Characteristics of coronary endothelial dysfunction in experimental diabetes

Mária Zsófia Koltai a, *, Pál Hadházy b, Ildikó Pósa a, Erzsébet Kocsis a, Gábor Winkler c, Peter Rösen d, Gábor Pogátsa a

a National Institute of Cardiology, PO Box 9-88, H-1450 Budapest, Hungary
b Department of Pharmacodynamics, Semmelweis Medical School, Budapest, Hungary
c St. John Municipal Hospital, Budapest, Hungary
d Diabetes Research Institute, Dusseldorf, Germany

Received 7 October 1996; accepted 23 January 1997

Abstract

Objective: To study the influence of diabetes on the endothelium-dependent vasodilation in the coronary arterial bed. Methods: The effects of acetylcholine ACh 2±36 pmol · kg−1; 18 nmol · l−1; 9.8 μmol · l−1; 0.1±10 μmol · l−1) and sodium nitroprusside (1 nmol · l−1; 100 μmol · l−1) were measured on coronary conductivity, vascular tone and cGMP release (RIA) in healthy and diabetic dogs. Results: ACh-mediated increase in coronary conductivity was reduced (P < 0.01) in the diabetic dogs in vivo, whereas no increase in cGMP release was observed in isolated diabetic coronaries (P < 0.05) which could not be enhanced by L-arginine (P < 0.05). Inhibition of cyclo-oxygenase after 20 min further impaired (P < 0.01) responsiveness to ACh in vivo and diminished the ACh response in isolated coronary strips of the diabetic dogs, but not in those of the controls. Relaxation in response to sodium nitroprusside was not altered by diabetes. Conclusions: Diminished vasodilation in diabetes is due to a defect in endothelial nitric oxide production and action. Vasodilating prostanoids do not sufficiently compensate this defect.

Keywords: Diabetes; Coronary artery; Nitric oxide; Prostaglandins; Dog

1. Introduction

The endothelium adapts to the local environment by releasing a large number of endogenous substances that regulate vascular tone [1]. There is biological and chemical evidence that one of its most important mediators is nitric oxide (NO) [2], which is a potent vasodilator and also cardioprotective [3,4]. Its synthesis can be triggered by two different mechanisms [5]. NO formation is enhanced either by receptor-independent mechanisms such as shear stress resulting in flow-dependent vasodilation, or by receptor-mediated processes in response to substances such as acetylcholine or bradykinin. In this way, NO contributes to the rapid adaptation of blood flow to various pharmacological and mechanical stimuli, thereby maintaining adequate tissue perfusion. Accordingly, defects in the generation and release of nitric oxide severely increase the cardiovascular risks. It is well known that endothelial function is altered in a number of pathological conditions such as atherosclerosis, hypercholesterolaemia, hypertension, reperfusion damage and diabetes [6].

There is experimental evidence that the endothelium-dependent relaxation induced by acetylcholine is markedly diminished in aortic rings of rats after 2 weeks of diabetes induction [7]. Similar observations were made in both type 1 and type 2 diabetic patients showing diminished reactivity of coronary arteries without angiographic abnormality [8,9].

These data indicate that the defect in nitric oxide function and activity develops very early in diabetes mellitus, and leads to altered vascular reactivity [10]. This phe-
nomogen can be recognized as an increased sensitivity to endogenous and exogenous vasoconstrictor agents and/or as a diminished responsiveness to vasodilators. It precedes the onset of both macro- and microangiopathy and is not associated with any morphological changes. Failure of vascular endothelium to evoke NO-mediated vasorelaxation may develop due to decreased flow, increased degradation, decreased sensitivity to the released NO or even a combination of these factors.

In the present work, to elucidate the underlying mechanisms, characteristics of NO-mediated vasorelaxation were studied both in vivo and in vitro, together with parallel determination of NO-dependent basal and stimulated 3',5' cyclic guanosine-monophosphate (cGMP) generation in alloxan-diabetic dogs and healthy controls. Furthermore, the role of substrate availability as well as the relation to other vasodilator (prostanoids, endothelium-independent) mechanisms was also studied.

