

Time-dependent hyperreactivity to phenylephrine in aorta from untreated diabetic rats: role of prostanoids and calcium mobilization

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Abstract

Diabetes alters vascular smooth muscle contractility. Changes in reactivity to phenylephrine (Phe) in aortas from controls and untreated 1- and 4-week streptozotocin (STZ)-induced diabetic rats were investigated. In 1-week diabetic (DB1) aortas, the maximum response (E_{\max}) and sensitivity (pD_2) to Phe were similar to controls (CT1), but in 4-week diabetic (DB4) aortas, the E_{\max} for Phe was increased compared to CT4 aortas (E_{\max} , DB4: $125 \pm 8.4\%$ vs. CT4: $89.8 \pm 4.5\%$, $P < .001$). Endothelial denudation increased the response to Phe, and E_{\max} was increased in the DB4 aortas compared to CT4 (E_{\max} , DB4: $156 \pm 4.2\%$ vs. CT4: $125 \pm 3.8\%$, $P < .001$). Pretreatment of CT4 and DB4 aortas with indomethacin reduced E_{\max} and pD_2 for Phe. After indomethacin treatment, no differences in E_{\max} and pD_2 to Phe were observed in either group. SQ 29548 did not alter the Phe actions in CT4 aortas. However, in DB4 aortas, E_{\max} was reduced to control level. CT4 and DB4 aortas incubated in free- Ca^{2+} solution plus Phe, contracted upon addition of $CaCl_2$, this response was increased in DB4 aortas. No changes were observed for acetylcholine (ACh) or sodium nitroprusside (SNP) responses. Nitric oxide (NO) release in response to Phe determined by acute L-NAME administration showed no differences in the percentage increase of the contraction in CT1 and DB1 aortas, but was enhanced in DB4 aortas. Results suggested that diabetes induces time-dependent changes in the vascular reactivity to Phe. This response is not related to a reduction of endothelium-derived NO but might be due to an increase in prostaglandin H_2 (PGH₂)/thromboxane A₂ (TxA₂) and/or an enhanced extracellular Ca^{2+} influx.

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1. Introduction

Complications due to cardiovascular diseases are among the major reasons for mortality occurring in diabetic patients (Garcia et al., 1974). However, the exact mechanisms of its development are not completely elucidated. Previous reports suggest that enhanced vascular reactivity to vasoconstrictor agents (Abebe et al., 1990; White and Carrier, 1990) or impairment of the vascular relaxation (Fortes et al., 1983; Pieper and Gross, 1988; Tesfamariam et al., 1989) contribute to the cardiovascular complications associated with diabetes mellitus. Enhanced vascular reactivity to α_1 -adrenoreceptor agonists was demonstrated in different vascular beds from diabetic animals (Abebe et al., 1990; White and Carrier,

1990; Inazu et al., 1991; Taylor et al., 1994). However, unchanged (Chang and Stevens, 1992; Heygate et al., 1995) or attenuated (Ramanadham et al., 1984; Oyama et al., 1986) responses have also been described. The reason for these differences is not entirely clear, but contributing factors may be differences in the species, duration of diabetes and type of vascular preparation studied. Moreover, the mechanisms responsible for an increased contractile responsiveness of diabetic arteries to vasoconstrictor agonists are not completely understood. However, alterations of endothelial function (Chang and Stevens, 1992; Özçelikay et al., 2000), enhanced calcium mobilization (Abebe et al., 1990; White and Carrier, 1990) and inhibition of Na^+ , K^+ -ATPase activity (Ohara et al., 1991; Davel et al., 2000) appear to contribute to these changes in vascular reactivity.

It is well established that diabetes is associated with an endothelial dysfunction, characterized by impaired responses to different endothelium-dependent agonists (Fortes et al.,

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1983; Pieper and Gross, 1988; Tesfamariam et al., 1989). The decreased endothelium-dependent relaxation observed in diabetes may be due to an impaired synthesis or release of endothelium-derived relaxing factors, increased release of constricting factors such as vasoactive arachidonic acid metabolites (thromboxane A_2 [TxA_2], prostaglandin H_2 [PGH_2]) and/or inactivation of nitric oxide (NO) by free radicals (Tesfamariam, 1994). These endothelial abnormalities may contribute to the enhanced responsiveness to vasoconstrictor agonists in vascular beds from diabetic animals.

Other mechanisms that could contribute to enhanced responses to vasoconstrictor agents in vascular beds from diabetic animals have been proposed. White and Carrier (1990) suggested that the enhanced responsiveness to norepinephrine might be due to an increased mobilization of extracellular calcium. Moreover, Abebe and MacLeod (1990a) demonstrated that phosphoinositide metabolism in response to norepinephrine is enhanced in the aorta from diabetic rats. Taken together, these results suggested that the enhanced vascular contractile response to α_1 -adrenoreceptor agonists in diabetes may be due to an alteration of synthesis and/or release of the vasoactive endothelium-derived substances, increased calcium influx and release in smooth muscle cells and increased protein kinase C (PKC) activity.

Several of these mechanisms noted above were elucidated in vascular beds obtained from animals with chronic diabetes. However, little is known about the vascular reactivity of α_1 -adrenoreceptor agonists in earlier stages of diabetes. Previous results from our laboratory demonstrated an increased reactivity to phenylephrine (Phe) in the tail vascular bed from 1-week streptozotocin (STZ)-induced diabetic rats (Davel et al., 2000). However, these alterations have not been studied extensively in other vascular beds in early stages of diabetes. Thus, the purpose of this study was to determine whether there are any changes in vascular reactivity to Phe in aortas from diabetic rats at an early and an intermediate stage of the disease (1 and 4 weeks, respectively). A second purpose was to evaluate the mechanism(s) responsible for such alterations. To address these questions, possible roles of the endothelium, of vasoconstrictor prostanoids and calcium mobilization pathways in the contractile response produced by Phe were investigated.

