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The impaired renal vasodilator response attributed to endothelium-derived hyperpolarizing factor in streptozotocin – induced diabetic rats is restored by 5-methyltetrahydrofolate

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Abstract

Aims/hypothesis. Endothelial dysfunction contributes to the development of diabetic vascular complications. A better understanding of the pathophysiology of endothelial dysfunction in diabetes could lead to new approaches to prevent microvascular disease.
Methods. Endothelium-dependent and endothelium-independent vasodilator responses were investigated in the renal microcirculation of streptozotocin-induced diabetic rats. We measured renal blood flow changes with an electromagnetic flow probe. In addition, the responses of the different segments of the renal microcirculation were evaluated with videomicroscopy using the hydronephrotic kidney technique. Because endothelial cells release different relaxing factors (nitric oxide, prostacyclin and an unidentified endothelium-derived hyperpolarizing factor), responses to acetylcholine were measured before and after treatment with the nitric oxide synthase inhibitor L-N^G-nitroarginine methylester HCI (L-NAME) and the cyclooxygenase inhibitor indomethacin. We evaluated with the effect of 5-methyltetrahydrofolate, the active form of folate, on the responses.
Results. The L-NAME- and indomethacin-resistant vasodilation to intra-renal acetylcholine was significantly reduced in the diabetic compared with control rats, suggesting impaired endothelium-derived hyperpolarizing factor-mediated vasodilation. The responses to the nitric oxide donor (Z)-1-[-2-(aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate) and to the K^+-channel opener pinacidil were similar in diabetics and controls, indicating intact endothelium-independent vasodilator mechanisms. The contribution of endothelium-derived hyperpolarizing factor to vasodilation induced by acetylcholine was greatest in the smallest arterioles. In diabetic rats, the response to acetylcholine was increasingly impared as vessel size decreased. Defective vasodilation in diabetic kidneys was rapidly normalized by 5-methyltetrahydrofolate.
Conclusion-interpretation. Endothelium-derived hyperpolarizing factor-mediated vasodilation is impaired in the renal microcirculation of diabetic rats, in particular in the smallest arteries. Treatment with folate restores the impaired endothelial function in diabetes. [Diabetologia (2000) 43: 1116–1125]

Keywords Endothelial dysfunction, renal microcirculation, diabetes, folate, endothelium-derived hyperpolarizing factor, acetylcholine, rat, renal blood flow, homocysteine

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Abbreviations: EDHF, endothelium-derived hyperpolarizing factor; 5-MTHF, 5-methyltetrahydrofolate; NO, nitric oxide; RBF, renal blood flow; SOD, superoxide dismutase; L-NAME, L-N^G-nitroarginine methylester HCI; DETA-NONOate, (Z)-1-[-2-(aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate

Dysfunction of the endothelium is an early and key event in the development of microvascular and macrovascular complications [1], which account for most of the morbidity and mortality of diabetes. Endothelial integrity is generally assessed by evaluating the vasodilator response of a blood vessel or a vascular bed to an endothelium-dependent agonist [2]. Endothelial cells relax the tone of the underlying vascular smooth muscle cells by releasing a number of vasodi-

lating substances, including nitric oxide (NO), prostacyclin and an unidentified factor that induces hyperpolarization of smooth muscle [3]. The latter substance has been termed endothelium-derived hyperpolarizing factor (EDHF) [4]. Although NO has generally been considered to be the principal mediator of endothelium-dependent relaxations, it has become increasingly clear that NO-independent endothelium-derived relaxing factors might have an important role in local vasomotor control. Evidence is mounting that EDHF is a major determinant of vascular tone in small resistance vessels [5].

Impaired endothelium-dependent relaxations have been reported in different types of blood vessels of different animal models of diabetes [6]. The majority of the studies were done in large conduit arteries such as the aorta and the endothelial dysfunction was generally attributed to a reduced production or increased inactivation of NO [7–11] or to an overproduction of endothelium-derived vasoconstrictors, opposing the effects of NO [12, 13]. These studies have, however, limited relevance to the alterations in the microcirculation and in the local control of tissue perfusion occurring in diabetes. There is limited information about endothelial cell dysfunction in the diabetic microvasculature. In particular, little attention has been paid to the potential role of an impaired release or action of EDHF in diabetes.

Improved understanding of the pathophysiology of endothelial dysfunction in the diabetic microvasculature might lead to the development of new approaches to prevent microvascular disease. Although intensive glycaemic control delays the onset and slows down the progression of microvascular complications [14], this goal cannot be completely achieved in all patients. There is, therefore, a need for additional preventive and therapeutic measures. Folate was recently reported to improve impaired endothelial function associated with hypercholesterolaemia [15, 16] and with hyperhomocysteinaemia [17], two conditions associated with increased oxidative stress. In vitro studies showed that folate reduced superoxide anion generation [15]. These observations raise the possibility that the beneficial effect of folate might be extended to other disorders associated with endothelial dysfunction and increased oxidative stress, including diabetes.

Against this background, we examined the endothelium-dependent vasodilator response of the renal microcirculation in a rat model of streptozotocin-induced diabetes. The kidney vasculature was chosen because it is an important target for diabetic microvascular alterations. To focus on the contribution of EDHF, we also did experiments after blockading NO synthase and cyclooxygenase because the residual response is assumed to be mediated by EDHF. We observed a pronounced blunting of the EDHF-mediated response to acetylcholine in the diabetic kidneys. To attain a more accurate understanding of the vessel type involved, conducted we a further segmental analysis using the split hydronephrotic kidney model, an intravital microscopy technique that allows direct in vivo evaluation of different segments of the renal microcirculation. Finally, we investigated the effect of rapidly infusing 5-methyltetrahydrofolate (5-MTHF), the active form of folate, on the impaired dilatory response and compared with that of vitamin C, superoxide dismutase (SOD) and catalase, agents with established antioxidant activity.

