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Endothelial dysfunction in patients with glucose abnormalities and coronary artery disease

Studies of pathogenesis and treatment

by

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*To my “fantastic four”,
Camilla, Thea, Stella and Linn*

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ABSTRACT

Background

Type 2 diabetes is associated with endothelial dysfunction, which is characterised by the reduced bioavailability of nitric oxide (NO). This is a result of increased oxidative stress and inflammation and the synthesis of endothelium-dependent vasoconstricting factors such as endothelin-1 (ET-1) caused by hyperglycaemia, insulin resistance and dyslipidemia. The dysfunction of the vascular endothelium is regarded as an important factor for the increased risk of cardiovascular disease seen in patients with type 2 diabetes and it is thought to play a major role in the pathogenesis of both micro- and macrovascular complications in this patient category. This thesis aims to further explore the pathogenesis and treatment options of endothelial dysfunction in patients with glucose abnormalities.

Studies I-II

The importance of the lipid-independent (pleiotropic) effects of statins was studied in 43 patients with dysglycemia and coronary artery disease. Intensive lipid lowering with either 80 mg of simvastatin or a combination of 10 mg of simvastatin together with 10 mg of ezetimibe improved macrovascular endothelial function and microvascular function (n=36) and reduced inflammation. No difference between the two treatment strategies was found, indicating that the improvements were mainly due to lipid lowering and not to the pleiotropic effects of statins.

Study III

The effect of endothelin-A-receptor blockade on nutritive skin capillary circulation in patients with type 2 diabetes and microangiopathy was studied. Intra-arterial infusions of an endothelin-A-receptor antagonist improved nutritive skin capillary circulation in patients with type 2 diabetes (n=10) but not in healthy controls (n=8). This finding suggests that ET-1 is involved in the pathogenesis of diabetic microangiopathy.

Study IV

The effect of L-arginine and tetrahydrobiopterin (BH₄) infusion on ischemia/reperfusion (I/R)-induced endothelial dysfunction following 20 minutes of forearm ischemia was studied in 12 patients with type 2 diabetes and coronary artery disease. L-arginine and BH₄ significantly attenuated I/R-induced endothelial dysfunction in comparison with placebo.

Conclusions

The present studies of patients with type 2 diabetes and vascular complications indicate that

- 1) lipid lowering is more important than the pleiotropic effects of statins for the improvement in macrovascular endothelial function and microvascular function and the reduction in inflammation,
- 2) targeting the ET-1 system might be of importance in the treatment of complications related to diabetic microangiopathy and
- 3) supplementation with L-arginine and BH₄ may represent a future treatment strategy to limit the I/R injury in patients with type 2 diabetes.

LIST OF ABBREVIATIONS

Ach	Acetylcholine
AGE	Advanced glycation end products
Apo A1	Apolipoprotein A1
Apo B	Apolipoprotein B
B	Biopterin
BH ₂	Dihydrobiopterin
BH ₄	Tetrahydrobiopterin
CAD	Coronary artery disease
CBV	Capillary blood cell velocity
CETP	Cholesteryl ester transfer protein
CVD	Cardiovascular disease
DAG	Diacylglycerol
E10/S10	Ezetimibe 10 mg/Simvastatin 10 mg
EDV	Endothelium-dependent vasodilatation
EIDV	Endothelium-independent vasodilatation
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FBF	Forearm blood flow
FMD	Flow-mediated dilatation
GLUT4	Glucose transporter isoform-4
HbA1c	Glycosylated haemoglobin A1c
HDL	High-density lipoprotein
I/R	Ischemia/reperfusion
IDL	Intermediate-density lipoprotein
IGT	Impaired glucose tolerance
LDF	Laser-Doppler fluxmetry
LDL	Low-density lipoproteins
LPL	Lipoprotein lipase
MAP	Mean arterial pressure
MI	Myocardial infarction
mRNA	Messenger ribonucleic acid
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PI-3 kinase	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
PU	Perfusion units
RAGE	Receptor for advanced glycation end products
ROCK	Rho-associated coiled-coil containing protein kinase
S80	Simvastatin 80 mg
SEM	Standard error of the mean
SNP	Sodium nitroprusside
Statin	HMG-CoA reductase inhibitor
WHO	World Health Organisation
VLDL	Very low density lipoprotein

LIST OF ORIGINAL PAPERS

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

I

Settergren M, Böhm F, Rydén L, Pernow J
Cholesterol lowering is more important than pleiotropic effects of statins for endothelial function in patients with dysglycemia and coronary artery disease.
European Heart Journal 29(14):1753-60, 2008

II

Settergren M, Böhm F, Rydén L, Pernow J, Kalani M
Lipid lowering *versus* pleiotropic effects of statins on skin microvascular function in patients with dysglycemia and coronary artery disease.
Journal of Internal Medicine in press 2009

III

Settergren M, Pernow J, Brismar K, Jörneskog G, Kalani M
Endothelin-A receptor blockade increases nutritive skin capillary circulation in patients with type 2 diabetes and microangiopathy.
Journal of Vascular Research 45(4):295-302, 2008

IV

Settergren M, Böhm F, Malmström RE, Channon KM, Pernow J
L-arginine and tetrahydrobiopterin protects against ischemia/reperfusion induced endothelial dysfunction in patients with type 2 diabetes mellitus and coronary artery disease.
Atherosclerosis doi.10.1016/j.atherosclerosis.2008.08.034, 2008

INTRODUCTION

General background

As a result of a sedentary lifestyle and an ageing population, diabetes is an emerging epidemic. In 2007, there were 246 million people living with diabetes worldwide and more than a third of the population are expected to develop diabetes within their lifetime.¹ Diabetes is classified into two major types, type 1 with no remaining insulin production and type 2 with predominantly insulin resistance and relative insulin deficiency.² Type 2 diabetes accounts for 80-90% of all cases.

Cardiovascular diseases (CVD), including coronary artery disease (CAD), retinopathy, nephropathy and retarded wound healing, are the principal causes of death and disability in patients with type 2 diabetes.³ Type 2 diabetes increases the risk of developing CVD two to four times⁴ and 20 to 30% of patients with CAD suffer from known type 2 diabetes,⁵⁻⁷ while an additional 30% are affected by subclinical glucose abnormalities.^{5,8} There is accumulating evidence of a relationship between glucose levels and cardiovascular events even below the diabetic threshold.⁹

The importance of type 2 diabetes for the development of CAD is further underlined by the finding that patients with type 2 diabetes without a previous MI run the same risk of future MI as non-diabetic patients with previous MI. Type 2 diabetes can therefore be regarded as a CAD equivalent.¹⁰ Patients with type 2 diabetes also have a poorer prognosis following myocardial infarction (MI) than their non-diabetic counterparts. Approximately 50% of diabetic patients die within 5 years after a MI compared with about 25% of non-diabetic patients.¹¹

The dysfunction of the vascular endothelium is regarded as an important factor in this increase in cardiovascular risk and it is thought to play a major role in the pathogenesis of both micro- and macrovascular complications in patients with type 2 diabetes.³ Understanding the mechanism behind endothelial dysfunction and restoring endothelial function is therefore of great importance for patients with type 2 diabetes. This thesis aims to further explore the pathogenesis of and treatment options for endothelial dysfunction in patients with glucose abnormalities.

The endothelium

The arterial wall consists of three functionally separate layers: the intima, the media and the adventitia (Figure 1). The intima is composed of endothelial cells and the media consists predominantly of smooth muscle cells embedded in extracellular matrix. The adventitia harbours nutrient vessels, nerves and dense fibroelastic tissue.¹² The endothelium is a monolayer of endothelial cells lining the lumen of all blood vessels and it has been reported to measure 1,000 m² and weigh 1.5 kg in a normal-sized adult.¹³ The endothelium was first thought to be an inert transportation tube, but it has become increasingly clear that the endothelium is a complex organ, releasing a number of autocrine and paracrine substances.¹⁴ It is therefore not only a barrier between the circulating blood and the tissue but also an important regulator of vascular tone and permeability, the balance between coagulation and fibrinolysis, the adhesion and extravasation of leukocytes and inflammatory activity in the vessel wall.¹⁵

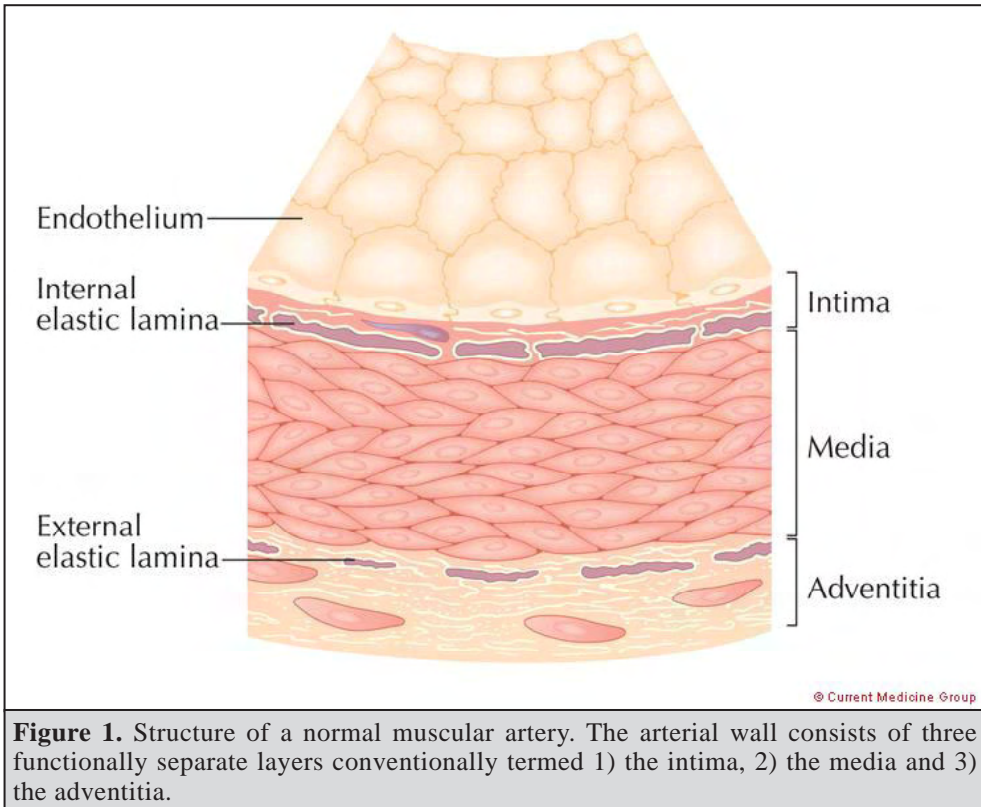


Figure 1. Structure of a normal muscular artery. The arterial wall consists of three functionally separate layers conventionally termed 1) the intima, 2) the media and 3) the adventitia.

Endothelial function and dysfunction

Certain important functions of the endothelium are mediated by a number of endothelium-derived factors which are summarised in Table 1. Nitric oxide (NO) is the key endothelium-derived factor that plays a pivotal role in the maintenance of vascular tone and the reactivity of the endothelium.¹⁶ NO is formed in endothelial cells from the amino acid L-arginine by endothelial NO synthase (eNOS) under the influence of the important co-factor tetrahydrobiopterin (BH₄).¹⁷ In addition to reducing vascular smooth muscle tone, NO serves to inhibit platelet and leukocyte activation and opposes the actions of endothelium-derived contracting factors such as endothelin-1 (ET-1) and angiotensin II. In the healthy endothelium, there is a delicate balance between vasodilating and vasoconstricting substances, resulting in balanced vascular tone and perfusion, as well as anti-thrombotic and anti-inflammatory effects. However, when exposed to the classical risk factors for atherosclerosis, such as hypertension, smoking and hyperglycaemia, this balance shifts towards increased vasoconstriction, thrombosis and inflammation. This endothelial activation is called endothelial dysfunction and it is primarily characterised by the reduced bioavailability of NO due to both the reduced synthesis and the increased degradation of NO. Endothelial dysfunction plays an important role not only in the initiation of atherosclerosis but also in its progression and clinical sequelae. Improving the endothelial function may therefore be important in preventing atherosclerotic disease and its complications.

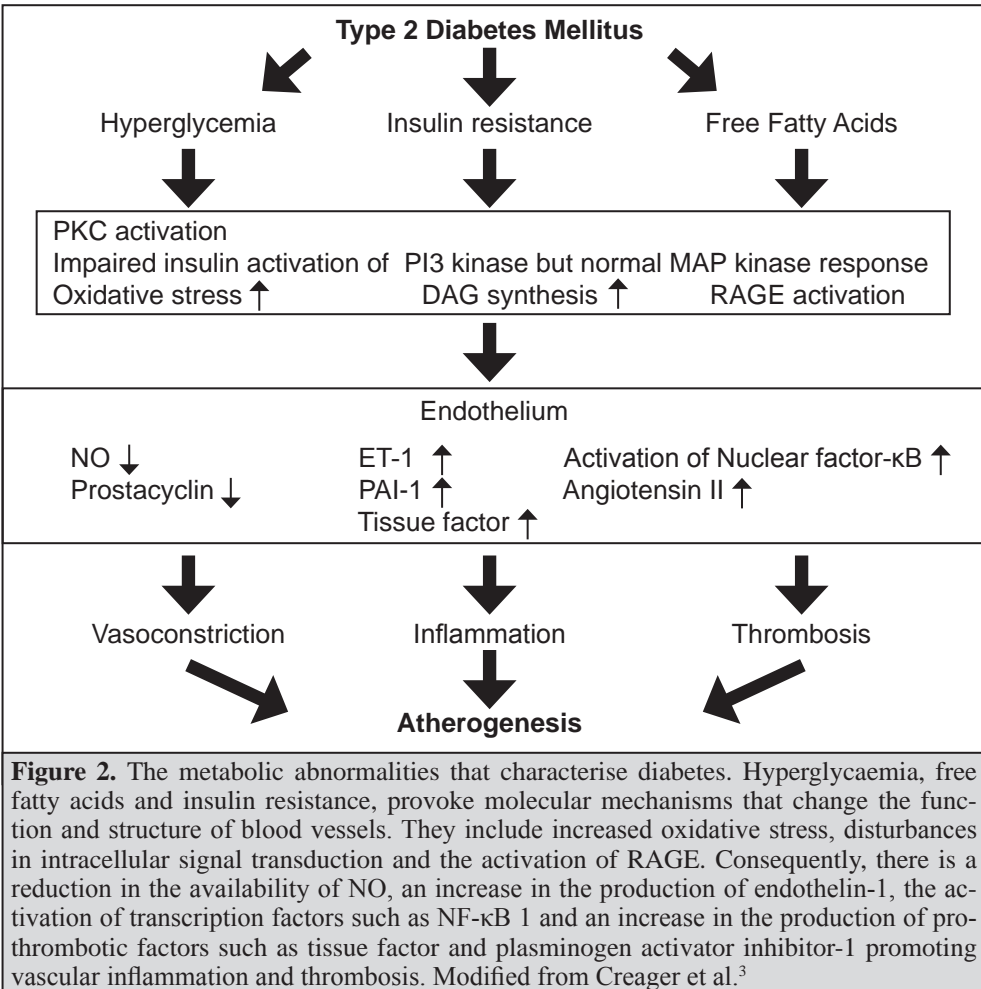
Table 1. Important functions of the endothelium and its mediators.

Functional targets of the endothelial cell	Specific cellular or physiological action	
Lumen	Vasoconstriction	Vasodilatation
	Endothelin-1	Nitric oxide
	Angiotensin II	Bradykinin
	Thromboxane A2	Hyperpolarising factor
	Prostaglandin H2	Prostacyclin
Growth	Stimulation	Inhibition
	Platelet growth-derived factor	Nitric oxide
	Fibroblast	Prostacyclin
	Insulin-like growth factor-1	
	Endothelin-1	
	Angiotensin II	
Inflammation	Pro-inflammatory	Anti-inflammatory
	Adhesion molecules	Nitric oxide
	VCAM, ICAM etc	
	Angiotensin II	
Hemostasis	Pro-thrombotic	Anti-thrombotic
	Plasminogen activator inhibitor-1 (PAI-1)	Prostacyclin
	Tissue factor	Tissue plasminogen activator
	Thromboxane	Nitric oxide

Endothelial dysfunction and type 2 diabetes

Type 2 diabetes is characterised by hyperglycaemia, but it also typically occurs in the context of a cluster of cardiovascular risk factors; abdominal obesity, hypertension, dyslipidemia, insulin resistance and chronic low-grade inflammation, all of which may impair endothelial function.⁴ Consequently, endothelial dysfunction has been found to be present in both cellular and experimental models of diabetes,^{18, 19} as well as in clinical studies of patients with type 2 diabetes.^{20, 21} The metabolic abnormalities that characterise type 2 diabetes and their consequences for endothelial function are summarised in Figure 2.

