Vascular compliance and reactivity in diabetes mellitus

Citation for published version (APA):

Huvers, F. C. (1997). *Vascular compliance and reactivity in diabetes mellitus*. [Doctoral Thesis, Maastricht University]. Universiteit Maastricht. <https://doi.org/10.26481/dis.19971218fh>

Document status and date:

Published: 01/01/1997

DOI:

[10.26481/dis.19971218fh](https://doi.org/10.26481/dis.19971218fh)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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Vascular compliance and reactivity in diabetes mellitus

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ISBN 90-901174-3

The scientific work was supported by grants from the Diabetes Fonds Nederland (91.103)
and Novo Nordisk Farma BV, the Netherlands.

Vascular compliance and reactivity in diabetes mellitus

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Mr. M.J. Cohen,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 18 december 1997 om 14.00 uur

door

F C Huvers

geboren op 06-08-1960 te Rotterdam

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mijn kleine prinsesjes,
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Chapter I

Introduction

Introduction

Diabetes mellitus is a chronic disease which was already described in 1500 BC by the Egyptians. The name diabetes mellitus is a composition of the Greek word *διαβαινειν*, meaning draining, and the Latin word *mellitus*, meaning sweet or honey. Diabetes mellitus is characterized by a chronic elevation of blood glucose levels caused by an absolute or relative deficiency of insulin. Glucose is an essential metabolic substrate, transported by blood from the gut to various organs. The uptake of glucose from the blood into organs, like muscle and liver, is stimulated by insulin, a hormone produced in the pancreas. Insulin was discovered by Banting and Best in 1921. The absolute deficiency of insulin is called insulin-dependent diabetes mellitus or type 1 diabetes. A relative deficiency of insulin is called non insulin-dependent diabetes mellitus or type 2 diabetes, as initially most of these patients can be treated not only by insulin but by various other medications as well.

The mortality rate caused by acute complications (hyperglycaemic or ketoacidotic coma) has dropped dramatically in type 2 diabetes and even more in type 1 diabetes, since proper treatment is available¹⁻³. However, morbidity and mortality from long-term complications, like diabetic angiopathy and nephropathy have increased³⁻⁶. Diabetic angiopathy can be present in both the macro- and microcirculation. The macrocirculation concerns central and conduit arteries, and veins. The microcirculation concerns arterioles, capillaries and venules. Macroangiopathy is characterized by premature atherosclerosis⁷. In fact, cerebral stroke, myocardial infarction and peripheral vascular occlusive disease are more frequent in diabetes mellitus patients, especially in type 2 patients, than in non-diabetic subjects⁸⁻¹⁰. Myocardial infarction is the main cause of death in these patients. Diabetic microangiopathy is characterized by changes in the basal membrane and cellular structures in the microcirculation¹¹⁻¹³. The consequences of diabetic microangiopathy are manifold, with retinopathy leading to blindness, and nephropathy resulting in proteinuria and renal insufficiency. In the kidney advanced nephropathy is characterized by glomerular sclerosis, loss of glomerular filtration and proteinuria. Advanced nephropathy is preceded by a phase of microalbuminuria, in which there is enhanced urinary excretion of albumin, in the face of normal or even increased filtration rate. The increased urinary albumin excretion is associated with widespread vascular disease, in both the micro- and macrocirculation. Therefore, microalbuminuria can be seen as an early marker of generalized vascular dysfunction, according to several authors¹⁴.

Beside structural abnormalities, also several haemodynamic abnormalities can be observed in both the macrocirculation and microcirculation. In type 1 patients the early microcirculatory abnormalities include increased flow in organs like skin,

limb, eye and kidney¹⁵⁻¹⁸. Experimental studies in diabetic animals and direct blood pressure measurements in nailfold capillaries of type 1 patients suggest that this hyperperfusion is accompanied by intra-capillary hypertension¹⁹. Both capillary hyperperfusion and hypertension fall during improvement of metabolic control¹⁹, which implies that in this stage the haemodynamic changes are functional rather than structural. The rises in capillary blood pressure and flow are probably due to pre-capillary vasodilation which is not compensated for by a decrease in post-capillary resistance. According to the "haemodynamic hypothesis" the early increased microvascular pressure and flow result in limitation of maximal blood flow, impaired autoregulation and, finally, in decreased resting blood flow in advanced microangiopathy. In accordance with this hypothesis Tooke et al observed particularly high (nailfold) capillary pressures in microalbuminuric patients¹⁹. The mechanism of these altered haemodynamics is unknown. In contrast to type 1 diabetic patients, resting skin blood flow and nailfold capillary pressure seem to be unaltered in type 2 patients²⁰. In these patients impaired maximal microcirculatory blood flow seems to be the most prominent finding²¹. However, presently data on microcirculatory haemodynamics in type 2 diabetes are relatively scarce.

Hypertension forms a major haemodynamic challenge to the macrocirculation in both type 1 and type 2 diabetes. Apart from the metabolic imbalances secondary to the diabetic state, the elevated blood pressure will act as an additional insult on the vessel wall in both macro- and microcirculation. The pathogenesis of hypertension in diabetes is largely unknown, but various potential mechanisms have been proposed²². Some authors, for instance, suggest that abnormalities in transmembrane cation transport play a central role²³⁻²⁵. According to this hypothesis changes in intracellular sodium, hydrogen and calcium concentrations would lead to enhanced vascular smooth muscle cell (VSMC) contractility and hypertrophy, left ventricular hypertrophy, and renal sodium retention. One model postulates that a genetic predisposition to abnormal cation transport is the common basis for hypertension and nephropathy²⁶⁻²⁸. In both type 1 and type 2 diabetes, hypertension is associated with micro-albuminuria²⁹. Several authors have suggested that a genetic predisposition for the development of hypertension may be a major determinant of the individual susceptibility to diabetic nephropathy. Increased sodium-lithium countertransport activity, which is thought to be a marker of the ubiquitous sodium-hydrogen antiport activity³⁰ and is largely genetically determined, has been reported in micro- and macroalbuminuric type 1 and type 2 patients^{26,27,31}. Indeed, increased sodium-hydrogen antiport activity has been reported in leucocytes and fibroblasts of type 1 patients with nephropathy³². The increased activity of this transport system may predispose to vascular smooth muscle cell hypertrophy, renal sodium retention and hypertension²². Finally, the abnormal cation transport in these subjects would, according to some authors, lead

to insulin resistance²⁴. However, the literature is conflicting as other authors could not confirm these findings³³.

Alternatively, according to another hypothesis, hypertension is a common feature of the "insulin resistance syndrome", which is further characterized by obesity, dyslipidaemia, atherosclerotic cardiovascular disease and impaired glucose tolerance³⁴. Insulin resistance is usually accompanied by compensatory hyperinsulinemia. The hyperinsulinemia could contribute to the rise in blood pressure by stimulating renal sodium reabsorption, by activating central nervous system, and by promoting VSMC proliferation³⁵⁻⁴⁰. Furthermore, various insulin resistant states are associated with enhanced blood pressure responses to systemic noradrenaline infusion^{41,42}. Insulin can affect trans-membrane calcium transport by several mechanisms and there is some evidence which suggests that insulin resistance is associated with an increased intracellular calcium⁴³. This rise in intracellular calcium could result in enhanced VSMC contractile responsiveness to noradrenaline and other agonists⁴³.

Beside abnormalities in the VSMC, experimental and clinical evidence indicates that endothelial abnormalities could play a central role in the development of microangiopathy, atherosclerosis, and, possibly, hypertension^{12,44-46}. The endothelium is an important regulator of permeability, haemostasis and fibrinolysis, vascular tone, VSMC growth, and matrix composition. In both micro- and macro-albuminuric patients, the increased renal permeability to albumin is associated with an increased transcapillary escape rate of albumin, compatible with a generalized impaired barrier function of the endothelium⁴⁷. In both type 1 and type 2 patients several abnormalities have been observed in factors which are produced by the endothelium and which are involved in haemostasis and fibrinolysis, like von Willebrand factor (vWF), tissue factor pathway inhibitor (TFPI), plasminogen activator-1 (PAI-1), and tissue plasminogen activator (t-PA)^{14,48-58}. These abnormalities are particularly observed in patients with (micro-)albuminuria¹⁴. The endothelium also produces several potent local dilators and constrictors, like nitric oxide (NO), and endothelin-1 (ET-1). There is some evidence that endothelial NO production is impaired in both type 1 and type 2 diabetes⁵⁹⁻⁶². Basal NO production in these studies was assessed by the vasoconstrictor response of the forearm vascular bed to local infusion of N⁸-monomethyl-L-arginine (L-NMMA), a specific blocker of NO-synthase. Stimulated NO production was assessed by the vasodilator response to acetylcholine or other muscarinic agonists. Most authors could not observe abnormal basal or stimulated NO production in type 1 patients without complications^{60,63}. However, Calver et al. reported abnormal L-NMMA responses in patients with type 1 diabetes of relative short duration⁶¹. Unfortunately, the absence or presence of microvascular complications was not specified in this study.

In type 1 patients with microalbuminuria, Elliott et al. observed that basal forearm NO production was reduced⁶⁰. Furthermore, in these same patients stimulated NO production was impaired compared to normoalbuminuric patients and healthy controls. Endothelium-independent vasodilation, studied by local infusion of sodium nitropruside (SNP) or nitroglycerin (NTG) in the forearm, was normal in most of the aforementioned studies^{59,60,63}.

In type 2 diabetes a different pattern of endothelium-dependent and -independent vasodilation was observed⁶². In one study, both basal and stimulated NO production were reduced in the forearm of patients without microalbuminuria⁶². Furthermore, in these patients the endothelium-independent vasodilation in the forearm arterioles was reduced⁶². In contrast, Avogaro recently observed normal stimulated NO production and normal endothelium-independent vasodilation in the forearm of uncomplicated type 2 patients⁶⁴.

Apart from changes in the VSMC or the endothelium, alterations in vascular viscoelasticity, leading to a loss of vascular compliance, could contribute to the development of hypertension in diabetes⁶⁵. Compliance can be defined as the change in volume capacity of the vessel for a given change in pressure. A decrease in arterial compliance contributes to a rise of systolic blood pressure by the reduction of systolic volume capacity of the central arteries. Indeed, systolic hypertension is disproportionally more frequent in type 2 diabetes^{66,67}. Several authors found reduced aortic compliance in normotensive type 2 patients, but others could not confirm these observations⁶⁸⁻⁷¹. These conflicting results are probably based on differences in patient characteristics, as aortic compliance is influenced by gender, aging, smoking habits, body weight, hypercholesterolaemia, and sodium intake^{65,72,73}.

Also, a reduction of the venous compliance could contribute to the development of hypertension, as the venous system plays a pivotal role in volume and blood pressure homeostasis⁷⁴. Approximately 60-80% of the total blood volume is located in the venous system in healthy man⁷⁴. The majority of this volume is haemodynamically inactive (unstressed volume). A reduction in venous compliance could lead to more centralization of the blood, with a subsequent increase in cardiac preload, followed by a rise in cardiac output, which could favor the development of hypertension⁷⁵.

Objectives of the studies described in this thesis

As outlined above several lines of reasoning lead to the view that alterations in endothelial function, or in VSMC cation transport, either primary or secondary to insulin resistance, will result in enhanced contractile responses in type 2 diabetic patients. To test the latter part of this hypothesis, the response of the blood pressure of the forearm arterial system and the dorsal hand vein was determined in normoalbuminuric, normotensive, type 2 patients. The results of these experiments are described in chapters 2 and 3. In earlier studies the noradrenergic response of the dorsal hand vein was enhanced in type 1 microalbuminuric patients, compatible with the view that microalbuminuria is associated with enhanced VSMC contractility⁷⁶. Accordingly, one would expect enhanced noradrenergic responsiveness of the forearm vascular bed in these microalbuminuric type 1 patients as well. Furthermore, one would expect enhanced noradrenergic responsiveness of the dorsal hand vein in type 2 microalbuminuric patients. These questions are addressed in chapters 5 and 2, respectively.

A number of studies has shown impaired vasodilator responses of the skin microvasculature and the forearm vascular bed in type 2 diabetes⁶². To determine whether an impaired VSMC dilator response of large conduit arteries and veins can be observed also in these patients, the effect of nitroglycerin on the femoral artery and forearm veins was determined, as described in chapter 4. Alterations in the vascular viscoelastic properties, both venous and arterial, could contribute to changes in vascular reactivity. Arterial and venous compliance in type 2 diabetic patients with and without microalbuminuria are described in chapters 2, 3, and 4.

The third aim of this thesis was to determine whether abnormalities in endothelium-derived NO production and/or bioavailability are associated with microalbuminuria and retinopathy. Given the potential confounding effects of hyperlipidemia, dyslipidemia, or hyperinsulinemia, these experiments were performed in type 1 patients only. The results of these experiments are described in chapter 5. According to several authors "endothelial dysfunction" plays a pivotal role in the development of diabetic microangiopathy^{56,57,60,61}. Following this line of reasoning, abnormalities in vascular permeability, endothelial NO production, and endothelium-dependent coagulation and fibrinolysis would be closely associated and tend to cluster within the same patients. This hypothesis was tested in type 1 patients, as reported in chapter 6.

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Chapter II

The venous system in type 2 diabetes:

Noradrenergic responsiveness and compliance

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Submitted for publication

Abstract

Introduction

Changes in the venous system could contribute to the development of hypertension by alteration of the volume homeostasis in type 2 diabetes. It is at present unknown whether venous noradrenergic (NA) responsiveness is increased or venous compliance is reduced in type 2 diabetes, especially in the presence of microalbuminuria. Therefore, NA responsiveness of a dorsal hand vein and venous compliance (Vcomp) of the forearm were studied in normo- and microalbuminuric type 2 diabetic patients as compared to healthy volunteers.

Patients and Methods

Eighteen normoalbuminuric type 2 diabetic patients (MA-), 10 microalbuminuric type 2 diabetic patients (MA+), and 14 healthy volunteers (HV), all with comparable age, blood pressure and body mass index (BMI) were studied. Diabetes duration was 4 years in the MA- group and 22 years in the MA+ group. NA responsiveness was assessed from the venous constrictor response to a cumulative local infusion of NA. Vcomp was measured by strain-gauge plethysmography.

Results

No differences were observed in the dose generating half maximal (ED50) and in maximal response (Emax) of the NA dose response curve between the MA-, the MA+ subjects and the HV: 1.16 (0.77-1.40) (median and interquartile ranges), and 0.92 (0.88-0.99) vs. 0.79 (0.51-1.03) log ngNA.min⁻¹; 104 (91-112), and 101 (83-110) vs. 90 (84-95) %, respectively. Vcomp was reduced in the MA+ subjects but not in the MA-subjects in comparison with the HV: 0.042 (0.029-0.054), $p < 0.03$, and 0.051 (0.043-0.059) vs. 0.058 (0.051-0.065) ml.100ml⁻¹.mmHg⁻¹.

Conclusions

As NA responsiveness of the venous system is normal in normotensive MA- type 2 diabetic patients and venous compliance is reduced in normotensive MA+ type 2 subjects with prolonged disease duration, the venous system plays only a secondary role in the development of hypertension in type 2 diabetes.

Introduction

Hypertension is frequently present in type 2 diabetes mellitus patients, in patients with other insulin resistant syndromes and in patients with microalbuminuria¹. Although the cause of hypertension in these hyperinsulinemic patients remains unknown, several potential mechanisms have been proposed². For instance, vascular tone may be enhanced by increased sensitivity to or increased production of noradrenaline (NA), the latter being due to augmented sympathetic outflow secondary to the hyperinsulinaemic state³. On the other hand, vascular tone may be raised as a consequence of reduced sensitivity to or deficient production of endothelium-derived nitric oxide^{4,5}. Finally, not only functional but also structural vascular changes can contribute to the development of hypertension. Arterial compliance, for instance, is reduced in non-diabetic hypertensive subjects⁶ and according to some also in type 2 diabetic patients⁷.

Most studies concerning functional and structural vascular changes in diabetes are limited to the arterial system. However, alterations of the venous system could also contribute to the development of hypertension⁸. The venous system contains 60-80% of the total blood volume⁹ and plays a pivotal role in blood volume and blood pressure homeostasis. A diminution of venous compliance can cause a rise in the filling pressure of the right heart with a subsequent increase in cardiac output, thus favouring the development of hypertension¹⁰. Theoretically, venous compliance could be impaired by enhanced venous tone secondary to increased noradrenergic responsiveness¹¹. Although systemic responsiveness to NA is enhanced in normotensive type 2 diabetic patients, no such data are available with respect to venous noradrenergic responsiveness.

Another possible cause for reduced venous compliance in diabetes is stiffening of the vascular wall due to non-enzymatic glycosylation, followed by advanced glycation end (AGE) product formation of collagen or other connective tissue proteins¹². The occurrence of irreversible cross-links between proteins would lead to altered mechanical properties¹³.

On the basis of these observations two questions emerge with respect to the venous system in type 2 diabetes mellitus. The first one refers to venous responsiveness to NA. Since arteriolar responsiveness to NA is increased in type 2 diabetes, one wonders whether venous responsiveness to NA is augmented as well. Furthermore, it remains to be determined whether this augmented NA responsiveness is more pronounced in patients with microalbuminuria (MA), similar to the situation in type 1 diabetes mellitus¹⁴. The second question is whether venous compliance is reduced in these same subjects, and whether this is related to an enhanced NA responsiveness. Therefore, in the present study we measured NA responsiveness of a dorsal hand vein in normotensive, normoalbuminuric and microalbuminuric type 2 diabetic patients and compared the results to those

obtained in healthy controls. In addition, we studied venous compliance of the forearm veins by strain gauge plethysmography in these same subjects.

Subjects and Methods

Subjects

Studies were performed in 28 type 2 diabetic patients and 14 healthy volunteers. The type 2 diabetic patients were divided into two subgroups, consisting of 10 type 2 diabetic patients with microalbuminuria (MA+) and 18 type 2 diabetic patients without microangiopathic diabetic complications nor diabetic neuropathy (MA-). Type 2 diabetes was defined as the onset of diabetes after the age of 40 years, initially treated with a diet and/or oral antidiabetic medication. Microalbuminuria was defined as an urinary albumin excretion (UAE) of 30-300 mg/24 hr. The presence or absence of autonomic neuropathy was assessed by measuring heart rate (R-R interval) variations during deep breathing and Valsalva manoeuvre, and heart rate and blood pressure responses to standing under standardized conditions as described by Ewing and Clarke¹⁵. The microalbuminuric patients were recruited from the outpatient department of four different hospitals. The normoalbuminuric patients were recruited from the outpatient department of the University Hospital Maastricht and by advertising in local papers. Control subjects (C) were recruited by advertising in local papers, had a negative family history of diabetes and were paid for their contribution.

Clinical characteristics of patients and controls are summarized in table 1. None of the subjects studied had evidence of cardiovascular disease or hyperlipidaemia on physical examination or routine biochemical screening. Supine blood pressure did not exceed 160/90 mmHg. Serum creatinine levels were all below 100 $\mu\text{mol.l}^{-1}$. Four MA+ subjects had one or more (borderline) abnormal autonomic function tests, but none of the subjects had an abnormal blood pressure response to standing. Also, none of the patients had symptoms or signs (abnormal tendon reflexes and vibration sense) of distal polyneuropathy. Metabolic control was achieved in all the MA- subjects by oral antidiabetic drugs and in the MA+ subjects by oral antidiabetic drugs (n=4), insulin (n=5) or a combination of insulin and an oral antidiabetic drug (n=1). Neither the type 2 diabetic patients nor the healthy volunteers used any medication (including NSAID's) for at least 4 weeks preceding the study, except for oral antidiabetic drugs or insulin by the diabetic patients.

Subjects were instructed to avoid caffeine-containing beverages, smoking and alcohol for 12 h preceding the study. All experiments were performed after an overnight fast. On the morning of the experiments the type 2 diabetic patients

omitted their anti-diabetic medication. All subjects gave written informed consent and the study was approved by the Ethical Review Committee of the University Hospital Maastricht. The investigations conformed with the principles outlined in the Declaration of Helsinki.

Protocol

Hand vein responsiveness to NA (Study I) was assessed by the linear variable differential transformer (LVDT) technique and venous compliance (Study II) by pletyhysmography. Both studies were performed in a randomised order on two separate days, at least 2 weeks apart.

LVDT technique

By this technique alterations in the distension of superficial hand veins during drug infusions of NA were measured¹⁶. Subjects were studied supine in a temperature-controlled room with a mean ($\pm$ SD) temperature of $26.0 \pm 0.5^\circ\text{C}$. In the non-dominant arm a dorsal hand vein was cannulated with a 23-Gauge butterfly needle. This arm was resting on a padded support, 30 degrees above the horizontal, so that the vein, which was above heart level, was completely collapsed. A cuff was attached around the upper arm. Changes in vein diameter were measured using the LVDT (model 100 HMR, Schaevitz Engineering, Pennsauken, NJ) and an ATA signal conditioner (Schaevitz Engineering). The LVDT was mounted on the back of the hand using a tripod. The tip of the steel core of the LVDT was positioned over the centre of the vein approximately 1 cm proximal to the tip of the butterfly needle. After an acclimatisation-period of 30 minutes, three baseline measurements of venous distension were performed with a cuff pressure of 40 mmHg. The difference between the collapsed and fully distended readings represented the maximum vein diameter, i.e. 0% constriction. Subsequently, a cumulative stepped infusion of noradrenaline, $0.3\text{--}160\text{ ng}\cdot\text{min}^{-1}$, dissolved in 0.9% saline, was delivered into the hand vein by a syringe driver pump (Terufusion syringe pump model STC-521, Terumo Corporation, Tokyo, Japan) at an infusion rate of $0.1\text{ ml}\cdot\text{min}^{-1}$. Each dose of noradrenaline (NA) was infused during 7 minutes with inflation of the cuff during the last 2 minutes. Vein diameter was measured during the 7th minute of the infusion.