2. Materials and methods

2.1. Animals

Male Wistar rats (260–300 g) were used in the present study. The care and use of laboratory animals were in accordance with NIH guidelines. All rats had free access to water and were fed with rat chow ad libitum.

Diabetes was induced by penile-vein injection of 50 mg/kg STZ (diluted in 0.1 M citrate buffer solution, pH 4.5) in

rats that had been anesthetized with ether. Two main groups were used in this study: 1- and 4-week STZ-treated rats and age-matched controls.

Blood glucose was measured by using a hemoglucotest (20-800 R-Boehringer Mannheim, Indianapolis, IN, USA) before and after STZ treatment. Blood pressure was measured by using a tail cuff method (IITC Model 29 Pulse Amplifier, IITC, CA, USA).

2.2. Tissue bath studies

At the end of each period, animals were anesthetized with sodium pentobarbital (35 mg/kg ip). A section of the thoracic aorta was removed and placed in cold oxygenated Krebs–Henseleit bicarbonate buffer (KHB). The buffer consisted of (in mM): NaCl 118; KCl 4.7; $NaHCO_3$ 25; $CaCl_2 \cdot 2H_2O$ 2.5; KH_2PO_4 1.2; $MgSO_4 \cdot 7H_2O$ 1.2; glucose 11 and EDTA 0.01. The aorta was cleaned of fat and connective tissue and was cut into rings of 5–6 mm length. Rings were mounted between parallel wires in tissue baths at 37 °C. The bath contained KHB gassed with 95% O_2 and 5% CO_2 to maintain the pH at 7.4. Rings from control and diabetic animals were stretched to an optimal resting tension of 1.0 g. Isometric tension was recorded by using an isometric force displacement transducer (GRASS FT03, RI, USA) connected to a data acquisition system (MP100 Biopac Systems, CA, USA).

2.3. Experimental protocols

After 45 min equilibration, each aortic ring was exposed twice to KCl (75 mM) to access its maximum contractility. Each ring was sequentially washed and re-equilibrated and was allowed to relax to baseline. Thirty minutes later, the rings were contracted with submaximal concentration of Phe and then acetylcholine (ACh, 10^{-5} M) was added to assess the integrity of the endothelium. A relaxation equal to or greater than 90% was considered as demonstrating the functional integrity of the endothelium. After 30 min, cumulative concentration–response curves were generated for Phe (10^{-10} – 3×10^{-5} M) on each ring. In other experiments, the Phe concentration–response curve was constructed in endothelium-denuded rings. The endothelium was removed by gently rubbing the intimal surface with a stainless steel rod. The effectiveness of endothelium removal was confirmed by the absence of the relaxation induced by ACh (10^{-5} M) in aortas precontracted with Phe.

NO production was determined either by measuring endothelium-dependent increases in tension to L - N^G -nitroarginine methyl ester (L -NAME, 100 μ M) in rings half-maximally contracted by Phe (10^{-7} M) or by measuring endothelium-mediated relaxation to ACh (10^{-9} – 3×10^{-5} M). Relaxation to sodium nitroprusside (SNP, 10^{-11} – 3×10^{-7} M) was also investigated to test the responsiveness of the vascular smooth muscle to exogenous NO.

Possible roles of cyclooxygenase–arachidonic acid metabolites were investigated in aortic rings from 4-week diabetic rats. Endothelium intact rings were preincubated with either indomethacin (a cyclooxygenase inhibitor, 10 μM) or SQ 29584 (an inhibitor of $\text{PGH}_2/\text{TxA}_2$ receptors, 1 μM) for 30 min, before generating concentration–response curves to Phe. In endothelium-denuded rings, the participation of smooth muscle-derived prostanoids was investigated in the presence of SQ 29548. In these experiments, concentration–response curves for Phe were performed in paired rings; one ring in the presence and the other in the absence of the antagonists.

To assess the relative role of the release of intracellular Ca^{2+} on the Phe-mediated contraction in control and diabetic aortas, contractile response to this agonist was obtained in calcium-free medium. In addition, the role of extracellular Ca^{2+} mobilization was investigated by CaCl_2 -induced contraction in the presence of Phe. Following the equilibration period in Krebs' solution containing 2.5 mM Ca^{2+} , arteries were incubated in calcium-free Krebs' solution containing 1 mM EGTA for 15 min before the addition of a single dose of Phe (10^{-6} M). When the tension stabilized, CaCl_2 (2.5 mM) was added to the bath in the presence of the agonist. This protocol was done in the absence and in the presence of SQ 29548 (1 μM). Calcium-free KHB was prepared by omitting CaCl_2 from Krebs' solution and was replaced by NaCl to maintain the osmolarity.

2.4. Drugs and reagents used

STZ, L-Phe hydrochloride, acetylcholine chloride, sodium nitroprusside dihydrate, L-NAME and indomethacin were purchased from Sigma (St. Louis, MO, USA); SQ 29548 was purchased by Biomol Research Laboratories (Plymouth Meeting, PA, USA) and sodium pentobarbital from Fontoveter (São Paulo, SP, BR). These drugs were dissolved in distilled water, except indomethacin, which was dissolved in Tris (hydroxymethyl aminomethane) buffer, STZ in citrate buffer and SQ 29548 in ethanol.

