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Study of Calcium Dobesilate in Diabetic Rats

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Abstract. According to the World Health Organization (WHO) 74% of diabetic patients die of vascular complications. Previous reports have shown that endothelium-dependent relaxation of diabetic vasculature is more sensitive to free radical-induced injury. Calcium dobesilate (DOBE) has been successfully used in the treatment of diabetic retinopathy. The aims of this study were to investigate the *in vivo* and *ex vitro* effects of DOBE on both contractile and relaxing responses in isolated diabetic rat aorta. Four groups of rats were used: Wistar rats (Group 0); spontaneously diabetic rats (BB/wor rats) (Group 1); BB/wor rats treated with DOBE 50 mg/kg/day (Group 2); and BB/wor rats treated with 500 mg/kg/day (Group 3). At 180 days after the development of diabetes, the animals were killed and the thoracic aorta were isolated, cleaned off, and mounted in an organ chamber. Two groups of experiments were carried out. In the first group (*in vitro*), incubation with DOBE 10^{-4} in aortic rings isolated from BB/wor rats decreased the contraction induced by noradrenaline (NA) 10^{-6} M (1.21 ± 0.11 g vs 0.67 ± 0.01 g $P < 0.01$, n = 8 in diabetic rings with or without the presence of DOBE 10^{-4} M, respectively), and this decrease was prevented by propranolol 10^{-6} M (1.20 ± 0.6 g). DOBE 10^{-5} and 10^{-4} M increased the endothelium-dependent relaxation induced by ACh in BB/wor rats [the maximal relaxation with ACh 10^{-5} M was 50.0 ± 5.1 vs 72.0 ± 11.0 ($p < 0.05$, n = 8) and 69.0 ± 7.8 ($p < 0.05$, n = 8) in BB/wor rats and after the incubation with DOBE 10^{-5} and 10^{-4} M, respectively], however, incubation with DOBE did not modify the endothelium-independent relaxation in these rats. In the second part of the study (*ex vitro*), we found an increase in the endothelium-dependent relaxation in arteries from diabetic rats treated with DOBE (Groups 2) compared with Group 1 (BB/wor rats) although we did not find any improvement in the endothelium-independent relaxation. Thus, in spontaneously diabetic rats, DOBE restored endothelium-dependent, but not independent, relaxation to normal and also decreased the contractile responses induced by NA through a mechanism that involves β-adrenergic receptors.

Introduction

It has been suggested that some manifestations of cardiovascular deterioration in diabetes are a consequence of altered sensitivity and/or responsiveness of vascular smooth muscle to neurotransmitters and circulating hormones [1]. In this way, the reactivity of the aorta of diabetic rats to contracting agents has been extensively studied. MacLeod and McNeill [2] demonstrated that contractile responses to noradrenaline (NA) of the aorta and mesenteric arteries from rats with streptozotocin-induced diabetes were enhanced. On the other hand, there are controversies about endothelium-dependent relaxation in arteries from diabetic animals. Wakabayashi et al. [3] reported that endothelium-dependent relaxation of aortas in response to acetylcholine was unaffected by the diabetic state. In contrast, Oyama et al. [4] reported that endothelium-dependent relaxation of diabetic aortas was attenuated with respect to control. Furthermore, McVeigh et al. [5] demonstrated an abnormal relaxation to acetylcholine (ACh) in the forearm of subjects with noninsulin-dependent (type II) diabetes mellitus.

Calcium dobesilate (DOBE) (calcium 2,5-dihydrosybenzene sulphonate, OM PHARMA, Meyrin/Geneva, Switzerland), one of the most active members of a series of cyclohexadienolic bisulfate derivatives, has been successfully used in the treatment of chronic venous insufficiency [6] and in diabetic retinopathy [7,8]. Previous studies carried out in our group have demonstrated that calcium DOBE enhances endothelium-dependent relaxation induced by ACh in rabbit-isolated aorta arteries [9,10]. The aim of this study was to investigate the *ex vivo* and *in vitro* effects of this drug on both contraction and relaxation responses in aortic rings isolated from spontaneously diabetic rats (BB/wor rats).