2. Methods

2.1. In vivo studies

Forty-six mongrel dogs (16–28 kg) of either sex were kept on the same diet consisting of 25% protein, 60% carbohydrate, 15% fat, vitamins and mineral salts. In 23 animals diabetes mellitus was induced by intravenous injection of 560 \( \mu \text{mol} \cdot \text{kg}^{-1} \) alloxan (alloxan tetrahydrate, Merck); the remaining dogs served as controls. By intravenous injection of 6 \( \text{mmol} \cdot \text{kg}^{-1} \) glucose, the plasma disappearance rate of glucose as well as fasting blood glucose and urea nitrogen levels were determined at the beginning of the study and subsequently once a month, whereas acetone and glucose excretion in the urine collected over 24 h was measured every week (for details of these techniques, see Ref. [10]). Three months after alloxan treatment (on the day before the experiment) all the above variables were redetermined.

Under pentobarbitone (133 \( \mu \text{mol} \cdot \text{kg}^{-1} \); Nembutal, Ceva) anaesthesia, the chest was opened and the animals were artificially ventilated (Respirator type: RO-5). Arterial blood pressure was measured via a cannula inserted into the thoracic aorta through the femoral artery with a Statham gauge (P23 Db) and coronary blood flow was detected by an electromagnetic flowmeter (Gould-Statham, SP 2202) with the help of a flow probe placed around the left anterior descending coronary artery (size: 1.0–2.0 mm in diameter). Alterations of the parameters were continuously recorded (Watanabe multiscriptor Mark VIII, Japan). Changes in conductance of the coronary arterial bed—expressed as a percentage of the basal value (flow/pressure ratio)—in response to intra-arterial infusion of acetylcholine (Sigma) (2.25, 4.5, 9, 18, 36 \( \text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) were determined both before and after intra-arterial injection of the cyclo-oxygenase inhibitor, acetylsalicylic acid (140 \( \mu \text{mol} \cdot \text{kg}^{-1} \); Sigma). Acetylcholine was infused cumulatively. The subsequent concentration was infused after reaching a steady state. The second exposure to acetylcholine was carried out 20 min after acetylsalicylic acid administration. Hence, the second acetylsalicylic infusion was performed approximately 30–40 min after the beginning of the first one. Furthermore, in another series of experiments \((n = 6, 6)\), the effect of sodium nitrite (0.5, 1.2, 2.5, 3.75 \( \text{pmol} \cdot \text{kg}^{-1} \); Biogal) was compared from the haemodynamic parameters in metabolically healthy and alloxan-diabetic dogs before and after intra-arterial injections of indomethacin (10 \( \mu \text{mol} \cdot \text{kg}^{-1} \); Sigma) or acetylsalicylic acid (140 \( \mu \text{mol} \cdot \text{kg}^{-1} \); Sigma).

2.2. In vitro studies

2.2.1. Measurement of vascular tone

The left coronary artery was removed, carefully cleaned of fat and connective tissue (to avoid damage to the endothelium), cut into strips (length 40 mm; width 2 mm) and suspended in an organ chamber containing 5 ml normal Krebs’ solution that was aerated with a mixture of 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \) at 37°C. The composition of the bathing fluid was (mM): NaCl 113, KCl 4.7, CaCl \(_2\) 2.5, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, NaHCO\(_3\) 25, glucose 11.5 and disodium-EDTA 0.04. Changes in vascular tone were measured by isometric transducers (Type DY 1; Ugo Basile, Varese, Italy) and recorded on a potentiometric recorder (Radelkis OH-814, Budapest, Hungary). The initial tension was adjusted to 7.5 mN. Effects were investigated after an equilibration period of 90 min.

The strips were precontracted by 2 \( \mu \text{mol} \cdot \text{l}^{-1} \) PGF\(_{2\alpha}\) (Chinoin; EC \(_{50}\)) and the relaxing effect was analyzed in this steady state; 100% was reached if the vascular tone equalled the pre-constriction level. The concentration–relaxation relationship was determined by cumulative addition of acetylcholine (Sigma) in a dose range of 18 \( \text{nmol} \cdot \text{l}^{-1} \)–9.8 \( \mu \text{mol} \cdot \text{l}^{-1} \). The dose–response curve was repeated 20 min after the addition of 3 \( \mu \text{mol} \cdot \text{l}^{-1} \) indomethacin or 10 \( \mu \text{mol} \cdot \text{l}^{-1} \) ibuprofen (Sigma).

The relaxant effect of sodium nitroprusside (Roche) was studied in 1 \( \text{nmol} \cdot \text{l}^{-1} \)–100 \( \mu \text{mol} \cdot \text{l}^{-1} \) concentrations.