Materials and methods

Laboratory animals. The studies were done in female Wistar rats (Iffa Credo, Brussels, Belgium) receiving care in accordance with National Institutes of Health (NIH) and our national guidelines for animal protection. Diabetes was induced by intravenous injection of streptozotocin (Pfanstiel, Davenham, UK, 65 mg/kg). Slow release insulin pellets (release rate 1U/24 h, Linshin, Scarborough, Canada) were implanted. Experiments were done 6 weeks later. In the rats with a hydronephrotic kidney, diabetes was induced 8 weeks after the ureter ligature. During each experiment plasma samples were drawn for measurement of glucose, fructosamine, total protein, cholesterol and total homocysteine concentrations.

Renal blood flow (RBF) measurement. The rats were anaesthetized with thiobutabarbital (Inactin, RBI, Natick, Mass., USA, 100 mg/kg i.p.). The trachea was intubated, a jugular vein was cannulated for continuous infusion of isotonic saline (3 ml/h) and giving drugs and a carotid artery was cannulated for continuous monitoring of mean arterial blood pressure. The right renal and suprarenal artery were exposed via a small abdominal incision. The suprarenal artery was cannulated for intra-renal application of drugs. A blood flow sensor with an inner diameter of 0.6 to 0.8 mm was placed on the renal artery, allowing continuous RBF monitoring by an electromagnetic square wave flow meter (Skalar Medical, Delft, The Netherlands) [18]. Calibration of the flow probe was conducted as described previously [19].

Experimental protocols. Series 1. The RBF response to intra-renal acetylcholine (Sigma Chemical Co, St. Louis, Mo., USA, 0.1 to 50 ng in bolus) was examined in diabetic ($n = 8$) and age-matched control rats ($n = 8$). The dose-response curve was repeated after intravenous L-N^G-nitroarginine methylester HCl (L-NAME) (Sigma, 10 mg/kg bolus followed by 20 mg · $kg^{-1} \cdot h^{-1}$). A third dose-response curve was done after intravenous indomethacin (Sigma, 4 mg/kg bolus followed by 8 mg · $kg^{-1} \cdot h^{-1}$) in addition to L-NAME. Before giving of the next dose of acetylcholine, RBF was allowed to return to baseline values.

Series 2. The RBF response to intra-renal acetylcholine, to the NO donor (Z)-1-[-2-(aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate) (Alexis, Grünberg, Germany, 16 to 112 μg in bolus) and to the K^+-channel opener pinacidil (Sigma, 25 to 175 μg in bolus) was examined, before and after intravenous L-NAME and indomethacin in diabetic ($n = 8$) and control rats ($n = 8$). The highest dose of acetylcholine, DETA-NONOate and pinacidil devoid of systemic blood pressure effects was chosen as the upper limit of the dose-response curve.

Series 3. The RBF response to intra-renal acetylcholine after intravenous L-NAME and indomethacin was examined before and 15 min after an intravenous bolus of 5-MTHF (Sigma, 200 μg in 0.5 ml NaCl 154 mmol/l) in diabetic ($n = 8$) and control rats ($n = 8$), and before and after a bolus of 0.5 ml NaCl 154 mmol/l without 5-MTHF in diabetic rats ($n = 8$). The same protocol was repeated in diabetic rats with vitamin C (Sigma, 10 mg) ($n = 6$) and with SOD (Sigma, 10.000 U) plus catalase (Sigma, 10.000 U) ($n = 6$).

Intravital microscopy of the hydronephrotic kidney. The techniques for induction of hydronephrosis and preparation of the hydronephrotic kidney have been described in detail previously [20, 21]. In brief, after anaesthesia with halothane (Fluothane, Zeneca, Destelbergen, Belgium) a unilateral hydronephrosis was induced by a permanent ligation of the left ureter. About 8 to 10 weeks after the induction of hydronephrosis, the renal parenchyma becomes a thin translucent tissue sheet due to tubular atrophy. The microcirculation remains, however, intact [22, 23] and is accessible for study by intravital microscopy. For the final experiments, the rats were anaesthetized with thiobutabarbital and cannulated as described above. The hydronephrotic kidney was exposed by a left flank incision and split with a thermal cautery along its greater curvature. The ventral half of the kidney was sutured to a semicircular frame and attached to the bottom of a plexiglas bath. The entry of the renal hilus into the bath was sealed with silicone grease and the bath filled with an isotonic, isocolloidal solution (Haemaccel, Hoechst Marion Roussel, Marburg/Lahn, Germany), maintained at 37 °C.

Glomeruli and renal vessels were visualized by transillumination microscopy (Axiotech Vario 100 HD, Zeiss, Jena, Germany) using water immersion objectives (Achroplan 10 ×, 40 ×, 63 ×). The resulting image was recorded by a high-speed video camera (Kodak Motioncorder Analyser, Eastman Kodak Company, San Diego, Calif., USA) and forwarded to the video recorder (S-VHS Panasonic AG-7355, Matsushita, Japan), either on-line or from the memory of the camera. Luminal diameters and erythrocyte velocities were analysed off-line, with image analysis software (Cap-Image, Ingenieurbüro Zeintl, Heidelberg, Germany). For erythrocyte velocity measurements, sequences were recorded with the high-speed camera at a rate of 600 fps and forwarded to the video recorder at a rate of 25 fps. The sequences were thus slowed with a factor 24, allowing analysis of the erythrocyte velocities with the line-shift-diagram method [24].