Hyperglycaemia is a major casual factor in the development of endothelial dysfunction in type 2 diabetes. The intracellular glucose concentration of endothelial cells mirrors that of the extracellular environment.²² An increase in intracellular glucose leads to the activation of protein kinase C (PKC) via the synthesis of diacylglycerol (DAG). PKC reduces the bioavailability of NO by reducing eNOS activity and increasing ET-1 synthesis.²³ Furthermore, PKC also regulates and activates NADPH oxidases with the subsequent production of superoxide anion. Superoxide anion is able to react with NO, which further reduces NO bioavailability. The interaction between superoxide anion and NO results in the formation of peroxynitrite



which can oxidise the eNOS co-factor BH₄ to dihydrobiopterin (BH₂) and biopterin (B).²⁴ As only the reduced form BH₄ is a co-factor for eNOS, the oxidation results in a situation in which eNOS produces superoxide instead of NO, a phenomenon referred to as eNOS uncoupling (Figure 3).²⁵ Superoxide anion also increases the production of advanced glycation end products (AGEs).²⁶ AGEs promote increased oxidative stress by increasing the production of oxygen-derived free radicals by activating the receptor for AGE (RAGE).^{27, 28} In addition, hyperglycaemia is able to further inhibit eNOS by increasing the levels of the endogenous eNOS inhibitor, asymmetric dimethylarginine.²⁹

Apart from hyperglycaemia, insulin resistance is also an important contributory factor to the development of endothelial dysfunction in type 2 diabetes. In healthy subjects, insulin increases NO production by stimulating eNOS activity by activating phosphatidylinositol-3 kinase (PI-3 kinase).³⁰ In the face of insulin resistance, the PI-3 kinase pathway is impaired, while insulin signalling via the MAP kinase pathway remains intact. This pathway may induce endothelial dysfunction by activating ET-1 production and increasing inflammation (Figure 4).^{31, 32} Insulin resistance is also associated with elevated levels of free fatty acids as a result of

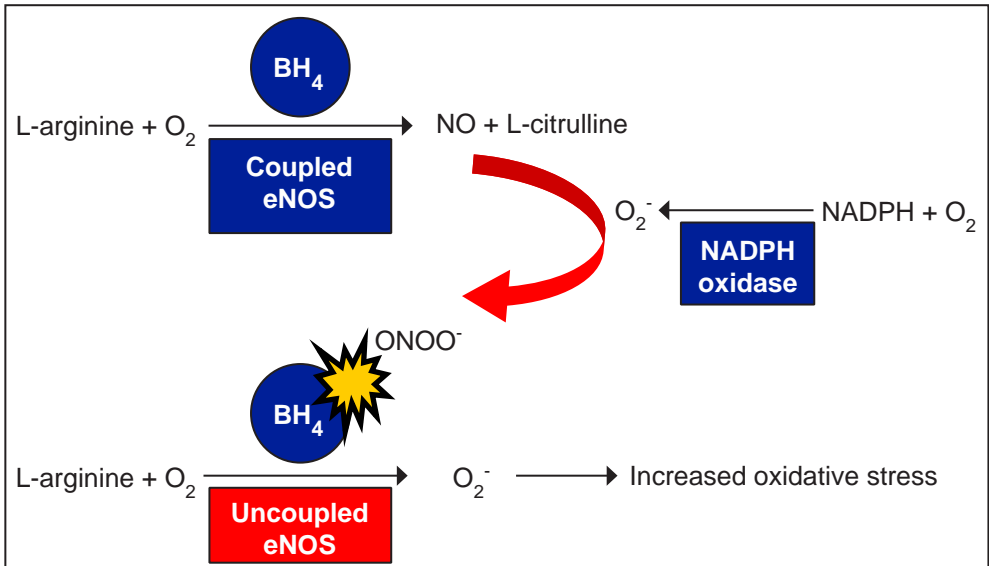


Figure 3. Potential mechanism for eNOS uncoupling in diabetes. NADPH oxidases are up-regulated in diabetes and the product, superoxide anion (O_2^-), reacts with NO to form peroxynitrite ($ONOO^-$). This oxidises BH_4 , the co-factor of eNOS. A functional eNOS is now converted into a dysfunctional O_2^- -generating enzyme that contributes to vascular oxidative stress.

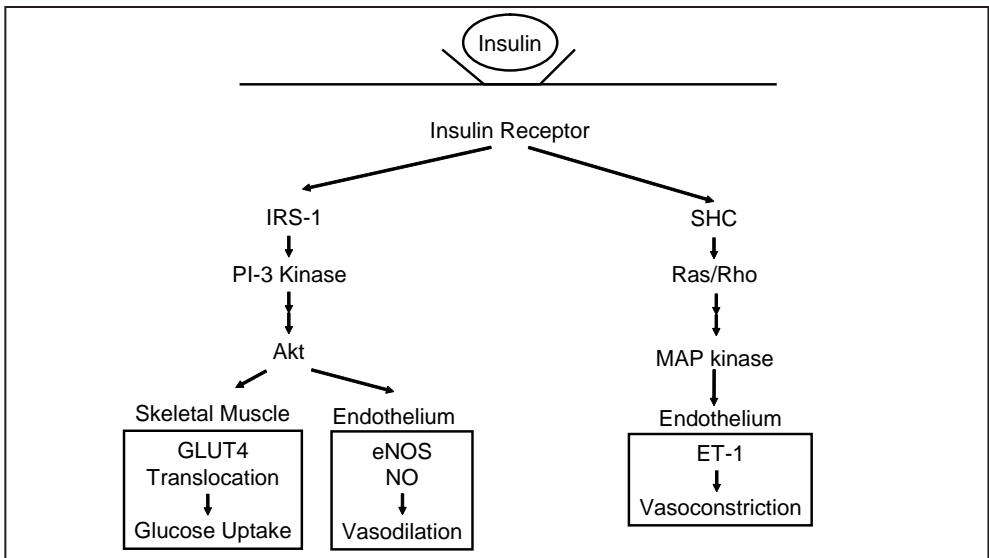


Figure 4. General features of insulin signal transduction pathways. Activation of the insulin receptor substrate-1 (IRS-1) stimulates the PI-3 kinase branch that regulates GLUT4 translocation and glucose uptake in skeletal muscle as well as endothelial NO production, resulting in vasodilatation in vascular endothelium. The MAP kinase branch of insulin signalling generally regulates growth and mitogenesis and controls the secretion of endothelin-1 in vascular endothelium. Modified from Kim et al.²²⁸

excess release from the adipose tissue and diminished uptake in the skeletal muscle.^{33, 34} Free fatty acids induce endothelial dysfunction by increasing the production of oxygen-derived free radicals, the activation of PKC and the exacerbation of dyslipidemia, as discussed below.^{35, 36} Obesity often co-exists with type 2 diabetes. The visceral adipose tissue is a highly active endocrine organ producing hormones, cytokines and enzymes that play a major role in affecting insulin sensitivity, creating a state of low-grade inflammation and inducing endothelial dysfunction.³⁷ The cytokines TNF- α , IL-6, PAI-1 and the adipokines adiponectin and leptin have received a great deal of attention for their association with inflammation, insulin resistance and CVD.³⁸⁻⁴⁰ TNF- α and IL-6 have been linked to endothelial dysfunction and inflammation. IL-6 is a potent stimulus for the production of CRP in the liver. CRP is regarded as an excellent marker of low-grade inflammation in the vascular wall, a well-recognised mechanism in the development of atherosclerosis.⁴¹

Adiponectin has antagonistic effects on the above-mentioned substances, it improves insulin sensitivity and has anti-inflammatory properties.³⁹ Accordingly, high levels of adiponectin have been associated with a lower risk of MI.⁴²

Endothelin-1

The endothelins were first described by Yanagisawa and co-workers in 1988.⁴³ Three different isoforms of endothelin have been found (ET-1, ET-2 and ET-3), of which ET-1 is regarded as the most important isoform for the cardiovascular system.⁴⁴ ET-1 is primarily produced in the endothelial cells, although in diseased states it is also produced in vascular smooth muscle cells, macrophages and leukocytes.^{45, 46} ET-1 exerts its effects by binding to ET_A and ET_B receptors.⁴⁷ The ET_A receptors are mainly located on the smooth muscle cells where they mediate vasoconstriction. Additional effects of ET_A receptor activation are increased inflammation and fibrosis. The ET_B receptors are mainly located on the endothelial cells where they stimulate NO production. They are also expressed on smooth muscle cells where they mediate effects similar to those induced by the ET_A receptor.⁴⁸ In the healthy vessel, the effects mediated by ET_A and ET_B receptors on the smooth muscle cells are partly opposed by the effects mediated by ET_B receptors on the endothelial cells. However, in atherosclerosis, there appears to be an up-regulation of the ET_B receptors on the smooth muscle cells, increasing the vasoconstrictor effect mediated by the ET_B receptors.⁴⁹ The effects mediated by ET-1 may therefore differ between physiological and pathophysiological conditions, depending on the change in receptor expression.

Increased plasma levels of ET-1 have been demonstrated in animal models of type 2 diabetes.^{50, 51} This finding has been confirmed in some⁵² but not in other⁵³ studies of patients with type 2 diabetes. These apparently conflicting findings may be related to the fact that plasma ET-1 levels may not truly reflect the activity of ET-1, since its secretion is largely towards the underlying smooth muscle and very little reaches the circulation.^{54, 55} Both hyperinsulinemia and hyperglycaemia may cause increased production of ET-1. Insulin has been shown to increase ET-1 expression and to enhance the release of ET-1 in both endothelial and vascular smooth muscle cells.^{56, 57} Hyperglycaemia stimulates ET-1 production in cultured endothelial cells.⁵⁵ In insulin resistance states, insulin will stimulate MAP kinase pathways, which leads to the increased production of ET-1 (Figure 4). Insulin has also been found to increase the number and binding sites of the ET receptors.⁵⁸ The increased expression of ET-1 and its receptors contributes to endothelial dysfunction and glucometabolic perturbations in type 2 diabetes. Administration of ET-1 results in impairment of endothelial function in healthy

volunteers and this effect is reversed by ET_A receptor blockade.⁵⁹ ET-1 may affect endothelial function through several mechanisms including the reduced expression and activity of eNOS, possibly involving the PKC pathway.⁶⁰⁻⁶² ET-1 may further reduce the bioavailability of NO by increasing the activity of NADPH oxidase and by uncoupling eNOS, with a subsequent increase in the production of superoxide.^{63, 64} Both selective ET_A receptor blockade and dual ET_A/ET_B receptor blockade have been shown to improve endothelial function in patients with CVD.^{49, 65} ET_A receptor blockade has been shown to acutely improve macrovascular endothelial function in patients with type 2 diabetes.⁶⁶ ET-1 may also exert important effects on glucose metabolism and insulin sensitivity. ET-1 increases the serine phosphorylation of IRS-1, leading to reduced PI-3 kinase activity in cultured vascular smooth muscle cells.⁶⁷ Furthermore, ET-1 has been shown to impair the insulin-mediated translocation of GLUT4, causing reduced glucose uptake and further insulin resistance.⁶⁸ These observations in cell culture studies have been confirmed in experimental human studies. The administration of ET-1 causes peripheral insulin resistance in healthy volunteers.⁶⁹ In addition, dual ET_A/ET_B receptor blockade, but not selective ET_A receptor blockade, acutely improves insulin sensitivity in patients with insulin resistance and CAD.⁷⁰ Collectively, these findings support the notion that ET-1 is involved in the pathogenesis of endothelial dysfunction and the regulation of insulin sensitivity in type 2 diabetes.

Microvascular function in type 2 diabetes

The principal function of the microcirculation is the exchange of nutrients and metabolites between blood and tissues.⁷¹ Disturbed microvascular function (i.e. microangiopathy) is a common and serious complication in type 2 diabetes. Complications that are related to microangiopathy are retarded wound healing, retinopathy, nephropathy and neuropathy. The pathogenesis of diabetic microangiopathy is complex and multifactorial. There also appears to be a difference in the pathogenesis of microangiopathy between type 1 and type 2 diabetes.⁷² Type 1 diabetes will not be further discussed here. The striking abnormality in type 2 diabetes is an early and profound reduction in microvascular vasodilatory capacity and an impairment in the autoregulation of capillary blood flow.⁷³ These changes cause absolute or relative ischemia in the perfused tissue, because the capillaries are no longer able to meet the metabolic needs of the tissue, often referred to as capillary ischemia. This effect has been shown to occur even in the absence of or in connection with only mild atherosclerosis in conduit arteries.⁷⁴ Possible defects that could account for this abnormality are reduced capillary density, basement membrane thickening, arteriolar hyalinosis, vascular smooth muscle abnormalities and microvascular endothelial dysfunction.⁷³ The development of microvascular endothelial dysfunction appears to be the most important factor and its development is strongly related to insulin resistance.^{75, 76} It has therefore been suggested that the impairment in autoregulatory function and vasodilatory capacity may be due to defective insulin signalling as described in insulin resistance where insulin via the MAP kinase pathway increases the synthesis of ET-1 and subsequently endothelial dysfunction.^{31, 76} This is further supported by the finding that circulating ET-1 levels are elevated in patients with type 2 diabetes and retinopathy or microalbuminuria.^{77, 78} The question of whether increased ET-1 production is of importance for the development of microvascular dysfunction and whether ET-receptor antagonists improve microvascular function in patients with diabetes has not yet been addressed, however.

Assessment of endothelial and microvascular function

In 1980, Furchgott and Zawadzki discovered the obligatory role of the endothelium in arterial relaxation in response to the administration of acetylcholine (ACh). The substance that mediated this relaxation was subsequently identified as NO.^{16, 79} Ludmer and co-workers adapted the findings made by Furchgott and Zawadzki to the catheter laboratory and were able to demonstrate the dose-dependent dilatation of the coronary arteries in response to ACh in subjects without CAD, whereas in patients with CAD a paradoxical vasoconstriction was observed, indicating endothelial dysfunction.⁸⁰ Quyyumi and colleagues later confirmed that the impaired response to ACh in patients with CAD was largely due to the reduced coronary bioavailability of NO.⁸¹ Since NO is an important mediator of both endothelium-dependent vasodilatation (EDV) and the anti-inflammatory and antithrombotic effects of the endothelium, EDV can be regarded as a “read out” of other important functions of the endothelium.⁸² The method of measuring endothelial function in the coronary arteries has been considered to be a “gold standard” against which other tests of endothelial function have been compared. This is, however, an invasive method that is restricted for use in patients undergoing cardiac catheterisation. Alternative tests of endothelial function involving the peripheral circulation have therefore been developed. Intra-arterial infusions of substances that release NO in the forearm vascular bed are commonly used. These substances include ACh, substance P and serotonin. At least 50% of the vasodilatory response to ACh in the forearm has been shown to be mediated by NO.⁸³ Endothelial function determined by the infusion of ACh in the forearm has been reported to correlate to that in the coronary circulation⁸⁴ and to predict cardiovascular events.⁸⁵⁻⁸⁷ The forearm is therefore considered to be a good model for evaluating endothelial function and also for investigating new pharmacological substances where the intra-arterial infusion produces local responses without affecting the systemic circulation.^{83, 87} However, the administration of ACh requires arterial cannulation which limits its repeatability and use in larger studies. In 1992, Celermajer and co-workers reported on a non-invasive, ultrasound-based test to assess conduit artery vascular function in the systemic circulation.⁸⁸ This method is based on the observation that conduit arteries dilate in response to increased flow (shear stress). The flow-mediated dilatation (FMD) of the brachial artery is stimulated by the reactive hyperemia obtained following a period of forearm ischemia and has been shown to occur predominantly as a result of NO release.⁸⁹ FMD has been shown to be correlated with coronary endothelial function⁹⁰ and to predict cardiovascular events in a number of studies.^{87, 91-96} The imaging technique is, however, technically demanding and requires experienced operators. In order to improve the limited resolution and high variability sometimes seen with FMD assessed by ultrasound, FMD using magnetic resonance imaging has been developed with promising results.⁹⁷

In addition to these two methods, a number of alternative non-invasive approaches for measuring endothelial function have been developed recently, including the analysis of arterial stiffness by radial artery tonometry or pulse contour analysis by digital photoplethysmography,⁹⁸ changes in augmentation index, reflection index⁹⁹ and digital pulse amplitude tonometry.¹⁰⁰ They all are promising, but further validation is required.