2.5. Statistical analysis

All values are expressed as means \pm S.E.M. Contractile responses are expressed as a percentage of the maximum response produced by 75 mM KCl. Relaxation responses to ACh and SNP are expressed as the percentage of relaxation of the maximum contractile response. The role of endothelium-derived NO on Phe-induced contraction was measured as percentage of increase in tension above the initial tension produced by Phe (10^{-7} M). The initial tension was considered as 100% and the additional tone caused by L-NAME was calculated on the basis of this initial contraction, estimating the percentage of increment in the tone induced by Phe. For each concentration–response curve, the maximum effect (E_{max}) and the concentration of the agonist,

which produced half of E_{max} ($\log \text{EC}_{50}$), were calculated using nonlinear regression analysis (GraphPad Prism Software, San Diego, CA). The sensitivity of the agonists is expressed as pD_2 ($-\log \text{EC}_{50}$).

In order to compare the effect of blockade of the cyclooxygenase or $\text{PGH}_2/\text{TxA}_2$ receptors on the responses to Phe in segments from control or 4-week diabetic rats, some results were expressed as “differences” of area under the concentration–response curves (dAUC) in control and experimental situations. AUC were calculated from the individual concentration–response curve and the differences were expressed as a percentual difference of AUC of the corresponding control situation.

Results were analyzed using Student's *t* test and analyses of variance (ANOVAs) followed by a Bonferroni's post hoc test. Differences were considered to be statistically significant for $P < .05$.

3. Results

All rats used in these experiments were normotensive (Table 1). Those rats treated with STZ exhibited severe hyperglycemia at 1 and 4 weeks after its administration and their body weights were smaller than those of age-matched controls (Table 1).

3.1. Vascular reactivity study

3.1.1. Vascular contraction to Phe and potassium chloride (KCl)

To assess maximal contraction for each preparation, rings were exposed to 75 mM KCl. Vasoconstrictor responses to this depolarizing solution were similar in all groups studied (1-week group: nondiabetic control: 2.82 ± 0.15 g vs. diabetic: 2.71 ± 0.16 g, nonsignificant, *t* test; 4-week group: nondiabetic control: 2.78 ± 0.12 g vs. diabetic: 2.72 ± 0.09 g, nonsignificant, *t* test).

One week after induction of diabetes, aortic rings with intact endothelium showed no difference in sensitivity

Table 1
Values of body weight, blood glucose and blood pressure in nondiabetic control and diabetic animals, after 1 and 4 weeks of STZ or citrate buffer administration

	Body weight (g)	Blood glucose (mg/dl)	Systolic blood Pressure (mmHg)
<i>One week</i>			
Nondiabetic	290 ± 4.0	87 ± 5.0	128 ± 2.0
Diabetic	$263 \pm 8.0^+$	$445 \pm 29^+$	133 ± 3.0
<i>Four weeks</i>			
Nondiabetic	331 ± 4.0	87 ± 4.0	122 ± 3.0
Diabetic	$236 \pm 5.0^+$	$590 \pm 25.0^+$	133 ± 4.0

Each value represents the mean \pm S.E.M. of all animals used in the present study.

⁺ $P < .0001$, Student's *t* test, diabetic vs. age-matched nondiabetic control.

(pD_2) or maximal contractility (E_{max}) to Phe between nondiabetic control and diabetic rats (Fig. 1A, Table 2). As shown in Fig. 1B and Table 2, at 4 weeks after STZ treatment, the E_{max} value for Phe was significantly greater in rings from diabetic animals than in rings from nondiabetic control animals. There were no differences in the pD_2 values for Phe among the 4-week groups. In all groups studied, contractions induced by 75 mM KCl were unaltered by endothelium removal (results not shown), however, it shifted the Phe concentration–response curves to the left of those obtained in preparations with the intact endothelium (Table 2). When the endothelium was damaged, there were no differences in E_{max} or pD_2 values for Phe in control and 1-week diabetic rats (Fig. 1C, Table 2). However, in the 4-week group, the E_{max} value for Phe, but not the pD_2 value, was increased in diabetic arteries compared to age-matched nondiabetic control (Fig. 1D, Table 2).

3.1.2. Endothelium-dependent and -independent responses to NO

To assess the contribution of endothelium-derived NO in response to Phe, precontracted aortas were exposed to a NO synthase inhibitor L-NAME. Endothelium-dependent increment in tension from aortic rings precontracted with Phe (10^{-7} M) is shown in Fig. 2. Rings from 1-week diabetic animals did not show any difference in this response compared with age-matched nondiabetic controls (Fig. 2). However, rings from 4-week diabetic animals responded to L-NAME with significantly greater tension development, indicating a significant increase of NO production in these vessels compared to rings from age-matched nondiabetic controls (Fig. 2). In endothelium-denuded aortas from all groups studied, L-NAME did not have any effect on Phe-induced contraction (results not shown).

The E_{max} and pD_2 values for the endothelium-dependent and -independent relaxations to ACh and SNP, respectively,