The renal vessels were identified according to their branching pattern from a selected glomerulus. The luminal diameters of the following vascular segments were measured: proximal arcuate artery (near the interlobar artery, diameter 45–55 μm), distal arcuate artery (near the interlobular artery, diameter 35–45 μm), proximal interlobular artery (near the arcuate artery, diameter 20–25 μm), distal interlobular artery (near the afferent arteriole, diameter 10–15 μm), proximal afferent arteriole (near the interlobular artery, diameter 8–12 μm), distal afferent arteriole (the narrowest segment before entering the glomerulus, diameter 6–9 μm), proximal efferent arteriole (whithin 50 μm of the glomerulus, diameter 8–12 μm), distal efferent arteriole (near the first branching, diameter 15–25 μm). Erythrocyte velocity (V_E) was measured in the efferent arteriole and glomerular blood flow (GBF) was calculated from the equation: $GBF = V_{RBC} \times \pi D^2/4$ with D = luminal diameter.

The kidneys were allowed to stabilize in the tissue bath for 1 h after completion of surgery, before control measurements of vascular diameters and glomerular blood flow were made. All drugs were applied locally and concentrations are expressed as final molar concentrations in the tissue bath.

Table 1. Characteristics and biochemical variables in control and diabetic rats

	Control	Diabetes
Body Weight (g)	269.8 ± 3.8	245.6 ± 2.9[a]
Mean arterial blood pressure (mm Hg)	121.4 ± 4.0	120.3 ± 2.0
Glycaemia (mmol/l)	9.1 ± 0.5	25.3 ± 1.2[a]
Fructosamine (μmol/g total protein)	1.78 ± 0.08	3.73 ± 0.24[a]
Cholesterol (mmol/l)	1.82 ± 0.07	1.87 ± 0.06
Homocysteine (μmol/l)	8.3 ± 0.5	6.0 ± 0.4[a]

[a] $p < 0.001$ vs control

Experimental protocols. Series 1. A dose-response curve to acetylcholine (10^{-8} to 10^{-5} mol/l) was done in diabetic ($n = 6$) and control rats ($n = 6$).

Series 2. Acetylcholine (10^{-5} mol/l) was added to the tissue bath before and after L-NAME 10^{-4} mol/l and after L-NAME 10^{-4} mol/l with indomethacin 10^{-3} mol/l in diabetic ($n = 6$) and control rats ($n = 6$).

Statistical analysis. The data are presented as means ± SEM. The RBF response to the different agonists is expressed as the area under the curve of the change in RBF ($ml \cdot min^{-1} \cdot min^{-1}$). The changes of vascular diameters and of glomerular blood flow are expressed as percentage changes from control values. Analysis of variance, paired and unpaired t tests were used as appropriate. The significance level was set at p less than 0.05.

Results

Characteristics of laboratory animals. Diabetic animals had significantly lower body weights and higher plasma glucose and fructosamine concentrations compared with the age-matched control rats (Table 1). Mean arterial blood pressure and plasma cholesterol concentration were no different between diabetic and control rats. Plasma total homocysteine concentration was significantly lower in diabetic than control animals. Homocysteine concentrations were no different before and after treatment with 5-MTHF, neither in control (8.9 ± 0.6 vs. 9.3 ± 0.6 μmol/l, $n = 8$) nor in diabetic rats (6.0 ± 0.2 vs 6.3 ± 0.9 μmol/l, $n = 8$). Homocysteine concentrations in diabetic rats were no different before or after treatment with NaCl 154 mmol/l without 5-MTHF (6.1 ± 0.5 vs 6.0 ± 0.9 μmol/l, $n = 8$).

RBF measurements. Systemic treatment with L-NAME increased blood pressure with 31.4 ± 2.7 % in control rats ($n = 8$) and with 22.9 ± 2.8 % in diabetic animals ($n = 8$) ($p < 0.05$ vs controls). Concomitant infusion of L-NAME and indomethacin increased blood pressure with 28.7 ± 2.9 % in control rats ($n = 24$) and 20.5 ± 1.3 % in diabetic rats ($n = 44$) ($p < 0.05$ vs controls).

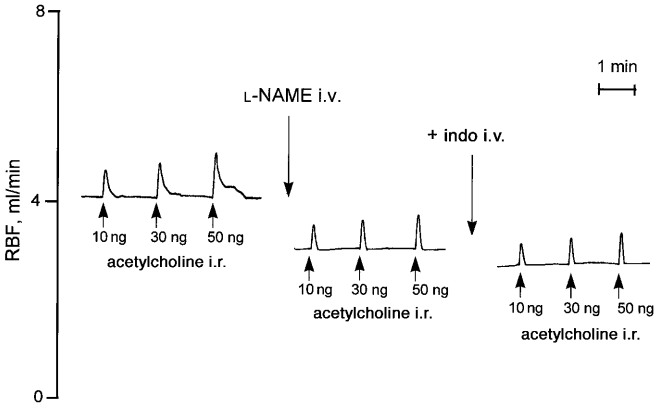

Fig. 1. Original tracing of the renal blood flow response to acetylcholine given intrarenally (i.r.) before and after systemic L-NAME and indomethacin (indo) in a control rat. The response has a typically biphasic pattern: a rapid, transient peak, largely resistant to L-NAME and indomethacin, followed by a more sustained component, which is abolished by L-NAME

Basal RBF was slightly higher in diabetic than control rats (4.21 ± 0.14 ml/min vs 3.85 ± 0.08 ml/min, $p < 0.05$). The decrease in RBF after systemic L-NAME infusion was more pronounced in diabetic than control rats ($34.5 \pm 1.9\%$ vs $23.7 \pm 4.6\%$, $p < 0.05$). After systemic L-NAME and indomethacin infusion, RBF was no different in diabetic and control animals (2.73 ± 0.14 ml/min vs 2.70 ± 0.08 ml/min).

