ON ENDOTHELIAL FUNCTION IN TYPE 2 DIABETIC PATIENTS WITH CORONARY ARTERY DISEASE

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Stockholm 2005
TO MY FAMILY
Abstract

Patients with type 2 diabetes have a poor outcome suffering a myocardial infarction (MI). Endothelial dysfunction may play a role in this poor prognosis. In type 2 diabetes, endothelial dysfunction is a salient feature coexisting with obesity and insulin resistance and may be linked by the same pathophysiology, i.e. low-grade inflammation. The aim of this work was to investigate endothelial function, i.e. flow-mediated vasodilation (FMD) and nitroglycerin induced vasodilation (NTG), in type 2 diabetic patients suffering a recent MI, in different working models.

Study I: In this prospective cohort study, we investigated temporal changes in endothelial function and inflammatory activity (C-reactive protein [CRP] and adiponectin) in type 2 diabetic and non-diabetic patients suffering an MI. Type 2 diabetic patients demonstrated a persistent endothelial dysfunction, which coincided with a persistent low-grade inflammation as reflected by elevated CRP levels. Changes in CRP negatively correlated with changes in FMD. Adiponectin levels were also lower in type 2 diabetic patients but showed no correlation to endothelial function.

Study II: This cross-sectional study comprised 20 type 2 diabetic and 20 non-diabetic patients with a recent MI. We investigated the association between lipids, CRP, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), insulin sensitivity (SI) and adipokines (adiponectin and resistin) and endothelial function. FMD and NTG were both impaired in type 2 diabetic patients concomitant with increased TNF-α and IL-6 levels and decreased levels of adiponectin. TNF-α concentrations and brachial artery diameter were negatively, whereas SI was positively associated with FMD.

Study III: This was a cross-over study, where we compared the acute effects of the endothelial nitric oxide synthase cofactor, tetrahydrobiopterin (BH4), between groups of 12 type 2 diabetic and 10 non-diabetic patients with a recent MI. Subjects were tested twice, one week apart, regarding FMD/NTG and SI during infusion of BH4 or placebo. BH4 improved glucose disposal in type 2 diabetic patients, without any effects in other groups. This beneficial effect of BH4 occurred without any discernable changes in FMD.

Study IV: Twenty-two male subjects, of whom 12 were type 2 diabetic patients with a recent MI and the remaining 10 were unmatched healthy subjects, took part in this randomized cross-over study. Subjects were tested twice, one week apart, regarding FMD/NTG and SI during short-term infusion of the emerging antidiabetic drug glucagon-like peptide-1 (GLP-1) or placebo. Also, we investigated whether GLP-1 receptors are expressed on endothelial cells. GLP-1 improved FMD in type 2 diabetic patients, without any effects in healthy subjects and the receptor for GLP-1 on endothelial cells was demonstrated. No effects on SI were noted after short-term GLP-1 infusion.

Study V: This study was conducted to investigate whether GLP-1 directly relaxes conduit vessels. It was found that GLP-1 relaxed femoral artery rings from male Sprague-Dawley rats ex vivo in a dose-response manner, via an endothelium-independent mechanism. The GLP-1 relaxation effect was completely attenuated by the specific GLP-1 receptor antagonist exendin(9-39), indicating the requirement for specific GLP-1 receptor occupancy.

Conclusions: In type 2 diabetic patients with a recent MI, prolonged endothelial dysfunction, pro-inflammatory activity and low plasma adiponectin concentrations coexist. FMD seems to be inversely associated with CRP and TNF-α and to some extent SI. BH4 enhances glucose disposal in type 2 diabetic patients, which may be due to a capillary recruitment mechanism. GLP-1 ameliorates endothelial dysfunction in type 2 diabetic patients with a recent MI and dose-dependently relaxes rat conduit vessels ex vivo. Improvement of insulin resistance and endothelial dysfunction may translate into beneficial effects on many cardiovascular risk factors and may thus have important clinical implications in preventing macroangiopathy in type 2 diabetes.

Key words: Type 2 diabetes, endothelial dysfunction, insulin resistance, coronary artery disease, nitric oxide, glucagon-like peptide 1, exendin, tetrahydrobiopterin, inflammation, adiponectin, resistin, C-reactive protein, tumor necrosis factor alpha, interleukin-6
List of original papers

This thesis is based on the following original articles, which will be referred to by their Roman numerals


II. Nyström T, Nygren A, Sjöholm Å. Is a more aggressive inflammation in coronary arteries behind myocardial infarction in type 2 diabetes patients? Relationship between endothelial dysfunction and tumor necrosis factor–alpha. *Manuscript*


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List of abbreviations

ACE-i  ACE inhibitor
ARB  Angiotensin receptor blockers
AT-II  Angiotensin-II
BH4  Tetrahydrobiopterin
BMI  Body mass index
BRIN  Insulinoma-derived cell line BRIN-BD11
CAD  Coronary artery disease
cGMP  cyclic GMP
CRP  C-reactive protein
CVD  Cardiovascular disease
DPPIV  Dipeptidyl peptidase IV
EC  Endothelial cells
ECG  Electrocardiogram
EDRF  Endothelium-derived relaxing factor
EDHF  Endothelium-derived hyperpolarizing factor
ELISA  Enzyme-linked immunosorbent assays
eNOS  Endothelial nitric oxide synthase
ET-1  Endothelin-1
FFA  Free fatty acids
FMD  Flow-mediated vasodilation
GLP-1  Glucagon-like peptide-1
GLUT-4  Glucose transporter isoform-4
HCAEC  Human coronary aortic endothelial cells
hsCRP  high-sensitive C-reactive protein
IFG  Impaired fasting glucose
IGT  Impaired glucose tolerance
IL-1  Interleukin-1
IL-6  Interleukin-6
KH  Krebs–Henseleit
L-NNA  N-nitro-L-arginine
MAP  Mitogen-activated protein
MI  Myocardial infarction
NO  Nitric oxide
NTG  Nitroglycerin-mediated vasodilation
PAI-I  Plasminogen activator inhibitor-I
PGH$_2$  Prostaglandin
PGI$_2$  Prostacyclin
PI3-kinase  Phosphatidylinositol 3-kinase
ROS  Reactive oxygen species
SMCs  Smooth muscle cells
SNP  Sodium nitroprusside
SU  Sulfonylurea
TBS-T  Tris-buffered saline Tween 20
TNF-a  Tumor necrosis factor-alpha
TZDs  Thiazolidinediones
WHO  World Health Organization
GLP-1 ................................................................. 27
Exendin(9-39) .......................................................... 28
Flow-mediated (FMD) and nitroglycerin-mediated (NTG) vasodilation .......... 28
Isoglycemic hyperinsulinemic clamp ................................................. 29
Arterial rings in organ baths .................................................................. 29
Cell culture and Western blotting ......................................................... 30
Statistical analyses ............................................................................. 30
Ethical considerations ......................................................................... 30

Results and Discussion ........................................................................ 31
   Endothelial dysfunction, insulin resistance and low-grade inflammation in type 2 diabetic patients with CAD (Paper I & II) .................................. 31
   Paper I ......................................................................................... 31
   Paper II ......................................................................................... 32
   Tetrahydrobiopterin promotes glucose disposal in type 2 diabetes (Paper III) .................. 34
   Effects of GLP-1 on endothelial function in type 2 diabetic patients (paper IV) ............ 35
   GLP-1 relaxes conduit arteries (Paper V) .......................................... 36

General discussion .............................................................................. 38
   Future directions ........................................................................... 39
   Limitations of the studies ................................................................. 41

Conclusions ....................................................................................... 42

Acknowledgments ............................................................................. 43

References ......................................................................................... 45

Paper I-V
Cardiovascular disease (CVD) is by far the most common complication of type 2 diabetes and also the most serious one. Suffering from type 2 diabetes not only dramatically increases the risk of CVD but is also associated with poor survival, both acutely and in the long term after a myocardial infarction (MI) (1). In fact, total mortality from coronary artery disease (CAD) in subjects with type 2 diabetes without a previous MI, is as high as that of non-diabetic individuals with a previous MI (2). Intense research efforts have thus been directed towards exploring the reasons for why particularly type 2 diabetic patients have such a poor prognosis suffering from CVD (1,3-12). Atherosclerosis, no matter which risk factors involved, is an inflammatory disease where endothelial dysfunction is a crucial factor in all stages of the atherosclerotic process (13). Endothelial dysfunction, widespread in type 2 diabetes and insulin resistant states (9), relates to the risk for an initial or recurrent cardiovascular event (14,15). Inflammation induced by the innate immunity may play a role in development in-insulin resistance and type 2 diabetes (16). Thus, the pathophysiology of endothelial inflammation and insulin resistance may share a common inflammatory basis (17). In insulin resistant states the contribution of hyperglycemia, dyslipidemia, obesity, hypertension and low-grade inflammation affect the endothelium negatively in a multiple and complex way (3,5). The combined effects of these factors on the endothelium may explain the poor outcome in type 2 diabetic patients suffering an MI.

THE METABOLIC SYNDROME

Most patients who develop CVD present a cluster of risk factors. These risk factors, e.g. hypertension, dyslipidemia and hyperglycemia, were noted by Reaven as a resistance to insulin-stimulated glucose uptake (21). He postulated that the changes associated with resistance to insulin-mediated glucose uptake comprise a syndrome, named syndrome X, which plays an important role in the etiology and clinical course of patients with type 2 diabetes, hypertension, dyslipidemia and coronary heart disease.
disease (22). In 1998, the WHO defined these clustered risk factors as the metabolic syndrome (20). This classification was based on the therapeutic challenge in a person with insulin resistance, hypertension, central obesity, dyslipidemia and microalbuminuria, with or without hyperglycemia, a person at very high risk of CVD (20). The definition by WHO of the metabolic syndrome is based on any sign of insulin resistance, i.e. impaired glucose tolerance (IGT), impaired fasting glucose (IFG) or diabetes, together with two of more of the components: hypertension, hypertriglyceridemia, low HDL-cholesterol, central obesity and microalbuminuria (table 1). Recently, the National Cholesterol Education Program's Adult Treatment Panel III (ATP III) report (23) identified six components of the metabolic syndrome related to CVD, namely abdominal obesity, atherogenic dyslipidemia, hypertension, insulin resistance, pro-inflammatory state and pro-thrombotic state. Based on these six components ATP III proposed in 2001 (23), that when three out of five factors are present (abdominal obesity, hypertriglyceridemia, low HDL-cholesterol, hypertension, and IFG), a diagnosis of metabolic syndrome can be made (table 1). The main differences between these two definitions are that the WHO considers insulin resistance in the syndrome, whereas the ATP III definition considers only IFG, which even needs not to be included, in its definition of the metabolic syndrome (24).

Approximately 80 % of all type 2 diabetes coexists with the metabolic syndrome (17) and a recent meta-analysis revealed that in a European based population 15 % have the metabolic syndrome, without having diabetes (25), a

Figure 1. Pathogenesis of type 2 diabetes

The pathophysiology of type 2 diabetes involves defects in several cornerstone organ systems, i.e. liver, pancreas, adipose tissue and skeletal muscle conspire together to produce abnormal glucose and lipid metabolism. At some point; the cells can no longer compensate causing insulin secretion to decline and fail to respond appropriately to glucose.
Table 1. Definition of the metabolic syndrome by WHO and ATP III criteria.

