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European Journal of Pharmacology 374 (1999) 221–227

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Plasma endothelin levels and vascular responses at different temporal stages of streptozotocin diabetes

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Received 14 January 1999; received in revised form 20 April 1999; accepted 26 April 1999

Abstract

While alterations in the release and action of endothelial derived vasoactive factors such as endothelin-1 and endothelial derived relaxing factor (EDRF) occur in diabetes mellitus, the nature and direction of these changes is controversial. We examined the role of diabetes duration on endothelin-1 plasma levels and vasoconstrictor responses to endothelin-1 in aortic rings from streptozotocin-induced diabetic rats. Endothelin-1 plasma levels were attenuated at 2-weeks, but conversely elevated at 14-weeks, after diabetes induction. Similarly, maximal vasoconstriction to endothelin-1 in aorta was exaggerated in 2-week, but attenuated in 14-week diabetic rats. Also, sensitivity of aorta to endothelin-1 was enhanced in the 14-week group. Neither nitric oxide synthase inhibition with nitro-L-arginine-methyl-ester, nor endothelium removal affected alterations in maximal vasoconstriction, but both abolished changes in sensitivity to endothelin-1. Endothelium dependent (acetylcholine-evoked) vasorelaxation responses were attenuated in 14-week, but not 2-week, diabetic rats, while endothelium independent (sodium nitroprusside-evoked) responses remained unchanged. Together, these data indicate a deficiency in EDRF production in the 14-week group. Elevated plasma glucose and attenuated insulin levels were present in both groups, but plasma cholesterol and triglyceride levels were elevated only in 14-week rats. We conclude that differences in the pre-existing duration of diabetes differentially affect both plasma levels and action of endothelin-1. These changes might be linked to coincident diabetes duration dependent changes in EDRF production and plasma lipid levels. Such a shift in the production and action of endothelial derived relaxing and contracting factors might contribute to the characteristic early and late stage cardiovascular complications of diabetes mellitus.

Keywords: Aorta; Endothelial derived relaxing factor; Endothelin-1; Streptozotocin diabetic, rat; Vascular responsiveness

1. Introduction

Early type I diabetes is associated with changes in vascular function leading to over-perfusion of the vasculature (Pieper, 1998), whereas the later stages are associated with atherosclerotic and hypertensive changes promoting the well-known late stage complications of the disease (Epstein and Sowers, 1995). While the mechanisms of these changes are not completely understood, it is possible that shifts in the actions of endothelial derived relaxing and contracting factors might contribute. In support of this, both the production and action of the highly potent vasoconstrictor/mitogenic peptide, endothelin-1, has been shown to be altered in various models of type I diabetes.

Both elevated and depressed endothelin-1 release and plasma levels, as well as vascular smooth muscle responses to the peptide have been observed in both human (Takahashi et al., 1990; Haak et al., 1992; Smulders et al., 1994; Malamitsi-Puchner et al., 1996) and animal (Fulton et al., 1991; Takahashi et al., 1991; Takeda et al., 1991; Frank et al., 1993; Hopfner et al., 1998a,b; Makino and Kamata, 1998) models of type I diabetes. The reasons for this inter-study variability are ill-defined. Such disparities might be explained by variability in metabolic and vascular factors that are known to contribute to endothelin-1 release and action. Such factors include endothelial function (Vermes et al., 1993), endothelial derived relaxing factor (EDRF) production (Boulanger and Luscher, 1990), insulinemia (Oliver et al., 1991; Frank et al., 1993; Hopfner et al., 1998a,b), glycemia (Hattori et al., 1991), and lipidemia (Lerman et al., 1993). Interestingly, varied duration of exposure to the metabolic dysregulation of diabetes has

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00316-7

Table 1
Metabolic variables in control and streptozotocin diabetic rats 2- and 14-weeks after diabetes induction