Reproducibility of LVDT: Prior to the study proper reproducibility experiments were performed to assess the within-day and the between-day coefficient of variance (CV) of the hand vein responses to NA. Two complete dose-response curves were made ($n=15$) with an interval of 30 minutes (within day). A third dose-response curve was made two weeks later (between day). The CV was calculated using the log NA dose (pD2) generating a half maximal response (ED50). The within day CV of the pD2 to NA was 22% and the between day of

the pD2 was 28%¹¹.

Venous compliance

Subjects were studied supine in a temperature-controlled room with a mean ($\pm$ SD) temperature of $24.0 \pm 0.5^\circ\text{C}$. Venous compliance was determined in the non-dominant arm, as described in detail elsewhere¹⁷. Briefly, forearm volume changes were measured using strain gauge venous occlusion plethysmography (Hokanson EC4, Hokanson, Issaquah, WA, USA). Changes in forearm volume (dV) and changes in intravenous pressure (dP), caused by 3-minute stepwise upper arm venous congestion, were measured at the following cuff pressures: 20, 25, 30, 35, 40, 50 mmHg. Each venous occlusion step was followed by a 2 minute interval of deflation of the cuff. Volume and pressure changes during each cuff pressure step were obtained from the values just before and after deflation of the cuff. The slope of the dV/dP relationship (Vcomp) is linear in the observed venous pressure ranges and is a measure of the visco-elastic properties of the peripheral venous system¹⁷. The CV of the venous compliance measured by this technique is 8.7% for two consecutive measurements (30 min interval), and 11.4% for two measurements on different days (interval greater than 5 weeks)¹⁷.

Assays

Fasting blood glucose concentration was measured by the glucoxidase method, cholesterol, triglyceride, creatinine by commercial methods, HbA1c using the Diamat HPLC-analyser and microalbuminuria by a commercial immunoturbidimetric method. United albumin excretion (UAE) was expressed as the mean of two 24-hr urine collections, taken within a 6-month period.

Calculations

Study I: Hand vein responsiveness to NA

The percentage reduction in maximal vein diameter in response to each dose of NA was calculated. For each subject an individual dose response-curve was constructed using Graphpad (Graphpad Software Inc.) after logarithmic transformation of the infusion doses. The maximal venous constrictor response (Emax) and the log NA dose generating a half maximal response (logED50) were calculated.

Study II: Venous compliance

The individual slopes of the dV/dP relation (Vcomp), were calculated by Pearson's linear regression.

Statistics

From the validation studies it was calculated that minimally 10 subjects per group

were needed to observe a difference of 35% in the pD2 NA dose with a power of 80%, with an $\alpha = 0.05$ ($Z\alpha=1.96$) and $\beta = 0.80$ ($Z\beta=1.28$). As the data were not distributed normally, all data are expressed as median values and interquartile ranges, unless otherwise indicated. Group comparisons were made using non-parametric (Mann Whitney U) tests. Statistical significance is defined as a p-value ≤ 0.05 .

Results

All groups were similar in terms of age, gender distribution, body mass index (BMI) and blood pressure (table 2.1). Heart rate was significantly higher in the MA+ group in comparison with the healthy volunteers and the MA- group. HbA1c was higher in the MA+ group than in the MA- group and in both diabetic groups HbA1c was higher than in the healthy volunteers. Diabetes duration was longer in the MA+ group in comparison with the MA- group: 22 (16-26) vs 4(3-7) yrs. Cholesterol and triglyceride levels were normal in all groups.

Table 2.1 Clinical and laboratory characteristics of subjects

	Type 2 diabetes		
	C	MA-	MA+
Male/female	10/4	13/5	7/3
Age (years)	59 (52-64)	59 (56-63)	57 (54-61)
BMI (kg.m ⁻²)	24.7 (23.2-26.5)	25.5 (24.8-27.7)	24.2 (23.5-25.5)
Diabetes duration (yrs.)		4 (3-7)	22 (16-26) *
HbA1c (%)	5.5 (5.1-5.8)	6.6 (6.2-7.2) †	8.8 (8.5-9.8) *†
Cholesterol (mmol.l ⁻¹)	4.9 (4.5-5.6)	5.4 (5.0-6.1)	5.4 (4.7-5.5)
Triglycerides (mmol.l ⁻¹)	1.0 (0.6-1.4)	1.2 (1.0-1.3)	1.1 (0.9-1.5)
UAE (mg/24hr)		11 (5-20)	130 (60-160) *
Blood pressure (mmHg)			
Systolic	147 (126-156)	142 (122-157)	143 (130-153)
Diastolic	68 (66-75)	77 (73-87)	80 (68-87)
MAP	96 (89-103)	99 (88-110)	100 (89-109)
Heart rate (BPM)	64 (60-68)	60 (57-64)	84 (75-85) *†

C: healthy controls; MA-: normoalbuminuric type 2 diabetic patients; MA+: microalbuminuric type 2 diabetic patients; UAE: urinary albumin excretion; BMI: body mass index; MAP: mean arterial pressure, calculated from the equation: [(systolic-diastolic)/3]+diastolic. Data are expressed as medians (interquartile ranges). Statistical significance: *p<0.01 vs MA-; †p<0.01 vs C.

Table 2.2 Noradrenaline (NA) responsiveness of a dorsal hand vein.

	Type 2 diabetes		
	C	MA-	MA+
ED50 NA (log ng.min ⁻¹)	0.79 (0.51-1.03)	1.16 (0.77-1.40)	0.92 (0.88-0.99)
E _{max} NA (%)	90 (84-95)	104 (91-112)	101 (83-110)

C: healthy controls; MA-: normoalbuminuria; MA+: microalbuminuria; E_{max}: maximal constrictor response; ED50: dose generating half maximal response. Data are expressed as median (interquartile ranges).

Blood pressure, heart rate and glucose levels remained stable during local infusions of NA (data not shown). No differences were observed in the dose generating half maximal (ED50) and in maximal response (E_{max}) of the NA dose response curve between the MA-, the MA+ subjects and the HV (table 2.2). In contrast, V_{comp} of the forearm was reduced in the MA+ subjects, but not in the MA- subjects, relative to healthy volunteers (figure 2.1). V_{comp} was negatively correlated with UAE: R=-0.47, p<0.01. V_{comp} was not correlated with mean arterial pressure, pulse frequency, HbA1c, age, or diabetes duration.

Figure 2.1 Venous compliance of the forearm. C: healthy controls; MA-: normoalbuminuric type 2 diabetic patients; MA+: microalbuminuric type 2 diabetic patients. Medians with interquartile ranges (boxes) and ranges (bars). * P<0.05 vs C.

Discussion

Hypertension is a major cardiovascular risk factor in patients with type 2 diabetes mellitus¹⁸. Although the cause of an elevated blood pressure in insulin-resistant

states such as type 2 diabetes and obesity is unknown, arteriolar pressor responses to exogenous noradrenaline and angiotensin II are potentiated¹⁹, suggesting enhanced responsiveness of the vascular smooth muscle cell (VSMC) to vasoconstrictor stimuli. Little is known, however, about venous responsiveness to NA. In the present study we used the hand vein as an *in vivo* model of an isolated vessel to study the interaction between the VSMC and exogenous NA. Using the LVDT-technique the local venous responsiveness can be measured during infusion of, for instance, minute amounts of noradrenaline, which do not elicit systemic and reflex responses. Furthermore, a complete dose-response curve can be obtained. As vascular responsiveness to vasoactive drugs is determined, amongst others, by basal vascular tone, all hand vein studies were performed in a warm and relaxing environment, such as to minimize the influence of basal sympathetic tone²⁰. In our hands, the within and between day variability of the ED₅₀ to NA appeared to be comparable to those in other studies^{20,21}. In the present study, we found that NA responsiveness of the dorsal hand vein in normo- and microalbuminuric type 2 diabetes patients was similar to that in healthy volunteers.

Bodmer and colleagues found, that venous NA responsiveness was markedly enhanced in microalbuminuric patients with type 1 diabetes¹⁴. This enhanced responsiveness is probably based upon a defect in venous α_2 -adrenoceptor activity¹⁴. No such abnormality was observed in normoalbuminuric type 1 patients¹⁴. In contrast, our data suggest that in type 2 diabetes venous NA responsiveness is similar in micro- and normoalbuminuric patients.

Several lines of evidence suggest that in diabetic patients, both type 1 and type 2, microalbuminuria can be seen as a marker of generalized vascular dysfunction^{22,23}. In type 1 diabetes the increased vascular permeability to albumin seems to be associated with enhanced venous NA responsiveness^{14,24}. However, in type 2 diabetes these two aspects of vascular function seem to be dissociated, thus weakening the concept that microalbuminuria is a marker of generalized, i.e. arteriolar and venous, vascular dysfunction.

Another aspect of our study concerned the measurement of venous compliance, which was calculated from the relationship between changes in forearm volume and intravenous pressure during venous congestion. Differences in capillary filtration, as observed in type 1 diabetes^{25,26}, could, theoretically, confound the measurements of forearm volume changes. Although capillary filtration is normal in type 2 diabetes patients²⁷, we determined forearm volume changes after deflation of the cuff to circumvent possible confounding by increased capillary filtration¹⁷. In an earlier study we observed reduced venous compliance in both normo- and microalbuminuric patients with long-term type 1 diabetes (mean diabetes duration 18 years)¹⁷. In the present study, however, venous compliance was reduced in the microalbuminuric, but not in the normoalbuminuric patients with type 2 diabetes. We even observed an inverse relationship between venous compliance and urinary

albumin excretion. Given the longer duration of diabetes in the micro- vs the normoalbuminuric type 2 patients (4 vs 17 years), we propose that the reduction in venous compliance is not related to urinary albumin excretion as such, but rather to the duration of the diabetic state. Indeed, both experimental and observational studies indicate that chronic hyperglycaemia can result in loss of the visco-elastic properties of the vessel wall^{11,28,29}.

Reduction of the visco-elastic properties of the venous vascular wall could be explained by structural changes resulting from non-enzymatic glycosylation of collagen and other connective tissue proteins, followed by advanced glycosylation end (AGE) product formation with irreversible cross-linking between ageing proteins¹². Previous studies by Monnier and coworkers showed a correlation between collagen linked fluorescence of the skin, as a measure of AGE products, and the visco-elastic properties of the aorta³⁰. On the other hand, it could be argued that the reduced venous compliance in the microalbuminuric type 2 diabetes patients is caused by increased venous tone, possibly as a result of enhanced sympathetic activity³¹. In fact, heart rate was higher in the microalbuminuric type 2 diabetes subjects, which is compatible with sympathetic overactivity, although this may also be due to reduced parasympathetic function³².

The present study clearly indicates that a reduction in venous compliance is not a sequela of hypertension in type 2 diabetes, as blood pressure was still normal in these microalbuminuric patients. However, since blood pressure in hypertensive patients with type 2 diabetes is frequently salt sensitive³³, loss of venous compliance may exaggerate the hypertensive effects of salt loading in these patients. When venous compliance is reduced, a minor increase in blood volume can lead to a relatively large increase in cardiac filling pressure⁹. When compensatory cardiovascular mechanisms are inadequate, this might result in an increase in cardiac output and blood pressure.

In conclusion, venous responsiveness to NA is unaltered in both normo- and microalbuminuric patients with type 2 diabetes. In contrast, venous compliance is reduced in normotensive microalbuminuric patients.

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Chapter III

**The enhanced pressor response
to noradrenaline in type 2
diabetes is not based upon a
generalized increase in
vascular responsiveness**

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Submitted for publication

Abstract

Objective

The present study was performed to discriminate between central and peripheral effects of noradrenaline (NA) in normotensive, non-obese, type 2 diabetic patients.

Methods

Study I: In 10 patients and 10 healthy volunteers(HV) cumulative doses of NA were infused intravenously until mean arterial pressure (MAP) rose with 20 mmHg, and subsequently the effects on the forearm blood flow (FBF) was measured. Also, the FBF response to intraarterial NA (0.025, 0.1, 0.4 $\mu\text{g}\cdot\text{min}^{-1}$) was measured.

Study II: In 13 patients and 14 HV the venous constrictor response to a cumulative local infusion of NA in a dorsal hand vein was determined.

Results

In *study I* the circulating plasma NA concentrations inducing a rise in MAP of 20 mmHg, were lower in the type 2 patients relative to the HV ($p<0.01$). The relationship between changes in pressure and changes in heart rate were similar in both groups. Moreover, FBF responsiveness to intraarterial NA was not different between the two groups. The slopes of the $\Delta\text{MAP}/\text{NA}$ regression lines were correlated with basal insulin levels and relative insulin resistance in the healthy volunteers ($R=0.77$, $p<0.01$, and $R=0.83$, $p<0.01$), but not in the type 2 diabetic patients. In *study II* no differences were observed in the dose generating half maximum (ED_{50}) and the maximum (E_{max}) response to NA between the type 2 patients and the HV.

Conclusions

Non-obese normotensive type 2 patients have an increased pressor response to NA, which is not based upon a defect in skeletal muscle resistance arterioles, peripheral veins, or a defect in the baroreceptor system. Therefore, in type 2 diabetes the noradrenergic responsiveness of other vascular beds, such as the splanchnic or renal, must be enhanced.

Introduction

Hypertension is a major cardiovascular risk factor in patients with type 2 or non-insulin-dependent diabetes mellitus¹. Moreover, hypertension and type 2 diabetes often coexist, which has led to the hypothesis that insulin resistance may play a central role in the pathogenesis of both derangements². However, the mechanisms by which insulin resistance affects blood pressure are unknown. Potential hypertensive effects of elevated insulin levels are increased sympathetic neural outflow³, augmented renal reabsorption of sodium⁴, and salt-sensitivity of blood pressure⁵. Finally, in insulin-resistant states such as type 2 diabetes and obesity, pressor responses to exogenous noradrenaline (NA) and angiotensin II (AII) are potentiated⁶, suggesting enhanced responsiveness of the vascular smooth muscle cell (VSMC) to vasoconstrictor stimuli. This enhanced responsiveness, in turn, could be related to abnormal VSMC calcium homeostasis⁴. On the other hand, increased pressor responsiveness could also be the consequence of impaired baroreceptor function, even without any abnormalities in VSMC contractility⁷. Indeed, Baron and co-workers suggested that in obese insulin-resistant subjects the increased pressor response to exogenous NA is based upon a defect in baroreceptor function⁸.

To our knowledge, no studies have been performed which address both local and systemic pressor responsiveness to exogenous NA in type 2 diabetes. Therefore, in the present study we assessed in the same type 2 subjects and healthy volunteers not only blood pressure and heart rate (as a measure of baroreceptor activity) responses to systemic infusions of NA, but also the vasoconstrictor responses of forearm resistance arterioles to local infusion of NA. In a separate group of patients and controls venous responsiveness to local NA was determined. Only normoalbuminuric subjects were studied, as most microalbuminuric patients have signs of autonomic neuropathy, which in itself could modify NA pressor responsiveness⁹. Furthermore, all participants were non-obese, as obesity may also enhance pressor responses to exogenous NA¹⁰. Finally, all subjects were normotensive, as we wanted to avoid exaggerated pressor responses due to hypertension-induced media hypertrophy.

Subjects and methods

Subjects

Control subjects were recruited by advertisements in local newspapers, while patients with type 2 diabetes were recruited from the outpatient department of the University Hospital Maastricht and by advertisements. Type 2 diabetes was defined

as the onset of diabetes after the age of 40 years, initially treated by diet and/or oral anti diabetic medication. Supine blood pressure in these patients did not exceed 160/90 mmHg. None of the subjects studied had evidence of cardiovascular disease, nephropathy, hyperlipidaemia or neuropathy on physical examination or routine biochemical screening. Serum creatinine levels were all below $100 \mu\text{mol.l}^{-1}$. Microalbuminuria, defined as an urinary albumin excretion (UAE) of 30-300 mg/24 hr, was excluded in all patients. The presence of autonomic neuropathy was excluded by measuring heart rate (R-R interval) variations during deep breathing and Valsalva manoeuvre, and heart rate and blood pressure responses to standing under standardized conditions as described by Ewing and Clarke¹¹. None of the patients had symptoms or signs (abnormal tendon reflexes and vibration sense) of distal polyneuropathy. Metabolic control in the type 2 patients was achieved by tolbutamide in eight patients, by gliclazide in three patients, by glimeperidine in one patient, by glibenclamide in three patients, while two patients were treated with diet only.

None of the type 2 patients or the healthy volunteers used any medication (including NSAID's) for at least 4 weeks preceding the study, except for oral antidiabetic drugs by the diabetic patients.

All subjects were instructed to avoid caffeine-containing drinks, smoking and alcohol for 12 h preceding the studies. Experiments were performed after an overnight fast and on the morning of the experiments the type 2 patients omitted their anti diabetic medication. All subjects gave written informed consent and the study was approved by the Ethical Review Committee of the University Hospital Maastricht. The investigations conformed with the principles outlined in the Declaration of Helsinki.

Protocol

Two different studies were performed in 17 healthy volunteers with a negative family history for diabetes or hypertension and in 17 type 2 diabetic patients. In the first study NA was infused intravenously to evaluate the effect of NA on blood pressure, heart rate and forearm blood flow (FBF) in 10 type 2 patients and 10 healthy volunteers (study I, FBF and pressor response). Subsequently, the vasoconstrictor effect of NA, administered into the brachial artery of the non-dominant arm, on FBF was determined in these same patients. In the second study the vasoconstrictor response of the dorsal hand vein to local infusion of NA was evaluated in 13 type 2 patients and 14 healthy volunteers (study II, hand vein). Seven healthy volunteers and 5 type 2 patients participated in both studies.

Study I

After local anaesthesia by 1.5 ml 1% lidocaine, a 20 gauge arterial catheter (Arrow, Reading, PA, USA) was inserted retrogradely into the brachial artery of the non-dominant arm for local NA infusions and blood pressure measurements. The catheter was kept patent with saline. Mean arterial pressure (MAP) was measured using a Hewlett Packard 78205C monitor (Boeblingen, Germany). FBF was determined simultaneously in both arms by ECG-triggered strain gauge venous occlusion plethysmography (Periflow, Janssen Scientific Instruments, Beerse, Belgium) as described earlier¹². The hand circulation was excluded during FBF measurements by inflating a wrist cuff to suprasystolic pressure, starting one minute before each FBF measurement. Hence, FBF measurements represent predominantly muscle blood flow¹³. Heart rate was derived from the ECG. All signals were stored on the hard disk of a personal computer by means of a custom build data acquisition system. Subjects were studied supine in a temperature-controlled room with a mean ($\pm$ SD) temperature of 24.0 ± 0.5 °C.

FBF and pressor response to systemic NA: After blood samples were taken for determination of basal arterial insulin, (nor)adrenalin, and glucose, NA was infused intravenously into the dominant arm. The starting dose was $30 \text{ ng.kg}^{-1}.\text{min}^{-1}$ followed by stepwise increases of $15 \text{ ng.kg}^{-1}.\text{min}^{-1}$ every 5 minutes, until the intraarterially measured MAP was elevated by 20 mmHg or a dose of $150 \text{ ng.kg}^{-1}.\text{min}^{-1}$ was reached (NAmax). FBF was measured in the non-dominant arm, before the infusion of NA and during the NAmax dose. At these same time points arterial blood samples were taken for determination of (nor)adrenaline.

FBF, before and during local intraarterial NA: FBF was measured during 3 minutes of baseline blood flow and during local infusions of three cumulative doses of NA (0.025 , 0.1 , $0.4 \text{ } \mu\text{g}.\text{min}^{-1}$, each dose for three minutes) in the brachial artery of the non-dominant arm. The doses and the duration of the drug infusions were such as to reach steady state vascular responses. The mean FBF value of the last minute of each drug step was used for calculations.

Study II

Hand vein: Venous responsiveness to NA was assessed by the linear variable differential transformer (LVDT) technique, which measures alterations in the distension of superficial hand veins during drug infusions¹⁴. The hand vein was used as an in-vivo model of an isolated vessel to study the interaction between the endothelium and the VSMC in response to NA. With this model local vascular responsiveness, independent of systemic and reflex responses, can be measured by infusion of minute amounts of NA. Subjects were studied supine in a temperature-controlled room with a mean ($\pm$ SD) temperature of 26.0 ± 0.5 °C, in order to

minimize vascular tone. In the non-dominant arm a dorsal hand vein was cannulated with a 23-Gauge butterfly needle. This arm was resting on a padded support, 30 degrees above the horizontal, so that the vein, which was above heart level, was completely collapsed. A cuff was attached around the upper arm. Changes in vein diameter were measured using the LVDT (model 100 HMR, Schaevitz Engineering, Pennsauken, NJ) and an ATA signal conditioner (Schaevitz Engineering). The LVDT was mounted on the back of the hand using a tripod. The tip of the steel core of the LVDT was positioned over the centre of the vein approximately 1 cm proximal to the tip of the butterfly needle. After an acclimatisation period of 30 minutes, three baseline measurements of venous distension were performed with a cuff pressure of 40 mmHg. The difference between the collapsed and fully distended readings represented the maximum vein diameter, i.e. the diameter at 0% constriction. Subsequently, a cumulative stepped infusion of NA, 0.3-160 ng.min⁻¹, dissolved in 0.9% saline was delivered into the hand vein by a syringe driver pump (Terufusion syringe pump model STC-521, Terumo Corporation, Tokyo, Japan) at an infusion rate of 0.1 ml.min⁻¹. Each dose of NA was infused during 7 minutes with inflation of the cuff during the last 2 minutes. Vein diameter was measured during the 7th minute of the infusion. Prior to the present studies the variability of the ED50 to NA was evaluated in healthy volunteers (n=18). The within-day coefficient of variance of the ED50 in these experiments was 28%, which is compatible with that in other studies¹⁵.