Material and Methods

Male genetically diabetic rats (BB/wor rats) weighing 300.00 ± 25.0 g were used. The Bio-Breeding (BB) rat was selected for use in this study because

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it is a model of spontaneous diabetes that closely resembles type I diabetes in man. It shares with the human disease an abrupt onset of hyperglycemia, glycosuria, ketonuria, and weight loss. In our study, 50% of the BB/wor rats developed severe diabetes between 70 and 120 days of the study.

The animals were randomly separated into four groups: Group 0, non-diabetic control rats (Wistar rats); Group 1, diabetic rats (BB/wor); Group 2, BB/wor rats + DOBE 50 mg/kg/day, and Group 3, BB/wor rats + DOBE 500 mg/kg/day. The treatment with DOBE was carried out for 45 days. Rats were housed separately and all the groups were given free access to food and water.

Glucose and ketones in urine were measured every morning, and when the glucose/ketone ratio was >1, a blood sample was taken by tail puncture to test for glycemia. A subcutaneous pellet of insulin was administered when the glycemia levels were more than 15 mmol/L.

Aortic Rings Preparation

A total of 180 ± 7 days after the development of diabetes, rats from each group were anesthetized with ethyl ether and killed by cervical dislocation. Aortic rings of approximately 3–5 mm in length were prepared from the section of the thoracic aorta between the aortic arch and the diaphragm. The rings isolated from rats of each group were rapidly placed in Godfraind solution of the following composition (mM): NaCl 121, KCl 5.8, $NaHCO_3$ 14.9, $MgCl_2$ 1.22, glucose 11, and $CaCl_2$ 1.25. Adherent fat and surrounding tissue were cleaned off and the rings were then suspended between two stainless steel hooks in organ baths containing 10 ml of Godfraind solution which was kept at 36 ± 0.5°C and gassed continuously with a 95% O_2-5% CO_2 gas mixture. The aorta rings were mounted after 1 g tension. Each preparation was allowed to equilibrate for 60–90 minutes. Contractile responses were measured isometrically by force-displacement transducers (Grass FT 03) and recorded on a Grass polygraph as previously described [11]. The isometric force was also digitalized by a MacLab A/D converter and stored and displayed on a Mackintosh computer [12].

In vitro *Experiments*

Agonist-induced Contraction. In Group 1 (BB/wor rats), we investigated the effect of the incubation with DOBE 10^{-5} or 10^{-4} M on the contractile responses induced by a single concentration of NA 10^{-6} M. The rings were exposed to NA (10^{-6} M) and, after being washed out, were incubated with DOBE (10^{-5} M or 10^{-4} M) for 45 minutes and the contraction with NA was repeated.

In view of the results obtained, we studied the implication of the L-Arg-NO pathway and β-receptors in the effect of DOBE. For this, we repeated the contraction induced with NA 10^{-6} M in the presence of DOBE (10^{-4} M) plus L-NAME 10^{-4} M (a NO-synthase inhibitor) or plus propranolol 10^{-6} M (a β-receptor blocker).

Agonist-induced Relaxation. In order to investigate whether diabetes altered both endothelium-dependent and independent relaxing responses and if so, whether incubation with several concentrations of DOBE could prevent these alterations, an initial contraction by NA 10^{-6} M was induced. When the plateau was reached, the following cumulative relaxation curves were made by adding aliquots of the relaxing agonist: (1) concentration-response to ACh (10^{-8}–10^{-4} M); (2) concentration-response curves to sodium nitroprusside (SNP) (10^{-8}–10^{-4} M).

After being washed out, the rings were incubated with DOBE 10^{-5} M or 10^{-4} M for 45 minutes and the previous procedure was repeated.

Ex vitro *Experiments*

Agonist-induced Relaxation. In other groups of experiments, we tested the effect of the different treatments on the endothelium-dependent and independent relaxation induced by either ACh of by SNP, respectively, in aorta arteries obtained from the different groups of rats. For this, an initial contraction by NA 10^{-6} M was induced. When the plateau was reached, the following cumulative relaxation curves were made by adding aliquots of the relaxing agonist: (1) concentration-response curves to ACh (10^{-8}–10^{-4} M); (2) concentration-response curves to SNP (10^{-8}–10^{-4} M).