Various non-invasive methods have been developed to assess microvascular function, particularly in the skin. Laser-Doppler fluxmetry (LDF) and capillaroscopy are two of the methods that are most frequently used for this purpose. LDF uses red laser that is transmitted to the skin. LDF measures total skin microcirculation, i.e. nutritional capillary blood flow, as well as non-nutritional subpapillary blood flow.¹⁰¹ In research, the microvasculature is provoked by transient ischemia or local warming. The mechanism behind the reactive hyperemia that follows is not fully understood, but it is thought to be endothelium-dependent.¹⁰² LDF has

been shown to predict ulcer outcome and a close relationship between post-occlusive reactive hyperemia and the Framingham risk score has recently been established.^{103,104} Capillaroscopy allows a 2D visualisation of the capillary network in real time. The capillaries can best be studied in the nail fold since the capillary loops run parallel to the skin surface. The capillaries are visualised by a light microscope which is connected to a computer through which the capillary blood cell velocity (CBV) can be calculated.¹⁰⁵ Capillaroscopy measures nutritional capillary blood flow. Jörnsekog and co-workers have shown that reduced CBV during reactive hyperemia predicts the development of ischemic foot ulcers in patients with diabetes and peripheral vascular disease.¹⁰⁶

Lipid metabolism

Lipoproteins play an essential role in the transport of cholesterol, fatty acids and fat-soluble vitamins from the liver and the intestines to the peripheral tissues and also in the reverse transport of cholesterol from the peripheral tissues to the liver. Lipoproteins contain a core of triglycerides and cholesteryl esters surrounded by phospholipids, unesterified cholesterol and proteins. The plasma lipoproteins are divided into five major classes: chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoproteins (LDL) and high-density lipoprotein (HDL). Dietary cholesterol, fatty acids and fat-soluble vitamins are absorbed in the small intestine and transported as chylomicrons to the peripheral tissue where the triglycerides are hydrolysed by lipoprotein lipase (LPL). The free fatty acids that form are encountered by the tissue where they are either oxidised to generate energy or re-esterified and stored as triglycerides. Endogenous lipoproteins are produced in the liver and secreted as VLDL particles that are hydrolysed in the peripheral tissue by LPL. The remnants of VLDL are IDL, which are remodelled by hepatic lipase to form LDL and cleared by LDL receptor-mediated endocytosis in the liver. All nucleated cells synthesise cholesterol, but only hepatocytes are able to excrete cholesterol from the body by conversion to bile acids. The liver and the intestine produce nascent HDL, which incorporates free cholesterol from the peripheral cells forming mature HDL. HDL can be taken up by the liver directly or transferred by cholesteryl ester transfer protein (CETP) to VLDL and chylomicrons that are taken up by the liver.

The lipoprotein abnormalities that are commonly present in type 2 diabetes include hypertriglyceridemia, reduced HDL and the formation of small, dense LDL.¹⁰⁷ This dyslipidemia is often present in pre-diabetes and in insulin resistance, but normal plasma glucose.¹⁰⁸ It is therefore suggested that abnormalities in insulin action and not hyperglycaemia are associated with the lipid abnormalities. This hypothesis is supported by the finding that thiazolidinediones, which improve insulin sensitivity, improve the lipid profile to a greater extent than other glucose-reducing agents.¹⁰⁹ Several actions of insulin are likely to contribute to diabetic dyslipidemia. They include effects on liver lipoprotein production, the regulation of LPL and CETP and peripheral insulin resistance.¹¹⁰

Statins

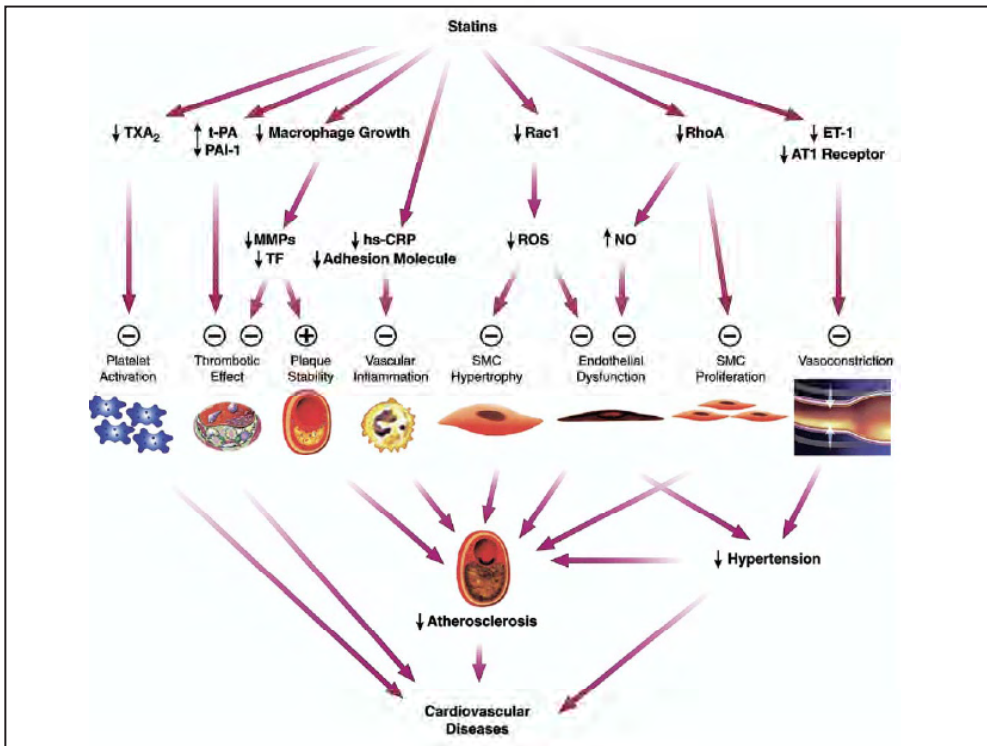
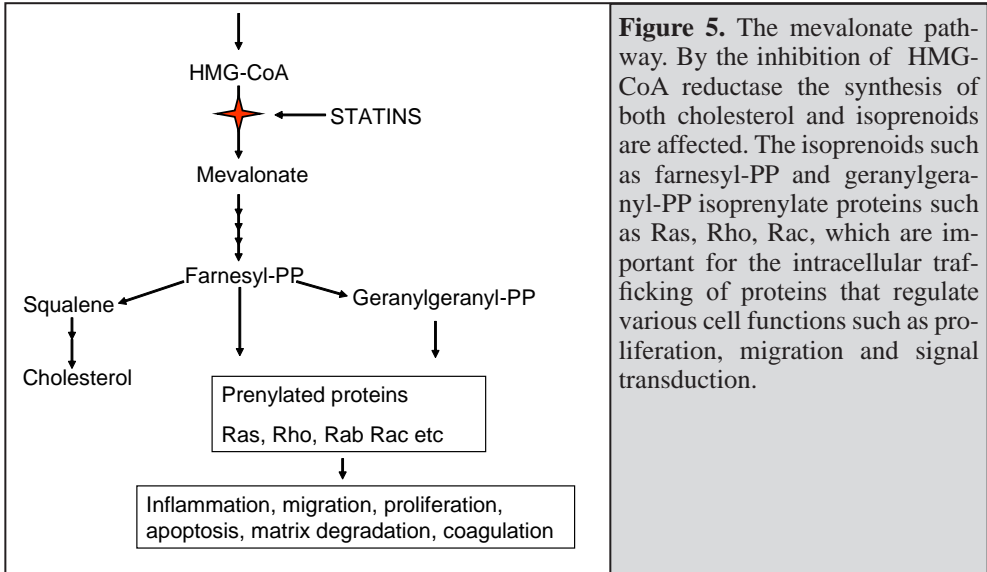
Hypercholesterolemia is an important risk factor for cardiovascular disease. Yusuf and co-workers analysed the relative contribution of risk factors to the occurrence of MI in the INTERHEART study. They found that more than 50% of the risk of developing MI could be accounted for by hypercholesterolemia.¹¹¹ Lipid-lowering therapy has been shown markedly

to reduce cardiovascular mortality in the primary and secondary prevention settings in patients both with and without diabetes.¹¹²⁻¹¹⁹ Furthermore, the STENO-2 study, investigating the effect of multifactorial therapy on patients with type 2 diabetes, revealed that more than 70% of the risk reduction was due to lipid-lowering therapy.¹²⁰

HMG-CoA reductase inhibitors (statins) are the most potent and widely used drugs for treating hypercholesterolemia and the introduction of statins is the main reason for the marked reduction in CVD associated with lipid-lowering treatment. Statins lower cholesterol by inhibiting the HMG-CoA reductase, which is the rate-limiting enzyme in cholesterol synthesis. This enzymatic pathway is involved not only in the synthesis of cholesterol but also in the production of isoprenoids, such as farnesyl-pyrophosphate and geranylgeranylpyrophosphate (Figure 5).¹²¹ These isoprenoids isoprenylate proteins such as Ras, Rho, Rac and Rap, which are important for the intracellular trafficking of proteins that regulate various cell functions such as proliferation, migration, signal transduction, eNOS and NO production.¹²² The inhibition of this pathway may therefore have several beneficial effects that may explain many of the beneficial effects of statins. Questions have therefore been raised about whether these effects are only due to lipid lowering or whether the lipid-independent, so-called pleiotropic, effects of statins are of clinical relevance. The most important effects of statins in relation to CVD are summarised in Figure 6. The possible pleiotropic effects of statins on endothelial function have attracted special interest. In experimental settings, statins have been shown to affect eNOS expression and activity through three different mechanisms that are related to the inhibition of isoprenoids.¹²³⁻¹²⁵ First, statins increase eNOS expression in a RhoA-dependent manner by prolonging the half-life of eNOS mRNA.¹²⁴ Second, statins reduce caveoline-1 abundance. Caveoline-1 binds to eNOS in the caveolae and thereby inhibits NO production directly.¹²⁵ Third, statins can activate the PI-3 kinase/Akt pathway and thereby increase eNOS activity.¹²⁶ Furthermore, statins have also been shown to reduce ET-1 gene expression and ET-receptor mRNA expression by a RhoA-dependent mechanism.^{127, 128} In some studies, statins have also improved endothelial function before any significant reduction in cholesterol levels occurs, suggesting that this improvement would be independent of lipid lowering.¹²⁹ These are all indications that statins could improve endothelial function independently of lipid lowering. On the other hand, there is a large bulk of evidence to indicate that hypercholesterolemia is associated with endothelial dysfunction.¹³⁰ The mechanism by which LDL cholesterol causes endothelial dysfunction involves a reduction in eNOS expression and reduced NO bioavailability due to an increase in reactive oxygen species (ROS).^{131, 132} In addition, oxLDL can recruit leukocytes and increase inflammation in the vascular wall.¹³³ The importance of LDL cholesterol for endothelial function is stressed by the findings reported by Tamai and co-workers, who found that a single LDL apheresis acutely improves endothelial function.¹³⁴ As a result, statins can improve endothelial function by both lipid-dependent and lipid-independent mechanisms, but it remains to be elucidated whether the lipid-independent effects are of relevance in the clinical setting.

Ischemia and reperfusion injury

MI is a result of ischemia of the myocardium due to a thrombus in a coronary artery. The restoration of blood flow is a prerequisite when it comes to salvaging jeopardised myocardium during MI. There is, however, evidence to suggest that reperfusion itself may cause damage to the myocardium by enhancing the formation of oxygen-derived free radicals and the



TXA₂, Thromboxane A₂; t-PA, Tissue Plasminogen Activator; PAI-1, Plasminogen Activator Inhibitor-1; MMPs, Matrix Metalloproteinases; TF, Tissue Factor; ROS, Reactive Oxygen Species; NO, Nitric Oxide; ET-1, Endothelin-1, AT-1 Receptor, Angiotensin-1 receptor; SMC; Smooth muscle cells.

inflammatory response.¹³⁵ It has been suggested that endothelial dysfunction, characterised by the reduced bioavailability of NO, is an important mechanism contributing to the damage after ischemia/reperfusion (I/R).¹³⁶ The reduced bioavailability of NO may be due to impaired NO synthesis as a result of diminished levels of its substrate L-arginine¹³⁷ and the important co-factor BH₄¹³⁸ or increased inactivation of NO by superoxide.¹³⁹ Patients with type 2 diabetes are known to have a poorer outcome following MI than non-diabetic patients¹⁴⁰, which may be due at least in part to increased susceptibility to I/R injury. This increased susceptibility may be explained by the fact that diabetic patients, as previously discussed, are known to have increased production of superoxide,¹⁴¹ as well as reduced levels of L-arginine¹⁴² and BH₄.¹⁴³ This will cause eNOS uncoupling which in turn results in the additional production of superoxide instead of NO. eNOS uncoupling can be avoided by BH₄ supplementation, which has been shown to improve endothelial function in patients with type 2 diabetes.¹⁴⁴ Both BH₄ and L-arginine have been shown to inhibit I/R-induced endothelial dysfunction in healthy subjects.^{145, 146} However, it is not known whether the effect of the combination of L-arginine and BH₄ prevents the development of I/R injury in patients with diabetes and CAD. This is of special interest, since the available data suggest that eNOS uncoupling may be of importance in patients with type 2 diabetes.¹⁴¹

AIMS

To investigate:

1. The importance of the pleiotropic vs. the lipid-lowering effects of statins on macrovascular endothelial function, microvascular function and inflammatory markers in patients with dysglycemia and coronary artery disease **(I, II)**
2. The involvement of ET-1 and ET_A receptors in the regulation of skin microcirculation in patients with type 2 diabetes and microangiopathy **(III)**
3. The effect of L-arginine and BH₄ on I/R-induced endothelial dysfunction in patients with type 2 diabetes and coronary artery disease **(IV)**

MATERIAL AND METHODS

Study subjects

The investigations were carried out in accordance with the Declaration of Helsinki and were approved by the ethics committee at Karolinska University Hospital or Karolinska Institutet. The participating patients gave their written informed consent.

Studies I-II

A total of 43 (I) and 36 (II) patients with type 2 diabetes or impaired glucose tolerance (IGT) and stable CAD were recruited from the Department of Cardiology, Karolinska University Hospital. The patients were classified as having type 2 diabetes mellitus or IGT, according to the WHO criteria.² The presence of stable coronary artery disease was defined by means of a coronary angiogram or a history of previous MI. The exclusion criteria were treatment with statins or other lipid-lowering agents during the preceding 12 weeks, changes in vasodilator drugs during the preceding six weeks, changes in medication during the study, age above 80 years, concomitant disease limiting the ability to complete the study protocol, MI or a coronary intervention within the last three months, known allergic reaction to acetylsalicylic acid, disturbed hepatic function according to a standard laboratory assessment, warfarin treatment or an international normalised ratio of > 2.0, untreated hypertension and participation in an ongoing study. The baseline characteristics of the study population are shown in Table 2.

Table 2. Baseline characteristics of subjects in studies I and II.

	Study I		Study II	
	E10/S10 (n=19)	S80 (n=20)	E10/S10 (n=15)	S80 (n=17)
Age, y	74 (66-77)	70 (62-74)	74 (66-77)	70 (67-74)
Male/female, n	11/8	15/5	9/6	13/4
Body mass index, kg/m ²	28 (26-29)	28 (25-31)	29 (27-30)	28 (26-30)
Smokers, n	4	4	3	3
HbA1C, %	6.2 (4.3-7.1)	5.5 (5.0-7.2)	5.9 (5.2-6.9)	5.5 (5.0-7.0)
Systolic blood pressure, mmHg	150 (140-160)	150 (120-160)	150 (150-162)	145 (120-160)
Diastolic blood pressure, mmHg	60 (50-70)	60 (50-75)	60 (60-65)	60 (57-70)
Type 2 diabetes/impaired glucose tolerance, n	19/0	17/3	15/0	14/3
Insulin treatment, n	6	5	4	4
Oral hypoglycaemic, n	8	9	6	8
Aspirin, n	16	20	14	17
Clopidogrel, n	2	3	0	3
Beta-blockers, n	15	18	12	15
Calcium channel blockers, n	6	8	6	8
ACE inhibitors, n	10	9	8	8
Statins, n	0	0	0	0

Values are median and quartiles. There were no significant differences between the groups.

Study III

Ten patients with type 2 diabetes and microangiopathy and eight non-diabetic control subjects were investigated. Microangiopathy was defined as the presence of microalbuminuria. The non-diabetic subjects were selected from individuals who had previously participated in screening programmes at Karolinska Institutet. The exclusion criteria were age > 80 years, ongoing warfarin treatment, participant in an ongoing study, unwillingness to participate and childbearing capacity. The baseline characteristics of the patients and the non-diabetic control subjects are presented in Table 3.

Study IV

A total of 12 patients with type 2 diabetes or IGT and CAD were recruited from the Department of Cardiology, Karolinska University Hospital. The patients were classified as having type 2 diabetes or IGT, according to WHO criteria. The presence of CAD was defined by means of a coronary angiogram or a history of previous MI. The exclusion criteria were age > 80 years, ongoing warfarin treatment, participant in an ongoing study, unwillingness to participate and childbearing capacity.

Table 3. Baseline characteristics of subjects in study III.

	Controls (n=8)	Type 2 diabetics (n=10)	p-value
Gender, male/female	7/1	8/2	ns
Age, years	60 (56-62)	60 (57-71)	ns
Smokers, n	1	2	ns
Diabetes duration, years		17 (11-30)	
BMI, kg/m ²	25 (24-27)	28 (26-33)	ns
Systolic arm blood pressure, mm Hg	130 (115-136)	152 (130-152)	<0.01
Diastolic blood pressure, mm Hg	80 (80-83)	78 (70-90)	ns
Systolic finger blood pressure, mm Hg	110 (100-130)	140 (130-152)	ns
S-creatinine, µmol/l	93 (75-102)	104 (86-126)	ns
S-total cholesterol, mmol/l	5.7 (5.3-6.4)	5.1 (4.0-6.7)	ns
S-LDL cholesterol, mmol/l	3.2 (3.0-4.0)	3.2 (2.2-4.7)	ns
S-HDL cholesterol, mmol/l	1.7 (1.4-1.9)	1.1 (0.9-1.2)	<0.001
S-triglycerides, mmol/l	1.3 (0.8-1.6)	1.6 (1.2-2.2)	ns
P-endothelin-1, pmol/l	3.8 (3.2-4.5)	4.9 (4.3-5.4)	<0.01
P-hsCRP, mg/l	0.8 (0.4-1.1)	2.1 (1.1-3.8)	<0.05
B-HbA1c, %	-	7.4 (5.9-7.9)	
IGFBP-1, µg/l	12 (5-27)	13 (6-38)	ns
ACE inhibitor/ARB, n	0	9	
Other antihypertensive treatment, n	1	10	
Statin treatment, n	2	7	
Insulin treatment, n	0	8	
Oral hypoglycemic, n	0	5	

Values are median and quartiles.

