Lack of endothelium-derived hyperpolarizing factor (EDHF) up-regulation in endothelial dysfunction in aorta in diabetic rats

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Abstract:
It is not known whether the impairment of nitric oxide (NO)-dependent vasodilation of the aorta of diabetic rats is associated with any changes in the endothelial production of vasoactive prostanoids and endothelium-derived hyperpolarizing factor (EDHF). Therefore, we analyzed the contribution of NO, vasoactive prostanoids and EDHF to the decreased endothelium-dependent vasorelaxation in Sprague-Dawley rats at 4 and 8 weeks after diabetes mellitus induced by streptozotocin (STZ).

The acetylcholine-induced (Ach) endothelium-dependent relaxation was significantly decreased in the thoracic aorta 8 weeks after the STZ-injection (Ach 10–6 M: 73.1 ± 7.4% and 56.7 ± 7.9% for control and diabetic rats, respectively). The sodium nitroprusside-induced (NaNP) endothelium-independent vasodilation was also impaired in the diabetic rats (8 weeks after STZ) (NaNP 10–8 M: 74.2 ± 11.4% and 35.9 ± 9.4% for control and diabetic rats, respectively). In contrast, the basal NO production, as assessed by the Nω-nitro-L-arginine methyl ester (L-NAME)-induced vasoconstriction was not modified in diabetes. Moreover, the amount of 6-keto-PGF1α (stable metabolite of prostacyclin / prostaglandin I2 / PGI2), 12-L-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT) and thromboxane B2 (TxB2) (stable metabolite of thromboxane A2 – TxA2) were significantly increased in the 8 weeks diabetic rat aorta. The EDHF-pathway did not change in the aortic endothelium during the development of STZ-induced diabetes.

Our results indicate that STZ-induced diabetes mellitus did not modify the basal NO production, but induced the impairment of acetylcholine- and sodium nitroprusside-induced vasodilation in the thoracic aorta. In parallel with the impairment of NO-dependent vasodilation, the basal PGF2α, 12-HHT and TxA2 synthesis were increased. The EDHF-pathway did not contribute to the endothelium-dependent relaxation either in control or diabetic aorta. The above alterations in the endothelial function may play an important role in the development of endothelial dysfunction and vascular complications of diabetes.

Key words: nitric oxide, prostaglandins, eicosanoids, EDHF, diabetes, aorta

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized not only by altered carbohydrate, protein and lipid metabolism but also by micro- and macroangiopathic vascular complications [9]. Endothelial dysfunction, determined as a decreased endothelium-dependent relaxation and/or increased vasoconstrictor capacity, contributes to the vascular inflammation that is responsible for the vascular complications of diabetes [5, 35, 37]. Accordingly, the development of endothelial dysfunction have been repeatedly demonstrated in various vascular beds in animal models of diabetes [10, 19, 29], as well as in patients with type I or type II DM [16, 22]. On the other hand, there are some studies that reported normal [24, 39], or even enhanced [2, 41] endothelium-dependent vasodilation.

The exact mechanisms that lead to the development of endothelial dysfunction during diabetes are still not known. The following mechanisms have been proposed to contribute to the impaired nitric oxide (NO)-dependent relaxation: reduced nitric oxide synthase (NOS) activity, decreased availability of substrate and co-factor (L-arginine and tetrahydrobioppterin) for NO synthesis, enhanced inactivation of NO (increased oxidative stress), impaired diffusion of NO to the underlying smooth muscle cells and decreased smooth muscle cell sensitivity to NO [3, 8, 15, 20, 28]. At the same time, many reports have debated the above mechanisms [3, 13, 17, 25].