Assays

Fasting blood glucose concentration was measured by the glucosidase method, cholesterol, triglyceride, creatinine by commercial methods, HbA1c using the Diamat HPLC-analyser. Serum insulin was determined by a modified RIA assay (Pharmacia Upjohn, Uppsala, Sweden) to exclude cross reactivity with human pro-insulin and with auto-antibodies. Plasma catecholamines were determined by HPLC followed by fluorescence detection. Microalbuminuria was measured by a commercial immuno-turbidimetric method. UAE was expressed as the mean of two 24-hr urine collections, taken within a 6-month period.

Calculations

Study I

FBF and pressor response to systemic NA: The individual slope of the MAP response to the respective doses of NA (dMAP/NA) and the individual slope of the change in heart rate to the change in MAP (dHR/dMAP) were calculated by Pearson's linear regression. The mean and 95% confidence intervals of the dMAP/NA and of the dHR/dMAP slopes were calculated using GraphPad (GraphPad Software Inc., San Diego, CA, USA). Forearm vascular resistance

(FVR) was calculated as MAP divided by concomitant FBF and expressed in arbitrary units (AU). Relative insulin resistance was calculated from fasting glucose and insulin according to the HOMA formula and expressed in AU^{16,17}.

FBF before and during local intraarterial NA: For each NA dose the FBF-ratio (FBF of the infused arm divided by FBF of the contralateral arm) was calculated. This calculated ratio corrects for systemic factors that affect the regulation of FBF in both arms (e.g. changes in blood pressure, level of arousal, hormonal changes etc.), and ensures that only the direct effects of locally infused substances on FBF are taken into account¹⁸. The stepwise NA doses were chosen in such a way that vasoconstrictor responses in the linear part of the dose-response curve were obtained. The response to the various NA doses are expressed as percentage change in FBF-ratio from baseline.

Study II

Hand vein: The percentage reduction in maximal vein diameter in response to each dose of NA was calculated. For each subject an individual dose response-curve was constructed, using GraphPad, after logarithmic transformation of the infusion doses. The maximal venous constrictor responses (Emax) and the log NA doses generating half maximal responses (logED50) were calculated.

Statistics

As the data were not distributed normally, all data are expressed as median values and interquartile ranges unless otherwise indicated. Group comparisons were made using non-parametric tests. Within group analyses of multiple related samples were made using Friedman's test (non-parametric analysis of variance), while the Wilcoxon paired rank test was used for paired analyses. The Kruskal Wallis test was used for multiple group comparisons and the Mann Whitney U test for two group comparisons. When multiple comparisons were made, Shaffer adjusted p-values were used¹⁹. Correlations were made by Spearman Rank tests. Statistical significance is defined as a p-value ≤ 0.05 .

Results

As expected HbA1c, fasting blood glucose, and serum insulin levels were higher in the type 2 patients ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively, table 3.1). Furthermore, no differences were observed between the groups in study I and II, except for triglyceride levels, which were higher in type 2 patients than in healthy volunteers ($p < 0.05$, table 3.1).

Table 3.1 Clinical and laboratory characteristics.

	Study I (systemic and in NA)		Study II (hand vein)	
	Healthy volunteers	Type 2 patients	Healthy volunteers	Type 2 patients
Male/female	10/0	10/0	10/4	9/4
smokers	3	3	1	2
Age (years)	59 (58 - 63)	59 (54 - 63)	59 (52 - 64)	58 (54 - 61)
BMI (kg.m ⁻²)	25.3 (24.0 - 25.7)	24.4 (24.2 - 25.3)	24.7 (23.2 - 26.5)	25.5 (23.9 - 28.1)
Diabetes duration (years)		8 (6 - 11)		4 (3 - 7)
Glucose (mmol.l ⁻¹)	4.6 (4.4 - 4.7)	8.5 (7.2 - 13.4) **	4.7 (4.3 - 4.8)	5.2 (4.9 - 8.0) *
HbA _{1c} (%)	5.4 (5.2 - 5.4)	8.1 (7.9 - 9.8) *	5.5 (5.1 - 5.8)	6.9 (6.3 - 7.9) *
Cholesterol (mmol.l ⁻¹)	4.5 (3.6 - 5.5)	5.4 (4.5 - 6.3)	4.9 (4.5 - 5.6)	5.5 (4.5 - 6.4)
Triglycerides (mmol.l ⁻¹)	0.8 (0.5 - 1.4)	1.9 (1.4 - 2.6) *	1.0 (0.6 - 1.4)	1.3 (1.1 - 1.5)
UAE (mg/24hr)	-	13 (7 - 16)	-	11 (5 - 20)
Insulin (mU.l ⁻¹)	6.4 (6.0 - 8.8)	10.1 (7.6 - 10.4) *	-	-
Blood pressure (mmHg)				
Systolic	130 (125 - 145)	135 (128 - 148)	123 (116 - 136)	126 (117 - 128)
Diastolic	80 (75 - 85)	80 (80 - 90)	85 (77 - 88)	80 (79 - 87)
MAP	97 (92 - 103)	100 (96 - 107)	96 (89 - 103)	94 (92 - 100)
Heart rate (bpm)	63 (59 - 67)	62 (58 - 69)	64 (60 - 68)	60 (57 - 64)

Abbreviations: UAE: urinary albumin excretion. BMI: body mass index. MAP: mean arterial pressure, calculated from the equation: diastolic + [(systolic-diastolic)/3]. Data are expressed as medians (interquartile ranges). *: $p < 0.05$ vs healthy volunteers. **: $p < 0.01$ vs healthy volunteers.

Study I

Pressor and FBF response to systemic NA: Systemic NA infusion resulted in a dose dependent increase in blood pressure in all subjects, both healthy volunteers (Friedman $p < 0.001$, figure 3.1) and type 2 patients (Friedman $p < 0.001$, figure 3.1). The increase in blood pressure was accompanied by a decrease in heart rate in the healthy volunteers and the type 2 patients (Friedman $p < 0.001$ and $p < 0.01$, figure 3.2). The circulating plasma concentrations of NA, inducing a rise in MAP of 20 mmHg, were lower in the type 2 patients in comparison to healthy volunteers: 13.1 (10.8 - 15.4) vs 20.0 (18.8 - 26.7) nmol.l⁻¹, ($p < 0.01$, table 3.2). The slopes of the dMAP/NA regression lines were steeper in the type 2 patients in comparison to the healthy volunteers: 0.27 (0.23 - 0.35) vs 0.16 (0.13 - 0.19) mmHg.ngNA⁻¹.kg⁻¹, ($p < 0.01$, figure 3.1). In contrast, the slopes of the dHR/dMAP regression lines were not different: -0.3 (-0.2 - -0.5) vs -0.2 (-0.1 - -0.3) BPM.mmHg⁻¹ (figure 3.2).

Basal FBF and FVR were not different between the healthy volunteers and the type 2 diabetic patients (table 3.2). FVR did not change during systemic NA infusion in either group, and there was no difference between both groups during the highest NA dose (table 3.2).

Systemic NA infusion resulted in a rise in arterial blood glucose levels in healthy volunteers from 4.6 (4.4 - 4.7) to 5.9 (5.2 - 6.8) mmol.l⁻¹ ($p < 0.01$). In type 2

patients blood glucose levels did not change: 8.5 (7.2 - 13.4) vs 8.3 (8.1 - 15.7) mmol.l⁻¹.

Figure 3.1 Pressor responsiveness to systemic noradrenaline (NA) in healthy volunteers and type 2 patients. MAP: mean arterial pressure. Mean regression line and 95% confidence interval. * $p < 0.05$ slope regression type 2 patients vs healthy volunteers.

Figure 3.2 Heart rate (HR) response to changes in mean arterial pressure (MAP) in healthy volunteers and type 2 patients. BPM: beats per minute. Mean regression line and 95% confidence interval.

Table 3.2 Haemodynamic parameters and catecholamine levels during systemic noradrenaline

	Study I, Healthy volunteers		Study I, Type 2 patients	
	before systemic NA	final dose NA	before systemic NA	final dose of NA
MAP (mmHg)	85 (82 - 96)	105 (99 - 117) *	92 (85 - 94)	111 (110 - 116) *
Heart rate (bpm)	63 (59 - 67)	56 (54 - 66) *	62 (58 - 68)	57 (55 - 66) *
FBF (ml.100ml ⁻¹ .min ⁻¹)	3.0 (2.4 - 3.3)	3.7 (2.8 - 4.6)	2.4 (2.0 - 2.7)	3.1 (1.7 - 3.5)
FVR (AU)	36.8 (34.0 - 42.0)	29.7 (27.0 - 40.0)	41.3 (33.9 - 45.9)	39.6 (30.2 - 66.3)
Noradrenaline (nmol.l ⁻¹)	0.9 (0.6 - 1.4)	20.0 (18.8 - 26.7) *	0.6 (0.5 - 0.8)	13.1 (10.8 - 15.4) *, †
Adrenaline (nmol.l ⁻¹)	0.26 (0.25 - 0.48)	0.23 (0.13 - 0.28)	0.13 (0.08 - 0.21)	0.11 (0.05 - 0.17)

Abbreviations: MAP: mean arterial pressure, measured intra-arterially. HR: heart rate. FBF: forearm blood flow. FVR: forearm vascular resistance. NA: noradrenaline. Data are expressed as medians (interquartile ranges). *: p<0.05 before NA vs final dose NA. †: p<0.01 type 2 patients vs healthy volunteers.

The slopes of the dMAP/NA regression lines were related to basal insulin levels (R=0.77, p<0.01) and to the relative insulin resistance (R=0.83, p<0.01) in the healthy volunteers, but not in the type 2 diabetic patients (figure 3.3).

Figure 3.3 Relationships (with 95% confidence interval) between basal insulin levels (left panel) and between relative insulin resistance (right panel), with sensitivity to noradrenaline in healthy volunteers (▲) and in type 2 diabetic patients (■). As no significant correlations were observed in the diabetic patients, only the correlations (with 95% confidence interval) in the healthy volunteers is shown in both panels. Slope: the slope of the MAP response to NA. MAP: mean arterial pressure.

Local intraarterial NA infusion: At the start of the intraarterial infusions FBF did not differ between healthy volunteers and type 2 patients: 3.2 (2.3 - 3.6) vs 2.6 (1.5 - 3.0) ml.100ml⁻¹.min⁻¹, p=0.15. Whereas blood pressure and heart rate did not change during intrabrachial NA infusion, this infusion caused a dose-dependent decrease in FBF-index in all subjects, both healthy volunteers (Friedman p<0.001, figure 3.4) and type 2 patients (Friedman p<0.001, figure 3.4). However, no difference could be discerned in FBF responses to the three NA-doses between the groups (figure 3.4).

Figure 3.4 Vasoconstrictor response of the forearm to intra-arterial infusion of noradrenaline (NA) in healthy volunteers and type 2 patients. FBF ratio=forearm blood flow infused/control arm. Data expressed as median and interquartile ranges (boxes) and ranges (T-bars).

Study II

Local venous NA infusion: Blood pressure and heart rate did not change during local intravenous infusion of NA (data not shown). The E_{max} and the ED_{50} of the dorsal hand vein to NA were not different between healthy volunteers and type 2 patients: 94 (83 - 100) vs 100 (85 - 100) %, and: 0.84 (0.52 - 1.4) vs 1.01 (0.69 - 1.25) $\log \text{ ng.min}^{-1}$, respectively (figure 3.5).

Figure 3.5 Vasoconstrictor response of the dorsal hand vein to local infused noradrenaline (NA) healthy volunteers (▲) and in type 2 diabetic patients (■). Data expressed as mean and standard deviation.

Discussion

The present study clearly indicates that the systemic pressor response to NA is increased in normotensive non-obese type 2 patients without microalbuminuria, thus confirming earlier studies^{6,20,21}. Moreover, in the healthy volunteers, but not in the type 2 patients, pressor responsiveness to NA was correlated with basal insulin levels and relative insulin resistance. Although pressor responsiveness to NA can be modified by sodium depletion²², we were merely interested in the localization of the defect that determines the enhanced responsiveness. Therefore, all subjects continued their habitual diet.

Theoretically, the difference in systemic pressor response to exogenous NA could be related to a decreased clearance rate of the administered NA, as can be observed in obese insulin-resistant subjects⁸. However, Tack et al. failed to find, in a recent study using the [³H]-noradrenaline tracer method, differences in total body clearance and forearm NA kinetics between normotensive type 2 patients and healthy volunteers²³. Moreover, in the present study circulating plasma levels inducing a MAP rise of 20 mmHg were clearly lower in the type 2 patients, indicating NA enhanced pressor responsiveness. Thus it is unlikely, that our results are due to reduced NA clearance in the type 2 patients.

NA raises arterial pressure primarily by increasing systemic vascular resistance⁹. At the same time, cardiac output falls as a consequence of baroreceptor stimulation⁷. From a theoretical point of view, therefore, an increased pressor responsiveness in type 2 diabetes could be related to impaired baroreceptor functioning. Such a mechanism has, indeed, been proposed by Baron and co-workers⁸. However, baroreceptor responsiveness to the NA induced rise in MAP was not different between type 2 diabetic subjects and healthy volunteers in the present study. We assessed baroreceptor performance from the relationship between the rise in MAP and the concurrent reduction in heart rate. For any given rise in blood pressure, we found that the fall in heart rate was similar in type 2 patients and controls, suggesting that in our patients baroreceptor function was intact. This could be the result of the meticulous selection of our type 2 patients. Since autonomous neuropathy was excluded in the type 2 patients, it is unlikely that the sympathetic nervous system was defective in these patients. Thus, our findings led us to conclude that an enhanced vascular reactivity (or sensitivity) was the main reason for the increased pressor responses in type 2 diabetes.

Our data further suggest that the increased pressor response is not the result of a generalized vascular defect. First of all, systemic administration of NA had no effect on FVR in type 2 patients, despite an adequate rise in arterial NA levels. Similar findings have been obtained in healthy subjects in whom FVR also remained unaltered or even decreased during systemic NA infusion^{24,25}. Secondly, local administration of NA elicited similar vasoconstrictor responses of forearm

arterioles in type 2 patients and controls. It could be argued, that during intrabrachial infusion far higher NA levels were reached in the forearm vascular bed in comparison to the levels reached during the systemic infusion. However, estimated NA levels during the local infusion of $0.1\mu\text{g}/\text{min}$ are similar to the levels measured during the highest dose of systemic NA administration. Finally, local administration of NA in a dorsal hand vein showed similar venoconstrictor responses in type 2 patients and controls. From these data we conclude that the increased systemic pressor response to NA is not related to an exaggerated vascular responsiveness in skeletal resistance arterioles and peripheral veins. It is tempting to speculate, therefore, that type 2 patients display an enhanced responsiveness of their visceral (i.e. splanchnic and/or renal) circulation. However, currently no data are available about the responsiveness of the splanchnic bed or renal vasculature to NA in type 2 diabetes.

Several mechanisms could be involved in the enhanced NA pressor responsiveness in type 2 diabetes. Firstly, treatment with sulphonylurea drugs could have influenced NA responsiveness in the type 2 patients. These drugs inhibit ATP-sensitive potassium channels, thereby stimulating Ca^{2+} influx and possibly enhancing vasoconstrictor responses²⁷. However, in study I (pressor response) only one patient was using glibenclamide, which seems to be the only sulphonylurea drug with such vascular effects in therapeutic concentrations²⁸. Secondly, glucose and triglyceride levels were different. Alterations in vascular noradrenergic responsiveness have been described in hyperglycemic, streptozotocin induced diabetic rats, a model for type 1 diabetes²⁹. However, studies in healthy volunteers showed that acute elevation of glucose and triglycerides have no effect on NA responsiveness^{12,30,31}, suggesting that hyperglycaemia and hypertriglyceridaemia are not important factors in NA pressor responsiveness. Thirdly, it is likely that the type 2 patients had elevated circulating levels of free fatty acids (FFA). Some data suggest that elevated FFA levels, which were not determined in the present study, could enhance the α -adrenergic responsiveness in man³². Further studies are needed to assess the potential role of FFA in the enhanced pressor response in type 2 diabetes.

Apart from sulphonylurea treatment, and differences in metabolic factors, hyperinsulinaemia and/or insulin resistance could play a role in the enhanced pressor response in type 2 diabetes. A striking finding in the present study were the strong correlations between NA pressor responsiveness and both basal insulin levels and calculated insulin resistance in healthy volunteers. Several authors suggest that hyperinsulinaemia or insulin-resistance is associated with enhanced VSMC contractility³³. This enhanced constrictor responsiveness could contribute to the enhanced NA pressor responses and could promote the development of hypertension³⁴. Insulin affects trans-membrane calcium transport by several mechanisms and there is some evidence which suggests that insulin resistance is

associated with an increase in intracellular calcium³³. This rise in intracellular calcium could result in enhanced VSMC contractile responsiveness to NA and other agonists³³.

Insulin also stimulates the production of endothelium-derived nitric oxide (EDNO), thereby increasing peripheral blood flow^{35,36}. In type 2 diabetics EDNO production and action are impaired in several vascular beds, including that of the forearm^{37,38}. In-vitro, EDNO attenuates NA constrictor responses³⁹ and it has been suggested that the impaired EDNO production in type 2 diabetes could contribute to enhanced VSMC contractility⁴⁰. However, as reported earlier in this journal (acute) hyperinsulinaemia induces vasodilatation in the forearm, without any effects on NA constrictor responses³⁶. Whether this also holds true for other vascular beds remains to be determined. As insulin levels in the splanchnic bed are up to three times higher than in peripheral vessels⁴¹, this vascular bed is exquisitely exposed to long-term hyperinsulinaemia.

In contrast to the healthy volunteers, no correlation was observed between basal serum insulin levels and calculated relative insulin resistance in the type 2 patients. At present this discrepancy is difficult to explain. It is unlikely that the increased insulin levels in the type 2 patients were (partly) based upon a rise in proinsulin, as a specific insulin assay was used. Relative insulin resistance was calculated using basal insulin and glucose levels¹⁶. Estimation of insulin resistance could, therefore, have been less accurate in the type 2 patients. Further studies, in which insulin resistance is quantified in more detail (eg. the hyperinsulinemic-euglycemic clamp technique), are needed to determine the relationship between insulin/insulin resistance and NA pressor responsiveness in type 2 patients. Alternatively, if the enhanced pressor responsiveness is based upon a severe defect in EDNO production, as discussed above, this could have resulted in a loss of association between insulin and pressor responsiveness in the type 2 patients.

In conclusion, our data suggest that the enhanced pressor responses to NA in type 2 diabetic patients are not based upon enhanced constrictor responses in the skeletal arterioles and veins. The potential role of the visceral circulation in blood pressure regulation in these patients needs to be explored.

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Chapter IV

Impaired arterial but not venous responsiveness to nitroglycerin in non-insulin- dependent diabetes mellitus

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The European Journal of Clinical Investigation 1997;27:360-365

Abstract

In earlier studies the response of the arterioles to nitroglycerin (NTG) was impaired in type 2 diabetes mellitus. NTG has major therapeutical effects in reducing cardiac after- and preload. Thus, the vasorelaxing efficacy of a therapeutic dose of 0.4 mg NTG sublingually (s.l.) was evaluated in conduit femoral artery and large veins of normotensive, normoalbuminuric type 2 patients. After NTG, the increase of the femoral artery diameter was significantly lower in the type 2 patients than in the control subjects: 0.49 (0.29-0.79) vs. 0.75 (0.47-1.00) mm respectively (median, interquartile ranges). NTG resulted in an increase of pulse wave transit time in the aorta in control subjects but not in the type 2 patients: from 64 (60-73) to 70 (63-80) ms, $p < 0.02$; and from 62 (53-69) to 66 (51-72) ms, $p = \text{NS}$, respectively. The reduction in venous tone of the forearm to NTG was similar in both groups. These results suggest, that the response to NTG is impaired in the arterial system but not in the venous system in well-regulated type 2 patients without diabetic complications.

Introduction

Large vessel disease is an important complication of type 2 diabetes mellitus^{1,2}. Several lines of evidence suggest that an abnormal production of and/or vascular sensitivity to endothelium-derived nitric oxide (EDNO) could play an important role in the atherogenesis in type 2 diabetes^{3,4}. Apart from its relaxant effects on the vascular smooth muscle cells (VSMCs), EDNO inhibits platelet aggregation and adhesion⁵. Furthermore, EDNO is an antiproliferative factor within the vessel wall⁵. In a recent study by McVeigh et al.⁶, the dilatory response of the forearm (arteriolar) vascular bed to intra-arterially infused nitroglycerin (NTG) was impaired in normotensive type 2 patients. This impaired arteriolar response to NTG, which is an exogenous NO donor⁷, could have important implications if present in the other vascular systems, as NTG is frequently used for the treatment of angina pectoris in type 2 patients.

At present, it is not known whether a reduced NTG-mediated response is limited to the arteriolar bed or is also present in the large arteries and the venous system. The therapeutic effect of NTG is based upon its modulation of both pre- and after-load^{8,9}. NTG-induced venodilatation decreases venous return, resulting in a decrease of cardiac preload¹⁰. Furthermore, NTG administration in an (oral) therapeutic dose dilates large and medium sized arteries^{10,11}. Moreover, NTG reduces the pulse wave velocity of the aorta by blunting pulse wave reflection in several (but probably not all) arterial beds^{11,12}. If the arterial or venous vessel wall were less responsive to NTG, this could seriously impair the therapeutic efficacy of this agent.