Blood flow measurements

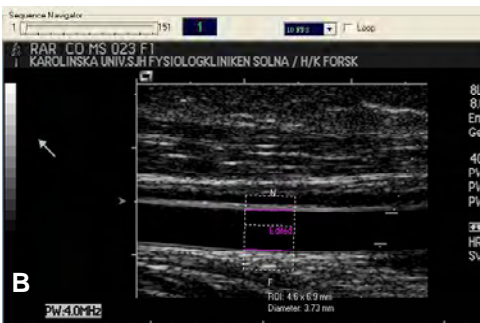
All the investigations were performed in the morning and the subjects were instructed to refrain from caffeine- and nicotine-containing products for 12 h.

Flow-mediated dilatation (f)

The patients were investigated in a quiet, dimly lit room in the supine position. A non-invasive examination of the brachial artery of the non-dominant arm⁸³ was performed by means of an 8 MHz linear-array transducer connected to a Acuson Sequoia® (Acuson Corporation, Mountain View, CA, USA) (Figure 7). Baseline images were saved every three seconds for one minute and a mean value was calculated from these values. Subsequently, a blood pressure cuff, positioned below the elbow, was inflated to 260 mmHg for five minutes. The artery was continuously imaged for three minutes during the hyperemia following the release of the cuff pressure to determine EDV. A mean value was calculated from three recordings at maximum dilatation. Endothelium-independent vasodilatation (EDIV) was determined following the sublingual administration of nitroglycerine (0.4 mg). All the images were analysed using proprietary software (Brachial analyzer®, Medical Imaging Applications, Iowa City, IA, USA) by a technician, blinded to treatment allocation. The maximum lumen diameter, found through beat-to-beat analysis, was measured using an automated contour detection system. The lumen diameter was defined as the distance between the intima of the far and near vessel walls. Dilatation was calculated as the maximum lumen diameter after ischemia or nitroglycerine minus the lumen diameter at baseline divided by the lumen diameter at baseline. The coefficient of variation for FMD determination on two study occasions was 18%.



Figure 7. Flow-mediated dilatation. Non-invasive examination of the brachial artery, performed by means of an 8 MHz linear-array transducer connected to an Acuson Sequoia® at baseline (B) and during hyperemia (C) to determine endothelium-dependent vasodilatation.



Venous occlusion plethysmography (I, IV)

Following the administration of local anaesthetics, a percutaneous catheter was inserted into the brachial artery of the non-dominant arm for drug infusions and the determination of blood pressure. Forearm blood flow (FBF) was measured simultaneously in both arms with venous occlusion plethysmography, using the mercury-in-silastic strain-gauge technique (Figure 8).¹⁴⁷ A venous occlusion cuff placed around the upper arm was inflated to 40 mmHg for 10 s to obtain recordings of arterial inflow, followed by deflation for 5 s. During recordings of blood flow, the circulation of the hands was occluded by a cuff inflated to 30 mmHg above systolic blood pressure. Heart rate was determined from an ECG recording. Average FBF values were obtained from four to eight inflow recordings during two minutes. Ach was infused into the brachial artery to assess EDV. This was followed by an infusion of the NO donor sodium nitroprusside (SNP) for the determination of EIDV. The NO-dependent property of the vasodilatation induced by Ach has been previously validated in this model.⁵⁹ The coefficient of variation between two consecutive determinations of EDV in one subject is 5.1%.



Figure 8. Forearm blood flow, measured simultaneously in both arms with venous occlusion plethysmography, using the mercury-in-silastic strain-gauge technique. Ach was infused into the brachial artery to assess endothelium-dependent vasodilatation and the NO donor sodium nitroprusside was infused to determine endothelium-independent vasodilatation.

Laser Doppler fluxmetry (II, III)

The investigations were performed after 20 minutes of rest with the patient in the supine position. All measurements were conducted in a temperature-controlled environment ($22\pm 1^\circ\text{C}$). Skin microcirculation was evaluated by LDF (PeriFlux 4001 Master, Perimed®, Järfälla, Sweden) on the dorsum of the foot, on the ulnar part of the forearm (II) and digit IV on the left hand (III) and is expressed as perfusion units (PU) (Figure 9). Post-occlusive LDF was measured during maximum hyperemia following a four-minute arterial occlusion at the ankle with a cuff pressure of 250 mmHg (peak LDF). The remaining LDF signal during an arterial occlusion was considered to be the biological zero value and was subtracted from the total LDF signal.¹⁴⁸ Heat LDF was measured at the end of a six-minute period of heating the skin under the LDF probe to 44°C on both the foot (heat foot LDF) and forearm (heat arm LDF) (PeriTemp 4005 with a thermostatic probe PF 457, Perimed®). The mean intra-individual CVs for the measurement of hyperemia post heating and following arterial

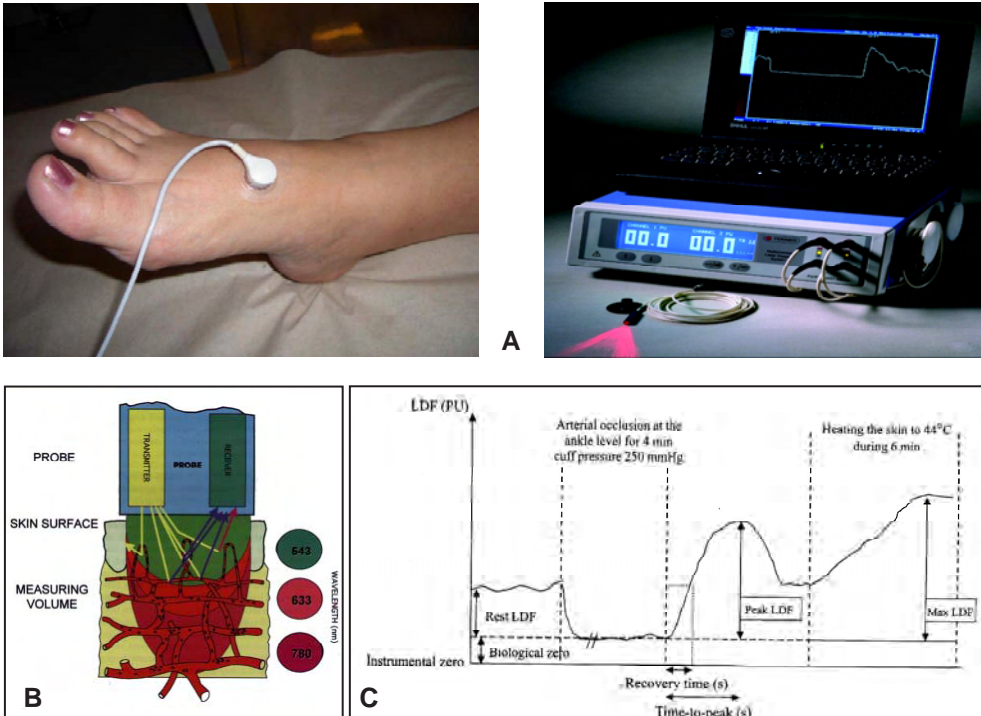


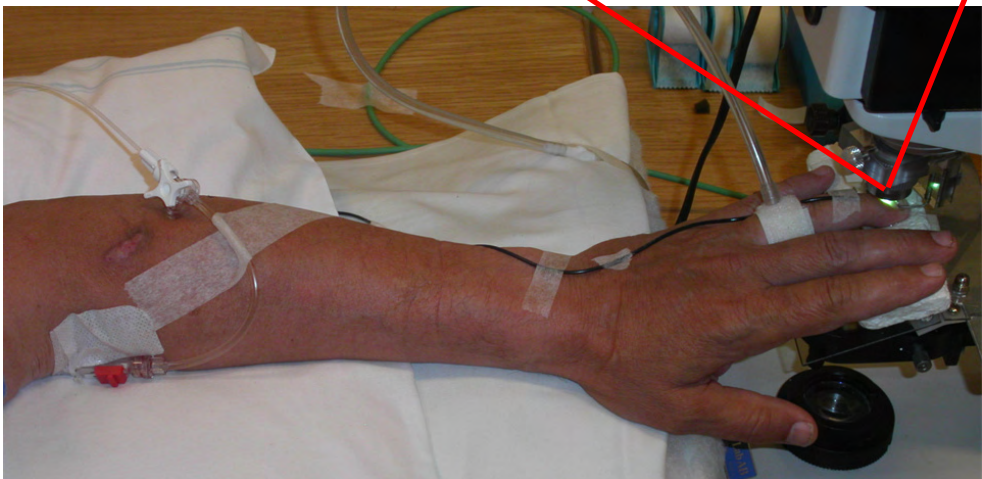
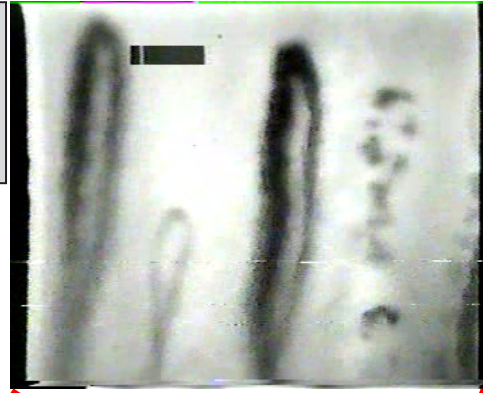
Figure 9. Non-invasive measurement of skin microcirculation by laser Doppler fluxmetry (LDF, A). LDF uses red lasers that are transmitted to the skin. Red blood cells moving in the skin back-scatter light from the laser, producing a frequency shift that is sensed by the probe (B). Variables of LDF measurements at post-occlusive reactive hyperemia and reactive hyperemia during heating of the skin (C).

occlusion were 7% and 18% respectively, determined from five subjects on two separate occasions. All LDF measurements were performed by an investigator who was blinded to treatment and the order of investigation (baseline or follow-up).

Capillaroscopy (III)

The skin microcirculation in the nail fold of digit IV on the left hand was investigated by capillary microscopy (Figure 10). A miniature cuff (20 mm wide) was applied to the proximal phalanx of the finger for arterial occlusions. The investigations were performed with the subjects seated and with the left arm supported at heart level on a table. The skin temperature of the finger nail fold was continuously recorded with an electronic thermistor (Exacon®, Copenhagen, Denmark). Nail-fold capillaries were visualised on a TV monitor by a Leitz Laborlux microscope [Leica (Leitz)®, Wetzlar, Germany] on which a video camera (ICD-44 DC, Ikegami®, Tokyo, Japan) is mounted. The image was stored on videotape for subsequent analysis. CBV was determined using a computerised, videophotometric, cross-correlation technique (Capiflow AB®, Stockholm, Sweden).^{149, 150} CBV was continuously computed for three minutes and the computer-integrated mean value during this period was termed “resting CBV”. Peak CBV and time to peak CBV were measured following a one-minute arterial occlusion induced by the miniature cuff inflated to 200 mm Hg. Coefficients of variation for peak CBV and time to peak CBV are 14% and 15% respectively.¹⁵¹

Figure 10. Capillaroscopy. Nail-fold capillaries were visualised on a TV monitor by a Leitz Laborlux microscope on which a video camera is mounted. Capillary blood cell velocity was determined using a computerised, video-photometric, cross-correlation technique.

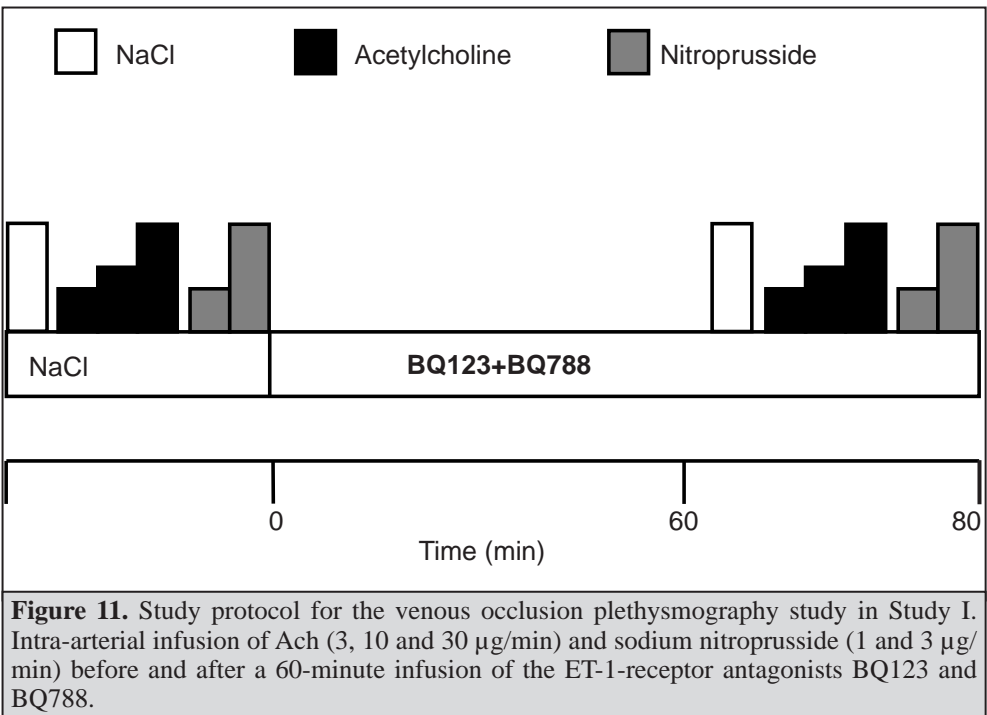


Study protocols

Studies I-II

This was a randomised, double-blind, controlled clinical trial and included two separate protocols. The patients arrived at the laboratory after 12 hours of fasting. Following blood sampling, the patients were served a standard breakfast consisting of a cheese sandwich and lingonberry juice. The patients were not allowed any caffeine-containing drinks or tobacco consumption on the day of the study. All drugs except aspirin, clopidogrel and glucose-lowering medication were withheld on the morning of the test. Protocol I (Study I) evaluated endothelial function determined by FMD and the effect of ET-1 on endothelial function using forearm venous occlusion plethysmography. Endothelial function assessed by FMD was performed as previously described. The effect of ET-1 on endothelial function was determined according to the protocol presented in Figure 11. Thirty minutes after the arterial cannulation, basal FBF was recorded during an infusion of saline. Thereafter, Ach (3, 10 and 30 $\mu\text{g}/\text{minute}$) was infused into the brachial artery to assess EDV. This was followed by an infusion of the NO donor sodium nitroprusside (1 and 3 $\mu\text{g}/\text{minute}$) for determinations of EIDV. Each dose was given for two minutes at a rate of 2.5 ml/minute. The ET-1-induced

vasoconstrictor tone was then assessed by infusions of the ET-1 receptor antagonists BQ123 (ET_A receptor antagonist) and BQ788 (ET_B receptor antagonist). The antagonists were infused for 80 minutes at a rate of 10 nmol/minute and FBF was determined every ten minutes. After 60 minutes of infusion, EDV and EIDV were re-assessed. The acute vasodilator effect of Ach and nitroprusside is expressed as the absolute change in FBF. The prolonged effect of ET-receptor blockade on baseline FBF is expressed as the percentage change in the ratio between the experimental and control arms according to previous recommendations.¹⁴⁷ Protocol II (Study II) evaluated microvascular function assessed by LDF, as described above. After completing these investigations, the patients were randomised to one of two treatment groups: 80 mg of simvastatin and placebo (S80) or 10 mg of ezetimibe/10 mg of simvastatin and placebo (E10/S10). All the drugs were given once daily in the evening. After six weeks of treatment, the patients were re-examined as above. Treatment compliance was checked through pill count. A flow chart for the patient population is shown in Figure 12.



Study III

Measurements of skin microcirculation were performed after 30 minutes of acclimatisation and the room temperature was kept between 22-24°C. A percutaneous catheter was inserted under local anaesthesia into the left brachial artery for infusions. Thirty minutes after the insertion of the catheter, basal measurements were recorded during an infusion of saline (1 ml/minute) for 15 minutes. This was followed by an infusion of BQ123 at a rate of 10 nmol/minute (1 ml/minute) for 60 minutes. BQ123 was diluted in 0.9% NaCl.