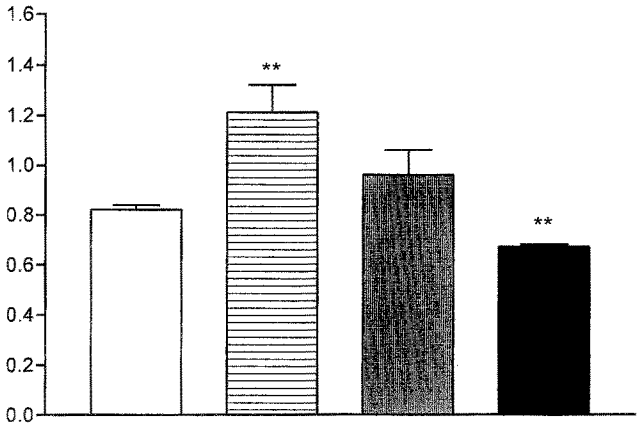

Fig. 1. Effect of DOBE (10^{-5} or 10^{-4} M) on contractions induced by NA 10^{-6} M in both groups of arteries, +E **(A)** and −E **(B)** in aortic rings obtained from diabetic (BB/wor) rats. Each bar shows the mean ± SEM of 7–9 experiments. $^{**}p < 0.01$, with respect to diabetic rings. □, wistar rats; ▤, BB/wor; ▩, DOBE 10^{-5} M; ■, DOBE 10^{-4} M.

Drugs

The following drugs were used: calcium dobesilate, noradrenaline bitartrate, sodium nitroprusside, propanolol, acetylcholine chloride, and N-nitro-L-arginine methyl ester. The drugs were dissolved in deionized water; working solutions were made in Godfraind solution. The concentrations for each chemical or drug are expressed as final concentrations in the chamber in terms of the salt. Ascorbic acid (10^{-4} M) was added to each daily prepared solution of NA.

Statistical Analyses

The results are expressed throughout as mean ± SEM of 7–9 rats in each group. All protocols concerning animals were approved by the Complutense University of Madrid. Comparisons among the different groups were performed by Student's *t*-test for unpaired data or two-way ANOVA when needed. Differences were considered significant at $p < 0.05$.

Results

In vitro *Experiments*

Effect of DOBE on Contractions Induced by NA. In the first group of experiments we studied the contractile responses induced by NA (10^{-6} M) arteries obtained from control and BB/wor rats, with or without the presence of DOBE (10^{-5} or 10^{-4} M). As shown in Figure 1, the contraction induced by NA increased in BB/wor rats as compared with control rats (1.21 ± 0.11 g vs 0.82 ± 0.02 g $p < 0.01$, n = 8, respectively). In rings obtained from BB/rats incubated with DOBE 10^{-5} M the contraction was 0.96 ± 0.10 g and with DOBE 10^{-4} M this contraction was 0.67 ± 0.01 g ($p < 0.01$, n = 8).

In order to rule out any action on endothelium, we tried to

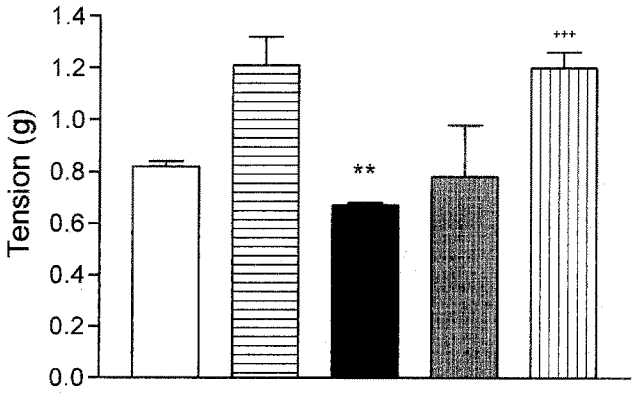

Fig. 2. Effect of L-NAME (10^{-4} M) or propranolol (10^{-6} M) on the preventive effect of DOBE on contractions induced by NA 10^{-6} M in aortic rings obtained from diabetic (BB/wor) rats. Each bar shows the mean ± SEM of 7–9 experiments. Diabetic rings, $^{**}p < 0.01$. +++ $p < 0.001$ with respect to NA-induced contraction in the presence of DOBE. □, wistar rats; ▤, BB/wor rats; ■, DOBE 10^{-4} M; ▩, DOBE + L-NAME 10^{-4} M; ◫, DOBE + propa. 10^{-6} M.

reverse the effect of DOBE in the presence of L-NAME (a NO synthesis inhibitor). Figure 2 shows that L-NAME did not significantly reverse the effect of DOBE on the contraction induced by NA (0.67 ± 0.01 vs 0.76 ± 0.20 in DOBE 10^{-4} M and DOBE plus L-NAME-treated arteries, respectively).