It is well known that endothelial cells produce not only NO, but a number of other vasodilators [bradykinin, prostacyclin (PGI2), PGD2, PGE2, 12-HHT and endothelium-derived hyperpolarizing factor (EDHF)] and vasoconstrictor metabolites (endothelin-1 and vasoconstrictor prostanoids). Therefore, it is plausible that a decrease in NO-mediated vasodilation does not fully account for the development of endothelial dysfunction in diabetes, but the reduced PGI2 and EDHF activity, and/or the increased synthesis of vasoconstrictor cyclooxygenase (COX) metabolites [thromboxane A2 (TxA2) and PGF2α] may be also involved.

Interestingly, some of the previous studies investigated only the NO-dependent relaxation in DM [19, 32]. Merely, in few reports the functional role of NO and prostaglandins [6] or NO and EDHF [11, 42] has been analyzed simultaneously, while in other studies [27, 43] only the alterations in prostaglandin production were demonstrated. To our knowledge there are still no reports that would have simultaneously analyzed the functional alterations in endothelial production of NO, vasoactive prostanoids and EDHF in aorta from diabetic rats. Accordingly, the aim of the present study was to assess possible changes in the production of vasoactive prostanoids (PGI2, PGE2, TxA2 etc.) and EDHF that are associated with the development of endothelial dysfunction in the thoracic aorta of streptozotocin (STZ)-induced diabetic rats.

Materials and Methods

Animals

The investigations conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The experiments were performed under a protocol accepted by the Ethics Committee for the Protection of Animals in Research, University of Szeged, Hungary.

Diabetes mellitus (diabetic group) was induced in male Sprague-Dawley rats (180–200 g) by a single injection of STZ (65 mg/kg) dissolved in citrate buffer into the tail vein. Age-matched control rats were injected with the similar volume of citrate buffer alone (control group). The animals were maintained under 12-h dark/12-h light cycles in a room at constant temperature (23 ± 1°C) with access to standard laboratory food and water ad libitum. The rats were used in experiments 4 or 8 weeks after the injection of STZ.

Analysis of serum glucose and HbA1c

Animals showing the typical features of DM (weight loss, polyuria) were used for the vascular experiments and the development of diabetic state was validated by elevated serum glucose and HbA1c levels. After overnight fasting, on the day of the experiments the blood samples were taken from the abdominal aorta of anesthetized rats (pentobarbitone, 60 mg/kg, ip) and were processed for serum glucose (Human GmbH, Wiesbaden, Germany) and level of HbA1c (BIO-RAD Laboratories GmbH, Germany) according to the manufacturer’s instructions.
Intravenous heparin (500 UI/kg) was given before anesthesia (pentobarbitone, 60 mg/kg, ip) to prevent blood clotting. The thoracic aorta was quickly removed and cleaned of connective tissue. Next, the aorta was cut into 3 rings each approximately 3 mm in length and transferred to organ chambers filled with 10 ml of freshly-prepared Krebs-Heinseleit solution (mM: NaCl 118, KCl 4.7, CaCl$_2$ 2.5, MgSO$_4$ 1.2, NaHCO$_3$ 25, KH$_2$PO$_4$ 1.2, glucose 10) maintained at 37°C, pH 7.4 and gassed with 95% O$_2$ and 5% CO$_2$.

Then, rings were mounted between 2 hooks attached to an isometric force transducer (UTC 2, Gould Sta-tham USA) connected to a data acquisition system (Powerlab 8SP, AD Instruments, Great Britain) for continuous recording of tension. After mounting the aortic rings, the resting tension was increased stepwise to reach a final value of 20 mN, which according to our preliminary experiments results in optimal and sustained vascular responses. Following one-hour equilibration period, the viability of the vessels was investigated by the contractile response to potassium chloride (KCl, 60 mM).

Aortic rings were then precontracted with phenylephrine (Phe, 2 × 10$^{-7}$ M) and after reaching a stable plateau phase, a cumulative concentration-dependent response for acetylcholine (Ach, 10$^{-8}$ to 10$^{-5}$ M) was induced.

The endothelium-independent vasorelaxation was evoked by sodium nitroprusside (NaNP, 10$^{-9}$ to 10$^{-6}$ M).