<table>
<thead>
<tr>
<th>Insulin resistance, identified by one of the following</th>
<th>Cut-off level</th>
<th>ATP III Risk factors</th>
<th>Cut-off level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 2 diabetes</td>
<td></td>
<td>Abdominal obesity, given as waist circumference</td>
<td></td>
</tr>
<tr>
<td>Impaired fasting glucose</td>
<td></td>
<td>Men</td>
<td>&gt;102 cm</td>
</tr>
<tr>
<td>Impaired glucose tolerance</td>
<td></td>
<td>Women</td>
<td>&gt;88 cm</td>
</tr>
<tr>
<td>Glucose uptake below the lowest quartile for background population under investigation under euglycemic hyperinsulinemic clamp conditions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus any two of following:</td>
<td></td>
<td>Hypertriglyceridemia</td>
<td>&gt;1.7 mmol/L</td>
</tr>
<tr>
<td>Antihypertensive medication and/or hypertension</td>
<td>&gt;140/90 mmHg</td>
<td>Low HDL-cholesterol</td>
<td></td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>&gt;1.7 mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low HDL-cholesterol</td>
<td></td>
<td>Hypertension</td>
<td>&gt;130/85 mmHg</td>
</tr>
<tr>
<td>Men</td>
<td>&lt;0.9 mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>&lt;1.0 mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obesity</td>
<td></td>
<td>Fasting plasma glucose</td>
<td>&gt;6.1 mmol/L</td>
</tr>
<tr>
<td>BMI</td>
<td>&gt;30 kg/m²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>and/or waist:hip ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&gt;0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>&gt;0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine albumin excretion rate</td>
<td>&gt;20 µg/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>or albumin:creatinine ratio</td>
<td>&gt;30 mg/g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aWHO; based on any sign of insulin resistance, i.e. impaired glucose tolerance (IGT), impaired fasting glucose (IFG) or diabetes, together with two or more of the components: hypertension, hypertriglyceridemia, low HDL-cholesterol, central obesity and microalbuminuria.*

*bATP III; When three out of five factors are present (abdominal obesity, hypertriglyceridemia, low HDL-cholesterol, hypertension, and IFG), a diagnosis of metabolic syndrome can be made.
much lower prevalence compared to the United States with a prevalence of the metabolic syndrome as high as 24% (26). Alone, each of the factors in the metabolic syndrome increases the risk for CVD. A combination of those risk factors dramatically increases the risk for CVD and together with type 2 diabetes this risk increases even more (24).

**CARDIOVASCULAR DISEASE, MYOCARDIAL INFARCTION AND TYPE 2 DIABETES**

Traditional risk factors for CVD and MI include family history, increasing age, hypertension, dyslipidemia, cigarette smoking, marked abdominal obesity, physical inactivity and diabetes (27). MI is defined as myocardial cell death due to a prolonged ischemia, and typically occurs when a fibrous cap in coronary artery vessel is ruptured followed by local thrombosis formation (13). The definitions of an acute MI are a typical rise and fall of biochemical markers of myocardial infarction together with at least one of following: ischemic symptoms and development of pathological Q-waves or electrocardiogram (ECG) changes indicative of ischemia (ST segment elevation or depression), according to the European Society of Cardiology (28). It should be noted that in patients with diabetes, the clinical symptoms of myocardial ischemia may be rather diffuse without typical chest pain (29). It is also rather common that ECG from diabetes patients reveal pathological Q waves without any history of chest pain, indicative of silent MI. This phenomenon may be due to the autonomic neuropathy seen in diabetic subjects, especially in those with poor metabolic control (29). CVD is the most common complication of type 2 diabetes and accounts for at least 70% of death in those patients (1). The occurrence of CVD is about 2-4 times higher in type 2 diabetic subjects compared with the general population (1). The prevalence of diabetes in patients with acute MI is high, approximately 30%, and is increased in older populations (30). Recent data reveal that the prevalence of diabetes (previously known or newly diagnosed) or IGT/IFG is extremely common in patients suffering an MI (31). High plasma glucose levels in patients with acute MI predict long-term outcome (32,33). Hyperglycemia during an acute coronary event may thus be a logical pathogenetic candidate for the poor outcome (34) and a lot of effort has been taken regarding lowering blood glucose in type 2 diabetes subjects to improve the poor outcome after an MI (7,35). However, intensive glycemic control does not significantly decrease CAD mortality in type 2 diabetes subjects (35). In contrast, insulin-glucose infusion followed by a multidose subcutaneous insulin treatment during hospital stay for acute MI decreases one-year mortality in type 2 diabetics (36), showing that tight glucose control during acute MI may improve long-term prognosis (37). Alternatively, the benefit conferred by this regimen may be due to non-glycemic actions (anabolic, lipogenic) of insulin. In type 2 diabetes also hypertension, dyslipidemia, obesity and insulin resistance are over-represented, all alone risk factors for CAD. In fact, intensified multifactorial intervention in patients with type 2 diabetes against all those risk factors markedly decreases cardiovascular complications (38). Despite significant declines in CVD risk associated with type 2 diabetes by intense and multifactorial treatment, these patients are still at an approximately 2-fold elevated risk of CVD events compared to those without type 2 diabetes (39). Thus, unknown diabetes-specific factors may be involved in the increases CVD risk.

**ATHEROSCLEROSIS**

Atherosclerosis is a chronic and multifactorial disease associated with a wide range of independent risk factors, including the traditional risk factors (27). Novel, non-traditional risk factors for atherosclerosis have been identified including a variety of factors, e.g. increased levels of lipoproteins, hyperhomocysteinemia, activation of the renin-angiotensinogen system, infectious disease, hyperproinsulinemia and insulin resistance (13,27,40). However,
there is now solid evidence that atherosclerosis, no matter which risk factors involved, is an immune-mediated inflammatory disease (13). The nature of the atherosclerotic process could be typified in four different phases, according to Ross definition (13):

First; early preceding formation of an atherosclerotic plaque
The earliest changes that precede the formation of lesions of atherosclerosis include increased endothelial permeability to plasma constituents, e.g. lipoproteins, mediated by vasoactive factors and up-regulation of specific adhesion molecules. This state is accompanied by leukocytes (monocytes and T lymphocytes) drawn to the vascular endothelium by cytokines, chemokines, migrating and colony-stimulating factors, maintaining an inflammatory milieu. Risk factors for atherosclerosis further increase leukocyte adhesiveness to the endothelium.

Second; fatty streak
Once adherent to the endothelium, monocytes transmigrate into the tunica intima, the innermost layer of the arterial wall. Within the arterial intima, monocytes are transformed to macrophages and begin to express scavenger receptors, which give rise to foam cells (lipid-laden macrophages). Macrophages play an important role, not only as lipid scavenger cells, but also as immunocompetent cells secreting the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-a) and interleukin-1ß, maintaining chemotactic stimulus for the ongoing process of adherent leukocytes (13,41).

Third; advancing fatty streak formation
The evolution of a fatty streak toward a complex lesion is typified by the proliferation of vascular smooth muscle cell (VSMC), migrating toward the intima and synthesizing collagen. This represents a type of healing or fibrous response to the injury. The fibrous cap covers a mixture of leukocytes, lipids and debris, forming a necrotic core. Continued release of cytokines not only perpetuates inflammation and lipid accumulation within the atheroma but also influences VSMC activity. Expansion of the lesion within the coronary arteries may result in lumen obstruction, causing a reduction of blood flow, which may present clinically as angina.

Fourth; rupture of the fibrous cap
The thin fibrous cap covers a melting pot of necrotic debris, which may rapidly rupture leading to a thrombosis and an acute coronary syndrome, a scenario that may account for as many as 50 percent of cases of MI. Erosion of the plaque surface exposes a vulnerable surface and a cascade of pro-thrombotic and pro-inflammatory mediators are released, contributing to an ongoing inflammatory process.

Inflammation participates in all steps of the atherosclerosis process and several discernible factors have been proposed to accentuate inflammation in the arteries, such as cigarette smoking, hypertension, dyslipidemia, infectious microorganisms, hereditary factors, oxidative stress and type 2 diabetes. Measuring inflammatory markers may provide clinicians with additional information regarding a patient’s risk for CVD.

ATHEROSCLEROSIS AND PLASMA INFLAMMATORY MARKERS
There are a lot of promising clinical markers proposed to link inflammation and atherosclerosis (42-44). Of these markers, C-reactive protein (CRP) and the cytokines TNF-a and interleukin-6 (IL-6) have been most widely studied. Although simple measurements of white blood cell count and its relationship to CVD have been reported (45), the most promising is CRP as a putative prognostic and predictive marker for cardiovascular events.

Circulating high-sensitivity CRP (hsCRP) is a strong predictor of cardiovascular death in a number of settings (46-48), although recently refuted (49). CRP is released by the cytokines IL-1, TNF-a and IL-6 from the liver as an acute phase reactant in response to inflammation.
Also, CRP may directly promote atherosclerosis and endothelial inflammation (50). CRP might thus directly trigger the development of a pro-inflammatory and pro-atherosclerotic state, leading to atherothrombosis (17).

TNF-α, a multifunctional circulating pro-inflammatory cytokine that releases IL-6 and CRP, is derived from endothelial cells (EC) and VSMC, as well as from macrophages and adipocytes (51,52). Also, TNF-α induces expression of adhesion molecules (42). TNF-α is persistently elevated among post-MI patients with increased risk for recurrent coronary events (53).

IL-6 is another multifunctional circulating cytokine mainly derived from adipocytes, but also from leukocytes and macrophages. IL-6 may be involved in the dysregulation of coagulation and endothelium (54). Serum levels of IL-6 may predict the risk of MI (55,56).

We are in a rapidly expanding phase of knowledge with respect to novel markers of vascular inflammation and endothelial dysfunction (42,43). Although studies of such inflammatory biomarkers provide substantial insights into the pathophysiology of atherosclerosis, the clinical utility of measuring these markers remains uncertain (57).

**ATHEROSCLEROSIS AND DIABETES**

In the atherosclerotic process, endothelial dysfunction is a key factor which precedes and creates a vulnerable environment in the arteries. Advanced endothelial dysfunction, widespread in type 2 diabetes, includes a vulnerable environment where leukocytes and other inflammatory cells more commonly infiltrate the intimal layer of the endothelium (30). Abnormalities in platelet function and coagulation are also commonly seen in patients with type 2 diabetes. The angiopathic processes are exaggerated in type 2 diabetes, associated with increased inflammation and intraplaque hemorrhage. Macrophage infiltration is increased in plaques and pro-coagulant factors are up-regulated in patients with type 2 diabetes. Taken together, these differences in plaque composition, coagulation, platelet function and inflammation may contribute to the advanced atherosclerosis seen in type 2 diabetes (58).

**THE ENDOTHELIUM**

Before discovering the complexity of the endothelium, it was proposed to be just an inert transporting tube. Today, it has become increasingly clear that the endothelium plays a crucial role in vascular homeostasis by modulating blood flow, delivery of nutrients, VSMC proliferation and migration, fibrinolysis and coagulation, inflammation, and platelet and leukocyte adherence (13,42,43).

The arterial vessel is outlined by three distinct layers; tunica intima – a single layer of EC, tunica media – which comprises the VSMC - and finally tunica adventitia, an elastic lamina with terminal nerve fibers and surrounding connective tissue. Each of these layers has distinct functions. Earlier investigations were most focused on VSMC functions within the vasculature bed, until Furchgott and Zawadzki demonstrated the important role of the endothelium for vasodilator activity (59). This vasodilator effect was demonstrated to be mediated by the endothelium-derived relaxing factor (EDRF), subsequently identified as NO (59). EC constitutively express NO synthase (eNOS) that after Ca²⁺/calmodulin binding generates NO using L-arginine as a substrate together with cofactors, e.g. NADPH and tetrahydrobiopterin (BH4) (60). NO then rapidly diffuses into VSMC and binds to a heme group of soluble 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 in VSMC, inhibiting the contractile machinery and thereby evoking vasodilation (61) (figure 2). The production and release of NO may be further increased by circulating factors, such as acetylcholine (ACh), bradykinin and serotonin. NO is also released by physical stimuli, e.g. shear stress and ischemia, which seem not to be Ca²⁺/calmodulin dependent.
Besides the potent vasodilator effect of NO, it mediates many other protective functions in the endothelium. It inhibits expression of pro-inflammatory cytokines, chemokines and leukocyte adhesion molecules, thereby limiting vascular recruitment of leukocytes and platelets (62). It also inhibits VSMC proliferation, an early sign of atherosclerosis (62).