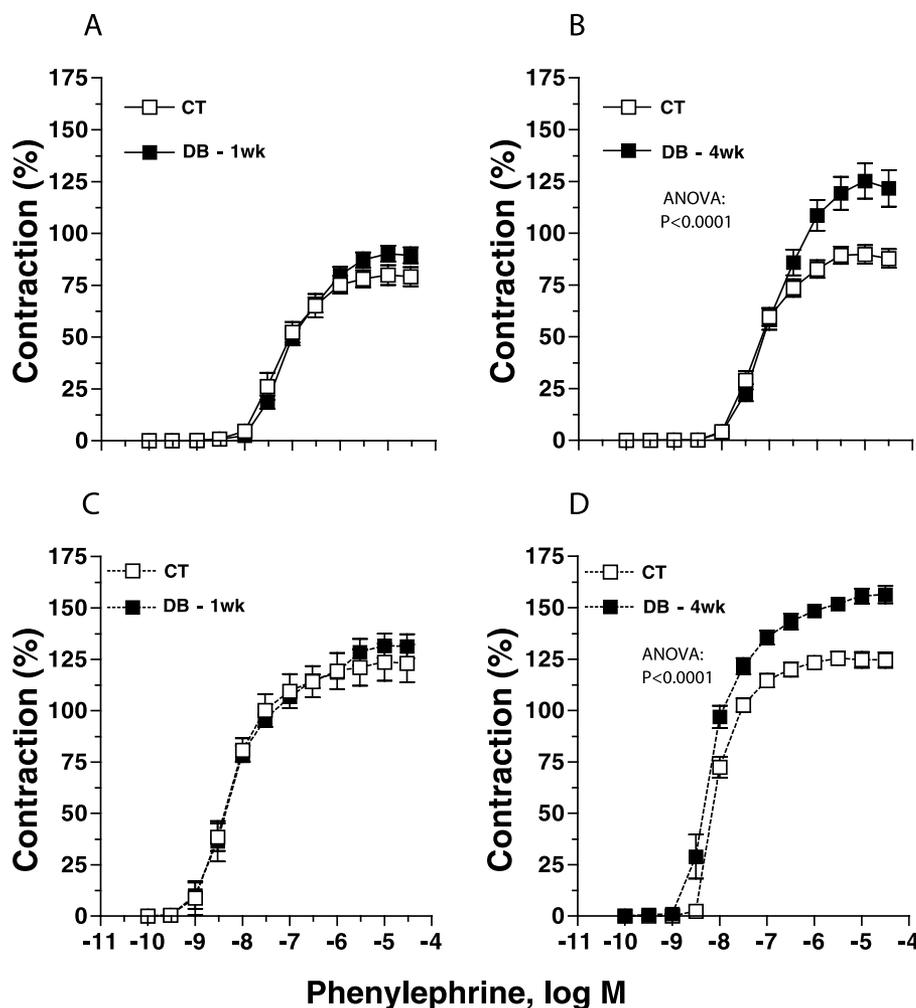


Fig. 1. Cumulative concentration–response curves to phenylephrine in endothelium intact (E+) and denuded (E–) aortic rings from nondiabetic control (CT) and diabetic (DB) rats, after 1 (Panel A: E+: CT, $n=7$; DB, $n=7$ and Panel C: E–: CT, $n=6$; DB, $n=6$) or 4 weeks (wk) (Panel B: E+: CT, $n=11$; DB, $n=11$ and Panel D: E–: CT, $n=7$; DB, $n=7$) STZ or citrate buffer administration.

Table 2

Time-dependent change in vascular response, pD_2 and maximum response (E_{max}) values, to Phe, with intact (E+) and denuded (E-) endothelium, ACh and SNP in diabetic and age-matched nondiabetic arteries

		Nondiabetic		Diabetic	
		pD_2	E_{max} (%)	pD_2	E_{max} (%)
Phe (E+)	1 week (7)	7.21±0.09	80.0±4.8	7.02±0.07	90.2±3.8
	4 weeks (11)	7.21±0.04	89.8±4.5	7.11±0.08	125±8.4 ⁺
Phe (E-)	1 week (7)	8.20±0.12*	124±9.1*	8.15±0.13 [#]	132±5.9 [#]
	4 weeks (7)	8.04±0.04*	125±3.8*	8.12±0.06 [#]	156±4.2 ^{#,+}
ACh	1 week (6)	7.24±0.19	99.2±0.83	7.20±0.16	100±1.18
	4 weeks (7)	7.35±0.12	92.6±1.47	7.38±0.08	99.5±1.19
SNP	1 week (6)	8.36±0.07	96.7±1.89	8.36±0.09	95.8±2.74
	4 weeks (7)	8.31±0.12	99.7±0.34	8.50±0.15	99.3±0.67

Each value represents the mean±S.E.M. Numbers in parentheses represent the number of experiments.

* Student's *t* test: $P<.001$, nondiabetic E- vs. corresponding nondiabetic E+.

⁺ Student's *t* test: $P<.001$, diabetic vs. nondiabetic.

[#] Student's *t* test: $P<.001$, diabetic E- vs. corresponding diabetic E+.

are shown in Table 2. These parameters were similar in diabetic and in respective age-matched nondiabetic controls (Table 2).

3.1.3. Role of the arachidonic acid–cyclooxygenase-derived products on the vascular response to Phe

To assess the role of cyclooxygenase–arachidonic acid metabolites on responses to Phe, the effects of indomethacin (10 μ M) and SQ 29584 (1 μ M) were evaluated in endothelium intact aortic rings. In the presence of indomethacin, pD_2 and E_{max} values for Phe in aortas from 4-week diabetic and nondiabetic control rats were reduced (Fig. 3A, Table 3). The comparison of dAUC values indicates that after cyclooxygenase blockade, the reduction

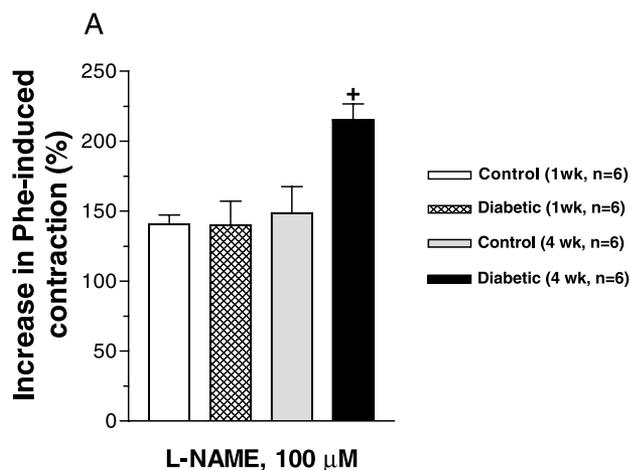


Fig. 2. Endothelium-derived NO (EDNO)-mediated response was assessed by L-NAME (100 μ M)-evoked increases in isometric tension of aortic rings contracted with phenylephrine (Phe, 10^{-7} M). EDNO release was significantly enhanced in 4-week STZ-diabetic rats. Data are expressed as % increase by L-NAME in initial tension induced by phenylephrine. $+P<.01$ vs. other groups.