In addition, in arteries incubated with DOBE (10^{-5} or 10^{-4} M) plus propranolol 10^{-6} M, the contractions induced by NA 10^{-6} M were 0.67 ± 0.01 vs. 1.20 ± 0.60 ($p < 0.001$, n = 8) in DOBE 10^{-4} M vs DOBE 10^{-4} M plus propranolol 10^{-6} M, respectively (Fig. 2).

Effect of DOBE on Endothelium-dependent Relaxation. In arteries obtained from Wistar rats, ACh (10^{-8}–10^{-4} M) caused a concentration-dependent relaxation, the maximal relaxation reached being 78.0 ± 9.0% with ACh 10^{-4} M. In arteries obtained from BB/wor rats, the concentration-response curve was significantly [$F(1,15) = 5.3$, $p < 0.05$] shifted upwards and to the left, the maximal relaxation being 50.0 ± 5.1. After incubation with DOBE (10^{-5} or 10^{-4} M) the concentration-response curve induced by ACh was significantly [$F(1,14) = 5.3$, $p < 0.05$ and $F(1,10) = 10.1$ $p < 0.05$ with DOBE 10^{-5} and 10^{-4} M respectively] shifted downwards and to the right, compared with control diabetic rats (Fig. 3).

Effect of DOBE on Endothelium-independent Relaxation. Figure 4 shows the endothelium-independent relaxation induced by sodium nitroprusside. Despite the maximal effects being similar among the groups, the sensitivity to SN decreased in diabetic rings and in diabetic rings incubated with DOBE, compared with control nondiabetic rings; thus, the EC_{50} values were 5.1 ± 0.2 × 10^{-8} M vs 4.9 ± 0.1 × 10^{-7} M ($p < 0.05$, n = 8) in control nondiabetic rings and diabetic rings, respectively, and 4.8 ± 0.2 × 10^{-7} M ($p < 0.05$, n = 8) and 5.2 ± 0.1 × 10^{-7} M ($p < 0.05$, n = 8 compared with control nondiabetic rings) in arteries isolated from diabetic rats incubated with DOBE 10^{-5} M and 10^{-6} M, respectively.

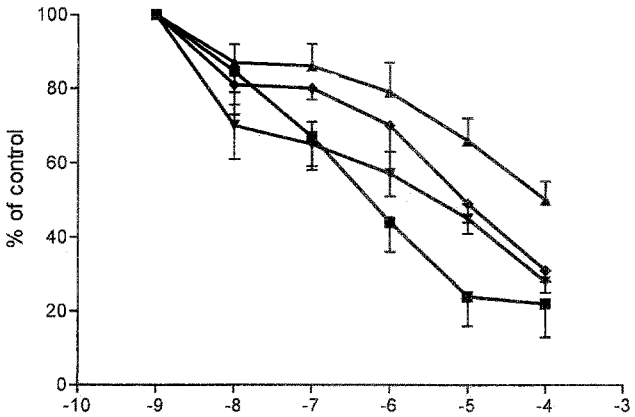

Fig. 3. Effect of DOBE (10^{-5} or 10^{-4} M) on endothelium-dependent relaxation induced by ACh (10^{-8}–10^{-4} M) in aortic rings obtained from diabetic (BB/wor) rats. Each point shows the mean ± SEM of 7–9 experiments. ■, Wistar rats; ▲, BB/wor rats; ▼, DOBE 10^{-5} M; ◆, DOBE 10^{-4} M.