	2-week		14-week	
	Control	Diabetic	Control	Diabetic
Body weight before induction (g)	388 ± 7	392 ± 7	396 ± 9	399 ± 6
Body weight at time of sacrifice (g)	420 ± 10[a]	374 ± 10[a]	511 ± 10[a]	281 ± 24[b]
Plasma glucose (mM)	5.3 ± 0.3	25.1 ± 2.0[d]	5.2 ± 0.2	25.0 ± 0.9[d]
Plasma insulin (pM)	533 ± 49	159 ± 18[d]	645 ± 81	124 ± 15[d]
Plasma cholesterol (mg dl^{-1})	67 ± 8	76 ± 4	85 ± 8	144 ± 11[c]
Plasma triglycerides (mg dl^{-1})	132 ± 18	191 ± 27	110 ± 18	1278 ± 204[d]

[a] $P < 0.05$; [b] $P < 0.01$ vs. respective weight before group.
[c] $P < 0.01$; [d] $P < 0.001$ vs. respective age matched control.
Data are mean ± S.E.M. from 8–10 separate rats.

been shown to variably alter many of these factors (Morff, 1990; Yoshino et al., 1992; Pieper and Siebeneich, 1998). Thus, it is possible that changes in endothelin-1 release and action may depend on the temporal stage of diabetes in which these parameters are measured. With this premise, the present study examines both plasma levels and vascular responses to endothelin-1 in conjunction with measurements of metabolic variables and endothelial function in streptozotocin diabetic rats examined at two different time points after diabetes induction.

2. Materials and methods

2.1. Animals

Diabetes was induced in 9-week old male Sprague–Dawley rats (150–200 g; Charles River Laboratories, Montreal, Canada) with a single dose of streptozotocin (diabetic group; 55 mg kg^{-1}) and confirmed 1 week later, as previously described (Hopfner et al., 1998b). Animals were separated into four separate groups: (a) streptozotocin treated rats killed 2 weeks after treatment (diabetic—2-week) and age-matched controls (control—2-week); and (b) streptozotocin treated rats killed 14 weeks after injection (diabetic—14-week) and age-matched controls (control—14-week). Animals were housed in animal quarters with a 24-h light–dark cycle and were fed standard rat chow ad libitum.

2.2. Analytical procedures

At the end of either 2 or 14 weeks after diabetes injection, animals were killed by decapitation under ether anesthesia. Blood was collected in tubes containing EDTA, centrifuged at $2000 \times g$ for 10 min, and stored at −70°C until plasma insulin, lipid, and endothelin-1 measurements were undertaken. Plasma glucose was measured on the day of sacrifice by glucose oxidase method (One Touch Basic, Lifescan, Vancouver, Canada), and plasma insulin and endothelin-1 were measured using RIA kits (Amersham, Oakville, Canada) as described earlier (Hopfner et al., 1998b). Plasma cholesterol and triglycerides were measured using standard enzymatic methods (Bucolo and David, 1973; Allain et al., 1974).

2.3. Isometric tension measurements

Ring preparations of the thoracic aorta (~5 mm) were dissected from each rat, cleared of adhering fat and connective tissue, and set up for isometric tension measurements in 20 ml organ baths containing modified Kreb's physiological salt solution [(in mM) NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgCl_2 \cdot 6H_20$ 1.2, $CaCl_2 \cdot 2H_2O$ 1.8, $NaHCO_3$ 25.0, and glucose 11.1] continuously oxygenated with 95% O_2/5% CO_2 at 37°C. Endothelium removal was facilitated in the specified groups by gentle rubbing of the ring between thumb and forefinger, and lack of responsive-