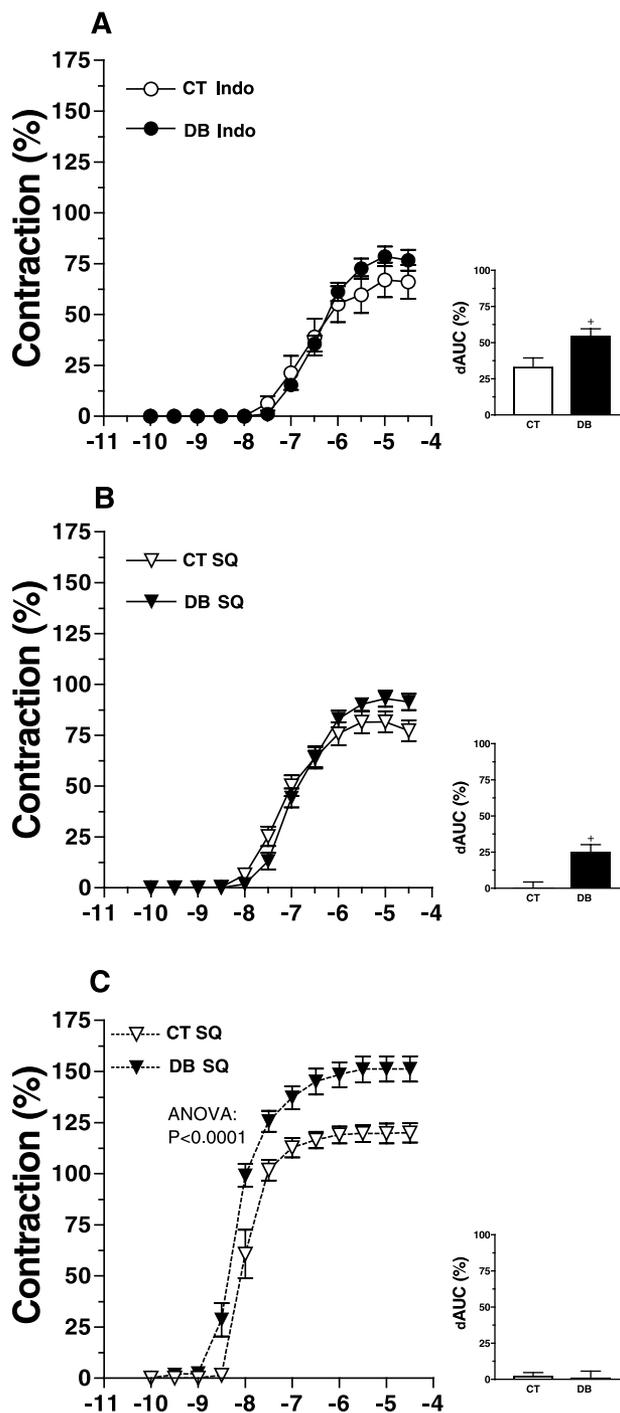


Fig. 3. Concentration–response curves to phenylephrine in endothelium intact (A and B, solid lines) and denuded (C, dashed lines) aorta isolated from 4-week diabetic (DB, $n=7-8$) rats and age-matched nondiabetic control (CT, $n=6-7$), preincubated with indomethacin (A, Indo 10 μ M) or SQ 29584 (SQ, B and C, 1 μ M) treatment. Inserts show differences in area under the concentration–response curve (dAUC) to phenylephrine in aortic segments with an intact endothelium (see Fig. 1B) and in segments pretreated with Indo (A) or SQ 29584 (B) from control and diabetic rats. dAUC are expressed as a percentual difference of the corresponding AUC for endothelium intact segments. $+P<.05$ vs. control.

Table 3

Effect of indomethacin ($n=6-8$) or SQ 29548 ($n=6-8$) treatment on pD_2 and E_{max} values on Phe contractility in endothelium intact aorta (E+) and denuded (E-) from 4-week diabetic and age-matched nondiabetics animals

	Nondiabetic		Diabetic	
	pD_2	E_{max} (%)	pD_2	E_{max} (%)
Control (E+)	7.21±0.04	89.8±4.5	7.11±0.08	125±8.4 ⁺
Indomethacin (E+)	6.58±0.10*	67.0±8.5*	6.44±0.05 [#]	76.7±5.1 [#]
SQ 29548 (E+)	7.16±0.06	82.0±5.2	6.95±0.08	93.0±3.9**
Control (E-)	8.04±0.04*	125±3.8*	8.12±0.06 [#]	156±4.2 ^{#,+}
SQ 29548 (E-)	8.00±0.05	120±4.6	8.15±0.04	151±6.2 ⁺

Numbers in each cell represent the mean±S.E.M.

* Student's *t* test: $P<.001$, nondiabetic: indomethacin or control E- vs. control E+.

** Student's *t* test: $P<.001$, diabetic: SQ 29548 vs. control E+.

+ Student's *t* test: $P<.001$, diabetic vs. nondiabetic.