The RBF response to acetylcholine had a typically bifasic pattern: a rapid, transient peak followed by a more sustained component (Fig. 1). Systemic L-NAME infusion resulted in a substantial decrease of the response to intra-renal acetylcholine, with a strong inhibition of the second component. After intravenous indomethacin in addition to L-NAME, a further modest decrease of the rise in RBF to acetylcholine was observed. A large portion of the first component of the response was, however, not inhibited by L-NAME or by indomethacin (Fig. 1).

In diabetic animals, the global RBF response to acetylcholine was not significantly different from that in control rats, except for the highest acetylcholine dose (Fig. 2). The L-NAME inhibited, however, the vasodilation to acetylcholine to a greater extent in the diabetic kidneys, showing a significantly impaired response compared with controls (Fig. 2). The residual L-NAME-resistant and indomethacin-resistant response was significantly lower in diabetic than in control rats (Fig. 2).

The RBF responses to DETA-NONOate and to pinacidil were no different between diabetic and control rats. Systemic infusion of L-NAME and indomethacin did not significantly influence the RBF response to DETA-NONOate and to pinacidil, neither in control nor in diabetic animals (Fig. 3).

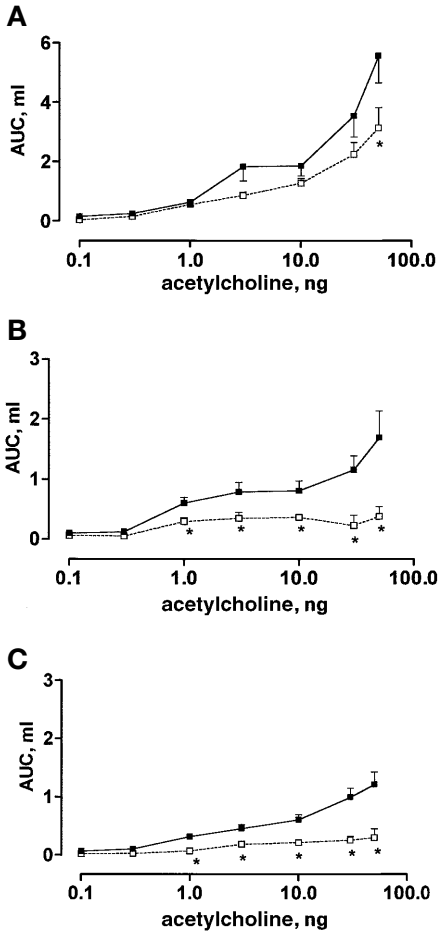

Fig. 2 A–C. The renal blood flow response to acetylcholine given intrarenally (**A**) in control (■, $n = 8$) and diabetic rats (□, $n = 8$). The same protocol was repeated after intravenous L-NAME (**B**) and after intravenous L-NAME with indomethacin (**C**). The area under the curve (AUC) of the change from baseline values was calculated for each bolus of acetylcholine and the data are expressed as means ± SEM. *$p < 0.05$

Intravital microscopy of the hydronephrotic kidney. Local application of acetylcholine, L-NAME and indomethacin in the tissue bath was devoid of systemic blood pressure effects. Basal vascular diameters and glomerular blood flow were higher in diabetic rats than in control rats (Table 2). The vasoconstriction after local application of L-NAME and of local L-NAME with indomethacin was more pronounced in the diabetic animals. After local L-NAME with indomethacin, vascular diameters and glomerular blood flow were not different between diabetic and control animals (Table 2).

In control rats, acetylcholine concentration-dependently dilated the preglomerular and postglomerular vessels and increased glomerular blood flow (Fig. 4). The vasodilation was most pronounced in the smallest arterioles: distal interlobular, proximal afferent, distal afferent and proximal efferent arterioles, whereas the larger preglomerular vessels and the distal efferent arteriole dilated only moderately

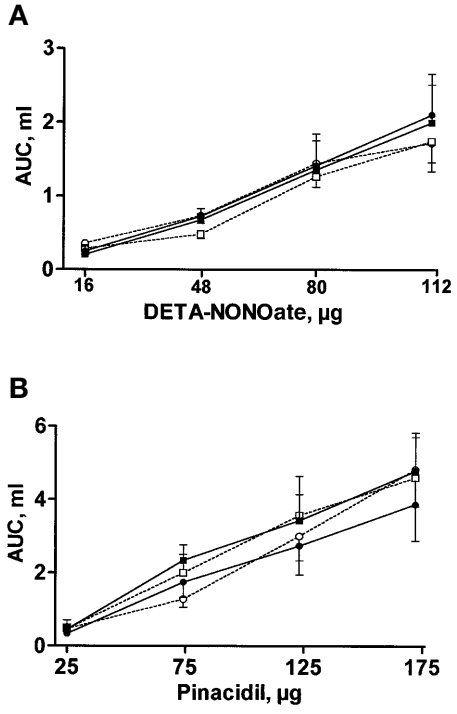

Fig. 3 A, B. The renal blood flow response to DETA-NONOate given intrarenally (**A**) and pinacidil given intrarenally (**B**) in control rats ($n = 8$) before (■) and after (●) simultaneous treatment with L-NAME and indomethacin and in diabetic rats ($n = 8$) before (□) and after (○) simultaneous treatment with L-NAME and indomethacin. The area under the curve (AUC) of the change from baseline values was calculated for each bolus of DETA-NONOate and pinacidil and the data are expressed as means ± SEM

in response to acetylcholine. The dilation to acetylcholine was impaired in the diabetic vessels (Fig. 4). In the arcuate and proximal interlobular arteries, the difference achieved significance only at the highest acetylcholine doses. In the smaller arterioles, the deficit was, however, significant at all doses. The increase in glomerular blood flow in response to acetylcholine was less pronounced in the diabetic vessels.