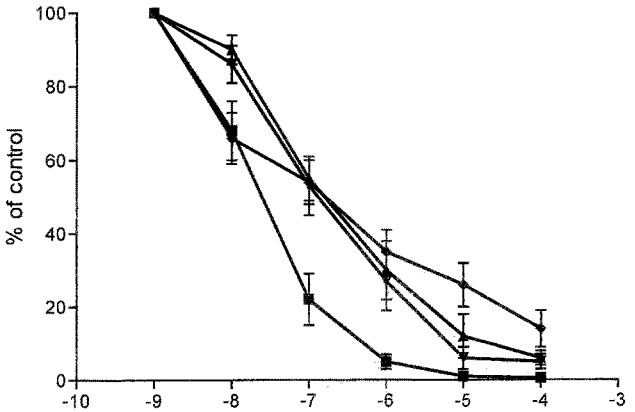

Fig. 4. Effect of DOBE (10^{-5} or 10^{-4} M) on endothelium-dependent relaxation induced by SNP (10^{-8}–10^{-4} M) in aortic rings obtained from diabetic (BB/wor) rats. Each point shows the mean ± SEM of 7–9 experiments. ■, Wistar rats; ▲, BB/wor rats; ▼, DOBE 10^{-5} M; ◆, DOBE 10^{-4} M.

Ex vivo *Experiments*

Effects of the Different Treatments on Endothelium-dependent and Endothelium-independent Relaxation. ACh caused a concentration-dependent relaxation in vessels precontracted with NA 10^{-6} M isolated from the different groups studied. The diabetic state (Group 1) induced a decrease in the endothelium-dependent relaxation induced by ACh, and the treatment with DOBE (50 mg/kg/day, Group 2) was able to restore the endothelium-dependent relaxation to normal. The maximal relaxations were 78.1 ± 5.0%; 50.0 ± 5.1% ($p < 0.05$, n = 7 for Group 0); 73.2 ± 6.1% ($p < 0.05$, n = 7 for the diabetic group) in Groups 0, 1, and 2, respectively (Fig. 5).

On the other hand, the endothelium-independent relaxation induced by SNP was shifted to the left in the diabetic group (Group 1) compared with the control group (Group 0) (the values of EC_{50} were 2.06 ± 0.001 × 10^{-8} M and 1.00 ± 0.02 × 10^{-7} M respectively ($p < 0.05$, n = 7). DOBE treatment

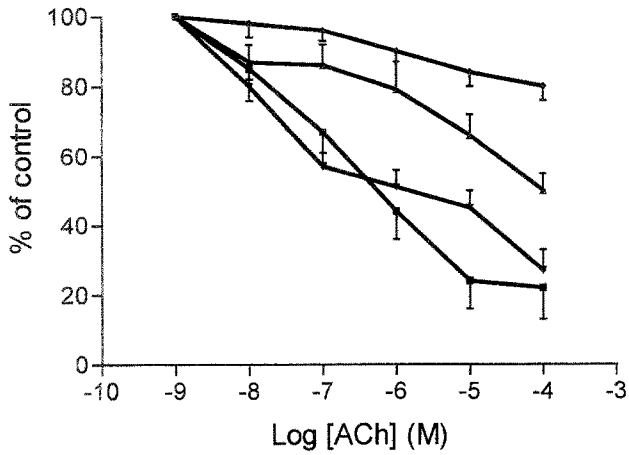

Fig. 5. Effect of DOBE treatment (50 or 500 mg/kg/day; Groups 2 and 3) on endothelium-dependent relaxation induced by ACh (10^{-8}–10^{-4} M) in aortic rings. Each point shows the mean ± SEM of 7–9 experiments. ■, Group 0 (wistar rats); ▲, Group 1 (BB/wor rats); ▼, Group 2 (BB + DOBE 50 mg/kg/day); ◆, Group 3 (BB + DOBE 500 mg/kg/day).

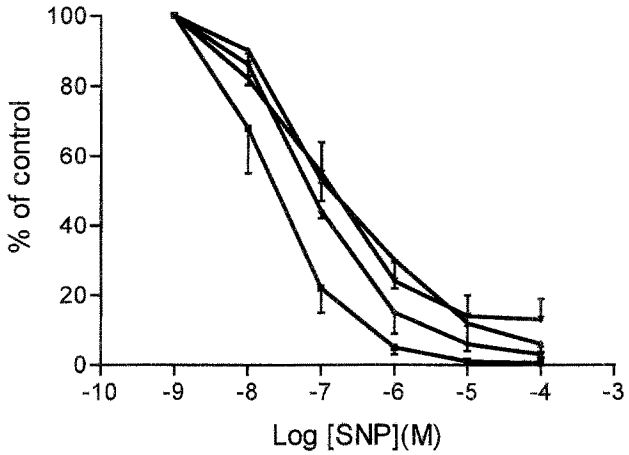

Fig. 6. Effect of DOBE treatment (50 or 500 mg/kg/day; Groups 2 and 3) on endothelium-independent relaxation induced by SNP (10^{-8}–10^{-4} M) in aortic rings. Each point shows the mean ± SEM of 7–9 experiments. ■, Group 0 (wistar rats); ▲, Group 1 (BB/wor rats); ▼, Group 2 (BB/wor + DOBE 50 mg/kg(/day); ◆, Group 3 (BB/wor + DOBE 500 mg/kg (/day).