The skin microcirculation in the nail fold of digit IV on the left hand was investigated by nail-fold capillary microscopy and LDF for measurements of nutritive skin blood flow and total

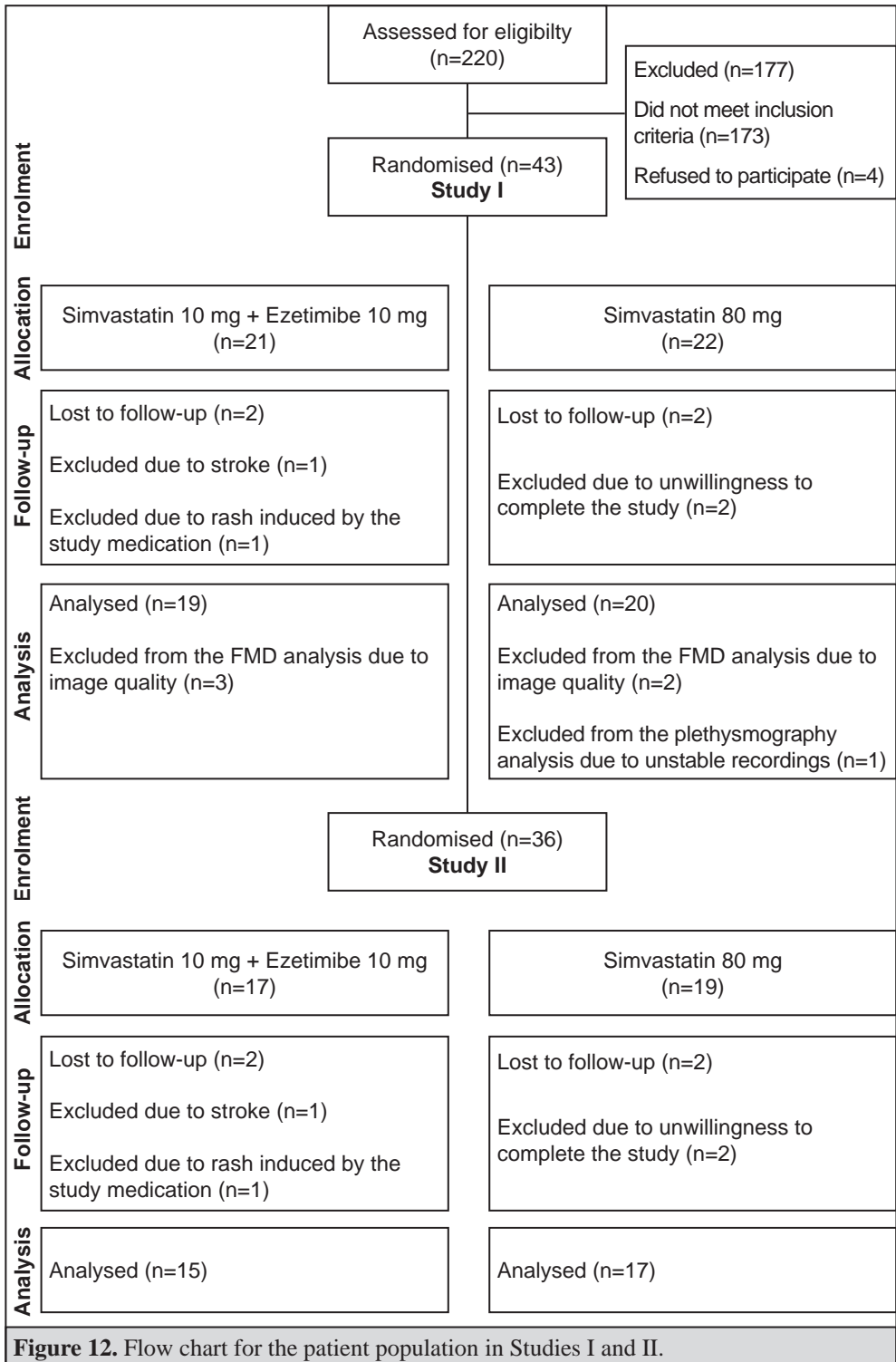
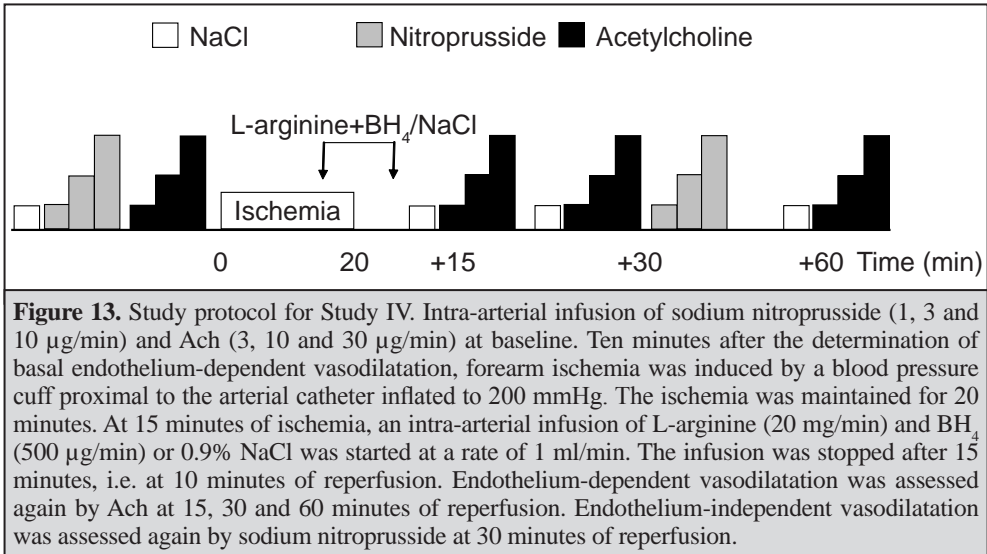


Figure 12. Flow chart for the patient population in Studies I and II.

skin microcirculation respectively, before and after saline infusion, and every 15 minutes during BQ123 infusion. A miniature cuff (20 mm wide) was applied to the proximal phalanx of the finger for arterial occlusions and measurements of finger blood pressure using LDF as the flow detector. The investigations were performed with the subjects seated and with the left arm supported at heart level on a table. The skin temperature of the finger nail fold was continuously recorded with an electronic thermistor (Exacon®, Copenhagen, Denmark).



Study IV

Using a cross-over protocol, each subject received either saline or L-arginine and BH_4 on the two study occasions. The order of administration was randomised and the patients were blinded to the treatment. Endothelial function was assessed by venous occlusion plethysmography. The protocol is summarised in Figure 13. Basal FBF was determined during a two-minute infusion of 0.9% NaCl at a rate of 2.5 ml/minute. EIDV was determined by an intra-arterial infusion of SNP (1, 3 and 10 $\mu\text{g}/\text{minute}$). EDV was assessed by an infusion of Ach (3, 10 and 30 $\mu\text{g}/\text{minute}$). Each dose was given for two minutes at a rate of 2.5 ml/minute. Ten minutes after the determination of basal EDV, forearm ischemia was induced by a blood pressure cuff proximal to the arterial catheter inflated to 200 mmHg. The ischemia was maintained for 20 minutes. At 15 minutes of ischemia, an intra-arterial infusion of L-arginine (20 mg/minute) and BH_4 (500 $\mu\text{g}/\text{minute}$) or 0.9% NaCl was started at a rate of 1 ml/minute. The infusion was stopped after 15 minutes, i.e. at 10 minutes of reperfusion. EDV was assessed again at 15, 30 and 60 minutes of reperfusion. EIDV was determined before ischemia and at 30 minutes of reperfusion. Deep venous blood was sampled at baseline and at 5, 20 and 50 minutes for the analysis of BH_4 and its oxidation products, dihydrobiopterin (BH_2) and biopterin (B). Blood pressure, heart rate and plasma glucose were measured before ischemia and at 20 and 60 minutes of reperfusion. In order to evaluate the effect of L-arginine and BH_4 on EDV and EIDV without prior ischemia, 10 patients, seven of whom also participated in the I/R protocol, were subjected to a similar protocol but without ischemia.

Biochemical analysis

Fasting plasma glucose, haemoglobin A_{1c} (HbA_{1c}), total serum cholesterol, LDL, HDL, triglycerides and apolipoprotein A1 and B (apoA1 and apoB) were assessed at baseline and at the end of Study I according to local laboratory routines. Plasma ET immunoreactivity was analysed by radioimmunoassay using commercially available antiserum (rabbit anti-ET-1 6901, Peninsula, Merseyside, UK) following ethanol extraction.¹⁵² High-sensitive CRP was measured using the Behring Nephelometer Analyzer II with a particle-enhanced immunonephelometric assay.¹⁵³ ICAM-1 and IL-6 were analysed on the Evidence® biochip array analyser (Randox Laboratories, Ltd., Crumlin, UK).¹⁵⁴ Total biopterin levels were determined by high-performance liquid chromatography with electrochemical and fluorescent detection in Oxford, UK.¹⁵⁵ Albuminuria, using the first morning sample of urine (10ml) and/or a 24 h sample of urine, was analysed using a Synchron LX Beckman Coulter AB machine. Normal reference levels are < 30 mg/l or 20 µg/minute. The IGFBP-1 concentration in serum was determined by radioimmunoassay according to the method of Povia et al.¹⁵⁶

Statistical analysis

In Studies I-III, the results are given as median and quartiles, while in Study IV they are given as the mean and standard error of the mean (SEM). Categorical data are expressed as numbers. A two-sided p-value of < 0.05 was considered significant. The effect of treatment on biochemical parameters and FMD was analysed using Wilcoxon's signed rank test (within-group comparison). Group comparisons with respect to clinical characteristics, laboratory results and FMD were made using the Mann-Whitney rank sum test. Statistical differences in plethysmography data were calculated using Wilcoxon's signed rank test, by comparing the mean of the dose response to Ach. Associations were assessed using the Spearman rank correlation. A two-way ANOVA was used to compare the dose-response curves for Ach and SNP at the different time points, the change in FBF, MAP and P-glucose following reperfusion between the two interventions in Study IV. In Study I, the number of patients in each group needed to detect a difference in FMD of 2% with a power of 80% and a two-tailed t-test at the 5% level was calculated to be approximately 22, assuming a normal shift model with a standard deviation of 2.4 for differences in FMD. Studies III and IV are exploratory in character and it is therefore difficult to perform accurate power calculations. Estimates based on previous studies were made using similar methods.^{59, 145, 157} Based on these results, we came to the conclusion that ten and twelve individuals are sufficient to detect a relevant difference. All the statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA).

RESULTS

Lipid lowering and vascular function

Effect of lipid lowering by different means on macrovascular endothelial function and inflammatory markers (I)

As shown in Table 2, the two groups were well balanced in terms of baseline characteristics. There were 17 patients with diabetes and three with IGT in the S80 group, while all the patients in the E10/S10 group had diabetes.

Serum lipids at baseline did not differ between the groups (Table 4). Total and LDL cholesterol and triglycerides decreased substantially and to similar degrees in both groups. HDL cholesterol did not change significantly. ApoB decreased in both groups, whereas apoA1 remained unchanged.

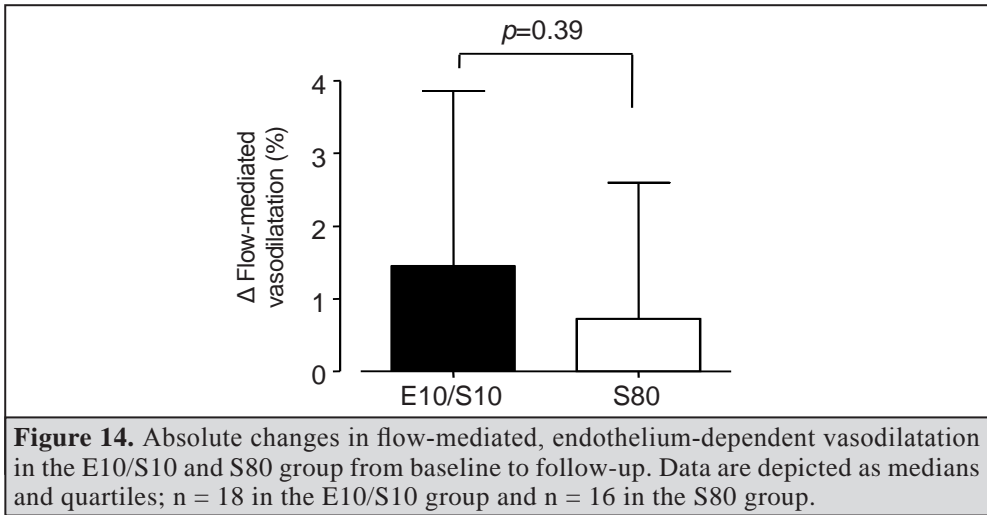
CRP, IL-6 and ICAM-1 decreased significantly in the entire study group, but there were no significant differences between the two study groups.

Because of image quality, FMD could not be analysed in two patients in the S80 group and in three patients in the E10/S10 group, leaving a total of 34 patients who were evaluated. Following six weeks of treatment, FMD in the entire study group increased from 4.3% (3.4-6.1) to 5.5% (3.4-6.6; $p=0.005$). FMD increased from 4.3% (2.7-6.0) to 5.8% (3.4-8.4) in the E10/S10 group and from 4.3% (1.7-6.2) to 5.2% (3.0-6.0) in the S80 group. There was no statistical difference in the increase in FMD between the two groups (Figure 14; $p=0.39$, 95%CI -0.7 to 1.9). EIDV induced by nitroglycerine did not change during treatment (11.5% (8.1-14.0) at baseline and 12.3% (7.5-16.0) at follow-up).

Table 4. Lipids, inflammatory markers and endothelin-1 at baseline and follow-up.

Variable	E10/S10		S80		<i>p</i> -value between groups
	Baseline	Follow-up	Baseline	Follow-up	
Total cholesterol, mmol/l	4.8 (4.7-5.2)	3.1 (2.9-3.6)	4.8 (4.2-5.6)	3.0 (2.5-3.4)	0.67
LDL cholesterol, mmol/l	3.1 (2.8-3.4)	1.5 (1.4-1.7)	3.0 (2.5-3.7)	1.3 (1.1-1.8)	0.47
HDL cholesterol, mmol/l	1.0 (0.9-1.2)	1.0 (0.9-1.2)	0.9 (0.8-1.2)	0.9 (0.8-1.3)	0.95
Triglycerides, mmol/l	1.4 (1.0-2.2)	1.0 (0.7-1.5)	1.8 (1.2-2.6)	1.1 (0.8-1.4)	0.68
ApoA1, g/l	1.4 (1.2-1.6)	1.4 (1.2-1.5)	1.4 (1.2-1.6)	1.4 (1.1-1.7)	0.38
ApoB, g/l	1.1 (1.0-1.6)	0.6 (0.5-0.8)	1.1 (1.0-1.3)	0.6 (0.5-0.6)	0.01
CRP, mg/l	3.1 (1.6-8.4)	2.8 (1.0-6.9)	2.7 (1.7-4.6)	2.3 (0.9-4.2)	0.60
IL-6, pg/ml	5.1 (2.4-11.5)	4.6 (2.0-6.0)	2.9 (1.8-8.8)	3.1 (1.7-6.9)	0.38
ICAM-1, ng/ml	338 (299-417)	344 (276-390)	378 (326-448)	378 (316-417)	0.89
Endothelin-1, pg/ml	6.0 (5.2-6.7)	5.8 (5.1-6.2)	5.2 (4.7-6.5)	5.5 (5.0-6.8)	0.05

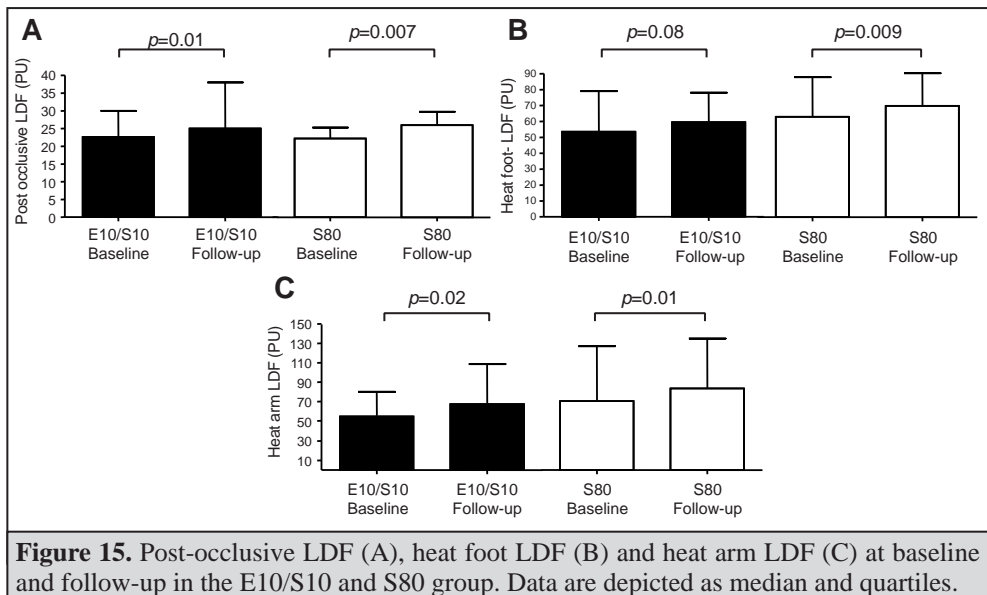
Values are median and quartiles.