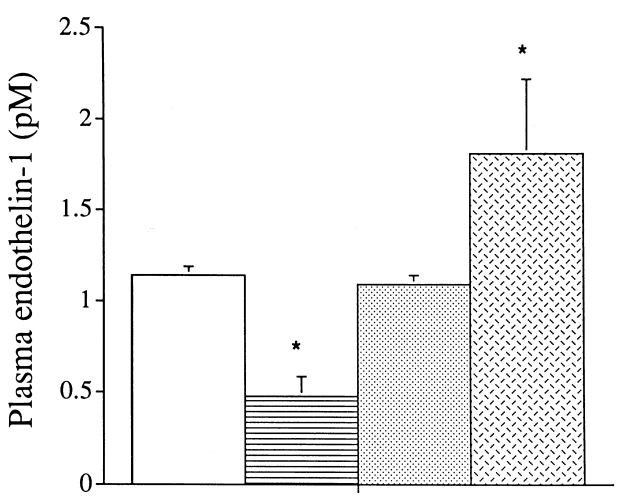

Fig. 1. Plasma endothelin-1 levels in control 2-week (open bar), diabetic 2-week (side-hatched bar), control 14-week (spotted bar), and diabetic 14-week (cross-hatched bar) groups. Bars represent mean ± S.E.M. of plasma samples from eight different rats in each group. * $P < 0.05$ vs. respective age matched control.

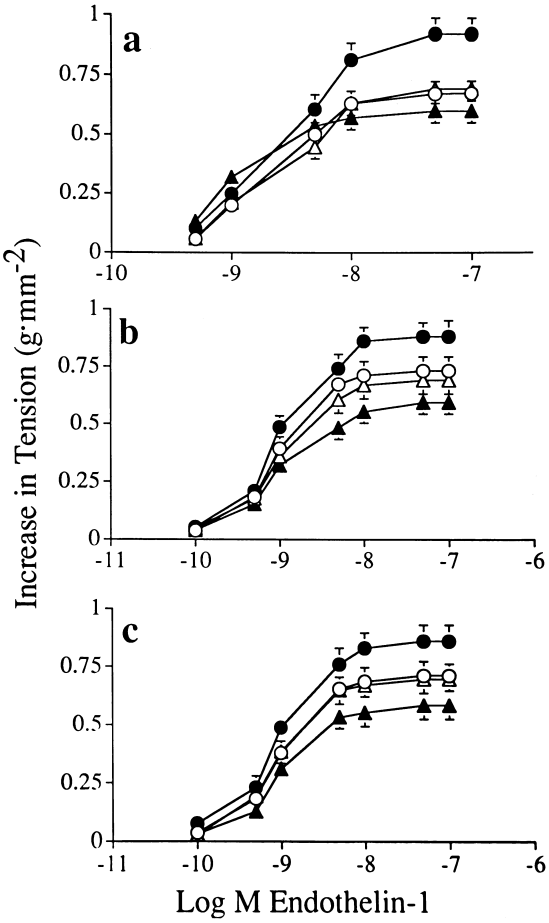

Fig. 2. Concentration–response curves for endothelin-1 (100 pM–100 nM) evoked vasoconstriction in isolated aortic rings obtained from 2-week (closed circles) and 14-week (closed triangle) diabetic and 2-week (open circle) and 14-week (open triangle) age-matched control rats. Isometric tension responses were recorded in aortic rings with both intact (a) and denuded (b) endothelium, as well as in preparations with intact endothelium in the presence of nitro-L-arginine-methyl-ester (L-NAME; 100 μM) (c) which was included in the buffer 30 min prior to endothelin-1 challenge. The ordinate scale depicts the increase in isometric tension development measured at the peak of the response for each concentration of endothelin-1. Each data point represents the mean ± S.E.M. of responses derived from aortic segments from 8–10 different rats in each group.

ness to acetylcholine was used to confirm denudation. Increases in isometric tension under basal (preload tension 2 g) and agonist-evoked conditions were recorded on a Grass 7E polygraph with FTO · 3 force transducers (Hopfner et al., 1998b).

Aortic rings were allowed to equilibrate for 1 h, after which a phenylephrine (30 μM) challenge was administered to stabilize the preparation. After return to baseline, cumulative concentration response curves were assessed in separate aortic segments from 8–10 rats in each group to the following vasoconstrictor stimuli: (a) endothelin-1 (100 pM–100 nM), (b) methoxamine (10 nM–100 μM) and (c) KCl (10–80 mM). Acetylcholine (5 nM–5 μM) and sodium nitroprusside- (1 nM–1 μM) evoked relaxations were determined in methoxamine (100 μM) preconstricted rings. In order to block nitric oxide synthase activity, nitro-L-arginine-methyl-ester (L-NAME; 100 μM) was added to the incubation buffer in specified ring segments 20 min prior to agonist challenge. Tension development was expressed in g mm^{-2} as described earlier (Hopfner et al., 1998b).