The present study was undertaken to determine whether the vasorelaxing properties of NTG are reduced in large arteries and the peripheral venous system in normotensive, normoalbuminuric, type 2 patients without manifest atherosclerosis relative to carefully matched healthy control subjects. As the vasodilator response to NTG is also determined by the basal diameter and the viscoelastic properties of the vessel wall, not only the vasodilator response to NTG, but also the dimensions and the viscoelastic properties of different vascular beds were measured first. The conduit arteries were studied using an ultrasound based vessel wall Doppler tracking (VWDT) system¹³. The VWDT allows local measurements of the vessel wall properties of a selected artery segment. In the present study the effect of NTG on vascular smooth muscle cells was determined in the femoral artery, a muscular vessel. Simultaneously, information was obtained on the pulse wave transit time (PWTT) in the carotido-femoral artery segment, representing the aortic viscoelasticity. Finally, venous viscoelastic properties and the venodilatory response to NTG were measured by forearm strain gauge plethysmography.

Patients and methods

Subjects

Eighteen normotensive type 2 patients with a known mean duration of diabetes of 4.4 years (SD 1.5 years) and 18 normotensive healthy control subjects were studied. Patients were recruited from the outpatient clinic, the healthy volunteers via advertisements in local newspapers. Patient characteristics are described in table 4.1. None of the subjects had signs or symptoms of peripheral neuropathy or autonomic neuropathy according to the Ewing criteria¹⁴. Furthermore, none of the subjects had microangiopathy except background retinopathy. None of the subjects had clinical signs of atherosclerosis. All patients had normal palpable arterial pulses, no auscultatory vascular murmurs and no vascular abnormalities at B-mode ultrasound. Furthermore, in none of the subjects blood pressure exceeded 160/90 mmHg (mean of three measurements by sphygmomanometer). Patients were in good metabolic control (table 4.1), which was achieved by diet alone (n=5) or by diet and oral antidiabetic medication (n=13). Anti-hypertensive treatment had never been used by any subject. No other drug therapy, except for the anti-diabetic medication by the type 2 patients, was allowed during 4 weeks preceding the study. All subjects gave written informed consent. The study was approved by the hospital's medical ethics committee.

Table 4.1 Clinical characteristics and laboratory results of type 2 patients and healthy subjects (Controls)

	Type 2 diabetics	Controls
Male/female	13/5	10/8
Age (years)	56 (50 - 61)	51 (47 - 62)
BMI (kg m ⁻²)	24.9 (23.6 - 27.4)	24.5 (23.4 - 25.8)
Cholesterol (mmol L ⁻¹)	5.4 (5.0 - 6.3)	4.8 (4.4 - 5.8)
HDL-cholesterol (mmol L ⁻¹)	0.9 (0.8 - 1.2)	1.1 (0.9 - 1.35)
Triglycerides (mmol L ⁻¹)	1.26 (0.97 - 1.73)*	0.94 (0.59 - 1.38)
Fasting blood glucose (mmol L ⁻¹)	6.1 (5.5 - 9.1)**	4.2 (3.8 - 4.7)
HbA1c (%)	6.5 (6.1 - 7.8)**	5.5 (5.1 - 5.8)
Microalbumin (mg albumin/mmol creatinine)	0.6 (0.5 - 1.7)	—
Systolic blood pressure (mmHg)	148 (129 - 158)	147 (127 - 156)
Diastolic blood pressure (mmHg)	75 (66 - 85)	71 (66 - 85)
Smokers (n)	4	6

BMI, body mass index. Values are expressed as median with interquartile ranges. *p<0.05 and **p<0.01 vs. controls.

Methods

All experiments started at 08.00 h. and were performed in a temperature-controlled room (24-25°C). The subjects were fasting from 22.00 h. in the evening before the experiment, and were studied in supine position. The morning medication was omitted. A 20-gauge catheter was inserted in a deep forearm vein for blood sampling and direct intravenous pressure measurements (Hewlett Packard, Boeblingen, Germany), the arm being at heart level. Measurements started 30 min after venepuncture. Arterial blood pressure was measured continuously at the third phalanx of the dominant hand using the Finapres blood pressure monitor (Ohmeda, Englewood, Co, USA).

Basal hemodynamic measurements

Arterial diameters and viscoelastic properties were studied by use of a Vessel Wall Doppler Tracking (VWDT) system¹³. VWDT is based on ultrasound M-mode detection (ATL ULTRAMARK IV) in combination with computerized data acquisition on a personal computer. This system measures the maximum (end-systolic) and minimum (end-diastolic) arterial diameter transcutaneously within a cardiac cycle. The process is triggered by an ECG-signal. Mean values of end-diastolic diameter (D), distension (maximum-minimal diameter= dD) and of the relative distensibility ($RD=dD/D*100\%$) were calculated from three cycles of five consecutive heart beats under basal resting conditions. RD is a measure of the strain exerted on the vessel wall uncorrected for the pulse pressure. Usually, RD is corrected for pulse pressure, resulting in the distensibility coefficient. As the non-invasive pressure measurements at the finger do not necessarily represent the intra-arterial pressures in the carotid and femoral artery, RD was not corrected for pulse pressure (PP). Mean arterial pressure (MAP) was calculated as diastolic blood pressure plus one-third PP . VWDT-measurements were done on the common carotid and the common femoral arteries, both left and right, which are good accessible for the technique. The ultrasound probe was positioned 2 - 3 cm proximal to the bifurcation of the common carotid and of the common femoral artery, respectively. The coefficient of variance of the femoral artery diameter measurements is 3%¹³.

As the aorto-femoral distance could not be measured accurately, pulse wave velocity could not be calculated. Thus, transit time of the pulse wave ($PWTT$) from the heart to the right femoral artery was determined as the delay time from the R-top of the ECG to 10% of the ascending limb of the distension waveform of the vessel wall (ultrasound). The effect of NTG on pulse wave velocity was expressed as the change in aorto-femoral pulse transit time.

Venous viscoelasticity of the non-dominant arm was measured by determining the venous compliance, as venous compliance, in the absence of sympathetic stimulation, is mainly determined by the morphological characteristics of the vessel

wall¹⁵. In short, forearm volume changes were measured using strain gauge venous occlusion plethysmography (Hokanson EC4, Hokanson, Issaquah, WA, USA). Change in forearm volume (dV) and change in intravenous pressure (dP), caused by 3-min stepwise upper arm venous congestion, were measured at the following cuff pressures: 20, 25, 30, 35, 40 and 50 mmHg. Each venous occlusion step was followed by a 2-min interval of cuff deflation. Volume and pressure changes during each cuff pressure step were obtained from the values just before and after deflation of the cuff. The slope of the dV/dP relationship is linear in the observed venous pressure ranges and is a measure of the viscoelastic properties of the peripheral venous system¹⁶. The within-day and between-day (more than 5 weeks between two measurements) coefficient of variance of the venous compliance measurements is 8.1 and 11.1 % respectively. Basal venous tone (VV30) was determined as the forearm volume increase during a cuff pressure of 30 mmHg above cuff zero (the highest cuff pressure that does not increase forearm volume) with a concomitant suprasystolic inflated pulse cuff¹⁷. The sequence of basal measurements, ultrasound or venous plethysmography, was randomized.

Effects of Nitroglycerin

Twenty minutes after basal haemodynamic measurements, the right femoral artery dimensions, PWTT and VV30 were measured again, as described above. Subsequently, a standard therapeutic dose of 0.4 mg nitroglycerin (nitrolingual spray) was administrated sublingually (s.l.). Thereafter, diastolic femoral diameter, PWTT, venous volume increase (ΔV -NTG) above VV30, MAP and heart rate were measured continuously for at least 7 min. For each parameter, the maximal change after NTG was calculated. The vasodilator effects of NTG were measured at the femoral artery only, as the femoral artery is mainly muscular and only one vessel could be investigated by the VWDT-system at a specific moment.

Laboratory

Fasting blood glucose concentration was measured by the glucoxidase method at the beginning and at the end of the experiment to enable assessment of the influence of major systemic glucose changes on the haemodynamic parameters. Cholesterol, triglyceride, creatinine were measured by commercial methods; HbA1c using the Diamat HPLC-analyzer; and microalbuminuria by a commercial immunoturbidimetric method.

Statistics

Statistical analysis was performed using non-parametric tests, as the data were not distributed normally. The Mann-Whitney *U*-test was used for two group

comparisons; paired-Wilcoxon tests were used for within-group comparisons. Correlations were performed with the Spearman rank test. The individual slopes of the dV/dP relation were calculated by linear regression. Statistical significance is considered as $P < 0.05$. Data are presented as median and interquartile ranges, unless otherwise stated.

Results

No differences in laboratory values were observed between the type 2 patients and the control subjects, except for blood glucose, HbA1c and triglycerides (table 4.1). Blood glucose did not change during the experiments in the type 2 patients (data not shown). Blood pressure and pulse pressure were not different between type 2 patients and healthy subjects. Only the data of the right carotid and femoral arteries are presented, as no differences were observed in comparison with the contralateral arteries in both type 2 patients and control subjects.

At baseline, no differences were observed in internal end-diastolic vessel wall diameters and relative distension of the carotid and of femoral arteries in type 2 patients in comparison with healthy subjects. The pulse wave transit time (PWTT) of the carotido-femoral artery segment was similar in the type 2 patients and the control subjects (table 4.2). Venous compliance (Vcomp) and venous tone (VV30) were not different between patients and healthy subjects (table 4.2).

Table 4.2 Basal end-diastolic diameter (D), relative distension (RD) of the carotid (Cr) and femoral (Fr) artery, pulse wave transit time (PWTT) in the carotido-femoral artery segment, venous compliance (Vcomp) and venous tone (VV30) of the forearm in type 2 patients and healthy subjects (Controls).

	Type 2 diabetics n=18	Controls n=18
CrD (mm)	5.8 (5.4-6.2)	6.0 (5.4-6.4)
CrRD (%)	6.0 (5.0-7.0)	6.1 (5.2-7.2)
FrD (mm)	7.4 (6.8-8.3)	8.1 (7.1-8.7)
FrRD (%)	3.7 (3.2-5.7)	3.6 (2.8-4.7)
PWTT (ms)	62 (53-69)	64 (60-73)
Vcomp (ml/100ml/mmHg)	0.050 (0.045-0.062)	0.053 (0.039-0.055)
VV30 (ml/100ml)	13.2 (10.3-17.5)	15.1 (10.5-20.8)

Values are expressed as median with interquartile ranges.

No changes in MAP were observed after 0.4 mg of NTG s.l. in the type 2 patients and minimal changes in the healthy subjects: 0 (-3-10) vs 5 (2-8) mmHg respectively. Heart rate increased similarly in both type 2 diabetic subjects and healthy control subjects after NTG from 60 (58-67) to 65 (60-72), $P<0.05$, and from 55 (54-60) to 60 (54-66) beats per minute, $p<0.05$, respectively. End-diastolic diameter of the femoral artery after NTG was increased from 8.9 (7.7-10.2) to 9.4 (8.1-10.6) mm ($p<0.01$) in the type 2 patients and from 8.4 (6.9-9.4) to 9.0 (7.4-10.2) mm ($p<0.01$) in the healthy control subjects. The diastolic diameter increase after NTG was less in the type 2 patients than in the healthy control subjects: 0.46 (0.29-0.79) vs. 0.75 (0.47-1.00) mm ($p=0.04$) (table 4.3).

After NTG, the pulse transit time of the carotido-femoral artery segment did not change in the type 2 patients, but increased in healthy control subjects, from 62 (53-69) to 66 (51-72) ms ($p=NS$) and from 64 (60-73) to 70 (63-80) ms ($p<0.02$) respectively (table 4.3).

No differences were observed in the venodilatory response after NTG (ΔV -NTG) between type 2 patients and healthy control subjects: 0.94 (0.78-1.17) vs. 0.98 (0.69-1.29) mL 100mL⁻¹ respectively (table 4.3). Furthermore, no differences were observed in the time between the administration of NTG and the initiation of the venodilatory response between the groups.

Table 4.3 Changes in end-diastolic diameter (ΔD) of the femoral artery and forearm venous volume (ΔV -NTG) in type 2 patients and healthy subjects (controls) after NTG, pulse transit time of the carotido-femoral artery segment before (PTTb) and after (PTTN) NTG.

	Type 2 diabetics n=18	Controls n=18
ΔD (mm)	0.46 (0.29-0.79)*	0.75 (0.47-1.0)
ΔV -NTG (mL 100 mL ⁻¹)	0.94 (0.78-1.17)	0.98 (0.69-1.29)
PTTb (ms)	62 (53-69)	64 (60-73)
PTTN (ms)	66 (51-72)	70 (63-80)**

Values are expressed as medians with interquartile ranges. * $p=0.04$, type 2 diabetics vs. controls. ** $p<0.02$, PTTb controls vs. PTTN controls.

Discussion

In the present study, the local dilatory response to NTG of the common femoral artery was reduced in well-regulated and normotensive type 2 patients without any long-term diabetic complications in comparison with matched healthy control subjects. Furthermore, NTG had no effect on the pulse wave transit time (PWTT)

in the carotido-femoral artery segment in the diabetic subjects, whereas PWTT increased in the healthy subjects. In contrast to the impaired response in the arterial system studied, the venodilatory response to NTG was unaltered in the type 2 patients. Finally, no differences were observed in the basal (arterial) diameters or the basal (arterial and venous) viscoelastic parameters.

The impaired dilatory response to NTG of the femoral artery has several theoretical causes. As we did not observe any abnormality in the any of the parameters of viscoelastic properties, the impaired NTG responsiveness in the present study suggests a reduced sensitivity of the vascular smooth muscle cells of the femoral artery to NTG, an exogenous NO donor. According to a recent study, this abnormality is also present in IDDM patients¹⁸. Furthermore, NTG-induced vasodilation is impaired in peripheral arterial resistance vessels in type 2 patients and is possibly based on a primary nitrate tolerance^{6,19}. The present data suggest that a similar defect in NTG sensitivity is present in the large conduit arteries, but not in peripheral veins, as NTG induced venodilation was unimpaired in the type 2 patients. Several different mechanisms could be responsible for an impaired NTG-induced arterial vasodilation in type 2 diabetes. NO could be quenched by advanced glycation end (AGE) products, which are formed by non-enzymatic glycation of amino-groups in the hyperglycemic state^{20,21}. Loss of viscoelasticity is a hallmark of AGE formation^{21,22}. As all vascular visco-elasticity parameters were normal, quenching of NO seems a less probable explanation for the impaired NTG vasodilatation. Alternatively, the decreased NTG response could be based on defect in guanylyl cyclase of the vascular smooth muscle cells, resulting in an impaired responsiveness to NO⁷. According to some, but not all studies the arterial response to sodium nitroprusside, a direct NO donor, is not reduced in type 2 diabetes^{19,23,24}. Finally, the efficacy of NTG as a NO-donor is dependent on intracellular thiol containing groups⁷. Reduced glutathione (GSH), a thiol containing molecule, was deficient in type 2 diabetes in one study²⁵, possibly because of impaired synthesis and/or increased oxidative stress²⁶. As the patients had not been prior exposed to NTG, a reduced primary availability of thiol-containing groups in type 2 patients could explain a decreased formation of NO from NTG. However, the normal venodilatory response to NTG in the present study limits this possibility to the arterial system only.

The present study has several limitations. Blood pressure was measured by Finapres. Finapres measurements are representative for the brachial artery blood pressure, but not for the femoral artery blood pressure. Thus, carotid and femoral viscoelastic properties were expressed as relative distensibility (RD), uncorrected for the pulse pressure, representing the strain of the vessel wall. As the MAP of both groups was similar and unchanged after NTG, within-group and between-group comparisons could be made.

The viscoelastic properties of the aorto-iliac artery were determined indirectly using the PWTT from the heart to the femoral artery. Both basal PWTT in the carotido-femoral artery segment and local measures of large artery distensibility were unaltered in the type 2 patients compared to healthy control subjects. In contrast to the present study, several studies have described an increased pulse wave velocity of the aorta in normotensive type 2 patients, suggesting a decreased distensibility of the vessel wall^{27,28}. We feel that the diabetes duration could explain this discrepancy, as the patients in the present study had a relative short (known) duration of diabetes, were well controlled, normotensive and of similar age as the control subjects. In IDDM patients, aortic viscoelastic properties are negatively related to the duration of diabetes, and this loss of viscoelastic properties is probably related to non-enzymatic glycation of collagen^{29,30}. Probably longer or more severe periods of hyperglycaemia are needed to induce a loss of viscoelastic properties of the carotid, femoral and carotido-femoral arteries in normotensive type 2 patients. In line with this hypothesis, venous viscoelasticity assessed as compliance was unimpaired in the 'short-term' type 2 patients, whereas we observed a decreased venous compliance in IDDM patients with long-term diabetes in an earlier study¹⁶. Finally, the large vessel wall properties are unchanged in uncomplicated short-term IDDM patients as well³¹.

In type 2 patients, NTG administration resulted in a reduced dilatation of the femoral artery and an unchanged PWTT of the carotido-femoral artery segment, compatible with an unchanged pulse wave velocity, which both would result in a attenuated decrease in cardiac afterload. These reduced effects on the femoral artery could be explained by changes in local blood pressure, which was not measured. However, even intra-arterial measurements of the local blood pressures would not have resolved this issue, since short-term variations in the viscoelastic properties of the femoral artery result in unpredictable relation between diastolic blood pressure and diameter in healthy subjects³². Using Finapres, we did not observe any significant change in mean arterial blood pressures (MAPs) after NTG administration. Based upon the data of the present study, the mechanism of this impaired effect on PWTT in the carotido-femoral artery segment is difficult to determine. The PWTT is affected both by the viscoelastic properties of the blood vessel studied and by the local pulse pressure, which could not be determined in the subjects studied for practical reasons.

In a study by others¹², 0.3 mg NTG had no effect on the viscoelastic properties of the aorta as the characteristic impedance was unchanged. However, we and others did observe a 10% decrease in the femoral pulse pressure, measured intra-arterially, after 0.4 mg NTG s.l. administration in young healthy subjects, while the brachial artery pulse pressure remained unchanged¹¹. This decrease in central pulse pressure after NTG is probably caused by a delay in pulse wave reflection secondary to NTG-induced dilatation of peripheral arterial system^{12,33}. The decrease

in peripheral pulse wave reflection will result in a decrease in central pulse pressure, which in turn will lead to an increase of the pulse transit time of the carotido-femoral artery segment. Thus, the absence of an effect of NTG on PWTT in the type 2 patients is probably related to the impaired dilatory response to NTG of peripheral arterioles, as observed by McVeigh et al⁶.

Finally only a single dose of NTG was administered. More detailed information can be obtained if several doses of NTG (and alternative exogenous NO donors) are infused locally into a large artery. Differences in the sublingually administered NTG absorption between the two study groups are unlikely since the venodilatory effect of NTG was unimpaired in the type 2 patients. Given the mixed effects of NTG on cardiac preload and afterload, further studies are necessary to determine the therapeutical efficacy of NTG in type 2 diabetes. However, the impaired arterial vasodilation, the blunted increase in pulse wave transit time and the similar venodilatory response to NTG suggest a primary NTG tolerance of the arterial system, but not of the venous system, in type 2 patients.

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Chapter V

Normal adrenergic vasoconstrictor response and normal endothelial nitric oxide production in type 1 diabetes under euglycemic conditions

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Submitted for publication

Abstract

1. Disturbances of vascular tone by an imbalance of vasoconstrictor reactivity and vasodilator factors generated by the endothelium could contribute to the development of microangiopathy. The aim of the present study was to evaluate the α_1 and α_2 adrenergic vasoconstrictor responses, as well as the basal and stimulated production of endothelium-derived nitric oxide (EDNO) of arterioles in type 1 diabetic patients with and without microvascular complications. Furthermore, the effect of exogenous L-arginine, the physiological precursor of EDNO, on a possibly reduced arteriolar EDNO production was studied in these subjects. All studies were performed under euglycemic conditions.

2. Thirty-four type 1 patients (11 without microangiopathy, 10 with (pre)proliferative retinopathy, 13 with microalbuminuria) were studied under euglycemic conditions, together with 12 healthy control subjects. Forearm vascular responses to brachial artery infusions of clonidine, phenylephrine, N^ω-monomethyl-L-arginine (L-NMMA), sodium nitroprusside (SNP), acetylcholine (ACh) in the presence and absence of exogenous L-arginine, were determined by strain-gauge plethysmography.

3. The responses to clonidine and phenylephrine were not different between type 1 patients and the healthy volunteers. Basal blood flow, NO sensitivity, basal EDNO production and the stimulated EDNO production were normal in the type 1 patients. Administration of exogenous L-arginine (10 mg/min) did not influence basal blood flow. In the type 1 patients maximal response to ACh was reduced after former endothelium-dependent vasodilatation despite the administration of exogenous L-arginine. When the 3 subgroups of diabetic patients were analyzed separately, the same pattern was observed, except in the retinopathic patients in whom the ACh + L-arginine response was larger than ACh alone ($p=0.05$).

4. These results suggest a normal noradrenergic arteriolar response and normal endothelium-dependent vasodilatation under basal euglycemic conditions in type 1 diabetes mellitus. The reduced maximal response to ACh in the presence of exogenous L-arginine after previous ACh induced vasodilatation alone, suggests a depleted intracellular L-arginine pool. This depleted L-arginine pool is possibly compensated by an increased NO-synthase activity in the retinopathic patients only.

Introduction

Vascular disease is one of the most important complications of type 1 diabetes mellitus. Several lines of research suggest that an abnormal regulation of microcirculatory blood flow and pressure contributes to the development of diabetic microangiopathy¹⁻³. Microcirculatory blood flow is determined by a balance of vasoconstrictor and vasodilator mechanisms⁴. Sympathetic adrenergic vasoconstrictor nerves are important central control mechanisms. Vasoactive agents produced by the endothelium, like endothelium derived nitric oxide (EDNO), play a pivotal role in local (auto)regulation⁵. EDNO is formed from L-arginine by NO-synthase and causes relaxation of vascular smooth muscle cells⁶. Finally, both pathways probably interact, as stimulation of endothelial adrenergic α_2 receptors can stimulate EDNO production^{7,8}.