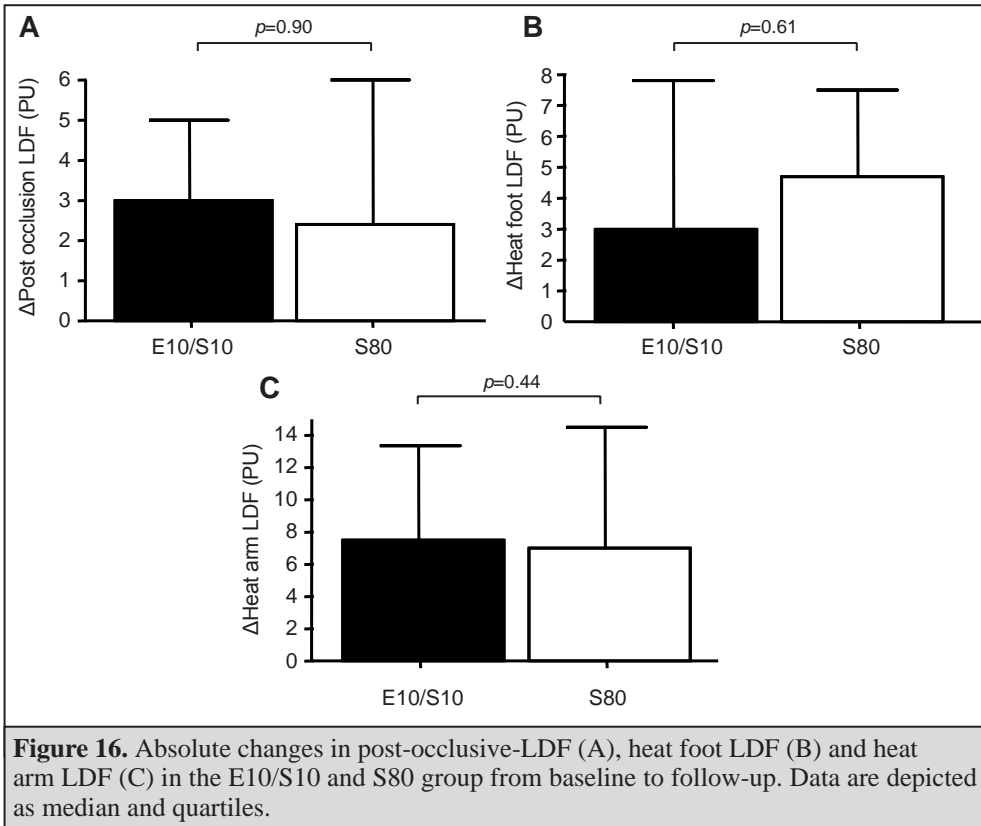


Effect of lipid lowering by different means on microvascular function (II)

There were no significant differences in LDF between the two groups at baseline. Following six weeks of lipid-lowering treatment, all the LDF parameters increased in both groups (Figure 15). Post-occlusive LDF increased by 18% and 14% in the S80 group and the E10/S10 group respectively. Moreover, heat foot LDF and heat arm LDF increased in both treatment groups (Figure 15). The changes in LDF parameters from baseline to the end of treatment did not differ significantly between the two groups (Figure 16).

Post-occlusive LDF at baseline correlated significantly to the insulin sensitivity markers HOMA ($r=0.37$, $p<0.05$), QUICKI ($r=0.37$, $p<0.05$) and IGFBP-1 ($r=0.37$, $p<0.05$). HOMA, QUICKI and IGFBP-1 did not change significantly following treatment in any group. There was no significant correlation between LDF parameters and HbA_{1c} or blood pressure.





ET-receptor blockade and vascular function

Effect of ET_A receptor blockade on microvascular function (III)

The baseline characteristics of patients and controls including medication are shown in Table 3. Patients with type 2 diabetes had significantly higher systolic arm blood pressure, concentrations of ET-1 and hs-CRP and lower HDL cholesterol than the non-diabetic controls. Diastolic blood pressure, systolic finger blood pressure, LDL cholesterol or triglycerides did not differ between the groups.

Capillaroscopy: At baseline, resting CBV, peak CBV, time to peak and skin temperature did not differ significantly between type 2 diabetes patients and controls. Resting CBV did not change during 15 minutes of saline infusion. During the infusion of BQ123, resting CBV increased in the diabetic patients throughout the infusion. In contrast, resting CBV remained unchanged in the controls (Figure 17). There was a significant difference in the change in resting CBV between the patients with type 2 diabetes and the non-diabetic controls during the infusion of BQ123 (Figure 17; Table 5). Peak CBV during post-occlusive reactive hyperemia also increased in patients during BQ123 infusion but not in the control group (Figure 18; Table 5). In line with the microvascular findings, the skin temperature increased in patients during the infusion of BQ123, whereas the temperature was unchanged in controls (Figure 19; Table 5). Accordingly, there was a significant correlation between the baseline skin temperature and resting CBV ($r=0.61$ $p<0.005$), as well as between the increases in

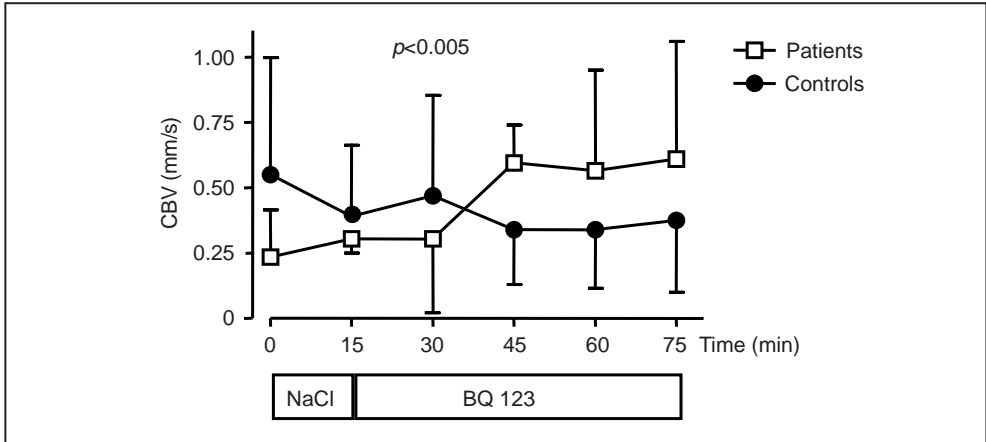


Figure 17. Effect of BQ123 on resting capillary blood cell velocity (CBV) in patients with type 2 diabetes (n=10) and non-diabetic controls (n=8). Data are depicted as median and quartiles. A significant difference between groups in changes in resting CBV following 60 minutes of BQ123 infusion is shown.

Table 5. Microcirculatory parameters during infusion of BQ 123.

BQ 123	Type 2 diabetics (n=10)		Controls (n=8)		p-value
	0 min	60 min	0 min	60 min	
Resting CBV (mm/s)	0.24 (0.20-0.34)	0.61 (0.46-0.88)	0.55 (0.10-0.68)	0.38 (0.13-0.88)	<0.005
Peak CBV (mm/s)	0.66 (0.49-1.01)	1.10 (0.94-1.16)	0.89 (0.84-1.22)	0.80 (0.39-1.15)	<0.05
Time to peak CBV (s)	11.9 (9.7-13.0)	12.1 (9.8-12.6)	12.0 (11.1-12.8)	13.0 (9.1-13.7)	ns
Skin temperature (°C)	25.8 (24.2-29.5)	29.8 (26.2-32.7)	30.4 (26.8-31.6)	31.1 (25.0-31.9)	<0.005
Resting LDF (PU)	18 (8-50)	16 (13-81)	30 (17-55)	62 (37-101)	ns
Peak LDF (PU)	75 (31-111)	71 (40-194)	66 (32-83)	129 (48-202)	ns
Time to peak LDF (s)	8.6 (4.7-15.6)	6.9 (4.3-13.4)	7.2 (4.9-10.8)	6.5 (3.8-7.3)	ns

Data are median and quartiles. The p-value shows statistical difference in change in microcirculatory parameters following 60 min of BQ123 infusion between the two groups. CBV, capillary blood cell velocity; LDF, laser Doppler fluxmetry; PU, perfusion units

skin temperature and resting CBV, during BQ123 infusion in the patient group. There was a significant positive correlation between BMI and the increase in CBV during BQ123 infusion in the patient group ($r=0.82$, $p<0.01$). There were inverse correlations between IGFBP-1 and BMI ($r=-0.74$, $p=0.03$) and an increase in CBV in the patients ($r=-0.92$, $p<0.001$; Figure 20). There was no significant correlation between systolic blood pressure ($r=0.38$, $p=0.11$), creatinine ($r=-0.43$, $p=0.25$) and HbA1c ($r=-0.14$, $p=0.71$) and the increase in resting CBV during BQ123 infusion.

LDF: At baseline, LDF, peak LDF and time to peak did not differ significantly between patients and controls. Although there was a trend towards an increase in resting and peak LDF during infusion of BQ123, the changes were not significant (Table 5).

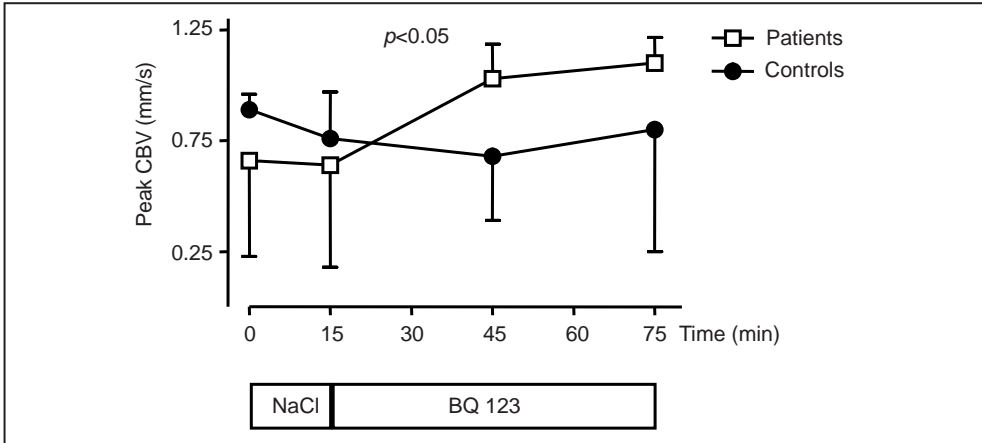


Figure 18. Effect of BQ123 on peak capillary blood cell velocity (CBV) following a one-minute arterial occlusion in patients with type 2 diabetes (n=10) and non-diabetic controls (n=8). Data are depicted as median and quartiles. A significant difference between groups in changes in peak CBV following 60 minutes of BQ123 infusion is shown.

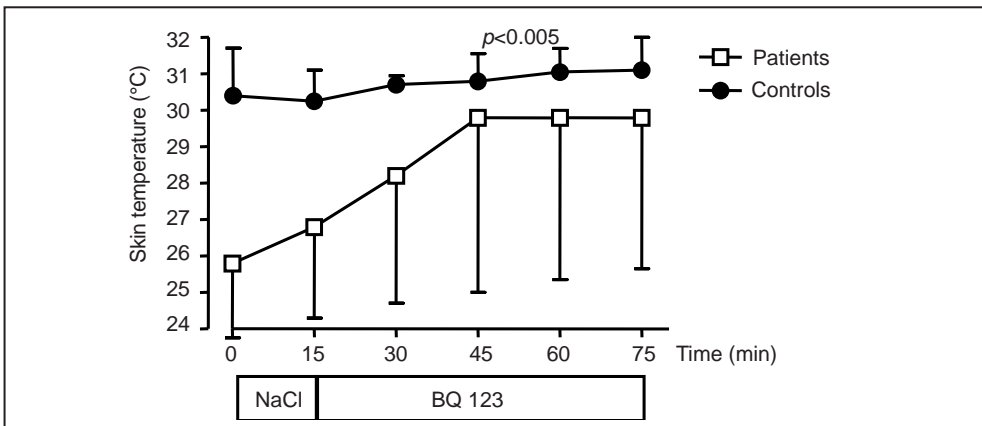


Figure 19. Effect of BQ123 on skin temperature in patients with type 2 diabetes (n=10) and non-diabetic controls (n=8). Data are depicted as median and quartiles. A significant difference between groups in changes in temperature following 60 minutes of BQ123 infusion is shown.

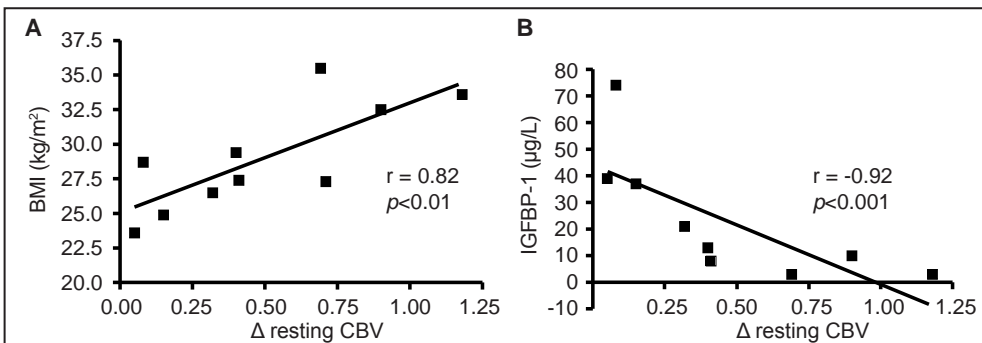


Figure 20. Correlation between Δ CBV and BMI (A, n=10) and IGFBP-1 (B, n=9).

Effect of ET_A - and ET_B receptor blockade on endothelial function before and after lipid-lowering treatment (I)

Flow recordings from one patient in the S80 group could not be analysed due to unstable recordings. As a result, 38 patients were available for the final analysis of the plethysmography recordings.

Infusions of BQ123 and BQ788 increased FBF by 20% (-5-45) ($p < 0.001$) in the entire study group at baseline and by 24% (7-43) at follow-up. The increase in FBF in response to BQ123 and BQ788 was similar in the two groups at baseline (27% (13-46) and 15% (-8-35) respectively) and at follow-up (22% (8-49) and 24% (-1-42) respectively). ET-receptor blockade caused a significant increase in EDV at baseline and this effect remained at follow-up (Figure 21). EIDV was not altered by ET-receptor blockade at baseline or follow-up (not shown). Furthermore, the two treatment strategies did not differ in terms of their effect on the improvement in EDV induced by ET-receptor blockade ($p = 0.66$) (Figure 21).

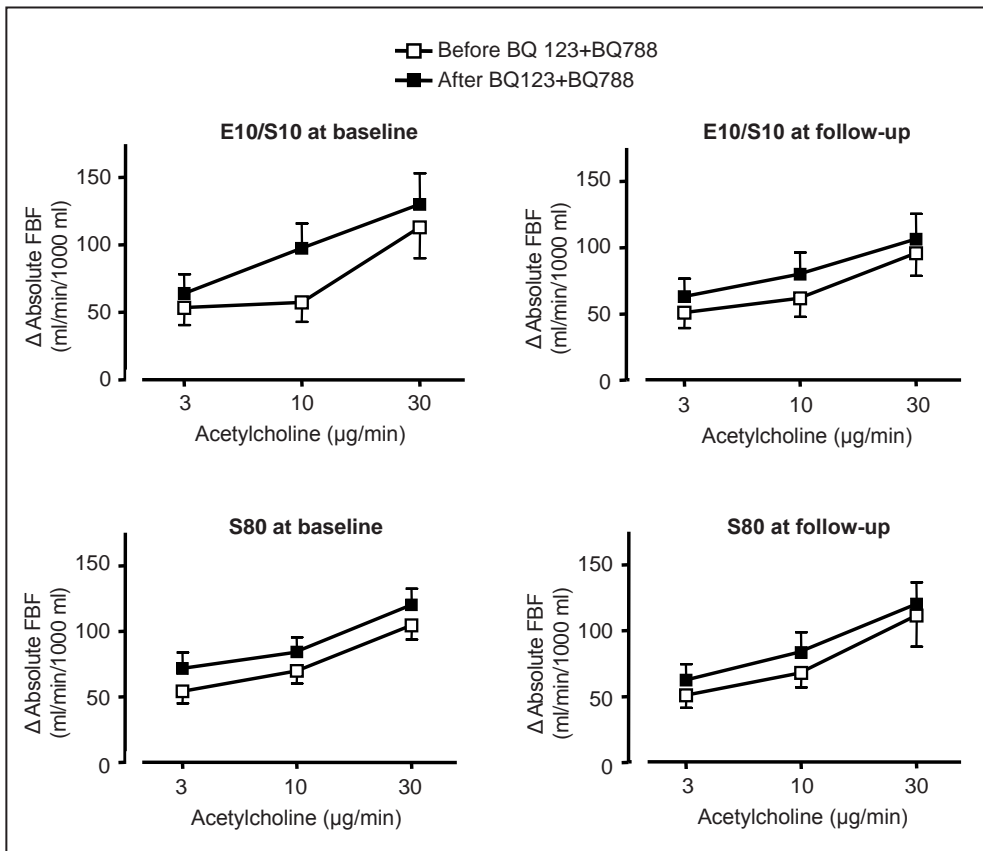


Figure 21. Effect of an intra-arterial infusion of Ach on forearm blood flow (FBF) before and during the co-administration of the ET_A receptor antagonist BQ123 and the ET_B receptor antagonist BQ788 at baseline and at follow-up in the E10/S10 and S80 group. Data are presented as median and quartiles; $n = 19$ in all groups. The two groups did not differ in terms of the effect on the improvement in endothelium-dependent vasodilatation induced by ET receptor blockade ($p = 0.66$).