Student's *t* test: $P<.001$, diabetic: indomethacin or control E- vs. control E+.

of the Phe-induced contraction in arteries from diabetic rats was greater than that observed in arteries from control rats (Fig. 3A), and this effect reflects a similar response to Phe in aortas from both groups in the presence of indomethacin (Fig. 3A).

Preincubation with SQ 29548 did not produce any changes in the concentration–response curve to Phe in nondiabetic control rings (see dAUC in Fig. 3B and Table 3). However, the modulation induced by PGH_2/TxA_2 was larger in diabetic aortas (see dAUC in Fig. 3B and Table 3). In the presence of this PGH_2/TxA_2 antagonist SQ 29548, E_{max} for Phe from rings of diabetic rats was reduced to values similar to the ones observed in nondiabetic aortas (Fig. 3B and Table 3). No changes in pD_2 were detected (Table 3). In endothelium-denuded rings from both diabetic and nondiabetic animals, preparations pretreated with SQ

29548 still showed the increased Phe-induced vasoconstriction. This response is similar to those observed in preparations without endothelium and no SQ 29548 treatment (see Fig. 3C and dAUC in the same figure).

3.1.4. Role of intracellular and extracellular calcium mobilization on the Phe-induced contraction

To assess intracellular calcium mobilization, preparations were studied in the presence of calcium-free medium containing 1 mM EGTA. Phe (10^{-6} M) induced a rapid phasic contraction that reached a peak and then returned close to baseline levels within 2–3 min. The magnitude of the peak phasic response was similar in diabetic and nondiabetic control arteries (Fig. 4A). Preincubation with SQ 29548 did not alter this response in both control and diabetic rats (Fig. 4A).

To assess extracellular calcium mobilization, preparations were investigated in the presence of 10^{-6} M Phe. Aortas incubated in calcium-free medium contracted in response to 2.5 mM $CaCl_2$. This response was greater in diabetic than in nondiabetic control aortas (Fig. 4B) and was not altered by pretreatment with SQ 29548 (Fig. 4B).

4. Discussion

Results presented here demonstrate that 4-week STZ-induced diabetes produced an enhanced responsiveness to Phe in aortas, although evidencing an increased production of endothelium-derived NO. On the other hand, in aortas from rats with early diabetes (1 week), no changes were detected in either responsiveness or sensitivity to Phe. This time-dependent hyperreactivity in the Phe-induced contrac-

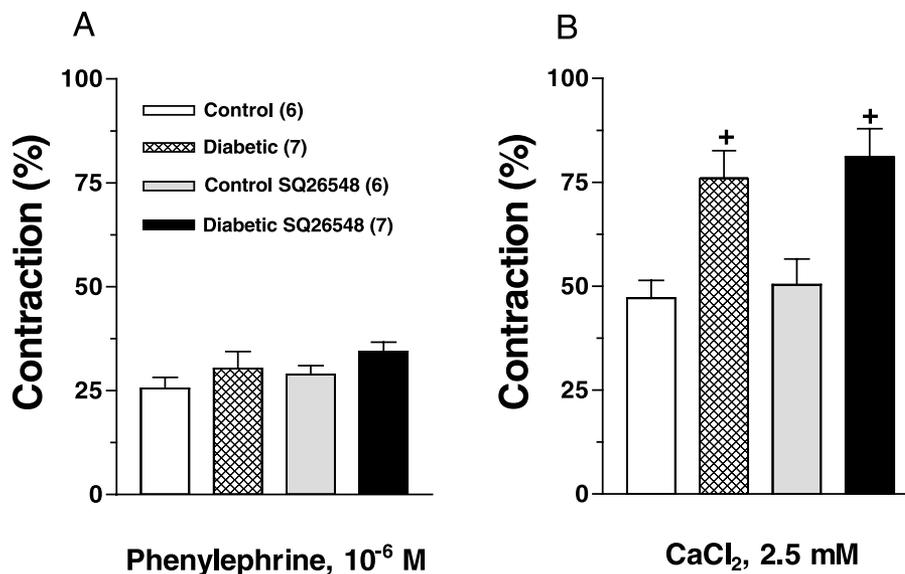


Fig. 4. Histograms showing the mean of the response induced by 10^{-6} M phenylephrine in Ca^{2+} -free Krebs' solution (A) and after subsequent addition of 2.5 mM $CaCl_2$ (B) in the continuous presence of phenylephrine. Numbers in parentheses represent the number of animals. Student's *t* test: + $P<.01$ diabetic vs. control.

tion seems to be maintained by the release of endothelium-derived vasoconstrictor prostanoids, probably PGH_2 and/or TxA_2 , and by a non-prostanoid-dependent increment of the extracellular calcium mobilization in aortas from diabetic animals.

It is well established that diabetes mellitus is associated with the development of vascular dysfunction. Previous reports have shown that acute or chronic hyperglycemia can cause vascular abnormalities, such as an impairment of endothelium-dependent vasodilatation (Fortes et al., 1983; Pieper and Gross, 1988; Tesfamariam et al., 1989), inhibition of Na^+ , K^+ -ATPase activity (Ohara et al., 1991; Davel et al., 2000) and increase in the response to vasoconstrictor agents (Abebe et al., 1990; White and Carrier, 1990).

Alterations of the reactivity to vasoconstrictor agonists have been demonstrated in isolated vascular beds from diabetic animals. However, these results are controversial. Actions of norepinephrine, Phe or serotonin have been shown to be enhanced (Abebe et al., 1990; White and Carrier, 1990; Miranda et al., 2000), attenuated (Ramanadham et al., 1984; Oyama et al., 1986) or unaltered (Chang and Stevens, 1992; Heygate et al., 1995). Reasons for these differences are not clear, although they could be explained by different experimental conditions including the type of artery, the type of agonist examined, the method used for vascular studies or the duration of diabetes.