**ENDOTHELIAL DYSFUNCTION**

The endothelium is able to sense changes in hemodynamic forces and blood borne signals, thereby synthesizing and releasing vasoactive substances as a response to the force or signal. The term endothelial dysfunction refers to a critical imbalance in the production of vasodilator factors, e.g. nitric oxide (NO), prostacyclin (PGI\(_2\)) and endothelial derived hyperpolarizing factor (EDHF), and vasoconstricting factors, e.g. endothelin-1 (ET-1), angiotensin-II (AT-II) and prostaglandin (PGH\(_2\)). When this balance is disrupted, it predisposes the vasculature towards a pro-thrombotic and pro-atherogenic milieu. This in turn may ultimately result in vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, pro-oxidation, impaired coagulation, vascular inflammation, atherosclerosis and thrombosis (63) (figure 3).

In the setting of risk factors for atherosclerosis, including diabetes and in atherosclerosis per se, arterial vasodilation is impaired and even a paradoxical constriction in coronary arteries has been reported, indicative of endothelial dysfunction (64).

Although the molecular basis of endothelial dysfunction is not completely understood, numerous studies point to the loss of NO bio-

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**Figure 2. The nitric oxide pathway in the vasculature**

Endothelial cells constitutively expressing nitric oxide synthase (eNOS) generate nitric oxide (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 (gc). This event results in formation of cyclic GMP (cGMP), activating a cGMP dependent protein kinase, which leads to an increased extrusion of Ca\(^{2+}\) from the cytosol inhibiting the contractile machinery and thereby evoking vasodilation. Production of NO can be further induced by e.g. acetylcholine (ACh) or by shear stress, which cause flow-mediated vasodilation. Nitrates, frequently used clinically in the management of angina, function as direct NO donors (here exemplified by sodium nitroprusside [SNP]) thereby causing vasorelaxation.

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**Figure 3. Imbalance in vasoregulating factors induces endothelial dysfunction**

The term endothelial dysfunction refers to an imbalance in the production of vasodilators, e.g. nitric oxide (NO), endothelial-derived hyperpolarizing factor (EDHF), prostacyclin (PGI\(_2\)), and vasoconstrictors, e.g. endothelin-1 (ET-1), angiotensin-II (AT-II) and prostaglandin (PGH\(_2\)). This imbalance may affect endothelial homeostasis and predisposing the endothelium towards a pro-thrombotic and pro-atherogenic milieu. Endothelial dysfunction is a key factor in all stages of atherosclerosis development.
logical activity and/or biosynthesis as a central mechanism (65). In the presence of suboptimal concentrations of substrate or cofactors for the synthesis of NO, eNOS may become uncoupled, resulting in the production of reactive oxygen species (ROS), e.g. superoxide anion, hydrogen peroxide, defined as oxidative stress (figure 4). In oxidative stress, there is an exaggerated generation of ROS, normally scavenged by multiple intra- and extracellular mechanisms. However, at high concentrations of ROS, this scavenging system is impeded and NO may rapidly react with certain ROS species to form peroxynitrite, exaggerating the oxidative stress further (66). The molecular causes of oxidative stress may be due to several underlying conditions, e.g. hyperglycemia, dyslipidemia, cigarette smoking, inflammation and insulin resistance.

ENDOTHELIAL DYSFUNCTION AND TYPE 2 DIABETES

Endothelial dysfunction is widespread in type 2 diabetic patients (5). The causes of endothelial dysfunction in subjects with type 2 diabetes seem to be multifactorial where several independent factors, e.g. hyperglycemia, insulin resistance, hypertension, dyslipidemia, abdominal obesity and low-grade inflammation, have all been associated with endothelial dysfunction (5).

Hyperglycemia

Hyperglycemia is proposed to be a crucial factor inducing endothelial dysfunction and several theories have emerged to explain the adverse effects of hyperglycemia on the endothelium, including the aldose reductase hypothesis, the advanced glycation end products hypothesis, the carbonyl stress hypothesis, the reductive stress hypothesis, the hyperperfusion hypothesis and the oxidative stress hypothesis (5). All these hypotheses overlap each other but the central mechanism may be that oxidative stress is the common disturbance, due to hyperglycemia (67,68). Also, hyperglycemia induces endothelial dysfunction in healthy subjects. This was, due to an attenuated response to methacholine, but not to calcium channel blocker infusion, indicating that the deficit involves endothelium-derived NO (69). Due to this, interest engendered in the possibility that L-arginine and/or BH4 may be deficient in various conditions associated with impaired endothelial function (70,71). In the setting of oxidative stress by hyperglycemia, BH4 depletion is seen and modulation of BH4 may regulate the ratio of peroxynitrite and NO, generated by eNOS (70,72,73). Treatment with BH4 has been shown to augment endothelium-dependent vasodilatation in humans with hypercholesterolemia, diabetes and in smokers (74-77). Also, L-arginine restores hemodynamic changes during acute hyperglycemia, suggesting that hyperglycemia may reduce NO availabil-

Figure 4. Coupled and uncoupled eNOS
In the presence of suboptimal concentrations of L-arginine or tetrahydrobiopterin (BH4), substrate and cofactor, respectively, for the synthesis of NO, eNOS may become uncoupled. This results in the production of reactive oxygen species, e.g. superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$), decreasing the bioavailability of NO which may ultimately lead to endothelial dysfunction.
ity (78). Finally, asymmetric dimethylarginine (ADMA), a strong competitive endogenous NOS inhibitor, may also be involved in the oxidative stress milieu and endothelial dysfunction characterizing type 2 diabetes (79).

**Insulin resistance**

Insulin resistance occurs when normal circulating concentrations of the hormone become insufficient to regulate glucose disposal appropriately. It is defined as an impaired glycemic response to either exogenous or endogenous insulin action. Initially, the pancreatic cells manage to compensate for insulin resistance by an increase in insulin secretion often seen in clinical practice as an increase in plasma insulin concentrations (11). A number of different altered metabolic states, e.g. glucose, insulin, lipid and cytokine metabolism, can lead to peripheral insulin resistance. The state seems to be fueled by, or perhaps to a certain extent the result of, obesity. The ensuing dysregulation of carbohydrate and lipid metabolism that occurs as a consequence of insulin resistance further exacerbates its progression, clinically characterized by obesity, dyslipidemia and hypertension. Obesity and insulin resistance, independently of other risk factors, are associated with endothelial dysfunction (80). Insulin also is an anti-lipolytic hormone and in the insulin-resistant state the normal suppression of free fatty acids (FFA) release from adipose tissue is impaired so that diabetic dyslipidemia occurs, i.e. hypertriglyceridemia, low HDL-cholesterol concentrations and elevation of FFA. Elevated circulating FFA and transient hypertriglyceridemia induce endothelial dysfunction in healthy subjects (81,82). Moreover, endothelial dysfunction has been demonstrated in patients with hypertension, another feature in the metabolic syndrome cluster (5). Finally, in insulin resistance and type 2 diabetes a state of pro-coagulability and low-grade inflammation occurs (e.g. low-grade inflammatory activity reflected by increased TNF-a, IL-6, plasminogen activator inhibitor-1 [PAI-1] and CRP levels), both of which have been coupled to endothelial dysfunction (5).

**Inflammation**

It has been suggested that type 2 diabetes may in part be precipitated or accelerated by an acute phase reaction as part of the innate immune response, in which large amounts of cytokines are released from adipose tissue, creating an inflammatory milieu (16). There is now increasing evidence that visceral adipose tissue constitutes a highly active endocrine organ, releasing a variety of secretory products, e.g. hormones, cytokines and enzymes with the propensity to impair insulin sensitivity (83). Of these secretory products, the cytokines (TNF-a, IL-6, PAI-1) and adipokines (adiponectin and leptin) have received a lot of attention, having been suggested to be associated with inflammation, insulin resistance and CVD (83,84). Adiponectin is inversely related to BMI and likely associated with both reduced insulin resistance and atherosclerosis, in an anti-inflammatory way (84). High levels of adiponectin are associated with lower risk of MI (85). Leptin receptors have been detected in EC and in atherosclerotic plaques, suggesting that leptin may be involved in the atherogenetic process (86). Also, the recently described protein resistin released from adipocytes in mice (87), but not from mature adipocytes in humans (88), may have a certain role in the inflammatory process, inasmuch as plasma levels of resistin correlate with inflammation and is an independent predictor of CAD (89). Keeping in mind that cytokines are involved in the atherosclerotic process (13), it has been hypothesized that obesity and insulin resistance, fueled by the cytokines, TNF-a, IL-6 and PAI-1, might sustain endothelial inflammation (3,17,54,90-96).

In general, visceral obesity leads to insulin resistance and endothelial dysfunction mainly through a cascade of released pro-inflammatory agents, e.g. cytokines and CRP. Insulin resistance leads to hyperglycemia that also promotes an inflammatory milieu, e.g. oxidative stress. The mechanisms by which these factors are interrelated are numerous and complex, depicted by Caballero in figure 5.
ASSESSMENT OF ENDOTHELIAL FUNCTION

Measurement of endothelial function in patients has emerged as a useful tool for atherosclerosis research. Studies showing that the severity of endothelial dysfunction relates to the risk for an initial or recurrent cardiovascular event (14,15,97-105). These studies include both the coronary and brachial vascular beds and it seems that endothelial dysfunction is a systemic process that can be identified in vascular beds remote from the coronary and cerebral circulations where events occur (106).

Figure 5. Mechanisms through which obesity, insulin resistance, and endothelial dysfunction are associated

Obesity can precipitate insulin resistance and endothelial dysfunction through a cascade of released pro-inflammatory agents, e.g. hormones, cytokines (including adipocytokines) and CRP. Insulin resistance leads to endothelial dysfunction and may contribute to obesity. Insulin resistance is frequently associated with other abnormalities that can affect endothelial function, such as hyperglycemia, hypertension, dyslipidemia, and deranged coagulation/fibrinolysis. Insulin resistance may itself impact the endothelium negatively by a disturbance in the insulin signaling pathway of the endothelium. Reprinted from ref 3, Copyright (2003), with permission from North American Association for the Study of Obesity (NAASO).

However, there are as of yet no studies demonstrating that correction of endothelial dysfunction directly decreases the mortality or morbidity in CVD.

Ever since the classical experiment by Furchgott and Zawadzki (59), revealing that the endothelium is responsible for vasodilation, the function of the endothelium has been a scientific focus in the study of vascular disease. It has been shown that NO is a key factor regulating the endothelium; therefore techniques to evaluate the release of NO in the coronary circulation as well as in the systemic circulation have been developed. Most endothelial func-
tion tests pertain to abnormalities in the regulation of the lumen of vessels. The action of EC may affect one or several functions, either simultaneously or in a temporal sequence, and thus cannot be considered a single, discrete, and uniquely defined entity. Consequently, it is hard to define any method for measurement of endothelial function superior to another, i.e. different techniques may measure different functions and are therefore complementary to each other. In general, endothelial function in humans is assessed experimentally by 1) methods that assess the functional consequences of EC activity, alone or complemented by 2) measurements of plasma concentrations of biomarkers of EC function.

In group 1, methods employed include the thermo-, or due-dilution, based on the Fick principle theory, as well as positron emission tomography (PET) scan, laser Doppler flowmetry, plethysmography and Doppler ultrasound. The common soil for all these methods is their ability to monitor the capacity of the endothelium to synthesize and release vasodilator compounds. Plethysmography and ultrasound, and measurements of biochemical markers, are described below.

**Plethysmography**

This is a commonly used method based on venous occlusion plethysmography for studying endothelium dependent and independent vasodilation in peripheral circulation, especially mapping dose-response relationships of endothelial agonists and antagonists. This technique uses infusion of ACh or other muscarinic receptor agonists, e.g. in the brachial artery and determines the vasodilator responses over a limb, e.g. forearm resistance vessels. Sodium nitroprusside (SNP) is usually used as a control substance in order to evaluate endothelium-independent vasodilation. The evaluation of changes in blood flow, contributed by the whole limb vessel portion, provides a measure of endothelial function.