2.4. Drugs

Acetylcholine chloride, nitro-L-arginine-methyl-ester (L-NAME), methoxamine hydrochloride, phenylephrine hydrochloride, sodium nitroprusside, and streptozotocin were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Human/porcine/rat endothelin-1 was obtained from American Peptide (Sunnyvale, CA, USA).

2.5. Data analysis

Cumulative concentration–response curves were determined individually in order to express concentration of

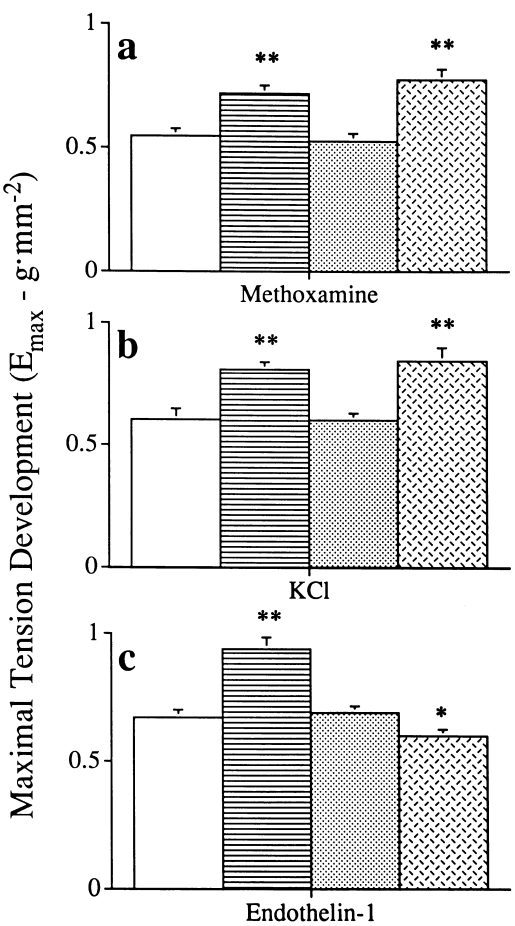

Fig. 3. Comparison of maximum evoked vasoconstriction (E_{max}) to (a) methoxamine; (b) potassium chloride and (c) endothelin-1 in isolated aortic rings from control—2-week (open bar), diabetic—2-week (side-hatched bar), control—14-week (spotted bar), and diabetic—14-week (mixed-hatch bar) groups. Each bar represents the E_{max} mean ± S.E.M. of isometric tension development examined by graded dose–response assessments in individual aortic segments from 8–10 different rats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. respective age matched control.