2.2.2. Measurement of cGMP formation

In further 5 healthy and 5 alloxan-diabetic dogs, the left anterior descending coronary artery was removed and cleaned of fat and connective tissues, cut into 1–2 mm rings and placed in an organ chamber containing 3 ml normal Krebs-Henseleit solution that was aerated with a mixture of 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \). The bathing fluid was replaced with fresh Krebs-Henseleit solution every 30 min. After an equilibration period of 2 h, samples were collected for assaying cGMP production 30 min after incubation with the fresh solution. The amount of cGMP was determined using a radioimmunoassay technique (Amersham) and expressed as fmol/mg tissue. Measure-
ments of cGMP in the superfusates of coronary arterial rings were performed under basal circumstances, after 30 min incubation with 0.1, 1.0, 10 μmol·l⁻¹ acetylcholine or with L-arginine (1 mmol/l; Sigma). The cGMP-releasing activity of sodium nitroprusside (1 μmol·l⁻¹) was determined in healthy and diabetic canine coronary arteries and expressed as a percentage of the basal value. No phosphodiesterase inhibitors were used to prevent degradation of cGMP since it might have changed vascular tone by accumulating cGMP in the parallel tension experiments.

2.3. Drugs

Most of the compounds were diluted in saline, while the solutions of indomethacin and acetylsalicylic acid contained distilled water and NaHCO₃ with a pH value of 7.0–7.2.

2.4. Statistical analysis

The data are given as means ± s.e.m. The results were compared by Student’s t-test, analysis of variance and regression line analysis. To evaluate the responsiveness of the coronary arterial bed to acetylcholine, linear regression was calculated. The slopes (b) of the y = a + bx regressions were compared by t-test. The b-values with the concomitant t- and P-values refer to whether the linear correlation between the given two groups is statistically significantly different or not. This type of comparison is supposed to detect and express even slight differences in vascular sensitivity to vasoactive drugs in diabetic early vascular dysfunction.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals (published by the US National Institutes of Health, Publication No. 85-23).

3. Results

Alloxan treatment induced a mild aketotic form of diabetes mellitus which was demonstrated by urinary excretion of glucose, a considerable increase in the fasting plasma level of glucose and a significant reduction in the plasma disappearance rate of this sugar (Table 1). Alloxan-treated animals with enhanced blood urea nitrogen level were excluded from the study.

Fig. 1. Effect of acetylcholine on the conductivity of the coronary arterial bed expressed as a percentage of the initial value in healthy and diabetic dogs. (A) Without acetylsalicylic acid. (B) After 140 μmol/kg acetylsalicylic acid pretreatment. n = 6, 6. The slopes (b) of the y = a + bx regressions were compared by t-test. The b-values with the concomitant t- and P-values refer to whether the linear correlation between the given two groups is significantly different or not. b₁ ≠ b₂, t = 4.68, P < 0.01; b₁ ≠ b₃, t = 6.48, P < 0.005; b₁ = b₂, t = 7.31, P < 0.001; b₁ = b₃, not significant.

Table 1

<table>
<thead>
<tr>
<th>Metabolic variables of dogs</th>
<th>Group</th>
<th>Body weight (kg)</th>
<th>Fasting blood glucose (mmol/l)</th>
<th>Plasma glucose disappearance rate (μmol/min)</th>
<th>Glucose excretion (mmol/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metabolically healthy dogs (n = 23)</td>
<td>19.0 ± 2.7</td>
<td>4.3 ± 0.5</td>
<td>15.0 ± 1.0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>Untreated alloxan-diabetic dogs (n = 23)</td>
<td>17.8 ± 2.0</td>
<td>14.4 ± 1.5 *</td>
<td>6.0 ± 2.0 *</td>
<td>46 ± 14 *</td>
</tr>
</tbody>
</table>

* P < 0.01 versus value in healthy controls.
3.1. Reactivity of vascular endothelium to acetylcholine

In vivo intracoronary administration of acetylcholine induced a dose-dependent increase in conductivity of the coronary arterial bed in both healthy and diabetic dogs (Fig. 1A). The basal haemodynamic parameters did not differ in the two groups (mean arterial blood pressure: healthy 107.1 ± 7.8 mmHg, diabetic 105.7 ± 15.2 mmHg; coronary blood flow: healthy 49.1 ± 7.8 ml/min, diabetic 52.7 ± 11.8 ml/min). However, vasodilatory responsiveness in the diabetic dogs proved to be significantly less than that in the control group. This difference may be a consequence of the diminished increase in coronary blood flow since mean arterial blood pressure was not affected differently by acetylcholine in the control and diabetic animals.