The aortic rings were then mildly precontracted by Phe to reach 10–20% of KCl-induced maximal vasoconstriction, and basal NO production was determined on the basis of the magnitude of contraction induced upon the administration of the nitric oxide synthase inhibitor N$_{ω}$-nitro-L-arginine methyl ester (L-NAME, 300 μM, 15 min).

In some experiments, we used the COX inhibitor indomethacin (2 μM, 30 min) to test the role of vasoactive prostanoids in the development of endothelial dysfunction.

EDHF-mediated relaxation was determined after the combined incubation with L-NAME and indomethacin.

Following sacrifice as described above 8 weeks after diabetes induction, the abdominal aorta was quickly removed from diabetic and control rats. Then, the aortic rings (15 mg wet weight/in each sample) were preincubated at 37°C for 10 min in 1 ml Medium 199 for tissue culture. The production of arachidonate metabolites was determined as described previously [23]. Briefly, the enzymatic reaction was started by the introduction of tracer substrate, [14C]arachidonic acid (3.7 kBq, 0.172 pmol), into the incubation mixture. Thirty minutes later, after stopping the enzymatic reaction by bringing the pH value to pH = 3 with formic acid, the samples were extracted with ethyl acetate (2 × 3 ml) and the organic phases were pooled and evaporated to dryness under nitrogen. The residues were reconstituted in 2 × 100 μl of ethyl acetate and quantitatively applied to silica gel G thin-layer plates. The plates were developed to a distance of 15 cm in the organic phase of ethyl acetate : acetic acid : 2,2,4-trimethylpentane : water (110:20:30:100) by means of overpressure thin-layer chromatography (Chrompres 25, Labor MIM, Hungary). Each 3-mm band of the chromatograms was then scraped off and the radioactivity was determined by liquid scintillation analyzer (TRI-CARB 2100TR, Canberra Packard, USA) using 5 ml of toluene containing 0.44% (w/v) of 2,5-diphenyloxazole, 0.02% (w/v) of 1,4-di-[2-(5-phenyl) oxazoyl] benzene and 10% (v/v) of ethanol. Radioactivity was expressed in disintegrations per minute (dpm). The radiolabeled products of arachidonic acid were identified with unlabeled authentic standards, which were detected by anisaldehyde reagent [18]. Assuming that the exogenously administered [14C]-arachidonic acid, as a tracer is converted in the same way as the endogenous source, our method allows to measure the relative amount of various prostanoids.

**Chemicals**

We purchased streptozotocin, phenylephrine hydrochloride, N$_{ω}$-nitro-L-arginine methyl ester hydrochloride, indomethacin and Medium 199 for tissue culture from Sigma-Aldrich (St. Louis, Mo, USA). Acetylcholine chloride and sodium nitroprusside were obtained from Fluka (Seelze, Germany). Pentobarbitone was purchased from Ceva-Phylaxia (Budapest, Hungary). Phenylephrine, acetylcholine, sodium nitro-
prusside and potassium chloride were prepared as stock solutions in distilled water. Indomethacin and Nω-nitro-L-arginine methyl ester were dissolved freshly in NaHCO₃ (0.6 M) and Krebs-Heineleit solution, respectively.

Statistical analysis

Vasodilator responses are expressed as a percentage of Phe-induced preconstriction. All results are expressed as the mean ± SEM. Comparison of the means was assessed by ANOVA and post hoc Scheffe test. Significant differences between the vasodilator and vasoconstrictor COX metabolites were established by modified Student’s t-test [40]. p < 0.05 was considered to be statistically significant.

Results

Development of diabetes

The plasma glucose, HbA1c level and the volume of 24-h urine were already increased four weeks after the STZ injection and these differences became more pronounced in animals 8 weeks after diabetes induction (Tab. 1.). Moreover, in the diabetic groups, body weight was reduced when compared to the corresponding non-diabetic controls.