**Ultrasound and Doppler**

Quantitative coronary angiography with an intra-coronary ultrasound and Doppler transducer has been proven to be ‘Gold Standard’ to assess endothelial function in the coronary circulation. By a stepwise infusion of ACh and SNP, the endothelium dependent and independent vasodilation can be quantified. However, this technique is complicated and invasive. Therefore, a simple non-invasive method suitable for repeated studies also for evaluating large groups of patients has been developed, first described by Celermajer, i.e. flow-mediated vasodilation (FMD) (107). FMD correlates to the endothelial function in the coronary circulation (106) and has been widely used in the past several years (108). FMD measurements are based upon the shear stress theory, whereby a short period of arterial occlusion increases flow in an artery. This stimulus is proposed to provoke the endothelium to release NO with subsequent vasodilation. Oral nitroglycerin is given to assess the endothelium-independent vasodilation (NTG). No specific receptor signaling pathways activated by shear stress have been reported, and the precise mechanisms for the acute detection of shear stress and subsequent signal transduction to modulate vasomotor tone are not fully understood (109). Thus, there is some redundancy in the system and several endothelial mediators other than NO are capable of acting as signals between the endothelium and vascular smooth muscle (9,109,110).

**Endothelial biochemical markers**

This method may be the simplest way to indirectly monitor endothelial dysfunction. When endothelial cells undergo inflammatory activation, and thereby endothelial dysfunction, an increased expression of biochemical markers has been detected. There is a wealth of markers linked to endothelial dysfunction, including selectins, integrins, cytokines, fibrinolytic and adhesion molecules, as well as CRP, which all promote the adherence of monocytes, thereby accelerating the atherosclerotic process described above (42,43).
**INSULIN**

**Insulin’s metabolic action**

Insulin is an anabolic hormone secreted by the pancreatic β-cells in response to increased circulating levels of glucose and amino acids after a meal. Insulin is essential for maintenance of glucose homeostasis and regulates glycemia by reducing hepatic glucose output through decreasing gluconeogenesis and glycogenolysis, and increasing the rate of glucose uptake, primarily into skeletal muscle and adipose tissue. In muscle and fat cells, clearance of circulating glucose depends on the insulin-stimulated translocation of the glucose transporter isoform-4 (GLUT-4) to the cell surface (111). In skeletal muscle, GLUT-4 is the rate-controlling step for insulin-stimulated muscle glycogen synthesis (111). Insulin also profoundly affects lipid metabolism, increasing lipid synthesis in liver and fat cells, and attenuating FFA release from triglycerides (112). Insulin action is initiated through the binding to and activation of its cell-surface receptor (113). The receptor then undergoes a series of intra-molecular transphosphorylation reactions where only one downstream signaling molecule appears unequivocally essential for insulin-stimulated GLUT-4 translocation, namely phosphatidylinositol (PI) 3-kinase that is highly necessary for insulin-stimulated glucose uptake (113). The targets of PI3-kinase action are the two classes of serine/threonine kinases known to act downstream of PI3-kinase, viz. protein kinase B (PKB) and protein kinase C (PKC) (113).

**Insulin’s vascular action**

For many years, research of the in vivo insulin action was focused on glucose and lipid metabolism. Recently, it has become increasingly clear that insulin also is a vasoactive hormone. Insulin, when administered intravenously, increases blood flow and vasodilation in a NO dependent manner (114,115). Although the vasoactive physiology of insulin has been vividly debated (116,117), the increase in blood flow evoked by insulin is much less then the classical endothelium dependent agonist ACh (116). The increase in blood flow elicited by insulin differs between different types of vessels, e.g. capillary and resistance vessels. Insulin recruits and immediately increases blood flow in capillaries, which may enhance nutritive uptake and glucose disposal in skeletal muscle (118-120). In contrast to capillary recruitment, it seems that insulin action on resistance vessels is slower in onset and requires at least several hours for a maximal effect (116). Whereas insulin effects on total blood flow are of physiological relevance has been debated, the interaction of insulin and NO may be of more interest (116). There is compelling evidence that insulin has a direct effect on endothelium by increasing NO production (121). Stimulation of NO production by insulin is mediated by signaling pathways involving activation of PI3-kinase, which activates eNOS phosphorylation (121-123). These data demonstrate that the metabolic and vascular actions by insulin share the same signaling pathway, i.e. PI3-kinase (figure 6). However, in the vasculature insulin may also activate the pro-atherogenic mitogen-activated protein (MAP)-kinase pathway, known to induce smooth muscle migration and PAI-1 production (3). In insulin resistant states an imbalance between these pathways has been suggested, leading to endothelial dysfunction (124). Finally, insulin deficiency or chronic hyperglycemia can increase the enzymatic activity of PKC and total diacylglycerol levels in the vasculature, resulting in endothelial dysfunction, which may be involved in the onset and progression of atherosclerosis (125).

**ASSESSMENT OF WHOLE BODY INSULIN SENSITIVITY**

The ability of insulin to lower blood glucose concentration by promoting glucose uptake (skeletal muscle) and suppressing its production (liver) can be quantified by different methods. There is a wealth of established methods for quantifying insulin sensitivity in humans (126), where the hyperinsulinemic clamp technique is considered ‘gold standard’. This tech-
Technique is the reference method for quantifying whole body insulin sensitivity first described by DeFronzo et al (127). The principle of the test is to keep a predetermined glucose level constant during a constant insulin infusion that stimulates glucose disposal. The glucose infusion rate is computed by a feedback controlled rate by measuring blood glucose frequently. Once a steady state has been reached, the degree of insulin sensitivity is positively related to the amount of glucose infused necessary to maintain the predetermined glucose level.

**STRATEGIES FOR REDUCTION OF ENDOTHELIAL DYSFUNCTION AND INSULIN RESISTANCE**

Because a relationship between insulin resistance and endothelial dysfunction exists (80,128-132), one hypothetical way to improve long term prognosis of diabetic patients would be to increase insulin sensitivity and thereby improve endothelial function. Thus, investigators have tested the possibility that physical activity or pharmacological agents may increase insulin sensitivity and improve endothelial function or vice versa. An important corollary to the hypothesis that endothelial dysfunction contributes to the pathogenesis of CVD, is the idea that reversing endothelial dysfunction will reduce cardiovascular risk. Although this corollary has not been tested directly, numerous studies have evaluated lifestyle and pharmacologic interventions to improve endothelial function, and many of these same interventions are known to limit cardiovascular risk (9).

**Lifestyle intervention**

Obesity is a key component of the insulin resistant state. Changes in lifestyle, e.g. physical activation and/or dietary modification, have proven to improve insulin sensitivity and endothelial function (133,134). Both weight loss and dietary modifications lead to a more favorable milieu in the body in terms of decreased lipid levels, blood pressure and inflammatory activity (93,135). Also, type 2 diabetes can be prevented by moderate lifestyle changes in high-risk IGT subjects (136-138). Smoking cessation ameliorates insulin resistance (139) and endothelial dysfunction in diabetic patients (140).

**Pharmacological agents**

*Insulin*

The use of high doses of insulin in patients with type 2 diabetes has been shown to improve insulin sensitivity and endothelial function (9). Contradictory, pharmacologically induced hyperinsulinemia causes endothelial dysfunction in healthy subjects (132,141). In the endothelium, insulin’s effects seem to be mediated via the PI3-kinase pathway activating eNOS, but also via MAP-kinase pathways inducing smooth muscle migration and PAI-1 production (3). Therefore, the controversy relating to putative atherogenic effects of insulin may be a matter of which pathway insulin chooses in the healthy or the insulin resistant endothelium.
In the healthy endothelium MAP-kinase may take overhand, whereas in the insulin resistant condition insulin may activate the PI3-kinase pathway (124). More importantly, this controversy may have arisen from the inability to distinguish between hyperinsulinemia per se and that reflecting insulin resistance. Also contributing to the confusion surrounding this issue might be the lack of separation of insulin from proinsulin, the latter known to be proatherogenic (40).

Sulfonylurea (SU)
Besides the hypoglycemic effects of SU, these drugs also have a non-glycemic effect, which mainly has been studied for gliclazide, a second-generation SU. Gliclazide improves endothelial dysfunction, independent from its hypoglycemic actions, via reduction of oxidative stress in aortic vessel rings from rabbits (142). This has also been shown in type 2 diabetes subjects, where gliclazide, but not glibenclamide, improved both antioxidant status and NO-mediated vasodilation (143).

Metformin
The mechanism of action of metformin is poorly understood, but includes mainly a decrease in hepatic glucose production. A key hepatic enzyme targeted by metformin was recently identified as AMP-activated protein kinase (144). In a subgroup analysis in the United Kingdom Prospective Diabetes Study, it was found that metformin in monotherapy, but not in combination with SU, may moderately reduce cardiovascular morbidity and mortality in overweight subjects with type 2 diabetes (145). Metformin slightly increases insulin sensitivity together with an improvement in endothelial function in type 2 diabetic subjects without CVD (146). Metformin may act in an anti-inflammatory way, showing a significant reduction in CRP levels in subjects with IGT, along with a reduction in the incidence of type 2 diabetes (138). Metformin may directly exert beneficial effects on the endothelium, improving markers of endothelial activation (147).

Thiazolidinediones (TZDs)
TZDs, whose mechanisms of action are probably largely explained via activation of the nuclear peroxisome proliferator-activated receptor-gamma, regulate gene expression mainly in adipose tissue. They are so called insulin sensitizers that improve whole body glucose uptake (148). Some studies show an improvement in both insulin sensitivity and endothelial function (149,150). However not all have reached the same conclusion (151). There are ongoing multicenter studies addressing whether TZDs have preventive effects on macrovascular events in type 2 diabetes (152).

Glucagon-like peptide-1 (GLP-1)
GLP-1 and its analogues are emerging new drugs in the armamentarium against type 2 diabetes (153,154). GLP-1 acts as an incretin, which means that it is released from intestinal cells after food ingestion and lowers blood glucose by augmenting insulin secretion, inhibiting glucagon secretion and also by inhibiting bowel motility and promoting satiety. GLP-1 is rapidly broken down by the ubiquitous enzyme dipeptidyl peptidase IV (DPPIV) so that its biological half life in the circulation is only 1-2 minutes. Therefore, long acting GLP-1 analogues have been created, as well as DPPIV enzyme inhibitors. Thus, the effects of the analogues are not expected to differ from the native compound, GLP-1. Several studies in humans demonstrate salutary effects of GLP-1 and analogues on glycemia (155-157), including enhanced insulin sensitivity independently of the islet hormones (158,159), but there are also studies that fail to show such effects (155). Besides GLP-1 receptor expression on the pancreatic cells, high-affinity receptors are also present in extrapancreatic and intestinal tissues, i.e. nervous system, heart, kidney and VSMC (157,160,161). There are some early and recent studies showing that GLP-1 may exert vasomotor effects (162-164). GLP-1 relaxes pulmonary artery rings in rats (161,165). Also, GLP-1 improves endothelial function in a salt sensitivity rat model of hypertension (166). GLP-1 directly protects the heart against ischemia (167) and
ameliorates severe left ventricular heart failure in humans suffering an MI (168).

ACE inhibitors (ACE-i) and angiotensin receptor blockers (ARB)
ACE-i and ARB have been shown in vitro and animal models to enhance insulin sensitivity (169). This effect may be due to augmentation of the insulin signaling pathway, inhibition of the renin-angiotensinogen-aldosterone axis and enhancement of the microcirculation in adipose tissue and skeletal muscle (169). However, human physiological studies investigating blood pressure independent effects of ACE-i and ARB on insulin sensitivity and endothelial function are sparse (9). ACE-i seem to have both anti-inflammatory and anti-apoptotic properties. In the Captopril Prevention Project trial, captopril decreased the risk of developing type 2 diabetes in hypertensive subjects (170). These results were later confirmed in the Heart Outcome Prevention Evaluation study testing another ACE-i, i.e. ramipril (171). Also, hypertensive and heart failure studies with ARBs are in line with a decreased risk of developing type 2 diabetes (17).

Statins
Treatment with statins to lower lipid concentration in hyperlipidemic states with or without coronary artery disease is well established. Statins may also have anti-inflammatory properties independent of the lipid lowering effects. In the West of Scotland Coronary Prevention Study pravastatin treatment reduced the incidence of new cases of diabetes by 30 %, suggested to be due to anti-inflammatory effects (17,172,173). Statins improve endothelial function in subjects with atherosclerosis secondary to improvement in NO bioavailability (174,175).

Aspirin
Inflammation adversely impacts both insulin sensitivity and endothelial function; therefore, agents that reduce inflammation would theoretically be an interesting approach for improving insulin resistance and endothelial dys-
AIMS

The general aim of this work was to study endothelial function in type 2 diabetic patients with CAD.