In isolated canine coronary strips, acetylcholine produced a similar degree of relaxation of the precontracted vessels (Fig. 2) from the healthy and diabetic animals.

Since the cGMP level may reflect nitric oxide formation, its level was determined. Under basal conditions the amount of cGMP in the superfusates of isolated coronary arterial rings was considerably (\( P < 0.05 \)) less in diabetic (1.08 ± 0.11 fmol · mg\(^{-1}\) vascular tissue) than in healthy (1.92 ± 0.17 fmol · mg\(^{-1}\) vascular tissue) tissues. Acetylcholine increased the cGMP level only in the control group, whereas it did not affect this parameter in the diabetic vessels (Fig. 3).

3.2. cGMP release in the presence of precursor of nitric oxide

\textit{L}-Arginine, the precursor of nitric oxide synthesis, in a dose of 1 mmol · l\(^{-1}\) enhanced the release of cGMP in healthy coronary arteries, but did not alter it in diabetic coronary arteries (Fig. 4).
3.3. Endothelial interaction between the NO system and vasoactive prostanoids

After pretreatment of anaesthetized dogs with 140 \( \mu \text{mol} \cdot \text{kg}^{-1} \) acetylsalicylic acid, the acetylcholine-induced elevation of coronary conductivity was not altered in the healthy dogs, whereas in the diabetic group acetylcholine induced a dose-dependent decline in coronary conductivity (Fig. 1B).

According to the in vitro observations, the dose–response curves of healthy and diabetic dogs did not differ markedly, whereas in the presence of either indomethacin or ibuprofen the relaxing effect of acetylcholine was considerably reduced in the coronary arteries of diabetic dogs (Fig. 2). Similarly to the in vivo observations, cyclooxygenase inhibition by either indomethacin or ibuprofen did not affect the alterations induced by acetylcholine in the isolated control vessels.

3.4. Sensitivity of vascular smooth muscle to directly acting dilators

Sodium nitrite, by relaxing directly the vascular smooth muscle cells, induced a dose-dependent increase in coronary conductivity and its effect was not different in healthy (from 110 ± 8% to 248 ± 42%) and diabetic states (from 121 ± 14% to 240 ± 38%). This reactivity was not influenced by indomethacin or acetylsalicylic acid in either group.

The dose-dependent decrease in vascular tone by sodium nitroprusside in vitro proved to be also similar in the healthy and diabetic groups (EC\(_{50}\); healthy 8.6 ± 0.4 nmol \( \cdot \) \( \text{l}^{-1} \), diabetic 8.3 ± 0.3 nmol \( \cdot \) \( \text{l}^{-1} \)).

Also, the cGMP level measured in the superfusates of isolated coronary arteries elevated by 1 \( \mu \text{mol} \cdot \text{l}^{-1} \) sodium nitroprusside did not differ significantly in the healthy (178 ± 32%) and diabetic (149 ± 28%) coronary arteries.

4. Discussion

In this study, the influence of diabetes on NO-mediated vasodilation of coronary arteries was investigated in vivo and in vitro. The vasodilation elicited by acetylcholine is impaired in the coronary arterial beds of diabetic dogs compared with that of controls studied in vivo. However, surprisingly, in vitro using isolated coronary vessels, differences in acetylcholine reactivity between vessels taken from healthy controls and diabetic animals were not observed. The acetylcholine-evoked, endothelium-dependent vasodilation in diabetic blood vessels was widely examined. The majority of data indicate that it is impaired both in large vessels [7,11–16] and in the microcirculation [17–19]. As far as the various regions of the vasculature are concerned, results are mostly obtained using aorta [7,12–14,16], cerebral vessels [11,17] and mesenteric arteries [18,19]. There is limited experience with coronary arteries. The discrepancy between our in vivo and in vitro data could be explained by the different types of vascular bed: in vivo, vascular reactivity is mainly determined by the whole coronary circulation and especially by the resistance vessels, whereas in vitro the large epicardial coronaries were studied.