did not improve the relaxation induced by SNP in the diabetic group. The EC_{50} values were $9.36 \pm 0.2 \times 10^{-8}$ ($p < 0.05$, n = 7 with respect to Group 0) and $6.70 \pm 0.1 \times 10^{-8}$ M ($p < 0.05$ n = 7 with respect to Group 0) in Groups 2 and 3, respectively (Fig. 6).

Discussion

The effect of the diabetic state on contractile responses in animal models is not well established. Thus, aortae from streptozotocin-induced diabetic rats have been found to show an attenuated maximal contractile response to noradrenaline [13], no changes [14], and even an increase in response to noradrenaline [4]. The results of this work show that noradrenaline-induced contractions are increased in aortic rings isolated from diabetic rats as compared with control (nondiabetic) rats. This fact implies that noradrenaline could induce the release of a contracting factor from endothelial and this factor could increase the noradrenaline-induced contraction.

Our results concerning the increasing contraction induced by noreadrenaline are in disagreement with other authors who found no difference between aortic rings from control and BB rats [15,16]. A possible explanation might be that these studies were carried out for 60–90 days whereas in our study the rats were maintained in a diabetic state for 180 ± 7 days. In this way, the results of some studies have suggested that the influence of diabetes on vascular reactivity varies with the duration of the diabetic state [2].

On the other hand, when diabetic aortic rings were incubated in the presence of DOBE, we found a decrease in the contractile responses induced by NA only with the highest concentration of DOBE (10^{-4} M). In order to rule out any action on NA-induced NO synthesis, we tried to reverse the effect of DOBE in the presence of L-NAME (a NO synthesis inhibitor). Figure 2 shows that L-NAME did not significantly reverse the effect of DOBE on contraction induced by NA. However, in another group of experiments, we tried to demonstrate the hypothesis that DOBE could act by modulating β-adrenergic receptors in vascular smooth muscle. For this, we repeated the contractile procedure in the presence of DOBE plus propranolol. Propranolol completely reversed the effect of DOBE on NA-induced contraction (Fig. 2), demonstrating that DOBE acts on β-adrenergic receptors.

On the other hand, it has been suggested that abnormal endothelium function may be a contributory factor to large and small vessel disease in diabetes mellitus [17]. Thus, impaired relaxation response to ACh has been demonstrated in the large blood vessels of animals with experimentally induced diabetes [4,18]. The majority of studies using BB/wor rats have shown a specific impairment of endothelium-dependent relaxation in conduit arteries such as the aorta [15,16]. In this work, ACh produced a concentration-dependent relaxation in both control and diabetic rings. The relaxation was significantly attenuated in diabetic rings compared with nondiabetic control rings (Fig. 4). Moreover, incubation with DOBE (10^{-5} M and 10^{-4} M) significantly improved this relaxation, suggesting that DOBE acts on NO synthesis, as we previously reported in rabbit-isolated aortic arteries [9,10]. Moreover, treatment with DOBE (50 mg/kg/day) improved the endothelium-dependent relaxation in rat aorta isolated from diabetic animals.

Furthermore, the effect of DOBE on endothelium-independent relaxation was also tested. We found an impairment in the sensitivity to sodium nitroprusside in diabetic rings compared with control nondiabetic rings, and neither the incubation with DOBE nor the treatment with the two doses of DOBE were able to improve this effect. It is well known that SNP, a NO donor, acts directly on soluble guanylate cyclase of vascular smooth-muscle cells, leading to vascular smooth muscle relaxation. These results suggest that not only is the L-Arg-NO pathway altered in the diabetic state but that the NO-cGMP pathway it is also altered.

In conclusion, in spontaneously diabetic rats, DOBE restored endothelium-dependent, but not independent relaxation to normal and also decreased the contractile responses

induced by NA through a mechanism involving β-adrenergic receptors.

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