More specifically, for each study, the aims were:

1 To compare type 2 diabetic patients with non-diabetic patients, following an acute MI, with regard to endothelial function, CRP and adiponectin.

2 To compare and investigate the association between lipids, CRP, IL-6, TNF-a, insulin resistance, adipocyte-derived factors and blood pressure upon endothelial function in patients with an established CAD with or without type 2 diabetes mellitus.

3 To investigate whether the critical eNOS cofactor BH4 improves endothelial function and whether such an effect is accompanied by increased insulin sensitivity in type 2 diabetic patients compared to non-diabetics, with established CAD.

4 To evaluate acute effects of GLP-1 on endothelial dysfunction in type 2 diabetic patients with established CAD and whether such an effect is accompanied by increased insulin sensitivity.

5 To investigate whether GLP-1 directly affects rat conduit vessel contractility ex vivo and to investigate the mechanism underlying such an effect, including the involvement of endothelium-derived NO.
SUBJECTS

All patients were recruited from the coronary care unit (CCU), Department of Cardiology or Diabetology, respectively, at Stockholm South Hospital. More precisely, for study I, 20 type 2 diabetic and 25 non-diabetic patients suffering an acute MI were consecutively recruited from the CCU and Department of Cardiology. For study II, 40 male patients with an established CAD were investigated, of whom 20 were type 2 diabetic and 20 were non-diabetic. This study was constructed from study III & IV and included 10 diabetic and 10 non-diabetic patients from study III, and 10 diabetic patients from study IV. Finally, 10 additional non-diabetic subjects were recruited from the Department of Cardiology. For study III, 32 male individuals with an established CAD were investigated. Twelve type 2 diabetic patients were matched regarding age and weight against 10 non-diabetic patients. Patients were recruited from the Department of Cardiology or Diabetology, respectively. The remainder consisted of 10 unmatched healthy individuals. These individuals had earlier volunteered for other experiments and were scheduled to participate in other studies at our metabolic laboratory. For study IV, 22 male subjects were investigated, of whom 12 were type 2 diabetic patients with an established CAD, recruited from the Department of Cardiology or Diabetology, respectively. The healthy group in this study was the same group as for study III. Baseline characteristics of patients and healthy controls in study groups are summarized in table 3. For study V, 14 healthy male Sprague-Dawley rats (weight 250–350 g) were used.

<table>
<thead>
<tr>
<th>Study</th>
<th>T2DM</th>
<th>Non-DM</th>
<th>T2DM</th>
<th>Non-DM</th>
<th>#Healthy controls</th>
<th>T2DM</th>
<th>#Healthy controls</th>
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<td>20</td>
<td>12</td>
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<tr>
<td>m/f</td>
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<td>17/8</td>
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<td>12/0</td>
<td>10/0</td>
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<td>59±2</td>
<td>60±2</td>
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<td>29±4</td>
<td>61±3</td>
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<td>3</td>
<td>1</td>
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<td>1</td>
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<td>sBP (mmHg)</td>
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<td>135±5</td>
<td>127±4</td>
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<td>111±1</td>
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<td>dBP (mmHg)</td>
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<td>HbA1C (%)</td>
<td>6.6±0.3</td>
<td>-</td>
<td>6.4±0.3</td>
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<td>6.1±0.2</td>
<td>4.8±0.1†</td>
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<td>§f-blood glucose (mmol/l)</td>
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<td>5.1±0.2†</td>
<td>6.5±0.5</td>
<td>5.0±0.2†</td>
<td>7.0±0.4</td>
<td>4.9±0.1†</td>
<td>4.6±0.1</td>
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<td>f-total cholesterol (mmol/l)</td>
<td>5.1±0.2</td>
<td>5.8±0.3*</td>
<td>4.2±0.1</td>
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<td>3.8±0.3</td>
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<tr>
<td>f-LDL-cholesterol (mmol/l)</td>
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<td>2.8±0.2</td>
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<tr>
<td>f-triglycerides (mmol/l)</td>
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<td>71±53</td>
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</table>

Data are means ± SE. T2DM, type 2 diabetic subjects; Non-DM, non-diabetic subjects; m, male; f, female; sBP, systolic blood pressure; dBP, diastolic blood pressure. *P<0.05, †P<0.01, compared with T2DM. ‡baseline data not statistically compared with T2DM or Non-DM. §for paper I, values are plasma glucose.
STUDY PROTOCOLS

Study I
This was a prospective cohort study. FMD and NTG, and fasting plasma levels of CRP, adiponectin, routine blood samples and glucose were studied in each patient at two separate occasions: a) acutely, investigation being performed within 1-3 days (from the onset of chest pain) and b) 60 days after AMI. Patients were examined in a supine position after 30 min at rest in a quiet, dark room with a temperature of 22ºC. Long-acting nitrates were withheld 24 hours before the study but no other drugs were withheld. Patients were asked to avoid coffee or tea and to refrain from smoking at least 12 hours before the ultrasonogram. Patients were not given any specific instructions on lifestyle modification during the study, nor did they take part in special exercise program. Between the two examinations patients were treated by physicians who were not aware of this particular study. Questionnaires at follow-up did not reveal any history of infection or inflammatory disease between the two procedures.

Study II
In this cross-sectional study we studied 40 male individuals, of whom 20 were type 2 diabetic and 20 were non-diabetic patients with an established MI. We investigated the association between lipids (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides), CRP, IL-6, TNF-a, glycemia (glucose, insulin, C-peptide and SI), adiponectin, resistin and blood pressure upon endothelial function (FMD/NTG). After a 12 h overnight fast, subjects arrived to our laboratory for the experiment at 8 a.m. Subjects were not allowed to eat or drink anything but water and they refrained from their medicines on the morning of the test day. Regarding subjects taking insulin, this was not given after 6 p.m. the day before the test. BH4 was infused 15 minutes after priming for the hyperinsulinemic clamp and throughout the clamp, thus in total for 105 min. Whole body glucose uptake was measured with a hyperinsulinemic isoglycemic clamp technique. Subjects were clamped with regard to their fasting blood glucose levels. If glucose levels differed between treatments (BH4 vs. placebo), correction was done either with pre-priming with insulin or glucose infusion to maintain exactly the same blood glucose level as for the week before. FMD and NTG were measured with ultrasonogram at onset and at 100 min in the steady state clamp procedure.

Study III
This was a randomized, cross-over conducted study. Subjects were tested twice, one week apart, regarding FMD/NTG and SI during infusion of BH4 or placebo (saline). After a 12 h overnight fast, subjects underwent infusion of i.v. infusion of BH4 or placebo in a cross-over random order and with a washout period for one week. Subjects were taking their medicines as usual between the test periods. Subjects were not allowed to eat or drink anything but water and they refrained from their medicines on the morning of the test day. Insulin was not given after 6 p.m. the day before the test. BH4 was infused 15 minutes after priming for the hyperinsulinemic clamp and throughout the clamp, thus in total for 105 min. Whole body glucose uptake was measured with the isoglycemic hyperinsulinemic clamp technique.

Study IV
This study had the same design as study III. After a 12 h overnight fast, subjects underwent i.v. infusion of GLP-1 or placebo (saline) in a random cross-over study and with a washout period of one week. Subjects were not allowed to eat or drink anything but water and they refrained from their medicines on the morning of the test day; otherwise subjects were taking their medicines as usual between the two procedures. GLP-1 was infused 15 minutes after priming for the hyperinsulinemic clamp and throughout the clamp, thus in total for 105 minutes.
min. FMD and NTG was conducted as depicted for study III.

**Study V**

Artery rings from male Sprague-Dawley rats were prepared and fixed in organ baths ex vivo. The contractile function of the vascular segments was tested by administration of phenylephrine. Endothelium-dependent and endothelium-independent relaxations were determined by administration of ACh and SNP, respectively.

**BIOCHEMICAL ANALYSES**

Venous blood or arterialized plasma samples were all drawn into EDTA anticoagulated vacuum tubes. Aliquots were placed on ice, centrifuged within half an hour, and separated plasma stored at -20°C pending analysis.

**Enzyme-linked immunosorbent assays (ELISA)**

All samples were run in a 96-well plate reader, according to the instructions provided by the manufacturers of the ELISA kits. The following manufacturers were used: adiponectin (R&D Systems, Abingdon, Oxfordshire, U.K.), hsCRP (ImmunoDiagnostics AG, Bensheim, Germany), C-peptide (Merckodia AB, Uppsala, Sweden), IL-6 (R&D Systems, Abingdon, Oxfordshire, U.K.), insulin (Pharmacia, Uppsala, Sweden), TNF-α (R&D Systems, Abingdon, Oxfordshire, U.K.) and resistin (BioVendor, Brno, Czech Republic).

**Routine laboratory methods**

For the determination of plasma glucose (study I), hemoglobin, creatinine, lipids, folate, vitamin B12 and HbA1c (as measured by the Swedish mono-S HPLC method), we used the accredited laboratories of Stockholm South Hospital.

**Blood glucose (used in the clamp method)**

Blood glucose was determined by the glucose oxidase method with a glucose analyzer (Yellow Springs Instruments 2300 STAT PLUS, Yellow Springs, OH).

**GLP-1**

Plasma GLP-1 levels were determined with a radioimmunoassay (RIA) using antiserum 89390, which is highly specific for the carboxyl terminus of GLP-1 and therefore measures the sum of GLP-1 (7-36) amide and its metabolite GLP-1 (9-36).

**Glucagon**

Plasma glucagon concentrations were determined with a RIA using an antiserum directed against the carboxyl terminus of the glucagon molecule (antibody code number 4305). For GLP-1 and glucagon analyses, plasma was extracted with ethanol (final concentration 70% by volume).

**TETRAHYDROBIOPTERIN (BH4)**

BH4 was purchased from Schircks Laboratories (Jona, Switzerland [purity >99.5 %]) and infused at a rate of 500 µg min⁻¹. BH4 was stored in ampoules in the freezer at -20°C pending the experiment. A few minutes before the infusion, the ampoule was broken and its content dissolved in 2 ml of oxygen-free 0.9 % NaCl (Apoteket AB, Sweden) and immediately transferred to a glass bottle containing 250 ml of oxygen free 0.9 % NaCl. The reason for this procedure was that BH4 is highly reactive in air.

**GLP-1**

For the human study (study IV), we used recombinant GLP-1(7-37) amide (rGLP-1), a generous gift from Restoragen Inc. (Lincoln, NE). Ampoules contained 1 mg/ml of rGLP-1 (purity 98 % and a bacterial endotoxin level of < 3 EU/mg). For the rodent study (study V), rGLP-1 was purchased from Neosystem (Strasbourg, France). Ampoules contained 1 mg/ml rGLP-1 (purity > 95 %). For both experiments, rGLP-1 was stored in ampoules (dry powder) in the freezer -20°C pending the experiments. In the human study (IV), rGLP-1 was dissolved in 3 ml 0.9 % NaCl (Baxter) and placed in an
insulin pump (Medtronic, Minneapolis, MN), used for the experiment. The insulin pump rate was then calculated from the experimental dose, which was 2 pmol kg\(^{-1}\) min\(^{-1}\). In the rodent study (V), rGLP-1 was dissolved in 0.9 % NaCl (Baxter) and serially diluted (10\(^{-12}\) - 10\(^{-7}\) mol/l), and kept on ice during the experiment.

**EXENDIN(9-39)**

Exendin(9-39), the highly specific GLP-1 receptor antagonist, was purchased from American Peptide Co. (Sunnyvale, CA) and stored at -20 C\(^\circ\) in the freezer as dry powder.