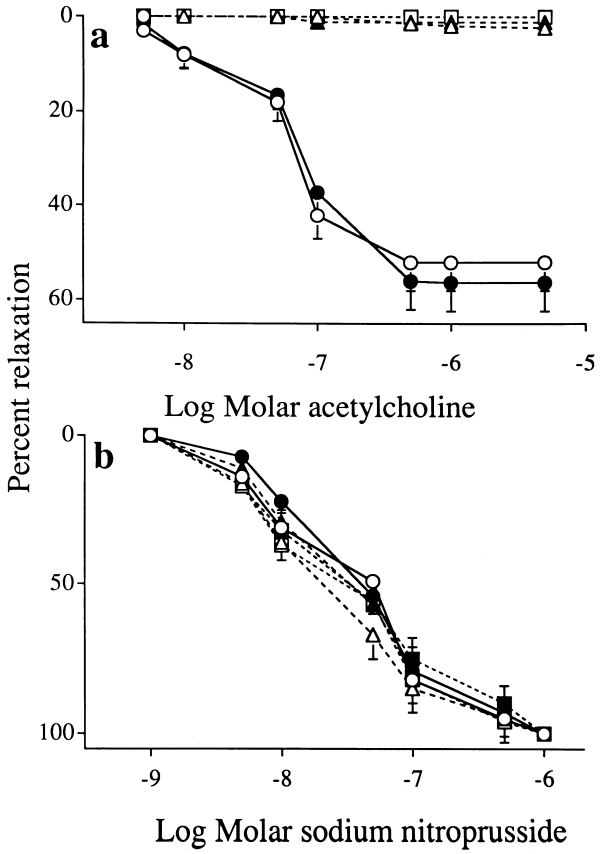

Fig. 4. Concentration–response curves to acetylcholine (5 nM–5 μM) (a) and sodium nitroprusside- (1 nM–1 μM) (b) evoked vasorelaxation of methoxamine (100 μM) precontracted aortic rings isolated from diabetic (closed points) and age-matched control (open points) rats 2 weeks after treatment. Rings were prepared either with intact endothelium (circles), denuded endothelium (triangles), or in the presence of intact endothelium and preincubated with L-NAME (squares; 100 μM; 30 min). The ordinate scale depicts the percent relaxation of maximal methoxamine evoked contraction. Data points represent the mean ± S.E.M. relaxation responses in individual aortic segments from 8–10 different rats in each group.

half-maximal response (pD_2) and maximal response (E_{max}) values as mean ± S.E.M. Acetylcholine- and sodium nitroprusside-evoked vasodilator responses were expressed as the percentage of inhibition of methoxamine-evoked contraction. Comparison of mean values amongst the various groups was performed by analysis-of-variance (SuperANOVA program-SAS Institute, San Francisco, CA, USA). Simultaneous multiple comparisons were examined by Scheffe's *F*-test.

3. Results

3.1. Metabolic variables

Table 1 depicts the metabolic characteristics in each group. Both groups of diabetic rats lost weight while control rats significantly gained weight over the same period. Plasma glucose levels were elevated and plasma insulin levels were attenuated to a similar extent in both 2- and 14-week diabetic groups. Plasma cholesterol and triglycerides were significantly elevated in the 14-week, but not the 2-week diabetic group.

3.2. Plasma endothelin-1

Plasma endothelin-1 levels were attenuated ($P < 0.05$) in the 2-week diabetic group, but were conversely elevated ($P < 0.05$) in the 14-week group compared to respective age-matched controls (Fig. 1).

3.3. Vascular responsiveness

Vascular response data for endothelin-1 and other agonists are shown in Figs. 2–5. Vasoconstrictor responses to endothelin-1 were shifted to the left (increased sensitivity) in aorta from 14-week, but not 2-week, diabetic rats