In type 1 patients with diabetic retinopathy an enhanced pressor response to exogenous noradrenaline has been observed, which could be related to an increased alpha-adrenergic responsiveness of resistance vessels⁹. In patients without microangiopathy the constrictor response to systemic and local noradrenaline is unaltered^{9,10}. Bodmer et al.¹¹ observed increased venous constrictor responses in type 1 patients with incipient nephropathy during local administration of noradrenaline¹¹. In subsequent studies venous sensitivity to phenylephrine (which mainly stimulates α_1 receptors) was unchanged, while the sensitivity to clonidine (mainly an α_2 agonist) was markedly increased in the microalbuminuric patients. No such abnormalities were observed in patients without this complication¹². Whether this enhanced α_2 adrenoceptor sensitivity is also present in other vascular beds is not known. Therefore, the first aim of the present study was to determine the vasoconstrictor response of the forearm vasculature to α_1 and α_2 agonists in type 1 patients without microvascular complications, with retinopathy or with microalbuminuria (and retinopathy).

Enhanced α_2 responsiveness could be related to impaired EDNO synthesis and indeed there is some evidence that EDNO production is reduced in type 1 diabetes^{13,14}. Basal EDNO production in these studies was assessed by the vasoconstrictor response of the forearm to local infusion of L-NMMA, a blocker of NO-synthase¹⁵. Stimulated EDNO production was assessed by the vasodilator response to infusion of acetylcholine (or other muscarinergic agonists). The literature is, however, somewhat conflicting. Most authors could not observe abnormal EDNO production in type 1 patients without complications, although Calver et al. reported abnormal L-NMMA responses in patients with diabetes of relatively short duration^{13,14,16}. Urinary albumin excretion rate, however, was not specified. Elliott suggested that impaired (extrarenal) EDNO production is limited to patients with incipient nephropathy¹⁴. It is, however, still unclear whether this abnormality can also be observed in patients with diabetic retinopathy. Therefore,

the second aim of the present study was to determine basal and stimulated EDNO production in the patients with and without microalbuminuria or retinopathy. As both adrenergic responsiveness and EDNO production was assessed in the same patients also the relationship between abnormalities in both systems could be studied.

The third aim was to determine whether stimulated EDNO production in the patients was dependent on the availability of L-arginine, the substrate for NO-synthase⁶. In atherosclerotic patients impaired EDNO production can be restored by exogenous L-arginine and the same phenomenon was observed in diabetic rats^{17,18}.

In the present study phenylephrine, clonidine and L-NMMA were infused directly into the brachial artery. Forearm blood flow was measured by strain gauge plethysmography. On a second occasion acetylcholine and sodium nitroprusside (as control for endothelium independent vasodilation) were infused. Finally, the acetylcholine infusion was repeated during simultaneous L-arginine administration.

Subjects and methods

Subjects

Experiments were performed in 12 healthy volunteers (HV) and 34 type 1 patients. Subject characteristics are depicted in table 5.1 and 5.2. Three subgroups of type 1 patients were studied: 11 subjects without complications (C-), 10 subjects with (pre)proliferative retinopathy previously treated by laser coagulation (R+) and 13 subjects with microalbuminuria (microalbumin excretion of 30-300mg/24hr; mean of 2 samples) with or without retinopathy (MA+). All groups were comparable in terms of age, gender and body mass index (BMI) and forearm volume measured by water volume displacement during submersion of the forearm. Supine blood pressure did not exceed 160/90 mmHg. None of the subjects had clinical signs or symptoms of atherosclerosis. No subject had hyperlipidemia or elevated serum creatinine levels ($>100\mu\text{mol/l}$). None of the patients had signs or symptoms of neuropathy. The subjects did not use any medication, including non steroid anti-inflammatory drugs (NSAID's) or angiotensin converting enzyme (ACE) inhibitors, for at least 4 weeks preceding the study, except for insulin in the diabetic patients. The study was approved by the medical ethical committee and all subjects gave written informed consent.

Methods

Forearm blood flow (FBF) was determined in both arms simultaneously using ECG-triggered strain gauge venous occlusion plethysmography (Periflow, Janssen Scientific Instruments, Beerse, Belgium) as described earlier¹⁹. The hand circulation

was excluded during FBF measurements by inflating a wrist cuff to suprasystolic pressure, starting one minute before each FBF measurement. Hence, FBF measurements represent predominantly muscle blood flow²⁰. FBF was measured during 3 minutes of baseline blood flow and during local infusions of three cumulative doses of vasoactive drugs. The mean FBF value of the last minute of each drug step was used for calculations.

Blood pressure was measured intraarterially with a catheter (Arrow, Reading, UK) in the brachial artery using a Hewlett Packard 78205C monitor (Boeblingen, Germany). Heart rate was derived from the ECG. All signals were stored on the hard disk of a personal computer by means of a custom build data acquisition system.

Protocol

Subjects were studied twice, day A and B, with an interval of minimal 2 weeks in males or one complete menstrual cycle in females. Female subjects were studied in the follicular phase of the menstrual cycle. All experiments were performed in a quiet, temperature-controlled room (24.5 ± 0.2 (SD)°C.) after an overnight fast. Subjects were not allowed to smoke, drink (except for water) or eat 10 hrs. preceding and during the study. Subjects were studied in supine position and precautions were taken to minimize external disturbances. They remained in bed during the experiment. Between the measurements they were allowed to sit upright. On the morning of the experiments the type 1 patients omitted their usual insulin injection. After local anesthesia by 1.5 ml 1% lidocaine, a 20 gauge arterial catheter was inserted retrogradely in the brachial artery of the non-dominant arm. In the diabetic patients an additional catheter was inserted into an antecubital vein of the other arm for infusion of insulin (Actrapid, Novo Nordisk) and/or 5% glucose, to keep blood glucose levels between 4 and 8 mmol/l. The amount of insulin infused was based on the individual daily insulin dose. Experiments were started when (near) normoglycemia had been reached for at least 30 minutes. The catheters were kept patent with saline.

The drug dose, duration and wash-out periods of the clonidine and phenylephrine infusions were based on pilot experiments in healthy controls. Both clonidine and phenylephrine consistently induced forearm vasoconstriction, which reached its maximum after 3 minutes, and at the highest dose resulted in a response on the flat part of the dose response curve in healthy subjects. All other infusions were based upon earlier studies^{19,21}. The duration of the drug infusions were such as to reach steady state in the vascular responses. The wash-out periods of the drugs were assessed in pilot studies.

On day A the vasoconstrictor response of the forearm vasculature to clonidine (Clo), phenylephrine (Phe) and finally Ng-monomethyl-L-arginine (L-NMMA) was studied. Clonidine (Boehringer Ingelheim, Ingelheim am Rhein, FRG), 0.05, 0.5,

5.0 $\mu\text{g}/\text{min}$ and phenylephrine (Centrafarm BV, Etten-Leur, the Netherlands), 0.3, 1.0, 3.0 $\mu\text{g}/\text{min}$ were infused intraarterially (each dose for four minutes). The sequence of the Clo and Phe infusions was randomized, each with a wash-out period of 75 and 60 minutes respectively. After the second wash-out period, L-NMMA (Clinalfa, Läufelfingen, Switzerland) 1, 2, 8 $\mu\text{mol}/\text{min}$ (each dose for 5 minutes) was infused.

On day B the vasodilator responses to acetylcholine (ACh; Dispersa AG, Hettlingen, Switzerland), 3, 10, 30 $\mu\text{g}/\text{min}$ and sodium-nitroprusside (SNP; Roche Nederland BV, Mijdrecht, the Netherlands), 1, 3, 10 $\mu\text{g}/\text{min}$ (each dose for three minutes) was measured. ACh and SNP were infused intraarterially in randomized order, each with a wash-out period of 30 minutes. Thirty minutes after the second wash-out period, all hemodynamic measurements were repeated and subsequently L-arginine (Fresenius, Bad Homburg, FRG), 10 mg/min , was infused intraarterially for twenty minutes. Immediately thereafter, the three doses of ACh were infused again under continuation of the L-arginine infusion.

Day A and day B were randomized. All drugs were dissolved in saline, except for SNP and L-NMMA. SNP was dissolved in 5% glucose and L-NMMA was dissolved with mannitol (1.54%) in saline. Fresh solutions were prepared just before each set of measurements.

Blood sampling

Blood samples were drawn, just before the first hemodynamic measurements. In the type 1 patients initial blood glucose levels were determined every 5 minutes with adjustment of the intravenous insulin infusion to establish steady state glucose levels between 4 and 8 mmol/l . At steady state arterial glucose samples were drawn every thirty minutes. Glucose was determined immediately by the glucose-oxidase based method (YSI, Yellow Springs, Ohio, USA) in arterial whole blood; serum total- and HDL-cholesterol and triglycerides were determined with enzymatic methods on a Cobas MIRA analyzer; LDL-cholesterol was calculated using the Friedewald formula; HbA1c and plasma (nor)adrenaline were determined by HPLC followed by fluorescence detection. Urinary albumin excretion was measured in two 24 hrs. collections by a commercial immuno-turbidimetric method.

Statistics and calculations

According to a power analysis 12 subjects per group are needed to discern a difference of 15% in FBF ratio between the groups with an $\alpha=0.05$ and $\beta=0.2$. All data are expressed as median values and interquartile ranges unless otherwise indicated. For each measurement period the ratio of the FBF was calculated (infused arm divided by the contralateral arm). This calculated ratio corrects for

systemic factors that affect the regulation of the blood flow in both arms (e.g. changes in blood pressure, level of arousal, hormonal changes etc.), and ensures that only the direct effects of locally infused substances on the blood flow of the forearm are taken into account²². The response to the various drugs are calculated as percentage change in FBF-ratio. Non parametric tests were performed since the data were not distributed normally. Within group data were compared according to Friedman and paired Wilcoxon. Between group comparisons were made according to Kruskal Wallis and Mann Whitney U-tests. For repeated measurements Shaffer adjusted, p-values were used²³. Statistical significance is defined as a p-value ≤ 0.05 .

Table 5.1 Characteristics of type 1 patients without complications (C-), with retinopathy (R+), with microalbuminaria (MA+) and healthy volunteers (HV).

	HV	C-	R+	MA+
Male/female	10/2	8/3	9/1	10/3
Smokers	5	3	3	5
Age (yrs)	31 (33-47)	32 (25-45)	38 (32-49)	38 (28-40)
BMI (kg/m ²)	23.3 (22.8-24.1)	22.7 (20.6-23.7)	23.6 (22.9-25.1)	24.3 (23.7-25.5)
Diabetes duration (yrs)		13 (11-22)	19 (17-23)	20 (17-26)
HbA1c (%)	-	8.4 (6.8-9.0)	8.8 (8.2-9.0)	9.6 (7.8-10.1)
Total cholesterol (mmol/l)	4.6 (3.5-5.3)	4.3 (4.0-5.4)	4.7 (4.1-5.1)	4.1 (3.5-4.5)
UAE (mg/24h)	-	10.91 (4.7-15.9)	11.4 (4.8-16.4)	149.5 (69.7-430.5)*
Psystolic (mmHg)	107 (103-122)	106 (98-117)	108 (100-133)	123 (116-140)**
Pdiastolic (mmHg)	70 (62-74)	67 (61-84)	75 (65-79)	80 (72-86)
Heart rate (bpm)	53(51-59)	67 (58-70)	66 (63-69)***	74 (64-83)***

UAE: urine albumin excretion; FBF: forearm blood flow, i=infused arm, c=control arm.

Data expressed as median (interquartile ranges). *: $p < 0.001$ vs R+ and C-; **: $p < 0.04$ vs R+, C-, HV;

***: $p < 0.01$ vs HV

Results

Subject characteristics

Sex ratio, age, smoking habits, BMI and lipid profiles were not different between the type 1 patients and the healthy volunteers (table 5.1). The diabetic groups had comparable metabolic control. HbA1c levels were not statistically different between

the groups (table 5.1). (Nor)adrenaline levels were not different between the type 1 patients and the healthy volunteers (table 5.2). Heart rate was higher in the R+ and MA+ patients, in comparison with the healthy volunteers (table 5.1). Systolic blood pressure was elevated in the MA+ patients (table 5.1). Diastolic blood pressure did not differ between the 4 groups (table 5.1). Blood glucose levels were between 4 and 8 mmol/l during the experiments in all type 1 groups, without major changes in glucose levels (mean 6.1 ± 1.6 mmol/l). Mean blood glucose levels during the experiments were not statistically different between the type 1 groups (data not shown).

Table 5.2 Basal adrenergic and haemodynamic characteristics of type 1 patients without complications (C-), with retinopathy (R+), with microalbuminuria (MA+) and healthy volunteers (HV).

	HV	C-	R+	MA+
Noradrenaline (nmol/l)	0.61 (0.51-0.81)	0.63 (0.39-0.95)	0.71 (0.66-0.77)	0.72 (0.57-0.81)
Adrenaline (nmol/l)	0.16 (0.15-0.21)	0.19 (0.09-0.32)	0.22 (0.18-0.35)	0.19 (0.13-0.29)
FBFi (ml/100ml/min)	2.8 (2.1-3.2)	2.5 (2.2-4.1)	3.0 (1.9-3.9)	2.8 (2.3-5.6)
FBFc (ml/100ml/min)	2.3 (1.8-3.4)	2.2 (1.8-3.1)	2.3 (1.8-3.2)	2.8 (1.8-3.3)

FBF, forearm blood flow, i=infused arm, c=control arm. Data expressed as median (interquartile ranges).

Responses to intraarterial drug infusions

Basal FBF was not different between the groups (table 5.2). Blood pressure and heart rate remained unchanged during and after the infusion of the various drugs (data not shown).

The alpha-adrenergic vasoconstrictor responses to Clo and Phe were not different between the 34 type 1 patients and the healthy volunteers. Subgroup analysis did not reveal any difference in the responses to Clo and Phe between the three type 1 groups (figure 5.1). Also, the vasoconstrictor responses to L-NMMA (endothelium dependent) are similar for the type 1 patients and the healthy volunteers, with no difference between the three subgroups (figure 5.1). The FBF response to the vasodilator drugs acetylcholine (ACh, endothelium dependent) and sodium-nitroprusside (SNP, endothelium independent) was not different between the type 1 patients and the healthy volunteers and no differences were observed in this FBF response between the three groups of diabetics (table 5.3). Administration of exogenous L-arginine (10 mg/min) for twenty minutes did not influence the basal FBF in the forearm in both the diabetic subjects and the healthy volunteers (data not shown).

Within-group analysis in the 34 diabetic subjects revealed that the vasodilator response of FBF (% index change) to the combined infusion of L-arginine and the

highest dose of ACh (30 $\mu\text{g}/\text{min}$) was reduced in comparison with the response to ACh (30 $\mu\text{g}/\text{min}$) alone: 140 (79-238) vs. 173 (102-391)%, $p=0.04$ (figure 5.2, left panel). When the different diabetic subgroups were analyzed separately, a similar pattern seemed to be present in the C- and MA+ patients (figure 5.2, right panel). In contrast, a different pattern was observed in the R+ patients: the FBF response to the combined infusion of L-arginine and ACh, 10 $\mu\text{g}/\text{min}$, was larger than ACh alone: 226 (88-287) vs. 80 (22-205)%, $p=0.05$ (figure 5.2, right panel). In the healthy volunteers no difference was observed between the vasodilator response to ACh alone and the combined infusion of L-arginine and ACh (figure 5.2; table 5.3). *Between group analysis* of the diabetic subgroups and healthy volunteers revealed no difference in the ACh response after exogenous L-arginine administration (table 5.3).

Table 5.3 Responses of the forearm blood flow (FBF), expressed as %change of FBF-index (FBF infused/FBF control arm) to sodium nitroprusside (SNP), acetylcholine (ACh), acetylcholine + L-arginine (ACh+L-Arg) of type 1 patients without complications (C-), with retinopathy (R+), with microalbuminuria (MA+) and healthy control subjects (HV).

	SNP 1 $\mu\text{g}/\text{min}$	SNP 3 $\mu\text{g}/\text{min}$	SNP 10 $\mu\text{g}/\text{min}$
HV	34 (16-62)	182 (145-217)	286 (208-417)
C-	43 (18-57)	209 (121-241)	305 (167-405)
R+	60 (24-99)	268 (135-343)	444 (154-615)
MA+	34 (16-66)	248 (172-279)	300 (183-483)
	ACh 3 $\mu\text{g}/\text{min}$	ACh 10 $\mu\text{g}/\text{min}$	ACh 30 $\mu\text{g}/\text{min}$
HV	23 (9-52)	100 (32-150)	158 (75-217)
C-	12 (3-40)	119 (40-145)	126 (91-253)
R+	29 (-2-37)	80 (22-205)	279 (93-387)
MA+	41 (10-138)	157 (67-489)	193 (151-424)
	L-Arg+ACh 3 $\mu\text{g}/\text{min}$	L-Arg+ACh 10 $\mu\text{g}/\text{min}$	L-Arg+ACh 30 $\mu\text{g}/\text{min}$
HV	31 (16-57)	126 (57-185)	153 (95-218)
C-	33 (15-82)	108 (48-168)	96 (63-176)
R+	33 (10-71)	226 (88-287)	221 (133-304)
MA+	29 (10-59)	131 (48-194)	131 (80-194)

Data expressed as median (interquartile ranges).

Figure 5.1 Vasoconstrictor response of the forearm blood flow to clonidine (Clo) upper panel; to phenylephrine (Phe) middle panel; to L-NMMA lower panel.
Response of the forearm blood flow (FBF), expressed as %change in FBF-index (FBF infused/FBF control arm). HV=healthy volunteers (n=12), C-= type 1 patients without complications (n=11), R+=type 1 patients with (pre) proliferative retinopathy (n=10), MA+=type 1 patients with microalbuminuria (n=13). Data are expressed as median, interquartiles (boxes) and ranges.

Figure 5.2 Vasodilator response of the forearm blood flow (FBF) to acetylcholine (ACh) before (●—●) and during (+—+) concomitant infusion of L-arginine (L-arg). FBF response expressed as %change in FBF-index (FBF infused/FBF control arm). Left panel: type 1 = patients with and without microvascular complications (n=34), HV= healthy volunteers (n=12). Right panel: subgroup analysis comparing C-= type 1 patients without complications (n=11), R+= type 1 patients with (pre) proliferative retinopathy (n=10), MA+= type 1 patients with microalbuminuria (n=13) and HV. Data are expressed as median. *: $p < 0.05$, ACh response during vs before L-arg.

Discussion

The present study clearly demonstrates, that under euglycemic conditions both the α_1 and α_2 adrenergic responsiveness of the forearm arterioles are intact in type 1 patients with or without microvascular complications. Also, the basal endothelium-derived nitric oxide (EDNO) production and the endothelium-dependent vasodilation of forearm resistance arterioles were undisturbed in these patients. However, during repeated ACh administration endothelium-dependent vasodilation was reduced, despite the administration of exogenous L-arginine. A limitation of the present study was of course that only vascular responses were studied in the forearm and it remains to be determined if the conclusions can also be extended other vascular beds. Furthermore, metabolic control was good to moderate in all subjects. Therefore, the present data cannot be extrapolated to patients with poor metabolic control.

Noradrenergic responses

Some studies suggest that adrenergic responsiveness can be increased as a consequence of diminished EDNO production by the endothelium^{7,8}. Human studies

on vascular adrenergic responsiveness in type 1 are, to our knowledge, scarce. In type 1 patients with retinopathy some indirect evidence suggests an abnormal adrenergic responsiveness, as in these patients the pressor response to systemic infusion of noradrenaline is increased⁹. However, it should be noted that the rise in blood pressure during systemic infusion of a pressor hormone is not only affected by vascular sensitivity but also by factors like baroreceptor activity. Local infusion of agonists circumvents these systemic effects. The unaltered α_1 and α_2 forearm adrenergic responsiveness in the type 1 patients without complications is in accordance with an earlier study by Halkin et al.¹⁰. In contrast to the present study, isolated arterioles of type 1 patients with retinopathy had a decreased maximal response to noradrenaline in comparison with healthy volunteers, while noradrenergic sensitivity was unchanged²⁴. It is of course unclear whether the top of the dose-response curve was reached in the present study, but important abnormalities in adrenoceptor activity of peripheral resistance arterioles are very unlikely in patients with retinopathy based upon the present study (fig 1).

In microalbuminuric type 1 patients Bodmer et al. observed a markedly elevated venous noradrenergic responsiveness¹¹. In a subsequent collaborative study with our group, this abnormality was found to be based upon an increased venous α_2 -adrenoceptor activity¹². The present study clearly indicates that arteriolar alpha adrenergic responsiveness of the forearm is unaltered in microalbuminuria. Limitation of this increased α_2 -adrenergic responsiveness to the post-capillary vascular bed, and in particular to the venules, could play an important role in the rise in intra-capillary blood pressure in microalbuminuric patients, as observed by Tooke et al. in the nailfold¹. Furthermore, skin blood flow is decreased in microalbuminuric patients, relative to patients without microalbuminuria³. A rise in post-capillary resistance, due to a selective increase in post-capillary adrenoceptor activity, could therefore be the basis of this decrease in flow with a concomitant elevation of pressure within the microcirculation. Further studies on pre- and postcapillary resistance are necessary to elucidate the significance of these observations with respect to the development of diabetic microangiopathy.