**FLOW-MEDIATED (FMD) AND NITROGLYCERIN-MEDIATED (NTG) VASODILATION**

The diameter of the brachial artery was measured from two-dimensional ultrasound images, using a 7.0 MHz linear array transducer and a standard 128XP/10 system (Accuson, Mountain View, CA) according to the method described by Celermajer et al (107). After a 60 min resting time, the subject’s left arm was immobilized and the transducer was fixed in the same position throughout the study with the assistance of a mechanical arm. The brachial artery was scanned longitudinally and the transmit (focus) zone was set to optimize images of the lumen arterial wall interface. The B-mode images were magnified by a resolution box and obtained with gating from the R wave of the electrocardiogram as trigging mode. The condition of reactive hyperemia was induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 300 mm Hg for 4.5 min, followed by release (endothelial-dependent vasodilatation [FMD]). Measurements were made at baseline (after 30 minutes of supine rest), and at 45 and 60 seconds after cuff release. Then, after ten min rest, 0.4 mg of nitroglycerine spray was applied and new images were obtained 4 min later (endothelial-independent vasodilatation [NTG]) (figure 7). Brachial artery diameter, the means of two images, was measured with an automated computerized analyzing system (182). In brief, this analyzing program is a PC/windows-based software with digitized ultrasound image. The starting point of the measurement area is set by the operator, and a 10 mm box is automatically drawn. The different echo interfaces are automatically outlined. If obvious errors are detected, it is possible to modify the measurement by marking a correct echo in the ultrasound image. In this case, only one or two manually marked points are needed to guide the automatic system to the correct interface.

Arterial flow velocity rates were obtained using a pulsed Doppler signal at 70\(^\circ\) angles to the vessel in the center of the artery. Ultrasound images were made 15 seconds after cuff release with the freeze mode. The volume flow

![Figure 7. Schematic drawing of high resolution ultrasound monitoring of endothelial function.](image)

Baseline images are first obtained, whereafter the cuff is inflated to 300 mmHg for 4.5 minutes, followed by release = FMD (flow-mediated vasodilation). Then, after ten min rest, 0.4 mg of nitroglycerine spray is applied and new images are obtained 4 min later = NTG (nitroglycerine-induced vasodilation).
was calculated by multiplying the velocity time integral of the Doppler flow signal from the mean of three pulse waves by the heart rate and vessel cross sectional area.

All calculations of blood flow and changes in arterial vasodilatation were performed by one investigator who was blinded to the identity of subject and sequence of the ultrasound image. Changes in brachial artery diameter was expressed both in absolute values (mm) and relative values using the formula \[ \text{diameter(after) - diameter(before)} / \text{diameter(before)} \times 100 \% \].

**ISOGLYCEMIC HYPERINSULINEMIC CLAMP**

Hyperinsulinemic clamps were performed according to DeFronzo et al (127). In brief, a superficial dorsal hand-vein was cannulated in retrograde fashion with a 21-gauge butterfly needle and kept patent by a slow infusion of saline solution. The hand was kept warm by an electric device for intermittent sampling of arterialized venous blood. After that, one intravenous catheter was inserted into the left antecubital vein for substrate (insulin/glucose) and drug infusion. During the 120 min of the test, insulin (Human Actrapid, 40mU·m⁻²·min⁻¹ NovoNordisk A/S) was infused along with 20% dextrose (Fresenius Kabi). The rate of dextrose infusion was adjusted to achieve a blood glucose level compared to subjects’ fasting glucose levels, on the basis of arterialized samples withdrawn every 5 min from the dorsal hand-vein catheter (heated-air box at 55°C, University of Nottingham Department of Physiology and Pharmacology). The glucose clamp-derived index of insulin sensitivity (SI) \[ 10^{-3}\text{dl·kg}^{-1}\cdot\text{min}^{-1}/(\mu\text{U}·\text{ml}) \] was calculated from glucose infusion rate (GIR), corrected for body weight, during the final 30 minutes as follows: SI = \( \frac{\text{GIR}_{SS}}{\text{G}_{SS}} \times \frac{1}{\text{I}_{SS}} \), \( \text{GIR}_{SS} \) is the steady state GIR (mg/min), \( \text{G}_{SS} \) is the steady-state blood glucose concentration (mg/dl), and \( \text{I}_{SS} \) is the difference between basal and steady state plasma insulin concentrations (\( \mu\text{U}·\text{ml} \)). This calculation is assumed to correct for differences in prevailing glucose and insulin concentrations.

**ARTERIAL RINGS IN ORGAN BATHS**

Femoral arteries from male Sprague–Dawley rats were carefully dissected free from surrounding tissue, removed and put in the organ bath with Krebs–Henseleit (KH) solution. Circular segments (1–2 mm in length) of the artery were mounted on two thin metal holders, one of which was connected to a force displacement transducer (model FT03, Grass Instrument Co, Quincy, MA) and the other to a movable device that allowed the application of a passive tension of 5 mN. The tension was recorded on a polygraph (model 7B, Grass). The mounted vascular segments were kept in 2 ml organ baths containing KH solution at 37°C and continuously bubbled with 5% CO₂ in O₂ to maintain a pH of 7.4. After preparation, the vascular segments were allowed to equilibrate for 60 min. The contractile function of the vascular segments was tested by administration of phenylephrine (10⁻⁵ mol/l) and with K⁺-rich (127 mmol/l) KH solution, prepared by replacing NaCl with equimolar amounts of KCl. Endothelium-dependent and endothelium-independent relaxations were determined by administration of ACh and SNP, respectively. ACh and SNP were added to the organ baths at cumulatively increasing concentrations \( 10^{-9} – 10^{-5} \text{mol/l} \) and \( 10^{-9} – 10^{-6} \text{mol/l} \), respectively) during a stable contractile tone induced by phenylephrine (10⁻² mol/l). The relaxatory response following preincubation with a studied substance was always compared to the preceding control response in the same vascular segment. In some experiments the endothelium was removed mechanically by a very gentle grip of the artery rings with a stainless forceps and then gently rolling the inner surface of the artery against the organ bath metal holders. This procedure was done under a microscope. Completeness of endothelium removal was checked by the absence of ACh-induced relaxation. All substances were added in 50 µl volumes.

GLP-1 was added to the organ baths at cu-
cumulative increasing concentrations (10^{-12}–10^{-7} mol/l) during baseline tension to evaluate contractile effects per se. Furthermore, GLP-1 (10^{-7} mol/l) was in separate experiments added ten minutes before a dose-response curve for phenylephrine to test for poteniations of phenylephrine-induced contractions. The relaxant effects of GLP-1 were evaluated by adding cumulatively increasing concentrations of GLP-1 to artery segments precontracted with phenylephrine (10^{-5} mol/l). The receptor specificity of GLP-1 was investigated by administration of the highly specific GLP-1 receptor antagonist exendin(9-39) (10^{-7} mol/l) ten minutes before adding GLP-1. In separate experiments, the NO synthase inhibitor N-nitro-L-arginine (L-NNA) (Sigma) in a final concentration of 10^{-3} mol/l was added 15 min prior to ACh to test for possible NO-dependent actions.

**CELL CULTURE AND WESTERN BLOTTING**

Human coronary aortic endothelial cells (HCAEC) were purchased from Clonetics Corp. (San Diego, CA) and cultured in endothelial growth medium according to the manufacturer’s instructions. A rat insulinoma-derived cell line BRIN-BD11 (BRIN), known to express the GLP-1 receptor (183), served as a positive control. Cells were maintained in sterile tissue culture flasks at 37°C in an atmosphere of 5% CO_{2}–95% air.

HCAEC and BRIN cells were harvested by gentle trypsinization. Cells were washed three times in phosphate buffered saline and were solubilized in SDS-PAGE sample buffer. Twice as much protein from HCAEC than from BRIN cells were subjected to SDS-PAGE under reducing conditions. Proteins in the gel were subsequently electrotransferred onto nitrocellulose membranes. The membrane was blocked overnight in Tris-buffered saline/0.05 % Tween 20 (TBS-T) with 5 % non-fat dry milk, followed by an overnight incubation with 5 μg/ml rabbit anti-N-terminal GLP-1 receptor antibody in TBS-T/BSA (1 %) at 4°C. The membrane was further incubated with horseradish peroxidase-labeled goat anti-rabbit IgG in TBS-T/BSA (1 %) for 1 h at room temperature. The immunostained proteins were visualized by enhanced chemiluminescence (184).

**STATISTICAL ANALYSES**

The results are given as means ± SE in all papers, except the percentage data. Statistical differences were calculated by Student’s unpaired two tailed t test for comparisons between groups regarding baseline data in study I-IV. The percentage data comparisons were tested by Chi square analysis in study I & II. For correlation data, Pearson correlation analysis was used for study I & II. Moreover, for study II a multiple regression analysis was undertaken to investigate the relationship between the single dependent variables, FMD and NTG. In study I, III & IV ANOVA for repeated measurements was used. The ANOVA tests were followed by post hoc Sheffe’s test. Finally, in study V, Friedman’s ANOVA test for repeated measurements was used.

**ETHICAL CONSIDERATIONS**

All human studies were conducted according to the Helsinki declaration and approved by the Ethics Committee of the Karolinska Institute. Study V 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 No. 85-23, revised 1985).
RESULTS AND DISCUSSION

ENDOTHELIAL DYSFUNCTION, INSULIN RESISTANCE AND LOW-GRADE INFLAMMATION IN TYPE 2 DIABETIC PATIENTS WITH CAD (PAPER I & II)

PAPER I

This study demonstrated a persistent endothelial dependent dysfunction with a concomitant persistent inflammatory activity in type 2 diabetic patients. Plasma CRP levels declined substantially over 60 days in the non-diabetic patients, whereas CRP remained elevated in type 2 diabetic patients. Plasma adiponectin levels were significantly lower in type 2 diabetic than in non-diabetic patients already at baseline, a pattern that did not change at follow-up. Moreover, between baseline and follow-up, changes in FMD followed changes in CRP concentrations significantly in non-diabetic patients with a borderline significance in type 2 diabetic patients (figure 8). In contrast, no such correlation was seen between temporal changes in FMD and adiponectin concentrations. Finally, changes in NTG between baseline and follow-up also did not reveal any significant correlation with the above variables in any group.

It has been proposed that CRP independently predicts cardiovascular risk (47,185,186), a claim that recently has been toned down in

Figure 8. Correlations between temporal changes in FMD and CRP.
This scatter diagram demonstrates that changes in CRP were accompanied by corresponding changes in FMD between baseline and follow-up.
the prospective Reykjavik study, showing just a moderate increase in the risk of CAD (49). However, there are 22 prospective studies, including the Reykjavik study, showing that high levels of CRP predict the risk of heart disease (187). Atherosclerosis is considered to be an inflammatory disease and there is evidence that type 2 diabetes and atherosclerosis may share a common inflammatory basis (16,188). Therefore, the persistent endothelial dysfunction seen in diabetic patients may be a consequence of low-grade inflammation, since CRP levels remained elevated in type 2 diabetic patients. A relationship between high CRP levels and endothelial dysfunction exists (189-191). Different explanations for the persistent endothelial dysfunction and inflammatory activity are feasible. It cannot be entirely excluded that putative subtle differences in drug therapy between groups may have influenced FMD and plasma levels of CRP or adiponectin. The effects of statins (174,175) and ACE-i (169) have been described in the literature to be mediated directly or indirectly by NO signaling pathways. Moreover, hyperglycemia may link low-grade inflammation and endothelial dysfunction, e.g. hyperglycemia is associated with increased levels of inflammatory markers (192,193). Hyperglycemia per se affects the endothelium negatively, e.g. by inducing vasoconstriction and endothelial dysfunction in man (69,78). Recent in vitro studies show that hyperglycemia, through the hexosamine pathway, impairs activation of the insulin receptor resulting in degradation of eNOS and decreased NO production (194,195). Furthermore, patients with type 2 diabetes are over-represented having the metabolic syndrome (24), including insulin resistance, dyslipidemia, hypertension and overweight, consistent with the current study. The metabolic syndrome has been conclusively shown to be associated with endothelial dysfunction and low-grade inflammation (3,5).

