moreover, angiogenesis can be of potential clinical benefit in patients with ischemic heart disease or peripheral arterial disease, but neovascularization and vasoproliferation are also parts of the pathological processes leading to diabetic retinopathy [258]. However, it has to be noted that proliferation of endothelial cells is just one part of the complex sequence leading to angiogenesis [259].

In the ex vivo setting (paper IV), we found vasorelaxant effects of GLP-1 and its metabolite (9-36) at slightly supraphysiological concentrations in a model of conduit arteries in non-diabetic rats, whereas no such effects of exenatide were noted suggesting vast differences in vasoactive

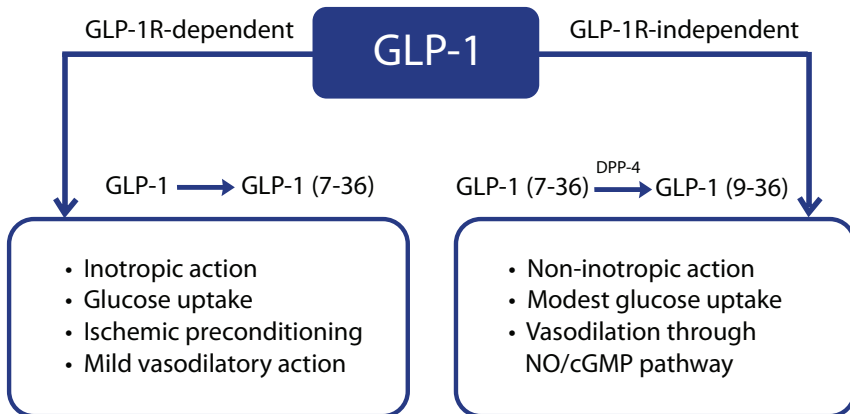


Figure 15. GLP-1R-dependant and GLP-1R-independent effects.

Model for explaining actions of GLP-1 (7-36) and GLP-1 (9-36) the vasculature and heart suggesting GLP-1 receptor - depending and - independent effects. Adapted from Circulation: Bank, Cardioprotective and Vasodilatory Actions of Glucagon-Like Peptide 1 Receptor - dependent and - Independent Pathways, Circulation, page 2340-2350, © (2008).

GENERAL DISCUSSION

potency between native GLP-1 and its metabolite on the one hand and the stable GLP-1 analogue exenatide on the other. This is supported by the finding that GLP-1 (7-36) and GLP-1 (9-36) but not exenatide exert vasodilatation in mesenteric arteries in wild type and in GLP-1R^{-/-} mice [197].

In our study (IV), exenatide failed to protect the arteries against triglyceride-induced endothelial dysfunction. These findings might be explained by differences in affinity to the GLP-1 receptors in the endothelium but this could not be experimentally proven, due to difficulties to detect any significant binding of GLP-1 in the endothelium. Another explanation for differences in cardiovascular effects between GLP-1 (7-36), GLP-1 (9-36) and exenatide may be the receptor-independent actions of GLP-1 proposed by Ban et al. [197] (figure 15). The concept of GLP-1R-independent cardiovascular effects was further supported in a study where the GLP-1R antagonist exendin (9-39) inhibited hindquarter vasodilatation induced by exenatide but did not affect the mesenteric vasoconstriction in Sprague-Dawley rats [211].

Study III demonstrated potent acute effects of exenatide on critical hemodynamic parameters in patients with T2DM hospitalized for decompensated heart failure. These effects appear to have occurred independent of changes in glycemia. The clinical impact of these findings, if any, is more difficult to interpret as we observed both positive, i.e. increase in CI and decreases of PCWP and RAP and potentially detrimental effects, i.e. increases in heart rate and NEFA levels). Recently published studies on a rodent model indicate that exenatide exerts tachycardia, vasoconstriction in mesenteric arteries and vasodilatation in hindquarters [211, 226]. The results in these studies were consistent

with the view that exenatide exerts cardiovascular effects, some of which involves sympathoadrenal activation [211, 226]. Therefore it seems prudent to believe that sympathoadrenal activation could have contributed to the increases in heart rate and NEFA levels that were observed in study III.