In a separate series of control and diabetic animals, the vasodilation of the different segments was evaluated only to the highest acetylcholine dose (10^{-5} mol/l). Again, the impairment of the vasodilation in the diabetic rats was most pronounced in the smallest vessels (Fig. 5). Local application of L-NAME decreased the response to acetylcholine especially in the larger preglomerular vessels, whereas an important residual response was apparent in the distal interlobular, proximal afferent and distal afferent arteriole (Fig. 5). In the diabetic rats, L-NAME greatly reduced the vasodilator response to acetylcholine in all vessels. Application of indomethacin in addition to L-NAME resulted in only a minor decrease of the response to acetylcholine in both control and diabetic vessels (Fig. 5).

Effect of 5-MTHF, vitamin C and SOD with catalase on L-NAME- and indomethacin-resistant RBF response to acetylcholine. Under conditions of systemic blockade of NO synthase and cyclooxygenase, injection of a bolus of 5-MTHF in the diabetic rats rapidly restored the impaired response of RBF to acetylcholine, to values that were not any more different from the response in control rats (Fig. 6). The 5-MTHF did not alter the L-NAME- and indomethacin-resistant response to acetylcholine in control rats (Fig. 6). A bolus injection of saline without 5-MTHF did not affect the L-NAME- and indomethacin-resistant response to acetylcholine in the diabetic rats (Fig. 6).

Giving vitamin C and SOD plus catalase did not change the L-NAME- and indomethacin-resistant response to acetylcholine in diabetic rats (Fig. 7).

Discussion

Our in vivo study found a profound impairment of the EDHF-mediated component of the endothelium-dependent vasodilator response to acetylcholine in kidneys of diabetic rats. The results further showed the reversal of this impairment by treatment with 5-MTHF, the active form of folic acid.

Impaired endothelium-dependent relaxations have been documented in experimental diabetes,

Table 2. Vascular diameters and glomerular blood flow in the hydronephrotic kidney of control and diabetic rats, in the basal state and after L-NAME with indomethacin

	Basal		After L-NAME + indomethacin	
	Control	Diabetes	Control	Diabetes
Proximal arcuate, µm	50.20 ± 1.98	57.17 ± 1.22[a]	29.87 ± 1.84	32.00 ± 1.39
Distal arcuate, µm	35.98 ± 1.66	45.24 ± 2.09[a]	22.45 ± 1.13	24.06 ± 1.84
Proximal interlobular, µm	22.61 ± 1.24	29.27 ± 1.48[a]	17.19 ± 0.8	18.69 ± 1.13
Distal interlobular, µm	12.29 ± 0.87	16.42 ± 1.3[a]	10.08 ± 0.65	10.36 ± 0.74
Proximal afferent, µm	9.36 ± 0.64	11.48 ± 0.6[a]	7.36 ± 0.48	8.12 ± 0.41
Distal afferent, µm	7.46 ± 0.25	8.91 ± 0.24[a]	6.27 ± 0.25	6.98 ± 0.33
Proximal efferent, µm	9.27 ± 0.38	11.21 ± 0.58[a]	6.23 ± 0.33	6.96 ± 0.28
Distal efferent, µm	17.84 ± 0.61	20.48 ± 0.78[a]	13.13 ± 0.42	13.99 ± 0.46
Glomerular blood flow, nl/min	38.1 ± 2.58	48.28 ± 1.77[a]	20.50 ± 1.61	22.89 ± 1.76