Vascular function of the aorta in diabetic rats

NO-dependent vascular function

Endothelium-dependent relaxation was assessed by a cumulative concentration-dependent response to Ach (from 10⁻⁸ to 10⁻⁵ M) (Fig. 1). Relaxation curves were identical at 4 and 8 weeks in control animals (Ach 10⁻⁶ M: 71.2 ± 6.1% and 73.1 ± 7.4% respectively); therefore, these animals were combined for further analysis. In contrast, 8 weeks after STZ injection, the Ach-induced endothelium-dependent relaxation was decreased (Ach 10⁻⁶ M: 56.7 ± 7.9%) as compared to control animals (Fig. 1).

Endothelium-independent relaxation was investigated as a cumulative concentration-response curve

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Tab. 1. Body weight, 24-h urine, blood glucose and HbA1c in control and diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
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<tr>
<td></td>
<td>4 weeks</td>
<td>8 weeks</td>
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<td></td>
<td>n = 7</td>
<td>n = 8</td>
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<tr>
<td>Body weight (g)</td>
<td>319.2 ± 27.3</td>
<td>395.7 ± 19.7</td>
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<tr>
<td>Urine (ml/24 h)</td>
<td>8.5 ± 3.1</td>
<td>10.0 ± 3.61</td>
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<tr>
<td>Blood glucose (mg/dl)</td>
<td>102.1 ± 13.1</td>
<td>101.2 ± 13.9</td>
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<tr>
<td>HbA1c (%)</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.1</td>
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Values are the means ± SEM. a p < 0.05 control at 4 weeks vs. control at 8 weeks; b p < 0.05 diabetic at 4 weeks vs. diabetic at 8 weeks; c p < 0.05 control at 4 weeks vs. diabetic at 4 weeks; d p < 0.05 control at 8 weeks vs. diabetic at 8 weeks.
for NaNP (from $10^{-9}$ to $10^{-6}$ M) (Fig. 2). The response induced by NaNP at a concentration of $10^{-9}$ to $10^{-8}$ M was significantly decreased 8 weeks after the STZ-injection. However, the maximal response induced by NaNP was similar in control (NaNP $10^{-6}$ M: $104.7 \pm 2.7\%$ and $104.4 \pm 5.5\%$ at 4 and 8 weeks in control rats, respectively) and in diabetic rats (NaNP $10^{-6}$ M: $104.7 \pm 4.2\%$ and $103.4 \pm 4.8\%$ at 4 and 8 weeks in diabetic rats, respectively) irrespective of the age of the animals.

Basal NO production, analyzed as the magnitude of contraction induced by L-NAME after a threshold concentration of Phe, was not different between control (30.4 ± 5.3\% and 29.2 ± 5.7\% at 4 and 8 weeks in control rats, respectively) and diabetic rats (31.4 ± 7\% and 31.1 ± 10.7\% at 4 and 8 weeks in diabetic rats, respectively).

EDHF-dependent relaxation in the aorta

The combined preincubation of aortic rings with L-NAME (300 \mu M, 15 min) and indomethacin (2 \mu M, 30 min) almost completely blocked the Ach-induced vasodilation in all experimental groups (<3\% both in diabetic and control rats, respectively).

The role of COX metabolic products in diabetic aorta

Indomethacin alone did not modify either the sensitivity or the maximal value of Ach-induced relaxation in control and diabetic rats.