Endothelium dependent vasodilation

In the type 1 patients without microvascular complications or with retinopathy the forearm constrictor response to L-NMMA was similar to the response in the healthy volunteers (fig. 2). As L-NMMA blocks EDNO synthesis selectively, basal forearm EDNO production seems to be unaltered in these patients during euglycemia. These data are in accordance with the study of Elliot et al., who also observed unaltered forearm L-NMMA responsiveness in uncomplicated type 1 patients during euglycemia¹⁴. In contrast, Calver et al. reported that L-NMMA was a less effective constrictor in type 1 patients, with a short duration of diabetes¹³. In this latter study basal forearm blood flow was increased in the patients relative to

the healthy volunteers, which theoretically could result in alterations in L-NMMA responsiveness. We feel this explanation is less plausible, as in healthy volunteers marked variations in forearm blood flow do not affect L-NMMA responsiveness¹⁹. In the study by Calver et al. patients were hyperglycemic (mean blood glucose 9 mmol/l) with relative insulin deficiency, while in the present study (and the study by Elliot) insulin was infused and patients were normoglycemic. This would suggest that short term changes in metabolic control can affect L-NMMA responsiveness. Hyperglycemia per se does not affect forearm responses to L-NMMA in healthy volunteers¹⁹. In contrast, insulin stimulates EDNO production and, as recently reported by our group, increases the forearm response to L-NMMA²⁵. The impaired L-NMMA responses during insufficient metabolic control as observed by Calver et al. could, therefore, be the consequence of insulin deficiency, but this hypothesis remains to be explored further.

In contrast to the present study, Elliot et al. reported a reduced vasoconstriction in response to L-NMMA in microalbuminuric patients, with characteristics very similar to the patients we studied. Possibly, differences in experimental protocol could explain some of the discrepancy between the present data and the study of Elliot et al. In both studies patients were investigated during euglycemia and similar constrictor responses to L-NMMA were reached in the healthy volunteers. In our protocol L-NMMA and acetylcholine were infused on different days, making carry-over effects very unlikely. In the study of Elliot et al. SNP, carbachol (like acetylcholine a muscarinergic agonist) and L-NMMA were infused with wash-out periods of 6-15 minutes, after which blood flow had returned to baseline¹⁴. Return of blood flow to baseline after stimulation of EDNO synthesis, however, does not preclude ongoing activation of EDNO synthesis. This activation can affect the subsequent response to L-NMMA, as recently shown by Rongen et al.²⁶. The present study clearly indicates that, under euglycemic conditions, basal EDNO synthesis can be unaltered in microalbuminuric type 1 patients. EDNO synthesis is impaired in essential hypertension according to one study²⁷. The aforementioned study of Elliot et al. would suggest that in type 1 diabetes the rise in systemic blood pressure in microalbuminuric patients is (partly) based upon a decreased EDNO production¹⁴. In the present study blood pressure was also elevated in the microalbuminuric patients. However, basal EDNO production was unimpaired, suggesting that disturbances in EDNO production are not an important factor in the rise in blood pressure in microalbuminuria.

Stimulated EDNO production; the response to acetylcholine and effect of L-arginine

In the type 1 subjects with or without microvascular complications the vasodilator response to acetylcholine (ACh) was unaltered. This finding is in accordance with earlier studies on the vasodilator response to muscarinergic agonists in both

euglycemic and hyperglycemic type 1 patients^{13,14,16}. In sharp contrast, many authors observed both in vivo or in vitro a clear alteration in ACh induced endothelium-dependent vasodilation in experimental diabetes^{28,29}. However, these studies are usually performed in animals during severe metabolic decompensation (glucose >20 mmol/l). Lowering of blood glucose levels to values more relevant for the diabetic patient by insulin therapy restores ACh induced vasodilation³⁰. In healthy volunteers L-arginine does not affect ACh mediated vasodilation, indicating unlimited substrate availability³¹. In hypercholesterolemic patients ACh-mediated vasodilation is reduced, but can be restored by exogenous supplementation of L-arginine, the substrate for NO-synthase³². In type 1 patients without microangiopathy or with microalbuminuria the maximal response to the second ACh infusion was reduced in comparison with the first ACh infusion, despite the presence of exogenous L-arginine. No such changes were observed in the healthy volunteers, suggesting that repeated ACh stimulation unmasks a subtle impairment in EDNO-synthesis (or action) in these patients with long-term type 1 diabetes. In contrast, in patients with (pre)proliferative retinopathy a markedly different pattern was seen, as repeated ACh infusion during L-arginine supplementation resulted in enhanced ACh responsiveness. The different endothelial behavior of the retinopathic patients in comparison with the other type 1 patients suggests, that these diabetic patients belong to a subset with a different pathogenetic pathway in the development of microvascular complications. However, these data should be interpreted with caution, as only a limited number of retinopathic type 1 patients was studied.

In conclusion

Adrenergic responsiveness of the forearm is unaltered in type 1 patients under euglycemic conditions. In contrast in earlier studies, venous adrenergic responsiveness activity was enhanced in type 1 patients with microalbuminuria. The enhanced post-capillary adrenergic responsiveness could contribute to the rise in intracapillary pressure in microalbuminuric type 1 patients. Further studies on the microcirculation are necessary to determine the role of altered adrenergic responsiveness and other abnormalities in post-capillary resistance in the development of intra-capillary hypertension in type 1 diabetes.

Both basal and stimulated EDNO production are unaltered in the forearm vasculature in type 1 patients with and without microvascular complications under euglycaemic conditions. However, in contrast to the healthy controls maximal EDNO production was depressed during repeated ACh stimulation (during concomittant L-arginine administration) compatible with exhaustion of EDNO production (or efficacy) during repeated maximal EDNO stimulation in type 1 diabetes.

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Chapter VI

Endothelium-dependent plasma markers in relation to the basal and stimulated endothelium-derived nitric oxide production in type 1 diabetes mellitus

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Submitted for publication

Abstract

1. The endothelium modulates haemostasis and vascular tone. It is unknown, whether and to what extent changes in various endothelial functions are simultaneously involved in the development of microvascular complications in type 1 diabetes. Therefore, endothelium-dependent haemostatic factors, basal and stimulated endothelium derived nitric-oxide (EDNO) production and endothelin-1 (ET-1) levels were measured in type 1 patients and healthy controls.

2. Plasminogen activator inhibitor type 1 (PAI-1) activity, tissue plasminogen activator (t-PA) antigen and von Willebrand factor (vWF) antigen levels, tissue factor pathway inhibitor (TFPI) activity and endothelin-1 (ET-1) levels were measured in thirty-four type 1 patients (11 without complications, 10 with (pre)proliferative retinopathy, 13 with microalbuminuria and retinopathy) under euglycemic conditions, and in 12 matched healthy volunteers (HV). Forearm vascular responses to brachial artery infusions of Ng-monomethyl-L-arginine (L-NMMA) and acetylcholine (ACh) were determined by strain-gauge plethysmography under euglycaemic conditions.

3. TFPI activity was elevated and PAI-1 activity was reduced in the type 1 patients. However, PAI-1 activity was positively correlated with microalbumin excretion ($R=0.48$, $p<0.01$). t-PA-ag concentration was reduced in the type 1 patients without complications only. vWF-ag levels, and ET-1 concentrations were normal in all type 1 subgroups. Furthermore, basal and stimulated EDNO production were normal in the type 1 patients and both were positively correlated with the mean arterial blood pressure. The vasodilator response to ACh was negatively correlated to PAI-1 activity ($R=-0.37$, $p<0.05$).

4. Subtle endothelial haemostatic changes are present in type 1 diabetes depending on the presence or absence of various microvascular complications. Elevated TFPI and reduced PAI-1 activity are present in the presence of normal basal and stimulated EDNO production in type 1 diabetes. Finally, PAI-1 activity seems related to EDNO production and microalbumin excretion.

Introduction

Diabetic angiopathy, in both macro- and microcirculation, is the major cause of morbidity and mortality in patients with type 1 diabetes. There are several indicators that damage of the vascular endothelium is a pivotal event in the development of atherosclerosis and diabetic microangiopathy.

The endothelium plays a central role in the regulation of haemostasis and fibrinolysis, vascular tone, vascular smooth muscle cell growth, matrix synthesis and permeability¹⁻⁶. Already early in the course of diabetes abnormalities have been observed in circulating endothelium-dependent factors involved in haemostasis and fibrinolysis, compatible with endothelial damage and/or activation. Furthermore, abnormalities in endothelium-dependent vasomotor function have been described in type 1 diabetes by some, but not all authors^{7,8}. The various studies are difficult to compare given the differences in ambient glucose levels, long-term metabolic control, lipid profiles, estrogen levels in women, and the various presentations of diabetic microangiopathy, i.e. retinopathy and microalbuminuria, all factors which could affect endothelial parameters. Moreover, most studies on the endothelial function in diabetes are limited to certain aspects, either haemostatic or vasomotor function, which are then used as marker(s) of generalized endothelial damage. However, it is still unclear whether disturbances in the various aspects of endothelial function, i.e. haemostatic and vasomotor, are synchronized in type 1 diabetes, as a consequence of endothelial damage or 'endothelial dysfunction'. Furthermore, the relationship of these disturbances with various forms of clinical microangiopathy are still a matter of debate.

Therefore, we determined in the present study the relationship between various aspects of endothelial function in type 1 patients with and without microangiopathy, and in healthy controls under controlled conditions. Simultaneously we measured plasminogen activator inhibitor type 1 (PAI-1) activity, tissue plasminogen activator antigen (t-PA-ag) and von Willebrand factor antigen (vWF-ag) levels, tissue factor pathway inhibitor (TFPI) activity and the two major vasoactive products of the endothelium, circulating endothelin-1 (ET-1) levels and endothelium derived nitric-oxide (EDNO) bioactivity in type 1 patients.

Subjects and Methods

Subjects

Experiments were performed in 34 type 1 patients and 12 age and sex matched healthy volunteers (HV). Patients were recruited from the outpatient clinics of the participating hospitals, control subjects by advertisements in local papers.

Retinopathy, both preproliferative and proliferative, was present in 23 type 1 patients. Microalbuminuria, defined as a urinary albumin excretion (UAE) of 30-300mg/24 hr, was present in 13 type 1 subjects with retinopathy. None of the subjects had clinical signs or symptoms of atherosclerosis or of neuropathy. Clinical characteristics are depicted in table 1. Supine blood pressure did not exceed 160/90 mmHg in any of them. Moreover, no one had hyperlipidaemia or elevated serum creatinine levels ($>100\mu\text{mol/l}$). The subjects did not use any medication (including NSAID's or ACE inhibitors) for at least 4 weeks prior to the study, except for insulin by the diabetic patients. The study was approved by the Medical Ethical Committee of the University Hospital Maastricht and all subjects gave written informed consent.

Methods

All experiments were performed in a quiet, temperature controlled room ($24.5\pm 0.2^{\circ}\text{C}$.) after an overnight fast. All female subjects were studied in the follicular phase of the menstrual cycle, or after one week of withdrawal for those using oral contraceptive drugs. Subjects were not allowed to smoke, drink (except for water) or eat from at least 10 hrs. before the experiment. On the morning of the experiments the type 1 patients omitted their insulin injection. After local anaesthesia with 1.5 ml 1% lidocaine, a 20 gauge artery catheter was inserted retrogradely into the brachial artery of the non-dominant arm for blood sampling and blood pressure recordings. The catheter was kept patent by (0.9%) saline. In the diabetic patients an additional catheter was inserted into an antecubital vein of the contralateral arm for infusion of insulin (Actrapid, Novo Nordisk) and 5% glucose, to keep blood glucose levels between 4 and 8 mmol/l. The amount of insulin infused was based on the required daily insulin dose of each patient. Blood glucose levels were determined every 5 minutes with adjustment of the intravenous insulin infusion, until steady state glucose levels were between 4 and 8 mmol/l. At steady state arterial glucose samples were drawn every thirty minutes.

Experiments started when (near) normoglycaemia had lasted for at least 30 minutes. Venous blood samples for determination of serum total cholesterol, HDL, TG, PAI-1-activity, t-PA-ag, vWF-ag, TFPI-activity, and ET-1 were drawn just before the first hemodynamic measurements. Subsequently, the vasoconstrictor response of the forearm vasculature to L-NMMA (1, 2, 8 $\mu\text{mol/min}$; Clinalfa, Lufelfingen, Switzerland) and the vasodilator response of the forearm blood flow to ACh (3, 10, 30 $\mu\text{g/min}$; Dispersa AG, Hettlingen, Switzerland) was measured by ECG-triggered strain gauge venous occlusion plethysmography (Periflow, Janssen Scientific Instruments, Beerse, Belgium) as described elsewhere⁹. Simultaneously, the forearm blood flow (FBF) of the contralateral arm, serving as an internal control, was measured to correct for systemic variations. Vascular reactivity data are expressed as %change in FBF-ratio (FBF infused arm divided by

FBF in the contralateral arm). This calculated %change in FBF-ratio ensures that only the direct effects of locally infused substances on FBF are taken into account.

Assays

Glucose was determined instantaneously by the glucose-oxidase based method (YSI, Yellow Springs, Ohio, USA) in arterial whole blood.

Blood samples for PAI-1, t-PA, vWF and TFPI were collected in tubes containing 3.25% sodium citrate (dilution 1:10) and were spun immediately at 5000g for 10 minutes at 4°C. Blood samples for ET-1 were drawn in EDTA cooled (0°C) vials and centrifuged at 2000g for 15 minutes at 4°C. All samples were stored at -70°C until further processing.

PAI-1 activity was measured photometrically (Kabi Diagnostica, Sweden; intra-assay CV 6.0 %); median (normal range) 8 (1-20) AU/ml. *t-PA-ag* was determined by an enzyme immunoassay (Innogenetics, Belgium; CV 7%); normal range 1.3-10.4 ng/ml. Plasma levels of vWF-ag were determined by an enzyme-linked immunosorbent assay using rabbit anti-human vWF (Dako A/S, Denmark; CV 7%); normal range 60-180%. *TFPI* activity was measured using the chromogenic assay according to Sandset et al.¹⁰ and is expressed in percentage with respect to standardized TFPI activity (%normal), measured in a plasma pool obtained from 45 healthy donors (CV 7.4% at the 100% level). *ET-1* was determined by an endothelin-1 high sensitivity Biotrak ELISA method (Amersham Nederland BV, Utrecht, the Netherlands) at 450 nm after a Sep-pak C18 column separation.

Serum total cholesterol, HDL and TG were determined with enzymatic methods on a Cobas MIRA analyser; LDL-cholesterol (LDL) was calculated using the Friedewald formula; HbA1c and plasma (nor)adrenaline were determined by HPLC. UAE was measured in two 24 hrs. collections by a commercial immuno-turbidimetric method.

Statistics and calculations

Data were analysed with the Statistical Package for Social Sciences (SPSS/PC Statistical Data Analysis, SPSS, Chicago, IL.). All group data are expressed as median values and interquartile ranges unless otherwise indicated. The healthy volunteers and the type 1 patients were compared using non-parametric tests. Between group data were compared according to Kruskal-Wallis and Mann-Whitney U-tests. Within group data were compared according to Friedman and paired Wilcoxon tests.

Correlations between the haemodynamic endothelial parameters and the other endothelial parameters were explored using only the responses to the highest doses of ACh (ACh30) and of L-NMMA (L-NMMA8) used, as these doses generate the maximum response¹¹.

The maximal vasoconstrictor response to 8 $\mu\text{mol/min}$ L-NMMA (L-NMMA8), the maximal dilator response to 30 $\mu\text{g/min}$ acetylcholine (ACh30), endothelin-1 (ET-1), PAI-1, t-PA ag, vWF-ag, and TFPI-act, were each correlated univariately (Pearson) with age, BMI, smoking habits (cigarettes per day), HDL-, LDL-cholesterol, triglycerides, HbA1c, mean arterial pressure (MAP) and the logarithmic transformed UAE (logUAE) to find possible determinants (eg. smoking) of endothelial function (eg. ET-1). To explore the presence of an association between endothelium-dependent haemodynamic parameters and endothelium-dependent haemostatic/fibrinolytic parameters, univariate correlations were performed between basal (L-NMMA8) and stimulated (ACh30) EDNO production, and ET-1 on one hand, and PAI-1, t-PA, vWF-ag, TFPI, and the endothelial barrier parameter logUAE on the other hand.

Multiple regression analyses were performed to explore the possible influence of diabetes and other independent variables on the various endothelium-dependent haemodynamic and haemostatic/fibrinolytic parameters. In this model, the presence or absence of diabetes, BMI, HDL-, LDL-cholesterol, TG, mean arterial blood pressure (MAP), age, gender, smoking habit (cigarettes per day), and the lipid-diabetes interaction variables, were used as independent variables.

In a separate analysis in the type 1 subjects only, these same models were used with logUAE as additional dependent variable, and HbA1c as additional determinant.

A p-value ≤ 0.05 is considered significant. When more than two comparisons were made, Shaffer adjusted p-values were used¹².

Results

MAP was higher and TG levels were lower in the type 1 subjects in comparison with the healthy volunteers (table 6.1). Subgroup analysis showed a significantly elevated MAP in the microalbuminuric subjects, 95 (89-99) mmHg, as compared to the normoalbuminuric subjects, 83 (76-88) mmHg, and to the healthy volunteers, 81 (73-85) mmHg, $p < 0.01$. Mean ($\pm$ SEM) glucose levels were 6.1 ± 0.5 mmol/l in the type 1 subjects during the experiments. Forearm volume, measured by water volume displacement during submersion of the forearm, was not different between the type 1 subjects and the HV.

Table 6.1 Subject characteristics

	HV	Type 1 diabetes
Male/female	10/2	27/7
Smokers	5	16
Age (yrs)	31(33-47)	32 (25-45)
BMI (kg/m ²)	23.3 (22.8-24.1)	23.6 (21.7-25.3)
MAP (mmHg)	81 (73-85)	87 (80-94)*
Diabetes duration (yrs)	-	18 (14-24)
HbA1c (%)	-	8.6 (7.7-9.4)
Total cholesterol (mmol/l)	4.6 (3.8-5.1)	4.3 (4.0-4.7)
HDL-cholesterol (mmol/l)	1.2 (1.0-1.3)	1.4 (1.1-1.5)
LDL-cholesterol (mmol/l)	3.3 (2.1-3.4)	2.9 (2.5-3.4)
Triglycerides (mmol/l)	1.3 (0.8-2.2)	0.7 (0.6-1.0)**
UAE (mg/24hr)	-	13 (5-72)

HV: healthy volunteers; BMI: body mass index; MAP: mean arterial pressure; UAE: urinary albumin excretion. Data expressed as median and interquartile ranges. *: $p < 0.05$, and **: $p = 0.01$, type 1 diabetes vs HV.

The vasoconstrictor response to L-NMMA and the vasodilator response to ACh were not different between the type 1 patients and the healthy volunteers (table 6.2). Circulating ET-1 levels were not different between the type 1 subjects and the healthy volunteers: 0.72 (0.64-1.41) vs 0.88 (0.76-1.14) fmol/l. In contrast, PAI-1 activity was reduced in the type 1 patients in comparison with the healthy volunteers: 6.0 (4.0-8.0) vs 10 (6.3-11.8) ng/ml, $p < 0.01$. TFPI-activity was elevated in the type 1 patients as compared to the healthy volunteers: 116 (108-125) vs 102 (97-108) %normal, $p < 0.001$. However, the other endothelial haemostatic/fibrinolytic parameters were not different between the type 1 subjects and the healthy volunteers, t-PA-ag: 3.4 (2.4-4.8) vs 5.2 (3.1-6.5) ng/ml, and vWF-ag: 95 (80-121) vs 106 (76-158) %normal, respectively.

The influence of microangiopathic complications in type 1 diabetes on the endothelium-dependent parameters was explored by subgroup analysis (table 6.3). When the different diabetic subgroups and the healthy volunteers were compared, PAI-1 activity showed a significant reduction ($p < 0.01$) in the retinopathic patients (R+) in comparison with the other type 1 subgroups and to the healthy volunteers (table 6.3). Subgroup analysis of the t-PA-ag showed a significant reduction in the

C-subjects in comparison with the healthy volunteers (table 6.3). The TFPI activity was elevated in the C- and MA+ subjects in comparison with the healthy volunteers (table 6.3).

Table 6.2 Percentage change in forearm blood flow ratio in response to acetylcholine (ACh) and L-NMMA.

	HV (n=12)	C- (n=11)	Type 1 diabetes	
			R+ (n=10)	MA+ (n=13)
ACh 3 µg/min	23 (9/52)	12 (3/40)	29 (-2/37)	41 (10/138)
ACh 10 µg/min	100 (32/150)	119 (40/145)	80 (22/205)	157 (67/489)
ACh 30 µg/min	158 (75/217)	126 (91/253)	279 (93/387)	193 (151/424)
L-NMMA 1 µmol/min	-4 (-18/-2)	-10 (-20/6)	-14 (-27/-2)	-12 (-20/-7)
L-NMMA 2 µmol/min	-22 (-33/-13)	-22 (-30/-12)	-23 (-27/-15)	-23 (-34/-17)
L-NMMA 8 µmol/min	-39 (-53/-20)	-26 (-52/-21)	-29 (-37/-15)	-43 (-57/-32)

Healthy volunteers (HV); type 1 patients without complications (C-), with retinopathy (R+) and with microalbuminuria (MA+). ACh: acetylcholine; L-NMMA: N^G-monomethyl-L-arginine. Data expressed as median (interquartile ranges).