Previous studies from our laboratory (Davel et al., 2000) using tail vascular bed preparations from 1-week STZ-treated rats showed an increased sensitivity to Phe as compared to age-matched controls. These data, in addition to our results, support the hypothesis that the alterations of the contractile response to vasoconstrictor agonists, as Phe, might be dependent on the vascular bed and/or the duration of the diabetic state.

Endothelial dysfunction plays an important role in the pathogenesis of diabetic vascular disease (Tefamariam, 1994; Cosentino and Lüscher, 1998). Whereas some reports showed normal (Mulhern and Docherty, 1989; Furman and Sneddon, 1993) or even enhanced endothelium-dependent relaxation (White and Carrier, 1986; Bhardway and Moore, 1988), impaired responses to various endothelium-dependent agonists have been demonstrated in different vascular beds from both human and experimental models of diabetes (Fortes et al., 1983; Pieper and Gross, 1988; Calver et al., 1992). Alterations of endothelium-dependent vasodilatation in diabetes also might be dependent on the vascular bed and/or the duration of the diabetic state. Davel et al. (2000) demonstrated an impairment of the endothelium-dependent relaxation induced by ACh in the tail vascular bed from 1-week STZ-induced diabetic rats. On the other hand, in the current study, no changes were detected in the ACh-induced relaxation. Our results are in agreement with Pieper (1999) who showed no changes in ACh-induced endothelium-dependent relaxation after 1 week of diabetes. However, after 8 weeks, the response to ACh was impaired (Pieper, 1999). In addition, it has been demonstrated that some

vascular beds, such as tail, basilar or mesenteric arteries, may present an endothelial dysfunction prior to other vessels such as the aorta (to review, see De Vriese et al., 2000).

Considering that the relaxation induced by ACh in the rat aorta is mainly dependent on NO production, our results suggest that the enhanced responsiveness of 4-week diabetic aortas to Phe was not due to an impaired release of NO. This conclusion is supported by our observation that the additional endothelium-dependent increment of the Phe-induced contraction produced by L-NAME is significantly enhanced in 4-week diabetic rats (see Fig. 2). This response reflects the amount of constitutively available NO that modulates contractile responses of the vascular smooth muscle (Hayashi et al., 1992). Our results reinforce previous findings showing an increased production of NO in the diabetic state (Fitzgerald and Brands, 2000). This increment of NO release may represent either a reactive response to enhanced oxidative stress (Kakkar et al., 1996) or a compensatory mechanism to the increased reactivity to α -adrenergic agonists, as demonstrated in the current study.

Removal of endothelial cells produced an increase of similar magnitude in both Phe responsiveness and sensitivity in nondiabetic control and diabetic arteries maintaining the enhanced Phe-induced contraction in 4-week diabetic aortas. Chang and Stevens (1992) reported that increased sensitivity to α -adrenergic agonist is dependent on the presence of endothelium in aortic rings from diabetic rats. However, they used rats after 52 weeks of diabetes and, at this stage, there was a marked impairment of endothelium-dependent vasodilatation induced by ACh, characterizing a marked endothelial dysfunction. In endothelium-denuded aortas from 1-week STZ-induced diabetic rats, similar to endothelium-intact condition, no changes were detected in either the maximum response or the sensitivity evoked by Phe. Although our results showed an endothelium-independent increased vasoconstriction to Phe and an evidence of increased production of NO in 4-week diabetic aortas, we cannot discard the possibility that the endothelium may contribute to this hyperreactivity by other mechanisms. Therefore, the additional protocols were performed only in aortic rings from 4-week diabetic rats and respective controls.

Vascular biosynthesis of some prostanoids, such as prostacyclin (PGI_2), PGH_2 and TxA_2 , was found to be altered in experimental diabetes and was shown to contribute to vascular dysfunction induced by acute or chronic hyperglycemia (Peredo et al., 1984; Tesfamariam et al., 1989, 1990). Tesfamariam et al. (1989) reported an increased production of TxA_2 by endothelium from diabetic rabbits, in steady state or stimulated conditions, compared to control animals. Others have confirmed these results in both diabetic animals and in vitro hyperglycemia (Koltai et al., 1990; Quilley and McGiff, 1990; Tesfamariam et al., 1990). We designed experiments to address whether arachidonic acid-cyclooxygenase-derived substances could

account for the changes observed on the agonist action of Phe in aorta from diabetic rats. Maximum contraction and sensitivity evoked by this agonist were significantly diminished by indomethacin in both diabetic and control aortas. It is important to observe that, after indomethacin treatment, no changes could be detected in either responsiveness or sensitivity to Phe between 4-week diabetic and nondiabetic control aortas. The participation of prostanoids derived from the arachidonic acid–cyclooxygenase pathway modulating the contraction induced by α -adrenergic agonist has been previously reported by other authors (Taberno et al., 1999) but this modulation, as shown in the present study, seems to be greater in the diabetic aorta. A competitive inhibitor of $\text{PGH}_2/\text{TxA}_2$ receptors, SQ 29548, also reduced the maximum response evoked by Phe in aortic rings from diabetic rats, but not from nondiabetic control rats. SQ 29548 treatment shifted the concentration–response curve for Phe from 4-week diabetic aortas to control levels. The lack of effect of SQ 29548 in the nondiabetic control aorta suggests that PGH_2 and/or TxA_2 do not normally modulate the actions of this agonist in this artery. Taken together, these results indicate that prostanoids, probably PGH_2 and/or TxA_2 , play a role in Phe-induced hyperreactivity in aortic rings from 4-week diabetic rats. This is in agreement with previous findings of Koltai et al. (1990) that demonstrated the participation of TxA_2 on the enhanced contractile response to norepinephrine in the femoral vascular bed from diabetic dogs. To evaluate if the hyperreactivity to Phe is modulated exclusively by prostanoids released by the endothelium, we investigated the response to Phe in endothelium-denuded aortas in the presence of SQ 29548. As described above, endothelium-denuded rings from 4-week diabetic animals exhibited an increased responsiveness to Phe compared to age-matched controls. Pretreatment with SQ 29548 did not alter this response, suggesting that other muscular mechanisms participate for the hyperreactivity to α -adrenergic agonist in the diabetic aorta. Based on the results described above, we suggest that the two mechanisms, endothelial (via prostanoids) and muscular, act independently to maintain the hyperreactivity to Phe in this diabetic vessel. We propose that, under conditions where the prostanoids were blocked, the enhanced production of NO in response to Phe in the diabetic aorta was able to maintain the response to this α_1 -adrenergic agonist at control levels despite an enhanced vasoconstrictor mechanism of the vascular smooth muscle.