L-arginine and BH₄ supplementation and I/R injury (IV)

All infusions, the ischemia and the reperfusion were well tolerated by all subjects.

Vascular responses: There was no difference in baseline FBF between the study occasions and basal FBF remained unchanged following reperfusion (Table 6). FBF in the control arm did not change throughout the protocol. Ach increased FBF by similar magnitudes at baseline on both study occasions. There was a trend towards an increase in mean arterial pressure (MAP) at 60 minutes of reperfusion compared with pre-ischemia on both study occasions (Table 6), but the increase was not significant and the change in MAP following reperfusion did not differ between the two study occasions. There were no differences in heart rate between the study occasions and heart rate did not change significantly during any of the studies (not shown).

Table 6.

Intervention	Infusion arm			Control arm				
	Pre-ischemia	Reperfusion			Pre-ischemia	Reperfusion		
		15 min	30 min	60min		15min	30 min	60 min
FBF (ml/min/1000ml)								
NaCl	30.7±4.4	29.4±4.2	29.5±3.4	29.4±4.1	26.1±4.2	22.8±2.7	24.1±2.5	22.1±3.1
L-arginine+BH ₄	29.3±3.8	27.8±4.2	27.9±4.0	26.8±3.3	25.3±3.0	23.5±3.3	22.0±2.7	23.0±2.6
MAP (mmHg)								
NaCl	87±4	91±4		93±6				
L-arginine+BH ₄	90±4	88±4		92±4				
P-glucose (mmol/l)								
NaCl	8.8±0.9	7.5±0.8		7.3±0.6**				
L-arginine+BH ₄	8.7±0.9	6.9±0.6**		6.4±0.5***				

Values are means±SEM. MAP; mean arterial pressure. Significant change from pre-ischemic values are indicated: * $p<0.05$, ** $p<0.005$, *** $p<0.001$

The increase in FBF induced by Ach was markedly impaired during the first 30 minutes of reperfusion on the saline study occasion ($p<0.001$). A significant attenuation of the vasodilator response to all doses of Ach at both 15 minutes ($p<0.05$) and 30 minutes ($p<0.01$) was observed (Figure 22). At 60 minutes of reperfusion, there was still an attenuation of the vasodilator response to Ach, but it did not differ significantly from the pre-ischemic value. By contrast, the vasodilator response to Ach was not significantly impaired during reperfusion compared with pre-ischemia following the administration of L-arginine+BH₄ (Figure 22). The impairment of the vasodilator response to Ach during reperfusion in comparison to pre-ischemia was significantly greater on the saline study occasion than following the administration of L-arginine+BH₄ (Figure 23).

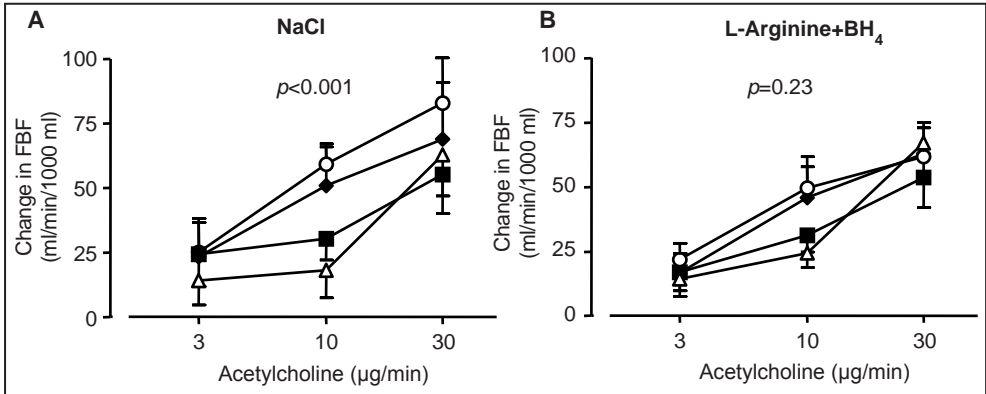


Figure 22. Effect of Ach on forearm blood flow (FBF) before ischemia (○) and at 15 (■), 30 (△) and 60 (◆) minutes of reperfusion on the saline (A) and L-arginine + BH₄ (B) study occasions. Data are mean and S.E.M.; n = 12. Significant differences from the pre-ischemic response are indicated.

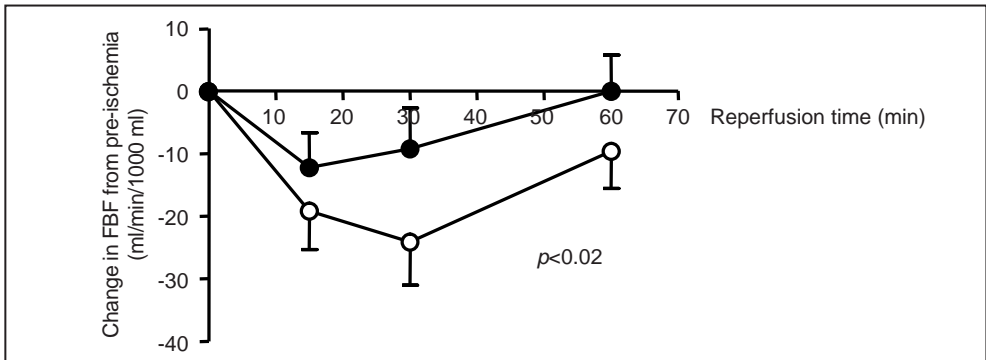


Figure 23. Change in forearm blood flow (FBF) from pre-ischemia following reperfusion on the L-arginine + BH₄ (●) and saline (○) study occasion. Data are mean and S.E.M.; n = 12. A significant difference between the treatments is indicated.

There was no difference in the forearm vasodilator response to SNP before ischemia compared with 30 minutes of reperfusion on any study occasion (not shown). L-arginine + BH₄ infusion without prior ischemia did not change the forearm vasodilator response to Ach or SNP (not shown).

Biopterins: There was no difference in baseline BH₄, BH₂ or B between venous and arterial samples, or between the different study protocols. During the infusion of L-arginine + BH₄, there was a significant increase in biopterins (BH₄, BH₂ and B) in deep venous samples from the infused arm in both the ischemic and non-ischemic protocols (Figure 24). There was still a significant increase in local deep venous plasma total biopterins compared with baseline at 20 minutes following infusion in the I/R protocol with L-arginine + BH₄ infusion (Figure 24). However, in the placebo protocol, there was no difference in BH₄, BH₂ or B levels during or following infusion. The arterial levels that reflect systemic levels also rose significantly at 20 minutes following infusion in the two protocols in which L-arginine + BH₄ was infused (not shown), but there was no difference between the two protocols. The local biopterins were about 10 times higher than the systemic biopterins at 20 minutes following infusion ($p < 0.001$).

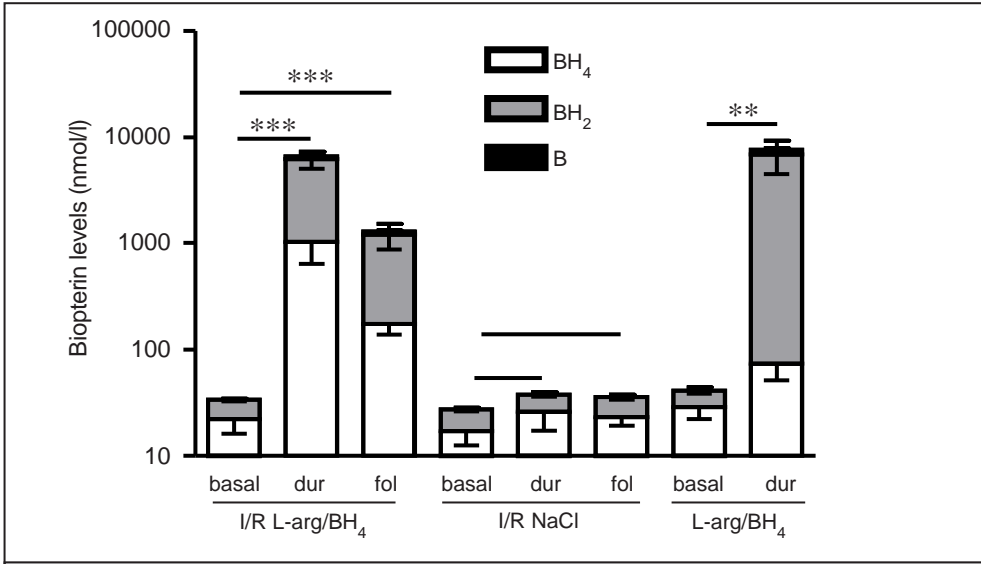


Figure 24. Local deep venous plasma biopterins (BH₄, BH₂ and B) in the infused forearm at baseline (basal), during infusion (dur) and 20 minutes following ischemia-reperfusion (fol) in the L-arginine/BH₄ protocol (I/R L-arg/BH₄; n = 9), the saline protocol (I/R L-arg/BH₄; n = 7) and in the protocol with L-arginine/BH₄ infusion without ischemia/reperfusion (L-arg/BH₄; n = 5).

GENERAL DISCUSSION

The prevalence and incidence of type 2 diabetes is increasing in both developed and developing countries.¹⁵⁸ CVD is the principal cause of death and disability among patients with type 2 diabetes.³ The presence of endothelial dysfunction, characterised by the reduced bioavailability of NO, and, as demonstrated in the present studies, the increased bioactivity of ET-1, is regarded as an important factor in this increase in cardiovascular morbidity and mortality. It is therefore important to understand the mechanisms behind endothelial dysfunction and to restore endothelial function in patients with type 2 diabetes.

Lipid lowering and vascular function

Hypercholesterolemia is probably the most important risk factor for CAD.¹¹¹ Consequently, lipid lowering, especially using statins, has been shown to reduce cardiovascular risk in numerous studies.¹¹²⁻¹¹⁹ Statins exert their effects by inhibiting HMG-CoA reductase, which converts HMG-CoA to mevalonate and is the rate-limiting step in the pathway resulting in the production of cholesterol. This inhibition reduces not only the synthesis of cholesterol but also the synthesis of isoprenoid intermediates, such as farnesylpyrophosphate and geranylgeranylpyrophosphate (Figure 6).¹²¹ In experimental models, the inhibition of isoprenoid synthesis has been shown to result in a vast number of effects including reduced inflammation,¹⁵⁹ antioxidant effects,¹⁶⁰ plaque stabilisation,¹⁶¹ reduced ET-1 expression,¹²⁷ the stimulation of endothelial progenitor cell recruitment¹⁶² and improved endothelial function.¹⁶³ Despite the ever-increasing number of studies investigating these lipid-independent/pleiotropic effects of statins, there is little evidence to support the hypothesis that the pleiotropic effects of statins are of clinical importance.¹⁶⁴⁻¹⁶⁶ This may be explained by the difficulty involved in distinguishing the effects related to lipid lowering from those caused by possible pleiotropic effects.

The potential pleiotropic effects of statins on endothelial function have attracted special interest. It is well known that hypercholesterolemia is associated with endothelial dysfunction¹³⁰ and that a single LDL apheresis improves endothelial function,¹³⁴ indicating that lipid lowering *per se* improves endothelial function. However, statins have been reported to increase eNOS expression via mechanisms that are independent of lipid lowering, such as the inhibition of RhoA and caveolin-1 and the activation of the PI-3 kinase/Akt pathway.¹²³⁻¹²⁵ Statins can therefore improve endothelial function by both lipid-dependent and independent mechanisms. Patients with type 2 diabetes are characterised by increased vascular inflammation, endothelial dysfunction and increased activity of ET-1, but in most cases their plasma LDL levels are only moderately elevated.^{4, 107} Type 2 diabetic patients may therefore be a patient category that would potentially benefit from the pleiotropic effects of statins. A new opportunity to distinguish the pleiotropic effects from the lipid-lowering effects of statins is offered by the introduction of ezetimibe, a pharmacological agent that lowers blood cholesterol by inhibiting intestinal lipid absorption without influencing the mevalonate pathway.^{167, 168} The efficacy of ezetimibe in reducing cholesterol as monotherapy is moderate, but it increases substantially if combined with a low-dose statin. The lipid-lowering effect of ezetimibe when combined with 10 mg simvastatin is comparable to that induced by 80 mg of simvastatin in monotherapy.¹⁶⁹

Macrovascular effects

In Study I, we confirmed in our patient cohort that the lipid-lowering effect of 80 mg of simvastatin as monotherapy and the combination of 10 mg of simvastatin and 10 mg of ezetimibe were comparable. Importantly, the two treatment strategies did not differ with regard to their effect on FMD and inflammatory markers. This suggests that lipid lowering *per se*, rather than a pleiotropic effect by statins, is important for the improvement in endothelial function and reduced inflammatory activity. This conclusion is supported by the finding that ezetimibe and simvastatin improve endothelial function and reduce inflammatory markers to the same extent in patients with rheumatoid arthritis,¹⁷⁰ that the equal lowering of LDL cholesterol by a low-dose statin plus ezetimibe or a high-dose statin induced the equivalent CRP reduction,¹⁷¹ together with similar effects on endothelial progenitor cells¹⁷² and on endothelial function in patients with the metabolic syndrome.¹⁷³ Furthermore, Bulut et al. recently demonstrated that switching from atorvastatin given as a daily dose of 40 mg to 10 mg together with 10 mg of ezetimibe induced a significant additional reduction in cholesterol and improved endothelial function in patients with the metabolic syndrome.¹⁷⁴ The conclusion that lipid lowering is more important than the potential pleiotropic effects of statins is further supported by the outcome of meta-analyses of clinical trials on lipid-lowering therapy, thereby underlining the fact that the reduction in LDL cholesterol explains the reduction in clinical events and CRP levels.^{164, 165, 175} The present data may appear to contrast to those reported in three other studies.¹⁷⁶⁻¹⁷⁸ Landmesser et al. compared the effect of 10 mg of simvastatin with that of 10 mg of ezetimibe as monotherapy on endothelial function in patients with heart failure. They found that EDV improved following simvastatin treatment but not following treatment with ezetimibe. One important difference between that and the present investigation is that Landmesser et al.¹⁷⁷ excluded patients with diabetes. Another difference is the use of ezetimibe as monotherapy, which only resulted in a 15% reduction in LDL in comparison with a 50% reduction when using the clinically approved combination of ezetimibe and simvastatin in the present study. In addition, the 15% reduction in LDL cholesterol in both groups is similar to that achieved with ezetimibe in other studies but half of that expected with 10 mg of simvastatin.¹⁶⁹ It is therefore possible to question whether the greater improvement in FMD with simvastatin reflects pleiotropic effects or whether it is due to a larger LDL reduction. More importantly, the effect of statin treatment in patients with heart failure has recently been investigated in two large clinical trials with neutral results.^{179, 180} Liu et al.¹⁷⁶ compared the effect of 40 mg/d of simvastatin, the combination of 10 mg of simvastatin and 10 mg of ezetimibe and placebo in three groups of patients with LDL cholesterol of >130 mg/dL and < 2 cardiovascular risk factors. The groups were compared in terms of changes in the activity of Rho-associated coiled-coil containing protein kinase (ROCK) as the primary endpoint and endothelial function as a secondary endpoint. They concluded that a high dose of statin given as monotherapy induces a greater reduction in ROCK activity and an improvement in endothelial function measured by FMD compared with the combination of the statin in a low dose combined with ezetimibe. Compared with our study, the patients in the study by Liu et al. ran a considerably lower cardiovascular risk, were not all naïve to statin treatment and patients with diabetes were excluded. The LDL reduction in the combination group was less pronounced than in our and other studies¹⁶⁹ with 10/10 mg/d of simvastatin/ezetimibe (35% vs. 50%). It is also important to note that it is not clear whether the change in endothelial function differed significantly between the two treatment groups. Although only the high-dose simvastatin group obtained a statistically significant improvement, a statistical analysis of the difference in change in FMD between the treatment

groups was not provided. Fichtlscherer et al.¹⁷⁸ studied the effect of ezetimibe and statin as monotherapy and in various combinations in patients with CAD. The interpretation of their data is hampered by the fact that the groups differed significantly in terms of LDL cholesterol levels at baseline and follow-up. This underlines the importance of well-matched groups regarding baseline cholesterol and treatment effects when addressing the relative impact of LDL reduction and pleiotropic effects of lipid-lowering agents.