[a] $p < 0.05$ vs control

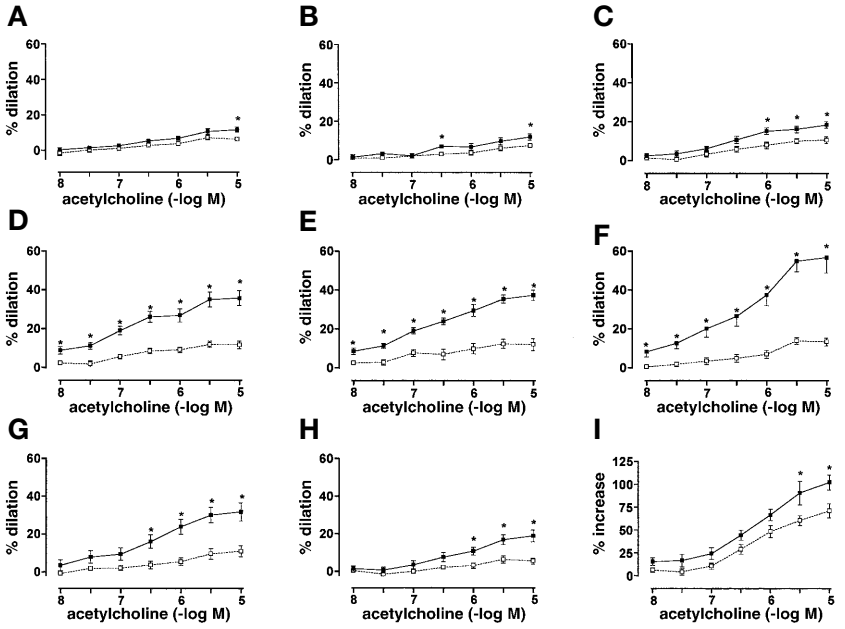

Fig. 4 A–I. Percentage changes of vascular diameters of the proximal arcuate artery (**A**), distal arcuate artery (**B**), proximal interlobular artery (**C**), distal interlobular artery (**D**), proximal afferent arteriole (**E**), distal afferent arteriole (**F**), proximal efferent arteriole (**G**), distal efferent arteriole (**H**), and of glomerular blood flow (**I**) in the hydronephrotic kidney of control (■, $n = 6$) and diabetic rats (□, $n = 6$) in response to local application of increasing acetylcholine doses in the tissue bath. Data are expressed as means ± SEM. *$p < 0.05$

mainly in isolated blood vessels [1, 6]. Our study aimed to analyse the influence of diabetes on the different components of the endothelium-mediated vasodilation in the renal microcirculation of the rat in vivo. To this end, two complementary techniques were used for the study of renal haemodynamics, providing information on the variations in total RBF as well as on the intra-renal distribution of the changes in vascular reactivity.

The acetylcholine-induced dilator response in the renal microcirculation is largely due to NO, as shown by the strong inhibition of the response by the NO synthase blocker L-NAME. Prostanoids, in contrast, do not seem to contribute substantially because additional blockade of cyclooxygenase with indomethacin produced little further inhibition. A considerable part of the acetylcholine-induced renal vasodilation is, however, resistant to inhibition with L-NAME and indomethacin. This component which is independent of NO synthase and cyclooxygenase is generally accepted to be mediated by EDHF [3]. This EDHF possibly comprises several different factors, with important species and regional heterogeneity [25]. Recently, strong evidence implicated the cytochrome P450 2C8-dependent metabolite 11,12-epoxyeicosatrienoic acid as an EDHF in porcine coronary arteries [26] and in hamster end arteries [27]. The factor(s) that mediate the NO synthase- and cyclooxygenase-resistant endothelium-dependent relaxation in many other vascular beds are still, however, to be identified.

The contribution of EDHF to relaxation is dependent on vessel size, being more prominent in smaller, physiologically more important, arteries than in larger ones [2, 28]. Hence, we did additional experiments using the split hydronephrotic kidney model, an intravital microscopy technique with which it is possible to examine the different segments of the renal microcirculation in vivo. As observed in these experiments, there is a clear gradient in the overall response to acetylcholine. It increases progressively from the proximal arcuate arteries to the afferent arteriole and decreases again in the postglomerular vessels. The pronounced vasodilation to acetylcholine in the smallest arterioles is largely mediated by EDHF, as was evident after inhibition of NO and prostaglandin synthesis.

In the diabetic animals, the global RBF response to acetylcholine tended to be lower but the difference was statistically significant only for the highest acetylcholine dose. When the NO-mediated and prostacyclin-mediated components of the acetylcholine response were inhibited, a profoundly impaired response was, however, uncovered in the diabetic animals. These results imply a decreased EDHF-mediated component in the diabetic kidneys. This decreased response cannot be explained by a decreased general responsiveness of the vascular smooth muscle cells because vasodilation in response to pinacidil and DETA-NONOate was not influenced under the same conditions.

The impaired EDHF-mediated response in diabetes is evidenced further in the experiments on the hydronephrotic kidney. The vasodilator response to acetylcholine was impaired in all vessels studied but