Adiponectin concentrations were significantly decreased in type 2 diabetic patients and did not change over time. Plasma levels of adiponectin are reportedly reduced in type 2 diabetes (196), and experimental studies have indicated anti-inflammatory and anti-atherogenic properties of this adipocytokine (197). Hence, a relative lack of adiponectin might be suspected to contribute to the pro-inflammatory state in diabetes. An inverse correlation between CRP and adiponectin concentrations has been reported (198), although this did not apply in this study. Very recently it was shown that high plasma levels of adiponectin are associated with lower risk of MI, without any relationship to CRP (85). This suggests that adiponectin may not correlate to CRP (85), but to other inflammatory markers. Adiponectin negatively correlates to TNF-a and IL-6 (197,199), two pro-inflammatory cytokines elevated in diabetes and involved in endothelial dysfunction. Furthermore, adiponectin stimulates production of NO in endothelial cells in a different way than insulin, involving phosphorylation of eNOS (200). However, adiponectin levels seem not to directly correlate with FMD in some (94,201), but not all (202) studies.

Acutely, we found a mild impairment of NTG in both groups but a significant recovery at follow up. Impaired vasodilatation responses to both nitroglycerin and ACh have been described in epicardial arteries in humans with CAD (203). It has also been suggested that smooth muscle dysfunction occurs independently of impaired endothelium-dependent vasodilatation in adults at risk of atherosclerosis (204). In contrast, it seems that the presently studied subjects retained much of their ability to react to organic NO and that responses were improved during two months of follow-up. The reason for this improvement in NTG is not clear, but functional changes may exist in the VSMC to account for the altered reactivity to NTG, e.g. decreased activity of intracellular guanylate cyclase, cGMP or Ca^{2+}-dependent relaxation.

**PAPER II**

In this study we demonstrated a reduced FMD and NTG in type 2 diabetic patients along with increased plasma levels of TNF-a and IL-6, indicative of pro-inflammatory activity (figure
TNF-a concentrations and brachial artery diameter were negatively, whereas SI was positively associated with FMD. Adjustment for age weakened the association for SI, whereas TNF-a and brachial artery diameter remained significantly associated with FMD after adjustment for group, age and BMI. For the NTG response, no correlation was seen with the above variables. Plasma levels of adiponectin were lower in type 2 diabetes patients, albeit without any correlation with endothelial function.

TNF-a may cause endothelial dysfunction in several different ways; it diminishes the ability of arterial rings to relax in response to the endothelium-dependent vasodilator ACh (205) and induces a transient reversible endothelial dysfunction in humans (206,207). Interestingly, anti-TNF-a treatment improves endothelial function in rheumatoid arthritis patients (208,209). However, not all studies show benefits on endothelial function after lowering plasma TNF-a levels (210). In humans TNF-a inhibits insulin-sensitive glucose uptake and endothelial function, and has been suggested to be a mediator between insulin resistance and endothelial dysfunction (211). TNF-a impairs intracellular insulin signaling, which improves after neutralization of TNF-a in a rodent model (212). However, we found no correlation between TNF-a and SI. Previous reports however indicate that TNF-a mRNA expression and secretion in adipose tissue are inversely associated with insulin sensitivity, whereas a poor correlation exists with circulating TNF-a plasma levels (213). Therefore, we cannot rule out that TNF-a still may be linked to insulin resistance and endothelial dysfunction in our patients. An alternate explanation is that insulin resistance and low-grade inflammation contribute to endothelial dysfunction in parallel (3).

There was no correlation between CRP and FMD, contradictory to our recent findings (paper I), where changes in CRP followed changes in FMD in patients suffering an MI. However, considering that the current work was a cross-sectional study, differences in study design may explain these apparent discrepancies. Very recently, two large cross-sectional studies, addressing whether inflammatory markers are related to endothelial function, have emerged. First, Verma et al. studied the correlation between CRP and FMD in a healthy population, and found no correlation at all (214). Interestingly, in the same study a weak correlation between CRP and FMD, which lost significance after adjustment for traditional CAD risk factors (215). They concluded that inflammation per se has no additional effects on FMD beyond those attributable to traditional risk factors but suggested that systemic inflammation may contribute to

**Figure 9. Impaired FMD and NTG concomitant with increased plasma levels of TNF-a and IL-6 in type 2 diabetic patients.**
Type 2 diabetic patients suffering an MI demonstrate endothelial dysfunction and signs of low-grade inflammation. *P<0.05 and †P<0.01, respectively, between groups at given time point. Bars indicate means ± SE.
impaired vasomotor function in forearm microvessels (215). Even if CRP has emerged as one of the most important predictors of CAD, it seems that the association with endothelial function is weak in healthy subjects (214,215). However, CRP correlates with FMD in subjects at high risk for CAD (214).

The association between whole-body glucose uptake and FMD has been demonstrated by some (128,132,216,217), but not all (218), groups. In our study, SI association with FMD revealed an age dependent association.

Type 2 diabetic patients are over-represented with the metabolic syndrome (24), consistent with our current work. BMI was positively correlated to TNF-a, whereas negatively to SI, and adjustment for BMI did not change TNF-a association with FMD. Although BMI and waist circumference did not differ between groups, it is conceivable that macrophages or even EC may have contributed to the differences of TNF-a between groups. Also, differences in visceral fat and its secretion products might in part contribute to differences in plasma TNF-a levels between groups. Plasma levels of adiponectin were lower in type 2 diabetic compared to non-diabetic patients, whereas no difference was seen for plasma resistin. Consistent with recent studies, no correlation between neither adiponectin nor resistin and FMD was noted (94,201). Interestingly, adiponectin positively correlated with SI and HDL-cholesterol, whereas negatively with TNF-a, triglycerides and BMI. Adiponectin significantly inhibits phagocytic activity and suppresses lipopolysaccharide-induced production of TNF-a (219). One enticing explanation remains, i.e. adiponectin may regulate insulin sensitivity and TNF-a production, thereby indirectly affect endothelial function.

In summary from papers I & II, patients with type 2 diabetes suffering an MI demonstrate a persistent endothelial dependent dysfunction, which in part may be explained by a concomitant persistent low-grade inflammation as reflected by an increase in CRP and TNF-a levels, and to some extent impaired whole body glucose uptake. Also, in type 2 diabetic patients, adiponectin levels are decreased compared to non-diabetic patients suffering an MI, but without any correlation with endothelial function.

TETRAHYDROBIOPTERIN PROMOTES GLUCOSE DISPOSAL IN TYPE 2 DIABETES (PAPER III)

The main and new finding in this study is that BH4 improves glucose disposal in type 2 diabetic patients, without any effects in non-diabetics or healthy subjects (figure 10). This beneficial effect of BH4 occurred without any discernable changes in FMD, at odds with other reports (74-77). Several potential reasons for this apparent discrepancy deserve consideration. Changes in glucose disposal may not mirror changes in FMD. Even if we were able to show an association between SI and FMD (paper II), this association may not be causal. Also, we cannot rule out that microvessels may have been affected by BH4, changes that may have been detected by other methods chosen for studying endothelial function (119,220,221). A number of studies have used total blood flow rates as a measure of insulin's vascular action, but this approach may mask a significant vascular effect of insulin. There are reports indicating that this effect occurs at least 60 min prior to any changes in total muscle blood flow.

![Figure 10. BH4 enhances insulin sensitivity in type 2 diabetic patients](image-url)

Insulin sensitivity index (SI) was measured at steady state (between 90-120 min) during the isoglycemic clamp. *P<0.05 compared to saline. Bars indicate means ± SE.
(221). Also notable is that microvascular flow closely follows changes in glucose infusion rate and not total muscle blood flow (222). One plausible explanation as to why the observed enhancement in glucose disposal evoked by BH4 was not paralleled by a corresponding increase in FMD and brachial artery blood flow would thus be that nutritive capillary recruitment may have occurred in response to BH4 infusion. Moreover, it should be noted that BH4 also serves as a cofactor for the aromatic amino acid hydroxylases (phenylalanine, tyrosine and tryptophane), independently of NO. Thus, we cannot rule out that mechanisms other than NO may also have contributed to the improvement in insulin sensitivity evoked by BH4. Furthermore, we cannot exclude that BH4 could have acted as a scavenger affecting microvascular endothelial function, thus producing the increase in glucose disposal. Nonetheless, Ihleman et al recently showed that improved endothelial function after BH4 treatment was not due to a scavenging effect, but attributable to the role of BH4 as a cofactor for eNOS (75).

It appears that BH4 improves insulin resistance only in the setting of hyperglycemia. The oxidative stress associated with hyperglycemia may influence EC function or skeletal muscle through a depletion of BH4 (70,223). BH4 supplementation significantly increases the vascular content of BH4 and restores NO production in aortas from fructose-fed rats and in mesangial cells cultured in high glucose (70,223,224). In the current working model, prolonged hyperglycemia in diabetic subjects may result in an alternative metabolism of glucose, e.g. through the polyol pathway which shifts the cytosolic NADH/NAD⁺ ratio towards an oxidative milieu. This altered redox ratio may limit the availability of BH4 and uncouple eNOS or neuronal NOS (nNOS), the predominant form of NOS in skeletal muscle, resulting in an increase in O₂⁻ production rather than NO. Therefore, BH4 may have restored an uncoupled state of the L-arginine-NO pathway in our patients, yielding NO instead of O₂⁻, thus alleviating insulin resistance via insulin-mediated capillary recruitment or by directly promoting glucose uptake in the skeletal muscle (225).

Although we have not yet pinned down the precise nature by which BH4 promotes glucose disposal in type 2 diabetic subjects, further research should be done to explore the mechanisms involved. There are several issues left to be addressed in this study and also some limitations, e.g. not measuring BH4 levels in plasma, short-time administration and most importantly the sole focus on a conduit vessel to monitor endothelial function.

**EFFECTS OF GLP-1 ON ENDOTHELIAL FUNCTION IN TYPE 2 DIABETIC PATIENTS (PAPER IV)**

The results from this study demonstrate a salutary effect of GLP-1 on FMD in type 2 diabetic patients, without any effects in healthy subjects (figure 11). We also demonstrated GLP-1 receptor expression on EC, which earlier has been described for many other extrapancreatic tissues but not the endothelium (157,160,161). It is premature to conclude that the beneficial effect is directly mediated by GLP-1, for several reasons. First, the improvement in FMD by GLP-1 was paralleled with a concomitant increase in C-peptide and decrease in glucagon levels. In fact, infusion of C-peptide increases basal levels of NO in subjects with type 1 diabetes mellitus (226). However, this effect of C-peptide has not been proven in type 2 diabetic subjects but we cannot exclude such an effect in the endothelium in these patients. Second, during the clamp endogenous insulin secretion was stimulated by GLP-1, as reflected by rising C-peptide levels in both groups which may reflect an increase in insulin clearance. Whether this may have affected FMD is not clear. Insulin has vasoactive effects, which also were seen in the current study with an increase in baseline brachial artery diameter and a trend towards an increase in FMD between onset and clamp. Even if plasma insulin did not differ between saline and GLP-1 infusions, we do not know whether GLP-1 may have potentiated insulin’s effect on the endothelium. Third, SI increased
by 16 % in type 2 diabetic subjects, albeit not attaining statistical significance. Although this increase was small, we cannot entirely rule out that this improvement in whole body glucose uptake also may partly have contributed to the improved FMD response seen in the type 2 diabetic subjects. However, in paper III SI increased by 30 % without affecting FMD, thus making this caveat unlikely.

GLP-1 treatment, both in short- and long term, (158,168) decreases FFA levels in man. Interestingly, transient hypertriglyceridemia and increased FFA acutely blunt the FMD response in healthy subjects (82). However, in our patients we did not measure FFA levels during the experiment but hypothetically GLP-1 may have affected lipid metabolism, and thereby improved FMD.

As far as I am aware there are no other human studies investigating whether GLP-1 affects endothelial function, but several studies show salutary effects by GLP-1 on the heart (168,227,228). Three days of GLP-1 infusion improves left ventricular heart failure in patients (of whom 50 % were diabetic) with acute MI and severe systolic dysfunction after successful primary angioplasty (168). This improvement was accompanied by changes in glycemia as well as insulin levels; therefore it remains elusive whether this beneficial effect of GLP-1 on cardiac function was directly mediated. Authors speculated that GLP-1 may have improved endothelial function and microcirculatory integrity as suggested by the higher peak creatine phosphokinase in the GLP-1 treated patients, despite comparable baseline regional and global left ventricular dysfunction in both groups (168). Also in a pacing-induced dilated cardiomyopathy canine model, GLP-1 improves left ventricular stroke volume and systemic hemodynamics, including coronary artery blood flow (228). This improvement was paralleled by an increase in myocardial glucose uptake and decreased plasma norepinephrine and glucagon levels (228). In contrast, the hemodynamic benefits by GLP-1 were not seen in normal dogs despite increases in myocardial glucose uptake, suggesting that GLP-1 has unique benefits in the diseased state (228). Very recently, it was also demonstrated that GLP-1 may protect the myocardium against ischemia/reperfusion injury (167). This action by GLP-1 was independent from other hormones (i.e. insulin, glucagon or norepinephrine) and abolished by an inhibitor of PI3-kinase, suggesting non-insulin dependent beneficial effects of GLP-1 on the myocardium via the PI3-kinase signaling pathway (167).