In summary, the studies in this thesis focus on potential effects of GLP-1 and exenatide in the vasculature and heart. We found that native GLP-1 (7-36), its main metabolite GLP-1 (9-36), and to a lesser extent exenatide, have direct effects on human coronary artery endothelial cells and rodent arteries in ex vivo settings. There are some contradictory findings in the preclinical experimental studies, i.e. paper IV and V. In study V, exenatide exerted activation of eNOS with a concomitant increase of NO production but we could not detect any activation of eNOS by exenatide in study IV. These differences might simply be explained by the fact that the studies were evaluating effects on different species and models, i.e. in vitro studies of HCAECs vs. ex vivo studies on rat femoral arteries.

We found potent hemodynamic effects of exenatide when administered to diabetic patients with worsened CHF. The observed effects were both beneficial and potentially detrimental, but the drug (exenatide) was fairly well tolerated in this group of hospitalized diabetic patients with decompensated CHF. Studies of the clinical implications of the use of exenatide in patients with decompensated heart failure are still in their infancy and therefore further prospective studies with clinical hard end-points, such as IHD morbidity and mortality, are very much needed. Although we have not compared the effects of native GLP-1 and exenatide in a clinical trial, our ex vivo study indicates that the

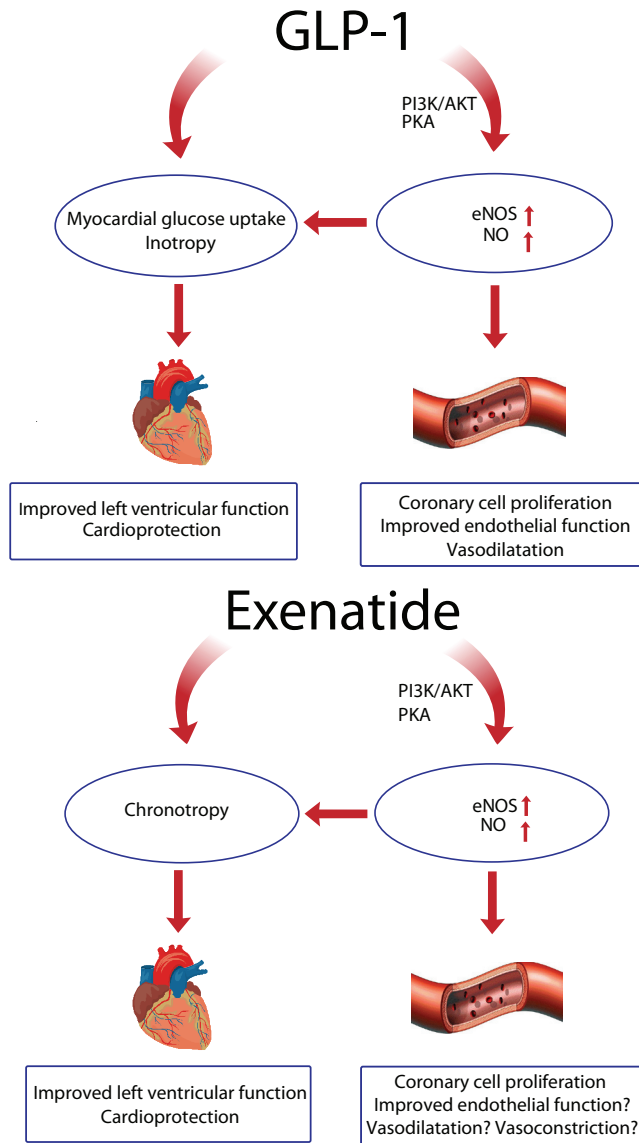


Figure 16. Cardiovascular effects of GLP-1 and exenatide.

Differences in cardiovascular actions between GLP-1 (A) and exenatide (B). Adapted from: Nyström T, The Potential Beneficial Role of Glucagon-like Peptide-1 in Endothelial Dysfunction and Heart Failure Associated with Insulin Resistance, *Horm Metab Res*, page 601, © (2008), with permission from Georg Thieme Verlag KG.

cardiovascular effects of GLP-1 (7-36) and GLP-1 (9-36) might be more pronounced than the effects of exenatide. These differences might be explained by both receptor dependent and receptor independent actions. Moreover, the molecular structure

of exenatide is clearly different from that of native GLP-1; in fact, exenatide only share 53% of the sequence homology of the native GLP-1. A unifying scenario of GLP-1 and exenatide cardiovascular effects is schematically proposed in figure 16.