The basal production of the total amount of vasodilators (6-keto-PGF$_{1\alpha}$, PGE$_2$, PGD$_2$ and 12-HHT) (9.52 ± 1.5 \times 10^3 dpm) COX metabolic products was significantly larger in aorta from diabetic rats, as compared with control animals (5.51 ± 0.5 \times 10^3 dpm). Indeed, the amount of 6-keto-PGF$_{1\alpha}$ (4.64 ± 0.9 \times 10^3 dpm) and 12-HHT (2.34 ± 0.4 \times 10^3 dpm) were significantly increased at 8 weeks in diabetic aorta when compared to the corresponding controls (2.23 ± 0.4 \times 10^3 dpm and 1.33 ± 0.2 \times 10^3 dpm for 6-keto-PGF$_{1\alpha}$ and 12-HHT, respectively) (Fig. 3). Moreover, the basal production of vasoconstrictor COX metabolic products (TxB$_2$ and PGF$_{2\alpha}$) was increased (2.74 ± 0.2 \times 10^3 dpm and 2.05 ± 0.1 \times 10^3 dpm for diabetic and control rats, respectively) and this could be attributed to an increased TxA$_2$ synthesis (1.33 ± 0.1 \times 10^3 dpm and 0.87 ± 0.05 \times 10^3 dpm for diabetic and control rats, respectively) (Fig. 4).
Discussion

The results of the present study indicate that functional alterations in the aortic endothelium induced by diabetes involve the impairment of NO-mediated vasodilation that is associated with the activation of COX-but not the EDHF-pathway. Indeed, the responsiveness of the vascular smooth muscle cells to exogenous NO was impaired and the Ach-induced relaxation was also decreased at 8 weeks in diabetic aorta. We observed an overproduction of vasodilator (PGI\(_2\) and 12-HHT) and vasoconstrictor (TxA\(_2\)) COX metabolic products in the aorta of diabetic rats. Moreover, we showed that diabetes did not induce the impairment of basal NO activity or the adaptive up-regulation of EDHF pathway, irrespectively of the age of the animals.

Although, NO has been generally considered as the principal mediator of endothelium-dependent relaxation, it has become clear that decreased NO-mediated vasodilation does not fully account for the development of endothelial dysfunction in DM [11]. Indeed, both the reduced bioavailability of different vasodilator (PGI\(_2\) and EDHF) and the increased amount of vasoconstrictor COX metabolic products (TxA\(_2\) and PGF\(_{2\alpha}\)) may contribute to the impairment of endothelium-dependent vasodilation in diabetes.

In previous studies investigating the mechanisms of endothelial dysfunction in DM, the functional role of NO, vasoactive COX products and EDHF were not analyzed simultaneously [1, 19, 27, 32]. Therefore, it is not clear whether the impairment of endothelium-dependent vasodilation in the thoracic aorta of STZ-induced diabetic rats is associated with the altered COX- or EDHF-pathways.

The majority of the previous studies demonstrated an impaired response to endothelium-dependent vasodilators in the presence of preserved endothelium-independent vasorelaxation [25, 30]. In the present experiments, we demonstrated that the endothelium-independent relaxation was also impaired in STZ-induced diabetic rats. This finding correlates with that of others [8, 26], who also reported an impaired endothelium-independent relaxation in diabetes. Moreover, our experiments suggest that the impaired vasodilation to sodium nitroprusside occurred subsequently to impaired response to acetylcholine. This may reflect that functional changes in both the endothelial and smooth muscle cells that may be involved in the development of impaired NO-dependent vasorelaxation. There was no difference between the magnitude of L-NAME-induced vasoconstriction irrespectively of the age of control and diabetic rats, which indicates that measurement of the basal NO production alone is not enough—at least in our experimental set-up—to detect the impairment of NO-dependent relaxation, as compared to the assessment of Ach-induced vasodilation.