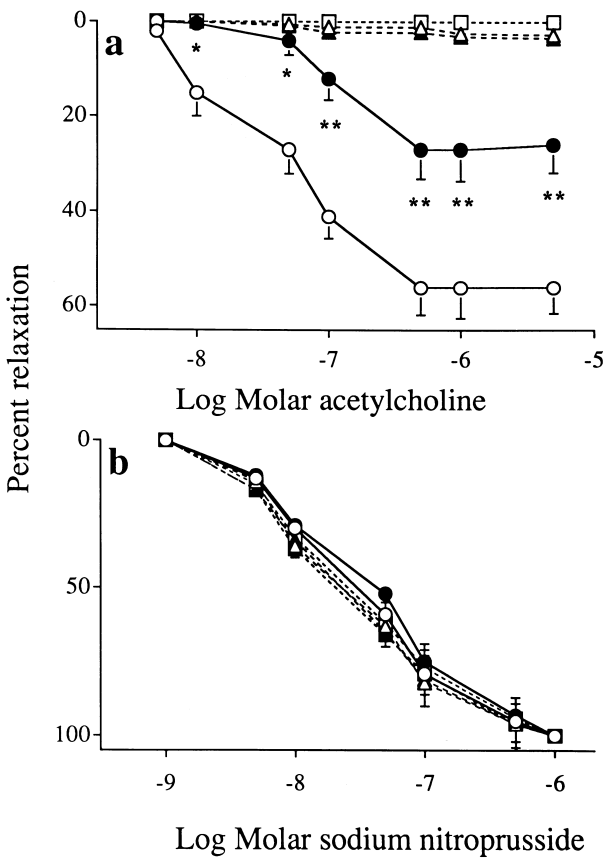

Fig. 5. Concentration–response curves to acetylcholine (5 nM–5 μM) (a) and sodium nitroprusside- (1 nM–1 μM) (b) evoked relaxation of methoxamine (100 μM) precontracted aortic rings isolated from diabetic (closed points) and age-matched control (open points) rats 14 weeks after treatment. Rings were prepared either with intact endothelium (circles), denuded endothelium (triangles), or in the presence of intact endothelium and preincubated (30 min) with L-NAME (squares; 100 μM). Each data point represents the mean ± S.E.M. percent relaxation of maximum methoxamine evoked contraction in individual aortic ring segments from 8–10 different rats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the same data point in 14-week control group.

Table 2
pD_2 ($-\log EC_{50}$) and E_{max} (g mm^{-2}) values for endothelin-1-evoked vasoconstriction in aortic segments from 2- and 14-week control and diabetic rats

Group	2-week		14-week	
	Control	Diabetic	Control	Diabetic
Endothelium intact				
E_{max}	0.67 ± 0.02	0.94 ± 0.04^b	0.69 ± 0.02	0.60 ± 0.02^a
pD_2	8.60 ± 0.03	8.56 ± 0.03	8.57 ± 0.02	8.87 ± 0.03^b
Endothelium denuded				
E_{max}	0.73 ± 0.03	0.88 ± 0.05^b	0.69 ± 0.02	0.59 ± 0.02^a
pD_2	9.08 ± 0.02	9.06 ± 0.02	9.05 ± 0.03	9.04 ± 0.02
Endothelium intact + L-NAME				
E_{max}	0.71 ± 0.04	0.86 ± 0.02^b	0.70 ± 0.01	0.58 ± 0.03^a
pD_2	9.00 ± 0.04	8.99 ± 0.03	9.00 ± 0.03	8.99 ± 0.03

$^aP < 0.05$; $^bP < 0.01$ vs. respective age matched control.

(Fig. 2a, Table 2). Both endothelium removal (Fig. 2b) and L-NAME (Fig. 2c) abolished this difference (Table 2), indicating that EDRF mediated mitigation of endothelin-1-evoked vasoconstriction is attenuated in 14-week rats.

Maximal vasoconstriction to both methoxamine (Fig. 3a; α_1-adrenoceptor agonist) and KCl (Fig. 3b; depolarizing stimuli) was elevated in aortic rings from both diabetic groups, indicating the presence of a non-specific increase in vascular contractility in diabetic rats regardless of the pre-existing duration of diabetes. However, while maximal vasoconstriction to endothelin-1 was similarly exaggerated in the 2-week diabetic group, it was actually slightly lower than control levels in the 14-week group (Fig. 3c). The alterations in maximal vasoconstriction persisted even after endothelial denudation or L-NAME pretreatment (Fig. 2b, c, Table 2), indicating that the source of this abnormality is at the vascular smooth muscle level—independent of either EDRF or endothelial function.

Maximal acetylcholine-evoked vasorelaxation was attenuated in aorta from 14-week (Fig. 5a), but not 2-week (Fig. 4a) diabetic rats. In addition, the dose–response curve was shifted to the right (decreased sensitivity) in the 14-week diabetic group (Fig. 5a). Both endothelium denudation and preincubation with L-NAME completely abolished vasodilator responses to acetylcholine (Fig. 4a-Fig. 5a), confirming the endothelium dependence, and more specifically, the EDRF dependence of this effect. Endothelium independent vasorelaxation was intact across all groups, as similar dose–response curves to sodium nitroprusside-evoked vasorelaxation were obtained in all of endothelium intact, endothelium denuded, and L-NAME pretreated aortic rings from both 2-week (Fig. 4b), and 14-week (Fig. 5b) diabetic rats.