Alterations in calcium mobilization pathways in vascular tissues from chronic diabetic rats have been demonstrated (Abebe et al., 1990; White and Carrier, 1990; Inazu et al., 1991). In order to address if the enhanced responsiveness to Phe, in aortas from 4-week diabetic rats, was associated with alterations in calcium mobilization pathways, the contribution of intra- and extracellular calcium was evaluated. In arterial smooth muscle, stimulation of α_1 -adrenoreceptors by Phe activates phospholipase C that causes the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG)

from phosphatidylinositol diphosphate (PIP_2). IP_3 can cause the release of Ca^{2+} from the sarcoplasmic reticulum (SR) and DAG stimulates the PKC activity. It is also accepted that Phe promotes the influx of extracellular Ca^{2+} into vascular smooth muscle cells by two main Ca^{2+} channels, voltage-operated calcium channel (VOCC) and receptor-operated calcium channel (ROCC) (for review, see Horowitz et al., 1996).

The contractile response of vascular smooth muscle incubated in the absence of extracellular Ca^{2+} to Phe is believed to result from IP_3 -induced intracellular Ca^{2+} release. In the present study, the contractile response to Phe (10^{-6} M) in the absence of extracellular Ca^{2+} plus 1 mM EGTA was not significantly different in diabetic aorta compared to controls. This result suggests that IP_3 -induced intracellular Ca^{2+} release did not contribute to the enhanced contractile response of diabetic aorta to Phe. This result differs from previous reports showing that the contractile responses and the increases in inositol phosphates produced by norepinephrine in the absence of extracellular Ca^{2+} were significantly increased in diabetic aorta and mesenteric arteries as compared to controls (Abebe and MacLeod, 1990a). Their results were obtained using animals after 12 to 14 weeks of diabetes and may represent yet another time-dependent change in alterations of vascular function caused by diabetes.

The response to added Ca^{2+} in the presence of Phe after depletion of intracellular Ca^{2+} stores was also examined. In the present investigation, the enhanced responsiveness to Phe was associated with an enhanced contractility to increasing extracellular Ca^{2+} . These results are consistent with previous reports showing that increased contractile response to norepinephrine in diabetic arteries is largely dependent on the presence of extracellular Ca^{2+} (Abebe et al., 1990; White and Carrier, 1990). In contrast, no changes were detected in the contraction of K^+ -depolarized aortas from diabetic rats as compared to controls. These results support the hypothesis that in the diabetic aorta there is no generalized increase of contractile response to extracellular Ca^{2+} . It is possible that in aortas from diabetic rats the increased response to extracellular Ca^{2+} in the presence of Phe may result from an increase in the efficacy of coupling of α -adrenoreceptors with their signal transduction system, which is known to influence the extracellular Ca^{2+} mobilization or the sensitivity of contractile elements to Ca^{2+} . This hypothesis is supported by previous results showing that diabetic mesenteric arteries show a similar increase in intracellular Ca^{2+} concentration in response to norepinephrine despite an increased contraction to this agonist compared to controls (Chow et al., 2001). Increased activation of PKC, which modulates the Ca^{2+} entering by sarcolemmal Ca^{2+} channels and the sensitivity of contractile elements to Ca^{2+} , appears to contribute to the enhanced contractile responses to norepinephrine (Abebe and MacLeod, 1990b). Additionally, we investigated a possible effect of endothelium-derived prostanoids ($\text{PGH}_2/\text{TxA}_2$) on intracellular and extracellular cal-

cium mobilization in smooth muscle, which could contribute to enhanced response to Phe in 4-week diabetic aortas. In the experimental protocol used here, we did not detect any effect of PGH₂/TxA₂ on Ca²⁺ mobilization pathways, which suggests that the enhanced response to extracellular calcium is not modulated by these endothelial prostanoids. Based on these results, we cannot discard that the role of these prostanoids on hyperreactivity to Phe can be induced by mechanisms that alter the mobilization or sensitivity to extracellular calcium. In the protocol used here, the rings were incubated in a free-calcium medium and we do not know if this manipulation altered prostanoid synthesis, masking a probable effect of prostanoids on calcium extracellular response in the diabetic rat aorta.

In summary, results suggest that diabetes induces time-dependent alterations of the Phe-induced contraction in rat aorta. Four-week diabetic rats, although not hypertensive, showed enhanced vascular responsiveness to Phe. This response does not seem to be related to a reduction of NO release, on the contrary, diabetic aortas showed evidences of enhanced endothelium-derived NO production. The Phe hyperreactivity seems to be maintained by two independent mechanisms: an increased release of endothelium-derived vasoconstrictor prostanoids and an enhanced Ca²⁺ influx and/or sensitivity of the vascular smooth muscle cells.

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