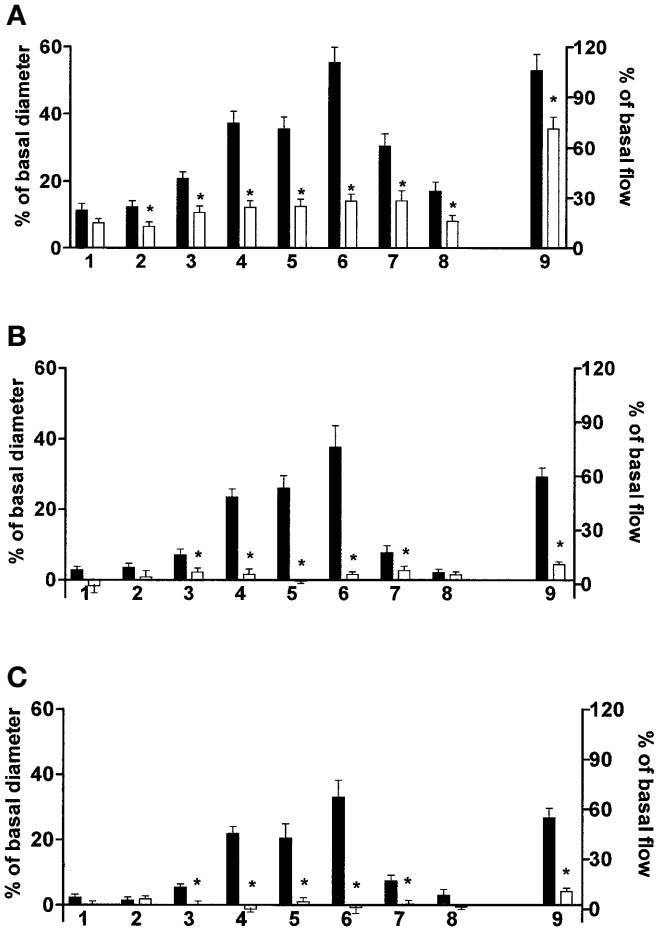

Fig. 5A–C. Percentage changes of basal vascular diameters and glomerular blood flow in the hydronephrotic kidney of control (■, $n = 6$) and diabetic rats (□, $n = 6$) in response to local application of acetylcholine 10^{-5} mol/l in the tissue bath (**A**). The protocol was repeated after local application of L-NAME 10^{-4} mol/l (**B**) and after local application of L-NAME 10^{-4} mmol/l with indomethacin 10^{-3} M (C). 1 = proximal arcuate artery, 2 = distal arcuate artery, 3 = proximal interlobular artery, 4 = distal interlobular artery, 5 = proximal afferent arteriole, 6 = distal afferent arteriole, 7 = proximal efferent arteriole, 8 = distal efferent arteriole, 9 = glomerular blood flow. Data are expressed as means ± SEM. *$p < 0.05$.

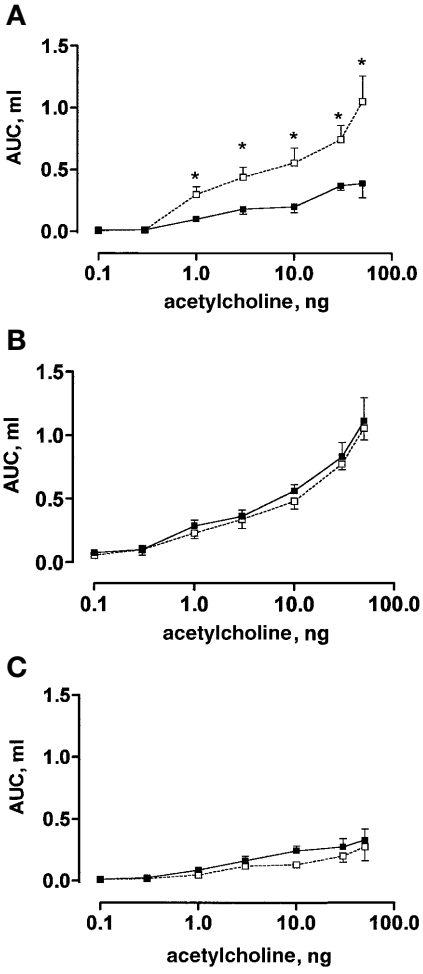

Fig. 6. The renal blood flow response to acetylcholine given intrarenally (**A**) in diabetic rats ($n = 8$) before (■) and 15 min after (□) an intravenous bolus of 200 μg 5-MTHF dissolved in 0.5 ml saline and (**B**) in control rats ($n = 8$) before (■) and 15 min after (□) an intravenous bolus of 200 μg 5-MTHF dissolved in 0.5 ml saline and (**C**) in diabetic rats ($n = 8$) before (■) and 15 min after (□) an intravenous bolus of 0.5 ml saline without 5-MTHF. The experiments were done under systemic NO synthase and cyclooxygenase blockade. The area under the curve (AUC) of the change from baseline values was calculated for each bolus of acetylcholine and the data are expressed as means ± SEM. *$p < 0.05$

the deficit increased as the vessel calibre became smaller. In the larger vessels the vasodilation was only mildly decreased with a statistically significant difference only for the highest acetylcholine doses, whereas a severe dysfunction was present in the smallest arterioles. Application of L-NAME and indomethacin almost abolished the response to acetylcholine in the latter vessels, thereby underlining that the endothelial dysfunction in the diabetic kidney is due to decreased action of EDHF.

The impaired NO synthase- and cyclooxygenase-resistant renal vasodilation in diabetes cannot be attributed to the differential effects of L-NAME on systemic blood pressure in diabetic and control rats because the RBF results were closely parallelled by the intravital microscopy findings where L-NAME was given locally and no systemic blood pressure changes were observed.

The absence of specific inhibitors of EDHF hampers the establishment of its physiological role. EDHF could provide a mechanism to induce a rapid dilation, followed by a relatively slow NO-mediated response [29]. A recent study showed that rhythmic alterations in intraluminal pressure induce the synthesis of EDHF [30]. Pulsatile pressure could thus be the physiological stimulus for EDHF release, in contradistinction to shear stress, a major stimulus for NO release [3]. Notably, the inhibition of EDHF led to a pronounced reduction of vascular compli-

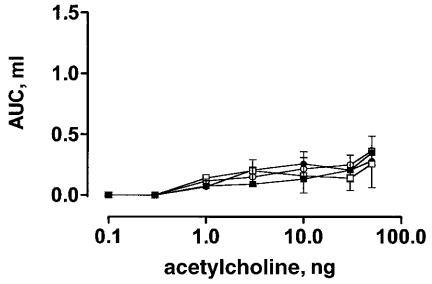

Fig. 7. The renal blood flow response to acetylcholine given intrarenally in diabetic rats ($n = 6$) before (■) and after an intravenous bolus of 10 mg vitamin C (□) and in diabetic rats ($n = 6$) before (●) after an intravenous bolus of 10000 U superoxide dismutase with 10000 U catalase (○). The experiments were done under systemic NO synthase and cyclooxygenase blockade. The area under the curve (AUC) of the change from baseline values was calculated for each bolus of acetylcholine and the data are expressed as means ± SEM

ance, as judged by the decreased amplitude of the diameter oscillations in response to the changes in intraluminal pressure [30]. In the light of these findings, our results could have important implications for the pathophysiology of diabetic microvascular complications and in particular of diabetic nephropathy. Increased blood pressure is injurious to glomeruli and predisposes to the development of diabetic glomerulosclerosis. A decreased action of EDHF in the small end vessels of the diabetic kidney could lead to a failure to protect the glomeruli from the deleterious effects of high blood pressure and thus contribute to the development of glomerulosclerosis.