In conclusion, GLP-1 may restore endothelial dysfunction in type 2 diabetic patients with CAD. The salutary effect on the endothelium may be a consequence of several additional effects. Several limitations of this study should be kept in mind, e.g. investigating direct GLP-1 effects on conduit vessels probably requires intra-arterial infusion, and semi-supraphysiological doses of GLP-1 may not reflect normal physiology.

GLP-1 RELAXES CONDUIT ARTERIES (PAPER V)

When GLP-1 was administered during a phenylephrine-induced contractile tone, a dose-dependent vascular relaxation of femoral artery rings was obtained. A significant relaxation was observed already at 10^{-11} mol/l of GLP-1. The maximal relaxation obtained with the highest concentration of GLP-1 (10^{-7} mol/l) was only 29 % compared to the 97 % relaxation achieved by ACh, revealing a weak vasorelaxant by GLP-1 consistent with other reports (161,165). The GLP-1 relaxation effect was completely inhibited by the specific GLP-1 receptor antagonist exendin(9-39), indicating the requirement for specific GLP-1 receptor occupancy for this action of GLP-1 (figure 12).

To further examine if the relaxation induced by GLP-1 was NO-dependent, the artery rings were preincubated with L-NNA at a concentration that markedly prevented the relaxation induced by ACh. L-NNA did not attenuate the relaxation induced by GLP-1. Furthermore, the relaxant effect of GLP-1 remained intact also after mechanical removal of the endothelium. The successful removal of the endothelium was
demonstrated by the significant attenuation of ACh-induced relaxation. This is not consistent with Richter et al., who showed an attenuated vasorelaxant effect by GLP-1 after denudation of the endothelium (161). An additional study demonstrated a NO-dependent relaxing effect of GLP-1 in precontracted rat pulmonary arteries (165). Also, Richter et al. were unable to demonstrate GLP-1 receptors on endothelial cells but on the VSMC in the vessel (161). Therefore, involvement of NO in the relaxant effect of GLP-1 may differ between arteries (conduit vs. pulmonary).

Taking paper IV & V together, it would be logical to conclude that GLP-1 effects on the endothelium in study IV were indirectly mediated (discussed above). However, the findings in study V are not consistent with other reports showing both an endothelium- and NO-dependent vasorelaxant effects by GLP-1 (161,165). The reasons for this may be inherent differences in artery vessel rings tested. The inconsistent findings (paper IV & V) may also be a consequence of differences in species (human vs. rat), vessel conditions (atherosclerotic vs. non-atherosclerotic), or experimental design (systemic in vivo vs. organ baths ex vivo). Nonetheless, these beneficial vascular effects of GLP-1 add yet another salutary property of the peptide, increasing its clinical utility in type 2 diabetic patients in whom endothelial dysfunction and hypertension are both salient features that adversely affect their survival.

**Figure 11. GLP-1 improves FMD in type 2 diabetic patients**

This box plot shows that FMD during isoglycemic hyperinsulinemic clamp was significantly increased by infusion of GLP-1. *P<0.05 vs. placebo.

**Figure 12. Vasorelaxation induced by GLP-1 is prevented by exendin(9-39)**

Relaxations of rat femoral artery ex vivo induced by GLP-1 alone (n=14) and in the presence of the receptor antagonist exendin(9-39) (10^-7 mol/l) (n=6).
Endothelial dysfunction is one major factor in the atherosclerotic progress and predicts CVD outcome in man (14,15,97-105). Endothelial dysfunction, widespread in type 2 diabetes, co-exists with obesity and insulin resistance and may explain the poor outcome in CVD in these patients (3). Therefore, any intervention affecting either endothelial dysfunction or insulin resistance may confer treatment benefit and perhaps improve survival in patients with type 2 diabetes. Also, subclinical chronic inflammation might be an important factor linking insulin resistance, obesity and type 2 diabetes with endothelial dysfunction and CVD (17). In this work we have investigated endothelial function in type 2 diabetic patients with different aspects in mind, e.g. MI, insulin resistance and low-grade inflammation.

First we demonstrated that type 2 diabetic patients suffering an MI show a persistent endothelial dysfunction, which in part may be due to a persistent low-grade inflammation as reflected by elevated CRP, TNF-a and IL-6 levels. More evidence for this view was inferred from the fact that changes in CRP negatively correlated to changes in FMD. However, this finding was not obvious in paper II, showing no correlation between CRP and FMD. This apparent discrepancy may be due to differences in study design (prospective vs. cross-sectional studies), i.e. changes in variables over time may have more predictive power than just one measured time point. Plasma levels of TNF-a and IL-6 were elevated in type 2 diabetic patients. Also, TNF-a was negatively correlated with FMD, whereas a weaker positive association between whole body glucose uptake and FMD was noted. Taking paper I & II together, it seems reasonable to conclude that prolonged low-grade inflammation in type 2 diabetic patients exists after an MI. It also appears that both TNF-a and CRP correlate to FMD and to some extent whole body glucose uptake, suggesting that the endothelium is negatively impacted in multiple ways by the diabetic state after an MI (4).

Compared to non-diabetic patients, type 2 diabetic patients were defined having the metabolic syndrome (24), consistent with dyslipidemia and glycemic disturbances, whereas BMI and waist circumference did not differ between groups. The metabolic syndrome and particularly insulin resistance might in turn give rise to the low-grade inflammation seen in type 2 diabetic patients. However, it is also conceivable that macrophages or even EC may have contributed to the differences in TNF-a, IL-6 and CRP levels between groups. Also, we cannot rule out that subtle differences in visceral fat and its secretion products may partly explain the differences in inflammatory markers noted.

Plasma levels of adiponectin were lower in type 2 diabetic compared to non-diabetic patients, whereas no difference was seen for resistin. High levels of plasma adiponectin concentrations are associated with lower risk of MI in humans (85). Consistent with recent studies, no correlations between neither adiponectin nor resistin and FMD were discerned (94,201). Therefore, it seems unlikely that there is a robust direct association between endothelial function and adiponectin or resistin levels.

Not only FMD was impaired in type 2 diabetic patients, but also NTG. The reason for this finding is not clear, but smooth muscle dysfunction in type 2 diabetic patients has been reported (229). The other interesting finding was that NTG seems to be impaired acutely following an MI, with a recovery after 60 days. For some reason it seems that the vascular smooth muscle is more responsive to nitroglycerin post-MI than in the acute state, a finding that to the best of my knowledge has not been described before.

The eNOS cofactor BH4 increased whole body glucose uptake in type 2 diabetic patients, but was inactive in non-diabetic patients or in healthy subjects. This enhancement in glucose
uptake was not accompanied by a corresponding improvement in FMD. The underlying mechanism for the improved insulin sensitivity remains elusive at this point, but several possible mechanisms should be considered. It seems that BH4 exerts its effect in particular in patients with hyperglycemia and pronounced insulin resistance. Therefore, BH4 may have restored the uncoupled state of eNOS or nNOS evoked by hyperglycemia. Moreover, improvement in whole body glucose uptake was not followed by an improvement in FMD, which may be too crude a measure of endothelial function. Whole body glucose uptake might not mirror conduit vessel physiology, e.g. microvascular flow closely follows changes in glucose infusion rate as opposed to total muscle flow (221). Hence, BH4 might have induced capillary recruitment secondarily to eNOS activation, without affecting macrovascular blood flow. Furthermore, other mechanisms than NO may also have contributed to the improvement in insulin sensitivity evoked by BH4. Nonetheless, these novel findings may be useful in designing novel drugs targeting the impaired insulin sensitivity characterizing patients with type 2 diabetes.

GLP-1 improved FMD in type 2 diabetic patients, without any effects in healthy subjects. Also, we demonstrated GLP-1 receptor expression on EC. Whether the salutary effect on the endothelium is directly mediated by GLP-1, remains elusive at this point. Several additional effects by GLP-1 in this setting were seen, i.e. changes in C-peptide and glucagon levels, and a small increase in SI, which all may have contributed to the improvement in insulin sensitivity evoked by BH4. Nonetheless, these novel findings may be useful in designing novel drugs targeting the impaired insulin sensitivity characterizing patients with type 2 diabetes.

FUTURE DIRECTIONS

Subclinical chronic inflammation might be an important pathogenetic factor in the development of insulin resistance and type 2 diabetes. More specific and sensitive biomarkers should be identified, which may predict early disturbances in insulin sensitivity and endothelial dysfunction. Also, inflammatory signaling pathways need to be explored in greater detail, and may form the basis of drugable targets against the epidemic of insulin resistance and atherosclerosis (17).

In atherosclerosis and diabetes, eNOS bioactivity is reduced and oxidative stress is increased, contributing to endothelial dysfunction. Salutary effects on endothelial function by BH4 have been demonstrated (231). Although we were unable to demonstrate any positive effect of BH4 on endothelial dysfunction in our diabetic patients, as opposed to insulin resistance, we believe that the insulin-sensitizing action of BH4 is an important finding. Improvement of insulin resistance may well translate
into beneficial effects on many CVD risk factors and may thus have important clinical implications in preventing macroangiopathy in type 2 diabetes.

GLP-1 is a promising emerging drug in the treatment of type 2 diabetes mellitus. Its widespread extrapancreatic effects reported, i.e. beneficial effects in heart failure and myocardial ischemia, are of extremely high interest (167,168,227,228,232). Another useful feature of GLP-1 is that it rarely induces hypoglycemia, a major clinical problem with insulin (36). As GLP-1 research has focused mainly on its glycemic actions, studies of the beneficial effects of GLP-1 on cardiovascular parameters and risk factors are just in its infancy but should be exploited thoroughly, given the magnitude of CVD in type 2 diabetes.
LIMITATIONS OF THE STUDIES

• Studies were small and have to be seen as pilot studies. No power calculations were provided.

• Except paper I, only male patients were studied, confining the results to men. Also, this thesis is based solely on patients with CAD.

• Papers I & II are small observational studies, which do not permit any mechanistic or causal insights.

• It would have been useful having measured small vessel function as well, e.g. resistance or even capillary vessels. This may have extended the conclusions, especially in paper III.

• In papers III and IV, only short-term infusions were used. This confines the results and conclusions to acute changes in endothelial function and insulin sensitivity, which may not necessarily translate into long-term changes.
CONCLUSIONS

- Prolonged low-grade inflammation (as reflected by persistently elevated plasma levels of CRP, TNF-a and IL-6) and low plasma adiponectin concentrations are present in type 2 diabetic patients with CAD.

- In type 2 diabetic patients with CAD, FMD and NTG are impaired, indicative of endothelial dysfunction. FMD (but not NTG) seems to be inversely associated with CRP and TNF-a and to some extent with insulin resistance. No direct association between FMD/NTG and adiponectin levels was observed.

- BH4 enhances glucose disposal in patients with type 2 diabetes, which may be due to a capillary recruitment mechanism. The precise nature of this mechanism should be investigated in future studies of type 2 diabetic patients, in whom insulin resistance is a salient feature.

- GLP-1 ameliorates endothelial dysfunction in type 2 diabetic patients with CAD. Whether this improvement is a direct effect by GLP-1 is not proven, but may nonetheless translate into significant cardioprotective influences in these patients at high risk of CVD.

- GLP-1 relaxes rat conduit arteries ex vivo via an endothelium-independent mechanism. The specific GLP-1 receptor antagonist exendin(9-39) completely blocks this effect, consistent with specific signaling through the GLP-1 receptor.
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