The lack of effect by lipid-lowering therapy on Ach-induced vasodilator response in forearm resistance vessels is in line with previous statin studies of patients with type 2 diabetes¹⁸¹⁻¹⁸³. One possible explanation for the differences in outcome when using FMD and venous occlusion plethysmography may be that FMD detects endothelial function in conduit arteries, whereas venous occlusion plethysmography reflects resistance vessel function. Accordingly, these two methods are not significantly related to each other.¹⁸⁴ Importantly, endothelium-dependent vasodilatation obtained with both methods is associated with cardiovascular events in patients with CAD and correlates with endothelial function in coronary arteries¹⁸⁵, as well as the Framingham risk score.¹⁸⁴

Microvascular effects

Disturbed microvascular function is a common and serious complication in type 2 diabetes. Complications that are related to microangiopathy are retarded wound healing, retinopathy, nephropathy and neuropathy. The pathogenesis of diabetic microangiopathy is multifactorial, but reductions in microvascular vasodilatory capacity and impairments in the autoregulation of capillary blood flow are important contributory factors.⁷³

Observational and cross-sectional studies demonstrate a correlation between microvascular complications and hyperlipidemia.¹⁸⁶ Lipid lowering induced by statins reduces the number of macrovascular events in patients with type 2 diabetes.¹¹³ In contrast, studies of the effect of lipid-lowering treatment on microvascular function in these patients have been neutral.^{182, 187} The studies were performed on a small number of patients with either long-standing, poorly controlled diabetes¹⁸² or with short diabetes duration and without cardiovascular complications.¹⁸⁷ The lack of effects seen in these two studies may therefore be explained by the fact that patients with either too advanced or limited disease were studied and possibly also by the limited number of patients included. Statins may also influence microvascular function by both lipid-dependent and lipid-independent effects. The pleiotropic effects of statins may be important for counteracting diabetic microangiopathy, as it may relate to a combination of endothelial dysfunction and increased vascular inflammation.

In Study II, microvascular function was studied by LDF, a measurement of total skin microcirculation, i.e. nutritional capillary blood flow, as well as non-nutritional sub-papillary blood flow. The maximum skin hyperemic response following local heating and arterial occlusion is impaired in patients with diabetes, a finding associated with macro- and microvascular complications and increased cardiovascular risk.^{148, 188} The maximum microvascular hyperemic response following local heating and arterial occlusion is likely to involve neurogenic and endothelium-dependent mechanisms.¹⁰² Our study revealed that both 80 mg of simvastatin and the combination of 10 mg of simvastatin and 10 mg of ezetimibe improved microvascular function. Since the two treatment strategies did not differ with regard to this effect, it can be assumed that lipid lowering rather than the pleiotropic effects of statins is also the main mechanism behind this effect.

In order to achieve intensive, equal lipid lowering in two study groups, it was necessary to combine ezetimibe with a low dose of simvastatin instead of using ezetimibe as monotherapy.

This may be regarded as a limitation, especially as the exact dose-response relationship for a potential pleiotropic effect of simvastatin or other statins remains uncertain. There are indications that statins exert biphasic dose-related effects on endothelial progenitor cells and angiogenesis.^{189, 190} The chosen combination is recommended for clinical practice, which is an important reason to study its impact on vascular function. Furthermore, if pleiotropic effects are important, some indication of a dose-response relationship might have been expected, as it is well known that increasing doses of statins improve cardiovascular outcome.¹⁹¹ The absence of a placebo group can also be regarded as a limitation. However, the study was designed to compare the effect of the two treatment strategies on endothelial function and a placebo group is not needed for this analysis. Furthermore, it would not be ethical to have a placebo group based on the current recommendations regarding statin treatment in patients with documented CVD and type 2 diabetes.

From Studies I and II, it is concluded that lipid-lowering treatment in patients with glucose perturbations by means of two different pharmacological strategies induced a comparable cholesterol reduction and a similar improvement in endothelial and microvascular function, thereby suggesting that the lipid-lowering capacity is more important than the pleiotropic effects of statins for this improvement.

ET-1 and vascular function

Circulating levels of ET-1 are increased in patients with type 2 diabetes, as demonstrated in Study III. Furthermore, there is a positive correlation between plasma ET-1 levels and microangiopathy in patients with type 2 diabetes.^{52, 66} In addition to the direct vasoconstrictor effects, enhanced levels of ET-1 may contribute to endothelial dysfunction through inhibitory effects on NO bioavailability.⁵⁹ Previous studies have shown that ET-1 impairs endothelium-dependent vasodilatation in healthy individuals⁵⁹ and that ET-receptor blockade improves macrovascular endothelial function in patients with atherosclerosis and type 2 diabetes.^{66, 192, 193} Furthermore, ET-receptor blockade improves EDV in healthy subjects with insulin resistance, indicating that endothelial dysfunction induced by ET-1 is an early phenomenon among subjects with glucose abnormalities.¹⁹⁴ There are several possible mechanisms behind the negative effect of ET-1 on NO bioavailability, including the down-regulation of eNOS expression,⁶² reduced eNOS activity⁶⁰ and the increased inactivation of NO by superoxide production generating superoxide.¹⁹⁵ Pretreatment with the antioxidant vitamin C has been shown to inhibit endothelial dysfunction induced by ET-1 in humans,¹⁹⁶ favouring a pro-oxidative effect of ET-1. In addition, statins have been proven in experimental settings to reduce the synthesis of ET-1, possibly through a lipid-independent mechanism.^{127, 128} It may therefore be hypothesised that part of the protective effect of statins on EDV may be related to the down-regulation of ET-1.

In Study I, we therefore investigated the effect of ET-receptor blockade on basal blood flow and EDV before and after lipid-lowering treatment. The vasodilator effect and improvement in EDV obtained by ET-receptor blockade at baseline were unchanged following lipid-lowering treatment in both groups. These findings indicate that: 1) the vascular tone mediated by ET-1 was unchanged and 2) ET-receptor blockade exerts beneficial effects on endothelial function on top of aggressive lipid-lowering therapy. However, the possibility that six weeks of treatment is too short to affect the vascular effects of ET-1 cannot be excluded. In addition to being a vasoconstrictor, ET-1 is a potent mitogen and effects of this kind may take longer to produce a result than vasoconstrictor effects.

No previous studies have investigated the effect of ET-receptor blockade on microvascular function in patients with type 2 diabetes. In Study III, we hypothesised that the enhanced ET-1-mediated vasoconstriction of precapillary resistance vessels leads to impaired blood flow through nutritive capillaries and increased arterio-venous shunting in patients with type 2 diabetes. Impaired nutritive skin microcirculation has been demonstrated in both the diabetic hand and foot and might contribute to the development of complications.^{151, 197-199} Skin nutritive microcirculation determined by nail-fold capillary microscopy increased markedly during ET_A receptor blockade. The hyperemic response, i.e. the peak value of CBV, following arterial occlusion also increased, indicating improved microvascular reactivity. Furthermore, skin temperature increased and this change correlated significantly to the increase in CBV. It is worth noting that the increase in CBV during BQ123 infusion in patients appears to be more pronounced in patients with a high BMI and low levels of IGFBP-1, supporting a close relationship between insulin resistance and increased ET-1 activity. The development of microvascular endothelial dysfunction appears to be related to insulin resistance.^{75, 76} It has therefore been suggested that the impairment in autoregulatory function and vasodilatory capacity is due to defective insulin signalling described in insulin resistance. This is characterised by the stimulation of the MAP kinase pathway by insulin, resulting in the increased synthesis of ET-1 and subsequently endothelial dysfunction.^{31, 76} Impaired microvascular function measured by laser Doppler and nail-fold capillaroscopy is associated with reduced insulin sensitivity in obese subjects^{200, 201}. Studies II and III extend these findings to the type 2 diabetic patient and also suggest that the microvascular endothelial dysfunction associated with insulin resistance appears at least in part to be mediated by an increase in ET-1 signalling.

There are several plausible mechanisms behind the observed effect of ET_A receptor blockade on the microcirculation in the patient group. One is the reduced shunting of blood through arteriovenous shunts due to reduced capillary resistance. Patients with type 2 diabetes and hypertension have increased capillary blood pressure. A reduction in capillary blood pressure, as a consequence of reduced arteriovenous shunt flow, and an increase in arteriovenous pressure difference might increase the capillary blood circulation²⁰². The effects of ET_A receptor blockade on skin microcirculation may be a consequence of blocking the precapillary constrictor effects of ET-1 mediated by ET_A receptors. Furthermore, ET_A receptor blockade may have improved microvascular endothelial function by increasing the bioavailability of NO,⁵⁹ which appears to be of importance for microvascular function in diabetes.^{3, 11, 73, 203} Another factor contributing to the changes in capillary flow may be that ET_A receptor blockade improves nerve conduction velocity and nerve blood flow in diabetic rats,²⁰⁴ which might result in the improved function of the autonomic nervous system and attenuated arteriovenous shunting. In addition to the increased levels of ET-1 in patients with diabetes as compared to non-diabetic controls found in the present and previous studies,^{205, 206} there are also indications that ET receptors are up-regulated in diabetes. The increased expression of both ET_A and ET_B receptors has been demonstrated in experimental models of diabetes²⁰⁵. The marked increase in nutritive capillary blood flow observed following BQ123 infusion in the diabetic patients, without any effect in the non-diabetic control subjects, may therefore be related to the increased production of ET-1, as well as the up-regulation of ET_A receptors mediating vasoconstriction.

There was no significant change in LDF in either group, although there was a trend towards increased flow. This may be related to the fact that LDF measures total skin microcirculation, i. e. both nutritive capillary circulation and deeper subpapillary microcirculation. The laser

Doppler signal is mainly generated by movements of blood cells in deeper subpapillary vessels and only a small part of the reflected light is derived from nutritive skin capillaries.²⁰⁷ The lack of significant change in LDF may therefore be related to the larger variation and relatively small number of patients in this study but also to the fact that the method partly detects blood flow in different areas compared with capillaroscopy.

From Study III, it is concluded that ET_A receptor blockade markedly increases nutritive skin microcirculation in type 2 diabetic patients with microangiopathy. The results suggest that ET-1 is involved in the pathogenesis of diabetic microangiopathy. Targeting the ET-1 system might be of importance in the treatment of complications related to diabetic microangiopathy. This needs to be confirmed in a placebo-controlled trial using the oral administration of an ET-receptor antagonist.

Ischemia and reperfusion injury

Patients with type 2 diabetes are known to have a poorer outcome following MI than non-diabetic patients¹⁴⁰ and this may be due at least in part to an increased susceptibility to I/R injury.¹³⁶ It has been suggested that endothelial dysfunction is an important mechanism that contributes to the injury after I/R.¹³⁶ The reduced bioavailability of NO during I/R contributes to poor reflow during reperfusion and pro-inflammatory effects. In vitro studies indicate that the reduced bioavailability of NO following I/R may be due to reduced synthesis as a result of diminished levels of L-arginine^{137, 145} and BH₄¹³⁸ or the enhanced inactivation of NO by superoxide.¹³⁹ Furthermore, superoxide has been shown to react with NO to form peroxynitrite, which may oxidise BH₄ and produce oxidative damage to eNOS. By this mechanism, eNOS may produce superoxide instead of NO, a phenomenon referred to as eNOS uncoupling.²⁰⁸ There is recent evidence to suggest that eNOS uncoupling may be more pronounced in the setting of type 2 diabetes than during normoglycaemia and that the supplementation of BH₄ leads to the re-coupling of uncoupled eNOS.^{208,141, 209}

In Study IV, we investigated the effect of L-arginine and BH₄ supplementation on I/R-induced endothelial dysfunction. We were able to demonstrate that there was a significantly larger reduction in EDV following I/R in the presence of saline than in the presence of L-arginine +BH₄. On the other hand, the effect on EIDV evoked by SNP was not affected at 30 minutes of reperfusion, suggesting that the ability of smooth muscle cells to relax in response to NO was unaffected by I/R. This observation suggests that L-arginine and BH₄ protect from I/R-induced endothelial dysfunction in patients with type 2 diabetes. L-arginine and BH₄ supplementation have previously been shown to improve endothelial function.^{144, 210} When using the protocol without ischemia, we were not able to observe any change in EDV at 15 and 30 minutes following L-arginine and BH₄ infusion. Importantly, this observation indicates that the effect on I/R-induced endothelial dysfunction by L-arginine and BH₄ is not due to an improvement in pre-ischemic endothelial function but to effects on injury induced by I/R.

Several possible mechanisms may underlie the protective effects of L-arginine and BH₄ on I/R-induced endothelial dysfunction. It may be argued that the beneficial effects seen after L-arginine supplementation are not due to critical concentrations of L-arginine, since the K_m of eNOS for L-arginine is about 3 µmol/l and L-arginine plasma levels hardly fall below 60 µmol/l.²¹¹ On the other hand, endothelial cells express arginase that competes with eNOS for the substrate and may thereby create a relative lack of L-arginine.²¹² Increased arginase activity has been found in diabetic subjects²¹³ and following I/R.²¹⁴ Accordingly, treatment with

both L-arginine and an arginase inhibitor partly restored EDV following I/R.²¹⁴ Interestingly, increased superoxide production in a rat model of diabetes was attenuated by both NOS and arginase inhibition, suggesting that increased arginase activity contributed to NOS uncoupling.²¹⁵ This uncoupling was most probably due to a relative deficiency of L-arginine. L-arginine has also been shown to increase eNOS activity following I/R in the rat heart.²¹⁴ There is a close correlation between NO synthesis and the intracellular concentrations of BH₄.²¹⁶ BH₄ is a potent reducing agent and is therefore subjected to oxidation in the settings of increased oxidative stress such as diabetes and I/R.²¹⁷ The reduction in BH₄ levels leads to eNOS uncoupling, resulting in an increase in the production of superoxide and the reduced synthesis of NO. Myocardial BH₄ levels are reduced by 92% during ischemia in experimental studies.²¹⁸ The supplementation of BH₄ may therefore increase NO bioavailability by recoupling eNOS.²¹⁹

Since L-arginine and BH₄ were combined, it is not possible to establish whether the beneficial effects were achieved by a single administration of either L-arginine or BH₄, or whether a combination is necessary. The rationale behind the combined administration is that BH₄ recouples eNOS and L-arginine increases substrate availability for eNOS. An increase in the activity of eNOS in the presence of low levels of BH₄ in diabetes may be detrimental during I/R.^{141, 208} Interestingly, supplementation with L-arginine alone had no effect or was detrimental in patients with peripheral arterial disease.²²⁰ Our data, on the other hand, suggest that the combination of L-arginine and BH₄ enhances the bioavailability of NO and thereby protects from endothelial dysfunction following I/R via multiple possible mechanisms. From Study IV, it is concluded that the local administration of L-arginine and BH₄ improves endothelial function following I/R in the forearm vasculature in patients with type 2 diabetes and CAD. L-arginine and BH₄ administration may thus represent a new treatment strategy to limit the I/R injury in this patient group.

Future directions

There are numerous animal and cell culture studies suggesting that the pleiotropic effects of statins play an important part in a vast number of effects that could potentially be beneficial for the treatment of CVD. However, meta-analyses of clinical trials on lipid-lowering therapy have not been able to confirm these results and clinical trials addressing this question have produced diverging results. This stresses the need for a larger, preferably multicentre study to shed more light on this important mechanistic issue. A study of this kind would be of clinical importance, as several studies support the notion of “the lower, the better” with regard to cholesterol levels.^{221, 222} It is important to clarify whether this intensive lipid lowering needs to be accomplished by high doses of statins, with the drawback of increasing side-effects,²²³ or whether the combination of a lower dose of statins with ezetimibe, achieving the same degree of lipid lowering, is an alternative.

ET-1 appears to be involved in the pathogenesis of diabetic microangiopathy, especially in the presence of insulin resistance. Previous studies have shown that patients with diabetes have impaired coronary microcirculation even in the absence of obstructive coronary atherosclerosis.^{224, 225} It has also been suggested that microangiopathy plays a major role in the pathogenesis of diabetic cardiomyopathy.²²⁶ It would therefore be attractive to study the effect of ET-receptor blockade on the coronary microcirculation and left ventricular function in patients with type 2 diabetes. Furthermore, the findings in Study III need to be confirmed in a larger study using oral treatment for a longer time period. An additional field that requires

further exploration is the pathophysiological importance of ET-1 in insulin resistance.⁷⁰ I/R injury is a common clinical concern. There are data showing that adequate restoration of myocardial tissue perfusion is not achieved in up to 25% of patients with acute myocardial infarction, despite coronary artery recanalisation. As yet, there is no pharmacological treatment that has been proven to be protective against I/R injury in clinical studies. Adenosine has been shown to reduce infarct size in patients with anterior myocardial infarction, but it had no effect in patients with other infarct locations and there was a trend towards an excess of clinical events in the adenosine group.²²⁷ The next logical step following Study IV would be to investigate the effect of L-arginine and BH₄ on I/R injury in a randomised clinical study of patients with acute myocardial infarction.

CONCLUSIONS

1. Lipid lowering is more important than the pleiotropic effects of statins for an improvement in macrovascular endothelial function, microvascular function and the reduction of inflammatory activity in patients with dysglycemia and CAD.
2. ET-1 is involved in the regulation of skin microcirculation in patients with diabetic microangiopathy. The blockade of ET receptors might be of importance in the treatment of complications related to diabetic microangiopathy.
3. L-arginine and BH₄ attenuate I/R-induced endothelial dysfunction compared with placebo in patients with type 2 diabetes and CAD. The supplementation of L-arginine and BH₄ may represent a future treatment strategy to limit I/R injury in this patient group.

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