So far, only a few studies have addressed the role of EDHF in diabetes and none of these were done in vivo. Endothelium-dependent hyperpolarization and the component of the relaxation to acetylcholine that is independent of NO synthase and cyclooxygenase are decreased in isolated mesenteric arteries from diabetic rats [31]. Other authors observed a more pronounced impairment of the acetylcholine-induced relaxations when NO synthase and cyclooxygenase were blocked in isolated mesenteric arteries [32] or a decreased NO synthase-resistant acetylcholine-induced relaxation in isolated renal arteries of diabetic rats [33] but they did not relate their findings to an impaired EDHF-mediated influence.

These studies and our results contrast with observations that an overproduction of endothelium-derived vasoconstrictors, such as prostanoids, accounts for the impaired endothelium-dependent relaxations in diabetic blood vessels [12, 13]. The existence of such an action of vasoconstrictor prostanoids in the kidney is not supported by our study, inasmuch as cyclooxygenase-inhibition did not restore the blunted relaxations observed in the diabetic animals. Other investigations showed an impaired acetylcholine-induced cyclic GMP generation [7, 8] or a restoration of the relaxation with L-arginine [9] or with free radical scavengers [10, 11], suggesting a reduced availability or an increased destruction of endothelium-derived NO. Another study specifically found no evidence for an impaired EDHF release in diabetes [34]. These in vitro studies were done in the aorta where NO is the principal mediator of endothelium-dependent responses. The discrepancies between these earlier observations and our study could therefore relate to the size of the vessels studied and illustrate the difference in relative contribution of NO and EDHF to endothelium-dependent relaxations.

In vivo studies examining the effects of EDHF have the drawback that hyperpolarization cannot be measured directly. Nevertheless, they can yield important information because they are carried out under physiological flow conditions. This is important because it is known that experimental conditions can determine the magnitude of the contribution of EDHF to endothelium-dependent vasodilation, by setting the resting membrane potential. In particular, depolarization of the vessel by pressurization will enhance the EDHF response [35].

If a reduced action of EDHF contributes to the occurrence of vascular complications, therapeutic measures reinforcing the action of EDHF would be beneficial. In our study, the impaired NO synthase-independent and cyclooxygenase-independent renal vasodilator response was restored to normal by systemic infusion of 5-MTHF, which is the active and circulating form of folic acid. The effect was specific for the pathological vessels because 5-MTHF did not augment the response to acetylcholine in control kidneys. The acute reversibility of the impairment in EDHF-mediated relaxations points to a functional defect, rather than to structural damage of the endothelial cells.

The mechanisms responsible for the beneficial effects of folate on vascular function are a subject of discussion. Folate is the principal therapy in patients with hyperhomocysteinaemia because it is the cosubstrate for the homocysteine-metabolizing enzyme methionine synthase. Hyperhomocysteinaemia is known to be associated with impaired endothelium-dependent vasodilation [36, 37]. Plasma homocysteine concentrations were, however, lower in the diabetic than in the control rats, as described previously [38], and were not influenced by treatment with 5-MTHF. Accordingly, folate has been shown to restore endothelial dysfunction during a methionine load test in healthy volunteers without affecting the rise in plasma homocysteine concentrations [17]. Other data have also supported vascular effects of folate independent of homocysteine lowering. A single intravenous bolus of 5-MTHF [15] and 4 weeks of treatment with oral folate [16] restored impaired endothelium-dependent vasodilation in the forearm circulation of patients with familial hypercholesterolaemia. Folate is known to stimulate the reduction of biopter-

in, the cofactor of endothelial NO synthase, to its active form [39]. In vitro NO production by recombinant endothelial NO synthase with or without addition of biopterin [15] was not, however, increased by 5-MTHF. In contrast, 5-MTHF reduced superoxide generation by both xanthine oxidase and recombinant endothelial NO synthase in vitro [15]. It was hypothesized that folate exerted its beneficial effects on endothelium-dependent vasodilation by preventing the oxidative breakdown of endothelium-derived NO [15]. Our experiments were done under systemic NO synthase blockade and, therefore, the effects of folate cannot be explained by an improved availability of endothelium-derived NO. Increased oxidative stress could, however, also have adverse effects on production or availability of EDHF or both. To investigate whether 5-MTHF acted as an antioxidant, we compared its effects with those of established antioxidant agents. Neither vitamin C nor SOD with catalase improved the L-NAME- and indomethacin-resistant vasodilation in diabetic rats. It thus seems unlikely that folate improves EDHF-mediated vasodilation through a direct antioxidant effect. Folate could have other, as yet unknown, effects on cellular metabolism that interfere with the synthesis or bioavailability of EDHF. Taken together, our results and these of others [15–17] suggest that folate has beneficial effects on endothelial function independent of its well-known role in homocysteine metabolism.

Our in vivo study shows that the EDHF-mediated vasodilator response is selectively and profoundly impaired in the renal microcirculation of diabetic rats, indicating substantial endothelial dysfunction. The effect of the deficit seemed to be largest in the smallest arteries. Because these vessels are involved in the local control of blood flow and the regulation of blood pressure, defective EDHF production could have an important role in the development of diabetic nephropathy and possibly of other diabetic microvascular complications. Treatment with 5-MTHF rapidly restores the defective endothelium-dependent vasodilation to normal in the diabetic kidney, providing support for a potential role for folate in the prevention or treatment of diabetic microvascular disease.

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