4. Discussion

There is considerable controversy regarding the effect of diabetes on endothelin-1 release and action. The present results demonstrate that the direction of these changes is dependent on the stage of diabetes at which these parameters are measured. Several lines of evidence support our observation that endothelin-1 release is attenuated in early stage diabetes, and elevated in the later stages. Plasma endothelin-1 levels in patients with type I diabetes have been shown to be directly correlated with age (Haak et al., 1992) and studies demonstrating elevated endothelin-1 plasma levels in diabetic rats used rats with diabetes of not less than 10-weeks duration (Takeda et al., 1991; Makino and Kamata, 1998). Conversely, studies demonstrating decreased endothelin-1 plasma levels tended to use models of diabetes in early stages. Attenuated plasma endothelin-1 levels were observed in children and adolescents and in patients with early-uncomplicated type I diabetes (Smulders et al., 1994; Malamitsi-Puchner et al., 1996) and studies in diabetic rats revealing attenuated endothelin-1 plasma levels used rats with diabetes of not greater than 5 weeks duration (Takahashi et al., 1991; Frank et al., 1993; Hopfner et al., 1998a,b).

Plasma endothelin-1 is thought to be derived primarily from the vascular endothelium (Lerman et al., 1991; Vermes et al., 1993). Thus, decreased plasma endothelin-1 in early diabetes likely reflects decreased endothelial production and release of the peptide. This is supported by a recent study demonstrating that endothelin-1 release is attenuated in aorta and mesenteric arteries from 2- and 4-week streptozotocin diabetic rats (Wu and Tang, 1998). Endothelin-1 is produced primarily in endothelial cells by gene activation of preproETmRNA and subsequent processing by proteolytic enzymes into the biologically active peptide (Yanagisawa et al., 1988). Suppression of endothelin-1 production may be mediated by metabolic factors such as hyperglycemia, or by attenuated plasma insulin levels, both of which are known to modulate endothelin-1 production in endothelial cells in vitro (Hattori et al., 1991; Oliver et al., 1991). Indeed, we have previously demonstrated that restoration of metabolic control restores attenuated plasma endothelin-1 levels to normal in 5-week diabetic rats (Hopfner et al., 1998b). An interesting possibility is that elevations in EDRF production, which have recently been demonstrated in the early stages of diabetes (Pieper and Siebeneich, 1998), might act to suppress endothelin-1 production (Boulanger and Luscher, 1990). Importantly, any such mechanism suppressing endothelin-1 is likely only relevant when endothelial function is intact.

In contrast to the 2-week group, endothelin-1 plasma levels are conversely increased in 14-week diabetic rats. Possible reasons for increased endothelin-1 plasma levels after chronic exposure to diabetes include: (a) a lack of suppression of endothelin-1 release secondary to attenuated EDRF production (Boulanger and Luscher, 1990); (b) endothelial damage itself contributing to increased endothelin-1 release (Vermes et al., 1993); or (c) elevated plasma lipoprotein stimulated increases in endothelin-1 production (Lerman et al., 1993). Indeed, the present study

demonstrates that 14-week, but not 2-week, diabetic rats exhibit both decreased EDRF and elevated plasma lipids —suggesting that one or both of these factors may be responsible for the shift in endothelin-1 plasma levels in the late stage group.