Measurements of cGMP levels in isolated coronary arterial rings demonstrate a diminished NO-mediated process in diabetic coronary preparations as compared to controls. On the one hand, this observation seems to be in accordance with those of most other authors [13,20] in rat aorta and glomeruli. On the other hand, diabetes was found to increase endothelial NO synthase activity, although the NO-mediated vasodilation in isolated rat heart was impaired [21]. This unexpected result has been explained by increased quenching of the elevated amount of free radicals and superoxide ions formed in diabetes. Differences in species and diabetes duration are suggested to be responsible for these contrasting observations. The duration of experimental diabetes does not seem to modulate NO-mediated processes in the same way. Two weeks after diabetes induction [7] a defect could be observed in receptor sensitivity. Twelve to 17 week diabetic vessels show less basal cGMP release (see Section 3), while after a longer period (26–52 weeks) of diabetes, diabetic vascular tissues are supposed to counteract it by increased NO synthesis up to the limitation of precursor quantity [21].

In non-diabetic canine isolated coronary arteries acetylcholine stimulates cGMP release (Fig. 3), but not in the diabetic group. This provides a good explanation for the diminished vasodilation in the diabetic coronary arterial bed in vivo (Fig. 1).

The defect in cGMP release could be caused not only by changes in NO synthase activity but also by the reduced availability of arginine as substrate for NO synthesis. Data demonstrate that the plasma concentration of arginine is decreased after diabetes induction [16,21]. Nevertheless, in contrast to controls, the coronary arteries of diabetic dogs preincubated with a significant amount of \( \ell \)-arginine do not show elevation of cGMP release (Fig. 4). Thus, these results indicate a defect of \( \ell \)-arginine utilization by NO synthase and/or in the coupling processes between receptor and NO synthase activity, and a reduced availability of substrate can be excluded.

Our following data are in agreement with those of others [11,15–17]: the non-receptor-mediated vasorelaxation induced by sodium nitroprusside, sodium nitrite or papaverine proved to be similar in control and diabetic blood vessels both in vitro and in vivo.

Previously, alterations in vascular prostaglandins were detected in diabetes [22]. Although in large coronary arteries basal prostacyclin release is not diminished, in diabetic coronaries the release of this prostanoid could not be stimulated either by adrenergic agonists [22] or by hypoxia [23]. In contrast, the synthesis of thromboxane by diabetic
coronary arteries is markedly elevated even under basal circumstances [22]. Not only the formation, but also the sensitivity of vascular smooth muscle to thromboxane [25] is augmented in diabetic myocardium. Inhibition of cyclooxygenase could not reverse endothelial dysfunction in diabetic rat aorta [14,26–29], mesenteric arteries [19], basilar arteries [11] or in macrophages [24]. A possible explanation for these data is that vasoactive prostanoids in certain vascular regions do not play an important role in vascular reactivity alterations [30]. Secondly, vasoconstrictor prostaglandins per se may not contribute significantly to the endothelial dysfunction in different diabetes models and in various parts of the vasculature. Accordingly, the entity of endothelial dysfunction might not be uniform in all vessel types. Indomethacin could also act by the inhibition of prostacyclin synthesis. We assume, therefore, that acetylcholine-induced vasodilation both in vivo (Fig. 1) and in vitro (Fig. 2) is further depressed in diabetic dogs due to the decreased formation of prostacyclin by indomethacin, ibuprofen or acetylsalicylic acid. We assume that, in controls, stimulation of NO formation can counteract and compensate the inhibited prostaglandin or more specifically the prostacyclin formation by cyclooxygenase inhibitors seen both in vivo and in vitro (Figs. 1 and 2). Although results suggest the presence of synergism between the actions of nitric oxide and prostacyclin [31] in the diabetic coronary circulation, the impaired capacity of both NO generation and that of prostanoids in the case of increased flow demand is not able to be compensated.

The present findings and conclusions can be summed up as follows: (1) The non-receptor-mediated vasorelaxation in coronaries is not altered in alloxan-diabetic dogs. (2) The sensitivity of the coronary arterial bed to acetylcholine is diminished in diabetes. (3) Basal NO synthesis estimated by cGMP release is markedly diminished in diabetes and could not be stimulated either by acetylcholine or by L-arginine. From these data we conclude that a primary defect involves the function and action of NO, which cannot be compensated by L-arginine and excludes insufficient substrate availability as cause. (4) Cyclo-oxygenase inhibition further impairs responsiveness to acetylcholine. Accordingly, the release of vasoactive prostanoids can partly compensate the defects in the function and action of NO in diabetic canine coronaries. Impaired endothelial function may be associated with defects in formation of NO, in its substrate utilization and in signal transduction between acetylcholine receptors and NO synthase activity, as well as with diminished compensating ability of prostacyclin.

Acknowledgements

This work was supported by a grant from the National Research Foundation OTKA T 6052.

References