Supporting a previous study [21], we also demonstrated that pretreatment with indomethacin had no effect on the Ach-induced vasorelaxation in either control or diabetic rats, suggesting that stimulated PGI\(_2\) release plays only a minor role in the Ach-induced vasodilatation of aorta. It has been reported that prostanoid production varies according to the time after the STZ-injection in rats [27, 31]. Indeed, the decreased production of PGI\(_2\) or the increased synthesis of TxA\(_2\) may reflect the severity of diabetes, especially the progression of vascular complications [12, 14]. Moreover, Peredo et al. [27] have shown that PGI\(_2\)/TxA\(_2\) ratio was decreased in the aorta 120 and 180 days but not after 30 days of diabetes. Here, we demonstrate that diabetes of 8 weeks duration induced an increased synthesis of PGI\(_2\), 12-HHT and TxA\(_2\) in the aorta. The overproduction of PGI\(_2\) may play a compensatory role in diabetic aorta, since PGI\(_2\) is a potent vasodilator and platelet inhibitor. Interestingly, it was reported that 12-HHT stimulates PGI\(_2\) synthesis [34] and that the primary metabolite of 12-HHT (12-oxoheptadeca-5(Z)-8(E)-10(E)-triene acid) has a TxA\(_2\) receptor antagonistic effect [33]. Therefore, one might suggest that increased 12-HHT synthesis, as measured in the present experiments, may contribute to the increased PGI\(_2\) production and decreased action of TxA\(_2\) at this relatively early stage of diabetes. Taking this into consideration, it is conceivable that the increased synthesis of PGI\(_2\) and 12-HHT may play a compensatory role at the early stage of diabetes, while these mechanisms do not operate at later stages displaying an increased activity of vasoconstrictor COX metabolites. However, it is worth noting that further experiments are needed to determine the changes in the absolute amount of various prostanoids in our experimental settings. Diabetes-induced alterations in the arachidonic acid content of membrane phospholipids as well as changes in the phospholipase A\(_2\) activity also need further investigations.

Only in a few studies, the functional changes in EDHF activity were investigated during the develop-
Role of NO, prostanoids and EDHF in diabetic rat aorta

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Impaired endothelium-dependent relaxation in isolated mesenteric arteries, but they did not link their findings to the impairment of EDHF-pathway [38]. It is known that the importance of EDHF increases as the vessel size decreases [36]; therefore, the majority of such studies are restricted to small resistance arteries. It is also known that NO plays a prominent role in the endothelium-dependent relaxation of aorta, however, as we previously reported, under pathological conditions in myocardial infarction (MI) the aorta might have the capacity for the compensatory synthesis of EDHF [7]. Indeed, the functional alterations of the rat aortic endothelium induced by coronary artery ligation involve the up-regulation of EDHF pathway. In that study, we have found that the up-regulation of EDHF activity was gender-unspecific in the early phase of post-MI remodeling, whereas in the late phase, the increased EDHF synthesis was preserved in female rats only. In contrast, in the present study, the combined preincubation with L-NAME and indomethacin completely blocked the Ach-induced vasodilation in all experimental groups suggesting that the up-regulation of EDHF-dependent vasodilation did not occur in the aorta of male DM rats. Moreover, to assess whether diabetes-induced changes in EDHF-mediated vasodilation depends on gender of the animals, we used female rats (data not shown) where the results were similar to males. Since the role of EDHF is not limited only to the regulation of vasomotor tone, but EDHF also displays potent anti-inflammatory action, one might speculate that lack of EDHF-pathway up-regulation may contribute to the development of endothelial dysfunction as well as the vascular complications in diabetes.

In summary, diabetes mellitus in rats did not modify the basal NO production, but induced the impairment of acetylcholine- and sodium nitroprusside-induced vasodilation in the thoracic aorta. Moreover, along with the impairment of NO-mediated relaxation the diabetes-induced alterations in the aorta are associated with the increased basal synthesis of PGI2, TXA2 and 12-HHT. Finally, the lack of EDHF-pathway up-regulation in the aorta may contribute to the impairment of endothelium-dependent relaxation and vascular complications of diabetes.

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References:

13. Heygate KM, Lawrence IG, Bennett MA, Thurston H: Impaired endothelium-dependent relaxation in isolated
41. White RE, Carrier GO: Supersensitivity and endothelium dependency of histamine-induced relaxation in mesen-


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