FUTURE DIRECTIONS

Finally it seems prudent to believe that GLP-1 does not act as a risk marker for CVD. However, it must be noted that study II was somewhat under-powered to conclude that alterations in GLP-1 levels cannot be associated with the incidence of CHF. There is evidence that NO exerts favorable effects on left ventricular diastolic distensibility [97]. Therefore, our observed association between GLP-1 concentrations and diastolic function could be explained by alterations in NO production as we have demonstrated that GLP-1 increases NO production in HCAECs. This might be mediated by PI3K and Akt signaling pathways. As we cannot prove any causality of the association between plasma GLP-1 levels and diastolic function there is a need for further longitudinal studies in this field of research.

Future directions

So far there is evidence of GLP-1 action on vessels and heart. However, it is puzzling that there seems to be substantial differences between native GLP-1 and stable derivatives thereof, i.e. exenatide that only partially share amino acid sequence homology with the native GLP-1. There is also evidence for cardiovascular effects of the main metabolite GLP-1 (9-36) that are almost identical to those of GLP-1 (7-36). This could be explained by a specific GLP-1 (9-36) receptor that mediates these cardiovascular effects (figure 15). The existence of such a receptor has already been suggested by Ban and coworkers [221]. More detailed investigation and characterization, e.g. sequencing and cloning of such a putative receptor could be of great value in the further exploration of this field of research. Furthermore, the mitogenic effects of GLP-1 and exenatide should be evaluated in retinal endothelial cells. Increased

proliferation of these cells could indicate an increased risk for retinopathy.

As existing incretin-based drugs do not increase the GLP-1 (9-36) concentrations, and taking into consideration the evidence of cardiovascular effects of this GLP-1 isoform, efforts aimed at finding drugs that increase both GLP-1 (7-36) and GLP-1 (9-36) may prove fruitful. Finally, it would be interesting to explore the putative effects of incretins on diastolic cardiac dysfunction in interventional prospective studies.

Limitations of the studies

- I&II:** These studies are based on elderly male patients, mainly Caucasians, which limits the generalizability of the results. In study II there were few events of CHF, which diminishes the power of the study.
- III:** Although a power calculation was made for this study, it was a small study with limited generalizability; the results cannot necessarily be extrapolated to a female, younger, non-diabetic or ethnically heterogeneous population receiving chronic treatment.
- IV:** It would have been convenient, although difficult, to include investigations on small vessels (resistance or capillary vessels), and to expand the work to include studies of chronic exposure, in vivo settings, both genders, and diabetic models.
- V:** We have only studied relatively short-term pharmacological effects of GLP-1, and derivatives thereof, on HCAECs in vitro from healthy subjects (non-diabetic individuals devoid of CVD). Further studies on conditional GLP-1R knock-out mice (soon to be acquired by our laboratory) and HCAECs in which GLP-1R is either over-expressed or silenced (by siRNA) and under varying glycemic states would have fortified the conclusions of this study.

General Conclusions

In agreement with our hypothesis, we have found evidence of broad cardiovascular actions of GLP-1 and related peptides in a translational perspective; we were able to demonstrate cardiovascular effects of GLP-1 and its derivatives in human cellular models in vitro, rodent ex vivo model and a randomized clinical trial in T2DM patients and also found correlative associations between plasma GLP-1 levels and CVD in epidemiologic cohort studies.

We did not find any evidence of GLP-1 as a predictor for CHD or CHF in a population-based longitudinal study of elderly men i.e. the ULSAM cohort. Nonetheless, we found a significant correlative association between measures of cardiac diastolic dysfunction and GLP-1 levels in a cross-sectional analysis of the ULSAM cohort.

The GLP-1 analog exenatide was found to exert potent and rapid hemodynamic effects in patients with T2DM hospitalized for CHF; exenatide increased CI and decreased PCWP and RAP significantly, but also increased heart rate and NEFA levels vs. placebo. As there seems to be both beneficial and potentially detrimental acute effects of exenatide in this group of patients, further prospective studies are needed. GLP-1 (7-36) and GLP-1 (9-36), but not exenatide, were found to exert vasorelaxant effects in femoral arteries ex vivo in non-diabetic rats. Exenatide failed to protect against triglyceride-induced endothelial dysfunction in this system.

Exenatide, GLP-1 (7-36) and GLP-1 (9-36) promoted mitogenesis and enhanced eNOS and Akt activation in HCAECs in vitro. These effects were prevented by PKA-, PI3K-, Akt- and eNOS-inhibitors, suggesting that the incretins stimulate cell proliferation through PKA-PI3/Akt-eNOS-dependent pathways.

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