Table 6.3 Endothelial plasma markers

	HV (n=12)	C- (n=11)	Type 1 diabetes	
			R+ (n=10)	MA+ (n=13)
PAI-1 activity (unit/ml)	10.0 (6.3-11.8)	7.0 (5.0-8.0)	4.0 (3.0-4.5)*	7.0 (5.5-8.5)
t-PA-ag (ng/ml)	5.2 (3.1-6.5)	2.4 (1.5-3.0)**	4.4 (2.9-6.1)	3.7 (2.7-5.4)
vWF-ag (%normal)	106 (76-158)	87 (70-145)	93 (84-136)	100 (83-112)
TFPI activity (%normal)	102 (97-108)	113 (108-125)**	109 (105-122)***	118 (114-127)**
ET-1 (fmol/l)	0.88 (0.76-1.14)	1.49 (0.67-2.79)	0.72 (0.64-0.84)	0.69 (0.55-1.30)

Healthy volunteers (HV); type 1 patients without complications (C-), with retinopathy (R+) and with microalbuminuria (MA+). PAI-1: Plasminogen activator inhibitor type 1; t-PA-ag: tissue plasminogen activator antigen; vWF-ag: von Willebrand Factor antigen; TFPI: tissue factor pathway inhibitor; ET-1: endothelin-1. Data expressed as median (interquartile ranges). *: p<0.01 vs. HV, C-, MA+; **: p<0.05 vs. HV; ***: p<0.06 vs. HV.

Univariate correlations

First, the endothelium-dependent parameters were correlated with several subject characteristics to find possible determinants of these parameters.

After univariate analysis ACh30, L-NMMA8 in all subjects, and the logUAE in the type 1 patients were all positively correlated with the MAP (table 6.4). TFPI activity was negatively correlated with age: $R=-0.41$, $p<0.01$. In the type 1 subjects, TFPI was negatively correlated with age ($R=-0.43$, $p<0.05$), with HDL ($R=-0.39$, $p<0.05$) and positively with TG ($R=0.43$, $p<0.05$). vWF-ag was negatively correlated with smoking habit ($R=-0.39$, $p<0.05$) in these patients. No further correlations were found between any other endothelium-dependent parameters and possible determinants, like blood pressure, age, lipids and smoking habit, after univariate analysis.

Second, the endothelium-dependent vasomotor parameters, the haemostatic/fibrinolytic parameters and the logUAE were correlated to explore the presence of synchronization of changes in these various parameters. Correlations within the endothelial parameters showed, that endothelium-dependent vasodilatation to ACh30 was negatively correlated with PAI-1 activity ($R=-0.37$, $p<0.05$). Furthermore, PAI-1 activity was positively correlated with logUAE in the type 1 patients ($R=0.48$, $p<0.01$; table 6.4).

Table 6.4 Univariate correlations

	MAP	AGE	PAI-1
ACh30	$R=0.31^*$	ns	$R=-0.37^*$
L-NMMA8	$R=0.41^{**}$	ns	ns
Log UAE	$R=0.53^{**}$	ns	$R=0.48^{**}$
TFPI activity	ns	$R=-0.41^{**}$	ns

ACh30: acetylcholine 30 μ g/min; L-NMMA8: N^G-monomethyl-L-arginine 8 μ M/min; UAE: urinary albumin excretion/24hr; TFPI: tissue factor pathway inhibitor; MAP: mean arterial pressure; PAI-1: Plasminogen activator inhibitor type 1. *: $p<0.05$; **: $p<0.01$.

Multivariate analysis

Multivariate analyses were performed to explore the influence of diabetes on the endothelium-dependent parameters, correcting for possible interaction of various subject characteristics. After multiple linear regression analysis TFPI activity was the only endothelium-dependent parameter determined by diabetes ($\beta=0.52$, $p<0.001$). Furthermore, TFPI was determined by BMI ($\beta=0.38$, $p<0.01$), by age ($\beta=-0.40$, $p=0.001$) and by LDL-cholesterol ($\beta=0.32$, $p<0.01$). All other endothelium-dependent variables were not determined by the presence of diabetes.

ACh30 was determined by the MAP ($\beta=0.34$, $p<0.05$) and BMI ($\beta=-0.30$, $p<0.05$). L-NMMA8 was only determined by the MAP ($\beta=0.41$, $p<0.01$). In the type 1 subjects logUAE was determined by the MAP ($\beta=0.46$, $p<0.01$). Furthermore, vWF was determined by smoking ($\beta=-0.40$, $p<0.05$) and HDL-cholesterol ($\beta=-0.38$, $p<0.05$) in the type 1 subjects. The other endothelium-dependent haemostatic/fibrinolytic parameters were not dependent on or affected by the explored subject characteristics.

Discussion

Several lines of evidence suggest a central role for the vascular endothelium in the development of diabetic microvascular complications^{7,8,13-21}. Endothelial cell damage in type 1 can result from various biochemical mechanisms, like nonenzymatic glycation, altered redox state and increased oxidative stress²². Of the several endothelium-dependent parameters determined in the present study, only TFPI activity and PAI-1 activity were significantly different between type 1 patients and healthy subjects. TFPI activity was most markedly elevated in the microalbuminuric patients and PAI-1 activity was correlated with urinary albumen excretion in the type 1 patients. Furthermore, PAI-1 activity was negatively correlated with acetylcholine stimulated endothelium-dependent vasodilation. However, subgroup and univariate analysis clearly suggested that no other consistent pattern of multiple endothelial abnormalities could be observed in type 1 patients without overt nephropathy. In our opinion the present data reject the current paradigm of a generalised endothelial dysfunction in type 1, in which one endothelium-dependent parameter can be used as a marker or substitute for alterations in other aspects of endothelial function.

Disturbances in vasomotor, haemostatic/fibrinolytic and barrier function of the endothelium in type 1 patients have been observed in various studies. However, frequently conflicting data have been reported and several factors not related to the presence of diabetes can affect endothelial function^{5,15,23-25}. The present study was controlled for factors like diurnal variation of physiologic parameters, estrogen status, physical activity and active smoking. Furthermore, all subjects had blood pressures under 160/90 mmHg and normal lipid profiles. Finally, all parameters were determined during short-term near normoglycemia to exclude possible confounding effects of frank hyperglycemia or insulin deficiency^{26,27}.

TFPI activity was elevated in the type 1 subjects, and in particular in the microalbuminuric patients, in accordance to earlier studies^{17,28}. TFPI is synthesized by the vascular endothelium²⁹. Subsequently, TFPI is bound to the endothelial surface and to a lesser degree it is released into plasma³⁰. The elevated TFPI activity in type 1 patients could, therefore, be a marker of early endothelial

activation or damage. In plasma TFPI is mainly transported by lipoproteins³¹, which probably explains the association between TFPI and LDL-cholesterol in the present study. The consequence of the rise in TFPI for coagulation is less clear.

Theoretically, an increased TFPI-activity could be beneficial, as this could result in a larger inhibition of coagulation activation by tissue factor³⁰. However, in an earlier study in type 1 patients levels of prothrombin fragments F_{1+2} were increased, indicating enhanced *in vivo* thrombin generation at the surface of the blood vessel wall, despite elevated TFPI levels²⁸. This could mean that in type 1 diabetes, either less TFPI is available at the endothelial surface, or that TFPI is less effective in inhibiting the early phase of coagulation.

PAI-1 is produced by the endothelium, hepatocytes and megakaryocytes, and inhibits fibrinolysis by complex formation with t-PA³². In the present study PAI-1 activity was lower in the type 1 patients than in the healthy volunteers. Furthermore, we observed a correlation between PAI-1 activity, a known cardiovascular risk factor³³, and urinary albumin excretion. These data are compatible with an earlier study¹³. However, no major abnormalities were observed in t-PA ag levels, which is also produced by the endothelium. As fibrinolysis is the result of a balance between various activators (eg. t-PA) and inhibitors (eg. PAI-1), it is not possible to draw conclusions on overall fibrinolytic activity from the present data. A new observation was the negative correlation between PAI-1 activity and acetylcholine stimulated endothelium-dependent vasodilation. This vasodilation is mainly mediated by EDNO, which also inhibits platelet aggregation and adhesion to the endothelium^{34,35}. The association between a rise in PAI-1 activity and a simultaneous decrease in EDNO production and/or bioavailability would favor the formation of thrombi at the endothelial surface. This thrombotic tendency would be aggravated by the (possible) reduced quantity or quality of TFPI at the endothelial surface, as described above. Also in type 2 diabetes and other insulin resistant states, a similar pattern in PAI-1 and EDNO production and/or bioavailability seems present, although these parameters were not studied simultaneously³⁶⁻⁴⁰.

Basal and stimulated EDNO production in type 1 patients with and without microvascular complications were unaltered in the present study. More importantly, basal and stimulated EDNO production were not related to any other endothelium-dependent parameter, except for the association between stimulated EDNO production and PAI-1 activity, as described above. In most studies endothelium-dependent vasodilation is unimpaired in normoalbuminuric type 1 patients without microvascular complications^{8,41,42}. In accordance with a recent study in non-diabetic hypertensive subjects with microalbuminuria⁴³, we observed an unimpaired endothelium-dependent vasodilatation in the microalbuminuric type 1 subjects. In contrast, Elliott found an impaired endothelium-dependent vasodilation in these latter patients⁸. This discrepancy in microalbuminuric type 1 patients is probably based upon differences in patient characteristics and/or experimental design.

An unexpected observation was the positive correlation between (both basal and stimulated) EDNO production and the intraarterial MAP after univariate and multivariate analysis. Mechanical and shear forces are important stimulators of EDNO production, enabling the endothelium to protect the vessel wall from excessive stress⁴⁴. The present data suggest, therefore, that EDNO production in normotensive type 1 subjects increases in response to a rise in blood pressure. However, excessive mechanical stress secondary to frank hypertension could finally damage the endothelium, resulting in a decreasing EDNO production as can be observed in normoalbuminuric hypertensive subjects⁴⁵. A limitation of the present study, of course, is that endothelium-dependent vasodilatation was determined in the forearm only. The present data of the endothelium-dependent vasodilatation cannot, therefore, be extrapolated to other vascular beds in kidney or retina.

In conclusion, the present study clearly indicates that no consistent pattern of multiple endothelial abnormalities can be observed in type 1 patients without nephropathy, when studied during (near) normoglycaemia. Elevation of TFPI was the most consistent marker of (early) endothelial damage. Prospective studies are needed to determine if TFPI can be used as a marker for the development of microangiopathic complications in type 1 diabetes. Both basal and stimulated EDNO production were positively correlated with blood pressure, suggesting that mechanical and not metabolic factors are important determinants of EDNO production in type 1 diabetes.

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Chapter VII

General Discussion

General discussion

In diabetes mellitus, haemodynamic changes can be observed in both the macro-circulation and in the micro-circulation. The present thesis focuses on the role of vascular compliance and reactivity in diabetes. Hypertension forms a major haemodynamic change in the macrocirculation in both type 1 and type 2 diabetes. Apart from the metabolic imbalances secondary to the diabetic state, the elevated blood pressure will act as an additional insult on the vessel wall in both macro- and microcirculation. The pathogenesis of hypertension in diabetes is largely unknown, but various potential mechanisms have been proposed¹. Several authors suggest that abnormalities in transmembrane cation transport play a central role²⁻⁴. In one model a genetic predisposition to abnormal cation transport is the common basis for hypertension and nephropathy^{1,5-7}. Indeed, increased sodium-lithium counter transport activity, the marker of the ubiquitous sodium-hydrogen antiport activity, has been reported in micro- and macroalbuminuric type 1 and type 2 patients^{5,6,8,9}. The abnormal cation transport in these subjects would, according to some authors, lead to insulin resistance³.

Alternatively, hypertension is seen as a common feature of the "insulin resistance syndrome"¹⁰. The secondary hyperinsulinemia contributes, according to this hypothesis, to the rise in blood pressure by stimulating renal sodium reabsorption, activating the sympathetic nervous system, and promoting vascular smooth muscle cell (VSMC) proliferation¹¹⁻¹⁶. Recently, a third mechanism of the hypertension in type 2 diabetes has been proposed¹⁷. In this model emphasis is placed upon an impaired vasodilator action of insulin¹⁷. Several clinical and experimental studies indicate that insulin not only lowers blood glucose levels, but also acts as peripheral vasodilator^{18,19}. De Haan et al. showed that local administration of insulin into the forearm, with insulin levels within the physiological range, increases flow by vasodilating skeletal muscle resistance arterioles without any effect on skin blood flow or venous tone²⁰. Impaired insulin vasodilation can be observed in insulin-resistant states, such as type 2 diabetes, hypertension and obesity, as shown by Baron et al. and other groups²¹⁻²³. The mechanism of the insulin-mediated vasodilation is still unclear, but several lines of evidence suggest that insulin-induced vasodilation is at least partly mediated by stimulation of NO production within the vessel wall^{24,25}. Tack et al. showed that in healthy subjects administration of ouabain, a specific inhibitor of the sodium-potassium pump, inhibited insulin-induced vasodilation in the forearm²⁶. These data suggest that activation of Na⁺-K⁺ ATPase could be an important factor in the mechanism of insulin-mediated vasodilation, either at the level of the endothelial cell or at the VSMC. Finally, recent studies suggest that also adenosine could be involved in this vasodilation^{22,27,28}. It seems reasonable to propose that insulin contributes to vasodilator tone in skeletal muscle, and that impaired insulin-mediated vasodilation

contributes to enhanced VSMC contractility. In summary, enhancement of VSMC contractility is thought to play a central role in several hypotheses on the development of hypertension in type 2 diabetes.

Several authors observed that intravenous administration of pressor agents like noradrenaline (NA) or angiotensin II in normotensive type 2 patients results in an exaggerated rise of blood pressure, compatible with enhanced VSMC contractility^{29,30}. However, systemic infusions of pressor drugs are difficult to interpret as abnormal blood pressure responses can also be due to alterations in baroreceptor activity or cardiac output. Indeed, Baron et al. suggested that the enhanced noradrenaline pressor responses in obese subjects is related to an impaired baroreceptor activity³¹. However, the present studies (chapter 3) clearly indicate that baroreceptor activity is intact in type 2 patients, suggesting that an enhanced reactivity (or sensitivity) is the main reason for the increased pressor response. Our results also show that NA constrictor responses are not enhanced in forearm arteriolar or venous beds in these patients, neither during systemic (intravenous) or local (brachial artery and hand vein) administration. These data indicate, that the increased pressor response is not the result of a generalized vascular defect or a defect of skeletal muscle resistance arterioles, as one would expect based upon the above described hypotheses on the development of hypertension in type 2 diabetes. Splanchnic and renal circulation are important determinants of total peripheral vascular resistance. Therefore, it is hypothesized, that the NA infusion resulted in enhanced constrictor responses in these vascular beds. Further studies are needed to resolve this issue.

In earlier studies the NA constrictor response of the dorsal hand vein was augmented in type 1 patients with microalbuminuria, compatible with the hypothesis that microalbuminuria is associated with enhanced VSMC contractility³². However, as described in chapter 2, NA responses of the hand vein in type 2 patients with microalbuminuria were similar to the responses in normotensive patients and healthy controls. In a hand vein study by Bodmer et al., using local infusions of clonidine and phenylephrine, it was shown that the enhanced venous NA reactivity in type 1 patients with microalbuminuria was based upon a defect in the α_2 adrenoceptor³³. In contrast, as described in chapter 5, clonidine (and phenylephrine) responses of the forearm vascular bed is unaltered in type 1 patients with microalbuminuria. Based upon these data it is concluded, that microalbuminuria itself is not associated with a generalized increase in VSMC contractility, and that augmented NA responses in microalbuminuric type 1 patients are limited to the venous system. If also present in other parts of the post-capillary vascular beds, in particular in the venules, this enhanced α_2 -adrenergic responsiveness could contribute to a rise in post-capillary resistance. This increase in resistance in its turn could play an important role in the rise of the intra-capillary

blood pressure in microalbuminuric type 1 patients, as observed by Tooke et al. in the nailfold³⁴. In another study, Houben et al. observed decreased skin blood flow in microalbuminuric type 1 patients, relative to patients without this complication³⁵. It is tempting to speculate that the decrease in flow with a concomitant elevation of pressure in the microcirculation is based upon a rise in post-capillary resistance, due to a selective increase in post-capillary adrenoceptor activity. Further studies are necessary in which these parameters are studied simultaneously within the same patients.

Beside alterations in constrictor tone, abnormalities in the vascular L-arginine nitric oxide (NO) system have been observed in diabetes. Several lines of evidence suggest, that impaired production of NO by the vascular endothelium, enhanced break-down of NO within the vessel wall, and impaired VSMC sensitivity to NO could play an important role in the development of microangiopathy, atherosclerosis, and, possibly, hypertension³⁶⁻³⁹.

In type 1 patients forearm vascular sensitivity to nitroglycerin or sodium nitroprusside, both NO donors, is unimpaired in most studies, as was also observed in the present thesis (chapter 5). In contrast, earlier studies have shown that the vasodilator responses of the arteriolar vascular bed in the forearm to nitroglycerin were depressed in type 2 patients⁴⁰. As described in chapter 4, also the vascular sensitivity of the large conduit vessels to NO can be depressed in these patients, given the impaired vasodilator response of the femoral artery to nitroglycerin. However, the efficacy of nitroglycerin as a NO-donor is dependent on intracellular thiol containing groups⁴¹. Glutathione, a thiol containing molecule was reduced in type 2 diabetes in one study, possibly because of impaired synthesis and/or increased oxidative stress⁴². Further studies are necessary using inorganic NO-donors, like sodium nitroprusside, to rule out the latter possibility. Given the normal responses of the peripheral venous system to nitroglycerin, the primary NTG tolerance in type 2 patients seems to be limited to the arterial system only.

Type 2 diabetes is frequently associated with lipid disturbances, which, apart from the effect of the diabetic state per se, can severely affect the vascular NO system⁴³. Therefore, the endothelial production and/or bioavailability of NO was studied in the present thesis in type 1 patients only. Given the potential effect of insulin deficiency on endothelial NO production, all patients were studied during intravenous insulin administration with (near) normoglycaemia. Under these conditions we could not observe any major abnormality in basal or stimulated NO production in type 1 patients with or without various signs of diabetic microangiopathy. The finding of a normal vascular NO system in type 1 patients without microalbuminuria is in line with most previous studies⁴⁴⁻⁴⁶. Neither the diabetic state nor hyperglycaemia per se (in healthy volunteers) seem to impair NO

production, bioavailability or sensitivity⁴⁷. Therefore, it is plausible to propose, that the abnormalities of the vascular NO system in type 2 diabetes are related to dyslipidemia, which is frequently associated with this disorder⁴⁸.

The finding of a normal vascular NO system in type 1 patients with microalbuminuria is in contrast to an earlier study by Elliott et al., who observed impaired forearm constrictor responses to L-NMMA in type 1 microalbuminuric patients⁴⁵. This discrepancy is difficult to explain. Possibly, differences in experimental protocol have resulted in different outcomes. In the study of Elliott et al. carbachol was infused to stimulate NO production. Basal NO production was determined by infusion of L-NMMA, which was administered relatively shortly (approx. 15 minutes) after the previous infusion of carbachol. Using this experimental design a carry-over effect of the previous carbachol infusion on the subsequent L-NMMA infusion cannot be excluded. Alterations in endothelial NO production in type 1 diabetes might, therefore, only become apparent after repeated stimulation of the NO synthase. Indeed, the vasodilator response to a repeated acetylcholine infusion was reduced in type 1 patients, despite exogenous L-arginine administration (as described in chapter 5).

A striking observation was the positive correlation between blood pressure and both basal and stimulated NO production in type 1 patients. Mechanical and shear forces are important stimulators of the endothelial NO production, enabling the endothelium to protect the vessel wall from excessive stress⁴⁹. The positive correlation between blood pressure and NO production suggests that mechanical and not metabolic factors are important determinants of NO production in type 1 diabetes. Furthermore, irrespective of the presence of microangiopathy, the endothelium seems to generate an adequate response when blood pressure rises to reduce shear stress. However, given the limited number of healthy volunteers in our study, no comparison could be made between type 1 patients and healthy subjects.

The endothelium is an important regulator of permeability, haemostasis and fibrinolysis, vascular tone, VSMC growth, and matrix composition. In both micro- and macroalbuminuric patients, the increased renal permeability to albumin is associated with increased transcapillary escape rate of albumin, compatible with a generalized impaired barrier function of the endothelium⁵⁰. In both type 1 and type 2 patients several abnormalities have been observed in factors, which are produced by the endothelium, and which are involved in haemostasis and fibrinolysis, like von Willebrand factor (vWF), tissue factor pathway inhibitor (TFPI), plasminogen activator-1 (PAI-1), and tissue plasminogen activator (t-A)⁵¹⁻⁶³. These abnormalities are observed in patients with (micro)albuminuria in particular⁵². According to several authors a generalised "endothelial dysfunction" plays a pivotal role in the development of diabetic microangiopathy. Following this

line of reasoning, abnormalities in vascular permeability, endothelial NO production, and endothelium-dependent coagulation and fibrinolysis would be closely associated and tend to cluster within the same patients. In contrast to this hypothesis, we only observed reduced PAI-1 activity and elevated TFPI activity, but normal endothelial NO production and normal levels of vWF, t-PA and ET-1 in type 1 patients, as reported in chapter 6. In conclusion, the present study clearly indicates that no consistent pattern of multiple endothelial abnormalities can be observed in type 1 patients without nephropathy, when studied during (near) normoglycaemia. Elevation of TFPI was the most consistent marker of (early) endothelial damage. Prospective studies are needed to determine whether TFPI can be used as a marker for the development of microangiopathic complications in type 1 diabetes.