Vasoconstriction to methoxamine and KCl is enhanced in aorta from both 2- and 14-week rats, but to endothelin-1 in 2-week rats only. Increased ex vivo vasoconstriction has previously been reported in streptozotocin diabetes and has been attributed to either modification of Ca^{2+} channel expression and action or hyperglycemia induced protein kinase C activation (Abebe and Macleod, 1990; White and Carrier, 1990). Thus, exaggerated vasoconstriction to endothelin-1 in 2-week diabetic rats might be a result of a non-specific elevation in vascular smooth muscle contractility. However, endothelin receptor changes cannot be ruled out since attenuated endothelin-1 release could theoretically lead to up-regulation of endothelin receptors. In contrast to the 2-week group, endothelin-1-evoked responses are selectively attenuated in aorta from 14-week diabetic rats. Others (Guillon et al., 1998) have also observed selective decreases in vasoconstriction to endothelin-1 in streptozotocin diabetes. Hyperglycemia mediated protein kinase C activation has been demonstrated to selectively down regulate both receptors for, and responses to, endothelin-1 (Awazu et al., 1991; Xu et al., 1993). Furthermore, increases in endothelin-1 plasma levels are known to cause homologous down-regulation of endothelin receptors (Cozza et al., 1990). Thus, receptor down-regulation and/or protein kinase C activation may be responsible for the selective suppression of endothelin-1-evoked vasoconstriction in 14-week diabetic rats.

Endothelium dependent vasorelaxation to acetylcholine is attenuated in 14-week, but not 2-week, diabetic rats. Since L-NAME pretreatment completely abrogated the response to acetylcholine, it is concluded that EDRF mediated vasorelaxation is attenuated in the 14-week group. Endothelium independent vasorelaxation to sodium nitroprusside was intact in both groups—suggesting that attenuated EDRF production, but not responsiveness, is attenuated in 14-week rats. In support of this, previous studies have shown that EDRF action becomes attenuated only after long term exposure to diabetes (Pieper, 1998), occurring secondary to progressive endothelial damage and other indirect actions of metabolic dysregulation (Epstein and Sowers, 1995). The fact that cholesterol and triglycerides are elevated only in the 14-week diabetic group attests to a possible role for lipoproteins in the progression of endothelial dysfunction and inhibition of EDRF release. Importantly, these data demonstrate a clear shift in the production of endothelial derived factors (i.e., increased endothelin-1 and decreased EDRF) favoring vasoconstriction in the late stage group.

Alterations in the balance of release and action of endothelial derived relaxing and contracting factors are thought to contribute to vascular dysfunction in a wide variety of cardiovascular disorders, including diabetes mellitus (Epstein and Sowers, 1995; Pieper, 1998). The present observation that endothelin-1 production is decreased in early streptozotocin-induced diabetes, in conjunction with the recent demonstration that vascular EDRF action is enhanced in early diabetes (Pieper and Siebeneich, 1998), suggests that a shift in the balance of endothelial vasoregulation favoring vasodilatation may contribute to increased capillary perfusion observed in early type I diabetes (Pieper, 1998). Conversely, elevated endothelin-1 release in conjunction with attenuated EDRF production in chronic diabetes may reverse this balance contributing to the hypertensive and atherosclerotic changes observed in the late stages of the disease (Epstein and Sowers, 1995). Further studies with endothelin antagonists are warranted to determine the role of this peptide in such changes in vascular function.

5. Conclusion

The present study shows, for the first time, that endothelin-1 plasma levels and endothelin-1-evoked vascular responses in diabetic rats are differentially altered at different stages of diabetes. Attenuated plasma levels of endothelin-1 prevail in early diabetes along with exaggerated vascular responses to the peptide. Later stage diabetes is associated with elevated plasma endothelin-1 levels in parallel with attenuated vascular responses to endothelin-1, suppressed EDRF action, and elevated plasma lipids. These observations attest to a pathophysiological role for a shift in the balance of endothelin-1 and EDRF in contributing to vascular dysfunction encountered in diabetes. Indeed, recent observations that endothelin-1 antagonists prevent some of the cardiovascular abnormalities in streptozotocin-induced diabetic rats indicate that alterations in the actions of this highly potent vasoconstrictor/mitogenic peptide might play a critical role in promoting vascular disease in type I diabetes (Stevens and Tomlinson, 1995; Cameron and Cotter, 1996; Benigni et al., 1998).

Acknowledgements

This work was supported by a grant from the Medical Research Council (MRC) of Canada (Grant no. MT-14377) and an MRC Doctoral Research Award to Mr. Rob L. Hopfner.

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