Apart from changes in the VSMC or the endothelium, alterations in the vascular visco-elasticity, leading to a loss of vascular compliance, could contribute to the development of hypertension in diabetes^{64,65}. In this thesis the compliance of the forearm veins, the carotid and femoral arteries, and aorta were measured in normoalbuminuric type 2 diabetic patients, as described in chapters 2,3 and 4. Both arterial and venous compliance were unaltered in normotensive normoalbuminuric type 2 patients with a relative short known duration of diabetes. In type 2 patients with microalbuminuria, venous compliance was reduced. However, these patients had diabetes with a long duration of disease. In earlier studies in type 1 patients, venous compliance was reduced in both micro- and normoalbuminuric patients with also a long duration of diabetes⁶⁶. Therefore, loss of venous compliance seems to be related to long-term chronic hyperglycaemia and not to microalbuminuria. Based on these earlier data and the present thesis, it is concluded that a reduction in venous compliance is not an important factor in the development of hypertension. Given the important role of the venous system in determining cardiac pre-load, alterations in blood volume would result in relative large alterations in cardiac output and blood pressure, when venous compliance is reduced.

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Chapter VIII

Summary

Summary

The present thesis focuses on modulation of vascular tone in diabetes mellitus. As described in chapter 1 vascular complications are the major cause of morbidity and mortality in diabetes, characterized by atherosclerosis in the macrocirculation, and in the microcirculation by retinopathy and nephropathy. In the present thesis the effects of diabetes on possible alterations in three basic vascular structures, the vascular smooth muscle cell (VSMC), the matrix and the endothelium, were studied. These alterations can result in changes of vascular tone, which, as we hypothesize, contribute to the development of vascular complications.

Chapter 2 describes the VSMC responsiveness to noradrenaline of a dorsal hand vein and the venous compliance of the forearm veins in type 2 diabetic patients with and without microalbuminuria. The noradrenergic responsiveness of the hand vein was not different between the type 2 patients and the healthy controls. In contrast, venous elasticity was reduced in the microalbuminuric type 2 patients in comparison with the healthy controls. From these data it is concluded that microalbuminuria in itself has no effect on the VSMC responsiveness in diabetes. The reduction in compliance in the microalbuminuric patients is probably related to the long duration of diabetes (20 years in the microalbuminuric patients and 5 years in the normoalbuminuric patients).

In chapter 3 the mechanisms of the increased blood pressure responsiveness to noradrenaline in normoalbuminuric type 2 patients is explored. Noradrenergic responsiveness of forearm vasculature, hand veins, and systemic blood pressure were determined in normotensive type 2 patients and healthy controls. Furthermore, baroreceptor responsiveness was studied. Systemic pressor response was elevated in the presence of a normal baroreceptor function in the type 2 patients. In contrast, noradrenergic responsiveness of the forearm arterioles and the hand vein was similar to healthy controls. From these data it is concluded that the noradrenergic responsiveness of other vascular beds must be augmented, possibly the splanchnic or renal vascular bed.

Chapter 4 describes a study on the vasodilator responsiveness of the femoral artery and the forearm veins to nitroglycerin (NTG) in normoalbuminuric type 2 patients and in healthy controls. Furthermore, compliance of the femoral artery and the forearm veins was measured. The vasodilator responsiveness of the femoral artery was reduced in the type 2 patients. Furthermore, after NTG pulse wave transit time in the aorta iliac artery segment increased significantly in the healthy controls, but not in the type 2 patients. In contrast, venous responsiveness to NTG was unaltered in these same patients. The impaired arterial vasodilation, the blunted increase in

pulse wave transit time and the similar venodilatory response to NTG suggest a primary NTG tolerance of the arterial system, but not of the venous system, in type 2 patients.

Chapter 5 describes a study to the α_1 and α_2 adrenergic responsiveness of the forearm arterioles during euglycaemia in type 1 patients with various forms of microangiopathy. Furthermore, basal and stimulated endothelium-derived NO production was measured. The responses to clonidine and phenylephrine were not different between type 1 patients and the healthy volunteers. Basal blood flow, NO sensitivity, basal EDNO production and the stimulated EDNO production were all normal in the type 1 patients in comparison with healthy controls. In the type 1 patients without complications or with microalbuminuria the maximal response to acetylcholine was reduced after former endothelium-dependent vasodilatation despite the co-administration of exogenous L-arginine. In contrast, in the patients with retinopathy a different pattern was seen with an enhancement of vasodilatation during co-administration of L-arginine with a submaximal acetylcholine dose.

These results suggest a normal noradrenergic arteriolar response and normal endothelium-dependent vasodilatation under basal euglycemic conditions in type 1 diabetes mellitus. The reduced maximal response to acetylcholine in the presence of exogenous L-arginine after previous acetylcholine induced vasodilatation alone, suggests a depleted intracellular L-arginine pool. This depleted L-arginine pool is possibly compensated by an increased NO-synthase activity in the retinopathic patients only.

In chapter 6 the concept of a "generalized endothelial dysfunction" in type 1 patients with various forms of microvascular complications is explored. Endothelium-dependent haemostatic factors, basal and stimulated endothelium-derived nitric-oxide (EDNO) production and endothelin-1 (ET-1) levels were determined in type 1 patients and healthy controls. TFPI activity was elevated and PAI-1 activity was reduced in the type 1 patients. However, PAI-1 activity was positively correlated with microalbumin excretion ($R=0.48$, $p<0.01$). t-PA-ag concentration was reduced in the type 1 patients without complications only. vWF-ag levels, ET-1 concentration were normal in all type 1 subgroups. Furthermore, basal and stimulated EDNO production were normal in the type 1 patients and both were positively correlated with the mean arterial blood pressure. The vasodilator response to ACh was negatively correlated to PAI-1 activity ($R=-0.37$, $p<0.05$). From these data it is concluded that changes in endothelium-dependent haemostasis and fibrinolysis are present in type 1 diabetes depending on the presence or absence of various microvascular complications. Finally, a generalized endothelial dysfunction, being the basis of cardiovascular complications in diabetic patients, does not seem to exist.

Chapter IX

Samenvatting

Samenvatting

Het voorliggende proefschrift heeft betrekking op modulatoren van de vaattonus bij diabetes mellitus patiënten. Zoals beschreven in hoofdstuk 1 zijn vasculaire complicaties de belangrijkste oorzaak van morbiditeit en mortaliteit bij deze ziekte. Deze vasculaire complicaties bestaan uit atherosclerose in de macrocirculatie en uit retinopathie en nefropathie in de microcirculatie. Een vroege uiting van diabetische retinopathie is het ontstaan van netvliesafwijkingen. Een vroege uiting van diabetische nefropathie is het toegenomen verlies van het eiwit microalbumine in de urine. In dit proefschrift zijn de effecten van diabetes mellitus op mogelijke veranderingen in drie vaatstructuren, te weten de gladde spiercel van de vaatwand (VSMC), het vasculaire bindweefsel (matrix) en de binnenste bekledingslaag van het bloedvat (endotheel), bestudeerd. Veranderingen in deze vaatstructuren als gevolg van diabetes mellitus kunnen leiden tot een veranderde vaattonus. Een veranderde vaattonus kan op zijn beurt weer leiden tot de ontwikkeling van vasculaire complicaties.

Hoofdstuk 2 betreft een studie naar de gevoeligheid voor de vaatvernauwer noradrenaline en naar de stijfheid van een handrugvene. In deze studie zijn type 2 diabetes mellitus patiënten met en zonder microalbuminurie en gezonde controle personen met elkaar vergeleken. De gevoeligheid van de handvene voor noradrenaline is niet verschillend tussen de type 2 patiënten en de gezonde controles. Daarentegen was de vasculaire stijfheid toegenomen in de type 2 patiënten met microalbuminurie. Op grond van deze data kunnen twee conclusies worden getrokken. Allereerst, microalbuminurie op zich heeft geen effect op de gevoeligheid van de VSMC voor noradrenaline in diabetes mellitus. Ten tweede, de toegenomen stijfheid van de handvene in de microalbuminurische patiënten is waarschijnlijk het gevolg van de lange diabetes duur (20 jaar in de microalbuminurische en 5 jaar in de normoalbuminurische patiënten).

In hoofdstuk 3 zijn verschillende mogelijke mechanismen voor de toegenomen gevoeligheid van de bloeddruk voor noradrenaline bij normoalbuminurische type 2 diabetes patiënten onderzocht. Daartoe zijn gevoeligheid voor noradrenaline van een handvene, het onderarmsvaatbed (vooral skeletspier) en van de bloeddruk onderzocht in normotensieve type 2 patiënten en gezonde controles. Voorts is de baroreceptor gevoeligheid bestudeerd. De systemische bloeddruk van type 2 patiënten reageert sterker op noradrenaline, terwijl de baroreceptor normaal functioneert. Voorts bestaan er in gezonde proefpersonen positieve correlaties tussen de gevoeligheid van de bloeddruk voor noradrenaline enerzijds en de basale insuline spiegels en de relatieve insuline resistentie anderzijds. Deze correlaties zijn afwezig in normotensieve type 2 patiënten. Daarentegen is de gevoeligheid voor noradrenaline in het onderarmsvaatbed en in de handvene niet verschillend. Op grond van deze data is geconcludeerd, dat de gevoeligheid voor noradrenaline in

andere vaatbedden verhoogd moet zijn, mogelijk het splanchnicus of niervaatbed. De afwezige correlaties in de type 2 patiënten suggereren een rol voor hyperinsulinaemie en insuline-resistentie bij het ontstaan van de verhoogde noradrenerge pressor respons.

In hoofdstuk 4 wordt een studie naar de vaatverwijdende respons op nitroglycerine (NTG) van een liesarterie, de arteria femoralis, en van het veneuze onderarmsvaatbed in normoalbuminurische type 2 patiënten en in gezonde controles beschreven. Tevens is de stijfheid van de betreffende bloedvaten gemeten. De mate van vaatverwijding op NTG in de arteria femoralis is verminderd in type 2 patiënten. Voorts nam de polsgolf looptijd in het aorto-iliacale traject na NTG toediening significant toe in de gezonde controles, maar niet in de type 2 diabeten. Daarentegen was de veneuze respons op NTG toediening niet verschillend tussen beide groepen. De verminderd vaatverwijding van de arteria femoralis, de verminderde en niet significante toename van de polsgolf looptijd in het aorto-iliacale traject en de gelijke veneuze vaatverwijding na NTG suggereren een primaire NTG tolerantie in het arteriële, maar niet in het veneuze systeem van type 2 diabeten.

Hoofdstuk 5 betreft een studie naar de α_1 en α_2 adrenerge respons van de onderarms arteriolen in type 1 diabetes mellitus patiënten met verschillende vormen van microangiopathie. Deze studies zijn verricht onder normoglycaemische condities. Voorts zijn bij dezelfde personen de basale en de gestimuleerde endotheliale NO productie gemeten. De respons op clonidine (α_2) en phenylefrine (α_1) was niet verschillend tussen type 1 patiënten en gezonde controles. Voorts waren de basale doorbloeding, de gevoeligheid voor NO, de basale en gestimuleerde NO productie normaal in type 1 patiënten in vergelijking met gezonde controle personen. In type 1 patiënten zonder complicaties of met microalbuminurie was de maximale respons op acetylcholine, een stimulator van de endotheliale NO productie, verminderd na een eerdere stimulatie, ondanks de toediening van extra substraat in de vorm van L-arginine. Daarentegen is de vaatverwijding toegenomen in de type 1 patiënten met retinopathie na een hernieuwde submaximale acetylcholine infusie in de aanwezigheid van extra L-arginine. Deze waarnemingen suggereren een normale adrenerge response en een normale endotheel afhankelijke vaatverwijding onder basale condities in type 1 patiënten. De verminderde vasodilatatoire respons na hernieuwde acetylcholine toediening in aanwezigheid van extra substraat, suggereert een uitgeputte intracellulaire L-arginine voorraad in type 1 patiënten met retinopathie. Deze uitgeputte L-arginine voorraad is mogelijk gecompenseerd door een toegenomen activiteit van het enzym NO-synthase.

In hoofdstuk 6 wordt het concept van een algehele endotheliale dysfunctie in diabetes mellitus onderzocht in type 1 patiënten met en zonder microvasculaire complicaties. Endotheel afhankelijke stollings en fibrinolyse factoren, zoals TFPI,

t-PA, PAI-1 en vWF, basaal en gestimuleerde endotheliale NO (EDNO) productie en endotheline-1 spiegels zijn gemeten in type 1 patiënten en gezonde controle personen. TFPI activiteit is verhoogd en PAI-1 activiteit is verlaagd in type 1 patiënten. De PAI-1 activiteit is echter positief gecorreleerd met de albumine excretie. De t-PA-ag concentratie is alleen verlaagd in type 1 patiënten zonder complicaties. Daarentegen zijn vWF-ag en ET-1 concentraties normaal in de verschillende patiënten subgroepen. Tenslotte zijn ook de basale en gestimuleerde EDNO productie normaal en zelfs positief gecorreleerd met de arteriële bloeddruk. Voorts is alleen de gestimuleerde EDNO productie negatief gecorreleerd met de PAI-1 activiteit. Op grond van deze data blijkt, dat veranderingen in de endotheel afhankelijke haemostase en fibrinolyse afhankelijk zijn van de aan- of afwezigheid van microvasculaire complicaties. Tenslotte lijkt een algehele endotheliale dysfunctie als basis van cardiovasculaire complicaties niet te bestaan in diabetes mellitus type 1.

Dankwoord

Dankwoord

Een proefschrift is zelden het werk van een persoon. Dit proefschrift vormt op die regel geen uitzondering. Veel dank ben ik dan ook verschuldigd aan degenen, die in de afgelopen jaren direct of indirect hebben bijgedragen aan de totstandkoming van dit proefschrift.

Prof dr PW de Leeuw, beste Peter, ongeveer tegelijkertijd begonnen wij in Maastricht. Aanvankelijk waren wij ieder beperkt tot onze werkgroepen en was er vooral samenwerking via de werkvloer en de dinsdagmiddag besprekingen. Later werd veel inhoudelijk gediscussieerd en ben je mijn eerste promotor geworden. Veel waardering heb ik voor je vermogen iemands ideeën richting te geven, zonder het gevoel te wekken iets uit handen te nemen. Je scherp analytisch inzicht en kennis maken, dat onervaren promovendi niet afdwalen en snel inzicht krijgen in complexe materie.

Prof dr AC Nieuwenhuijzen Kruseman, beste Arie, als geen ander wist jij in een vroeg stadium de randvoorwaarden noodzakelijk voor de afronding van alle onderzoeken veilig te stellen. Je gaf me tevens de mogelijkheid de opleiding endocrinologie te volgen. Jouw optimisme was een belangrijke steun in de afgelopen jaren.

Dr NC Schaper, co-promotor. Beste Nicolaas vele uren hebben wij de afgelopen jaren besteed aan alle onderzoeken. Aanvankelijk begon ik op jouw kamer, met jouw bureau, telefoon en computer aan de verschillende studies. Jouw inzet grenst aan het bezetene, je werk is nog steeds je grootste verslaving. Als geen ander weet je met je creatieve geest onderzoeksdata op ongelooflijke wijze combineren tot mogelijk nieuwe inzichten, dit het liefst in de late avonduren. Congresbezoeken waren hiervoor altijd de beste momenten. Onze samenwerking van de afgelopen jaren wordt hopelijk op interfacultair niveau voortgezet.

Dr AJHM Houben, paranimf. Beste Boy vele uren hebben we de afgelopen jaren besteed aan de wetenschap. Samen onderzoek verricht, het lab ontwikkeld en programma's uitgetoetst. Meestal wisten we wel het nuttige, eten, met het aangename, wetenschap, te verenigen. Of was het andersom? De vriendschap, die tussen ons als collega's is gegroeid, zal ik node missen. Zeer vereerd ben ik met je ondersteuning als paranimf.

Mw Drs CHA de Haan, paranimf. Claudia, dat jij mijn paranimf bent is zeer voor de hand liggend. Immers, de afgelopen jaren ben je al informeel mijn paranimf op het lab geweest. Altijd was je weer in staat proefpersonen over de streep te trekken om mee te doen met onderzoeken. Je geheugen voor namen is fenomenaal. Nog enkele jaren en ook jouw proefschrift is daar. Zet hem op!

Mw M Fuss, Mw E Lambrichts, Mw G Frijns. Beste Monique, Ellen en Gusta, jullie vormen de drie gratiën van het circulatie laboratorium. Altijd waren jullie er voor de nodige ondersteuning van het moreel en de inwendige mens. De samenwerking met jullie was voortreffelijk.

Hr P van Es. Beste Paul, hoewel je al enige tijd het verre Maastricht hebt verlaten, heb ook jij bijgedragen aan dit proefschrift. Als er weer eens een ruimte probleem was, wist jij altijd wel een oplossing te vinden, zodat onderzoeken voortgang konden vinden. Je gevoel voor humor was een belangrijke factor in het ontstaan van circulatie lab.

Dr BHR Wolffenbuttel, opleider aandachtsgebied endocrinologie. Beste Bruce, als echte

Rotterdammer is jouw credo "geen woorden, maar daden". Met veel inzet en visie heb je dan ook het opleiderschap van Arie overgenomen en de belangen van de gehele werkgroep behartigd.

Dr JP Sels, honoré confrère en kamergenoot. Beste Jean-Pierre, jij bent de enige echte dokter en allround clinicus van de werkgroep. Met jouw humor, realiteitszin en vooral je bescheidenheid was het een genot om allerhande zaken te bespreken. Ik heb veel van je wijsheden geleerd.

Ciska Buijs, Jobje Haarman, Jacqueline Zinkweg en François van Dielen, jullie hebben vele uren in het zweetkamertje doorgebracht, teneinde de LVDT studies te volbrengen. De volharding heeft François zelfs niet alleen wetenschappelijke, maar ook amoureuze prestaties opgeleverd.

Dr BJ Looij, internist Maasland ziekenhuis Sittard. Beste Bert-Jan, altijd was je bereid mee te werken aan het zoeken naar proefpersonen. Jouw database heeft onschatbare waarde. Dr A Spijker, internist Laurentius ziekenhuis Roermond. Beste Anton, was altijd goed verzorgd door je secretaresse Daniëlle. Ook al was de expeditie tevergeefs, een goede grap nam ik altijd mee terug naar Maastricht. Toch wil ik je biljard nog eens bewonderen. Drs W Venekamp, internist Wever-Gregorius ziekenhuis, locatie Brunssum. Beste Wim, jouw praktijk was een onschatbaar potentieel. De samenwerking was voortreffelijk en wordt helaas niet verder uitgebouwd. Hoe moet dat nu met die diabetes insipidus.

Mw Dr M Blonk en Dr B Bravenboer, internisten Catharina ziekenhuis Eindhoven. Beste Marion en Bert, jullie waren mijn redders in de nood. Toch nog de patiënten voor de studies. Wat "Lichtstad" al niet betekent.

Vele vrijwilligers, Uw inzet maakte het mogelijk de studies uit te voeren. U kwam de Brunssummer hei, Reuver, Swalmen en zelfs tot uit Best. Zo heb ik vele plaatsen in Limburg en Brabant leren kennen. Fantastisch.

Diabetes Fonds Nederland. Zonder de financiële ondersteuning van vele studies in dit proefschrift beschreven door het DFN, was dit proefschrift niet tot stand gekomen.

Mw T Wouters, computer junk. Beste Tiny, vele uren, sigaretten en koekjes heb jij ingezet voor de tot standkoming van dit proefschrift. Jij leerde mij daarnaast ook en passant Limburgs. Uiteindelijk ben jij degene, die de meeste proefschriften heeft gemaakt. Lieve ouders, jullie hebben mij de mogelijkheid geboden om academisch gevormd te worden, zodat een promotie mogelijk was.

Lieve Liesbeth, vele malen heb je mij voor gek verklaard met de uitvoering van alle studies, maar ik ben nu eenmaal eigenwijs. Jij zorgde ervoor, dat ons gezin door liep, als ik weer eens weg moest of laat thuis was. De opvoeding van onze dochters is daardoor grotendeels jouw verdienste. Laura, Paulien en Marjolein, mijn schatten, eindelijk hoeft pappa niet meer zoveel op zijn computer te spelen. Het kan nu eens wat vaker kinderdag zijn. Het konijnenhok zal pappa zelf voor jullie maken, dat beloof ik jullie.

Curriculum Vitae

1. Name: [Name] 2. Address: [Address] 3. City: [City] 4. State: [State] 5. Zip: [Zip]

Curriculum Vitae

Frank Christiaan Huvers werd op 6 augustus 1960 geboren te Rotterdam. Na het behalen van het gymnasium- β diploma aan het Christelijk Lyceum te Dordrecht, studeerde hij geneeskunde aan de Erasmus Universiteit Rotterdam. Van februari tot augustus 1990 was hij werkzaam als student-assistent binnen de Division of artificial organs van de University of Utah (Hoofd: Prof dr WJ Kolff). Van 1990 tot 1993 verrichte hij onderzoek als student-assistent in het endocrinologisch laboratorium van interne III (hoofd: Prof dr G Henneman) onderzoek naar de cellulaire opname van L-thyroxine. Na het behalen van het arts-examen in september 1985, was hij in opleiding tot internist in het Academisch Ziekenhuis Maastricht (opleiders Prof dr A Struyvenberg en Prof dr J van der Sluys Veer). Hij werd op 1 oktober 1990 ingeschreven in het specialistenregister en was nadien werkzaam als internist in het Maria ziekenhuis te Tilburg. Van 1992 tot 1997 was hij werkzaam binnen de werkgroep endocrinologie van Academisch Ziekenhuis Maastricht (hoofd: Prof dr AC Nieuwenhuijzen Kruseman; waarnemend hoofd: Dr BHR Wolffenbittel), alwaar hij onderzoek verrichtte naar vasculaire veranderingen bij diabetes mellitus. Deze onderzoeken hebben geleid tot deze dissertatie. Voorts was hij in deze periode werkzaam als internist-endocrinoloog en intensive care coördinator. Registratie als endocrinoloog volgde in november 1996. Sinds september 1997 is hij werkzaam in het Radboud ziekenhuis Nijmegen, afdeling algemeen interne geneeskunde, en in de St. Maartenskliniek te Nijmegen.