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Vasodilator effects of cromakalim and HA 1077 in diabetic rat aorta

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Summary

BACKGROUND: Impairment of the vasorelaxant responses have been reported in diabetes mellitus. In this study, the roles of the K_{ATP} channel and rho kinase pathway were evaluated by using the K_{ATP} channel opener cromakalim and Rho-kinase inhibitor HA 1077 in diabetic rat aorta.
METHODS: Adult male Wistar rats weighing (250–300 g) were divided into diabetic and control groups. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 55 mg/kg/i.p).
RESULTS: Vasodilator responses induced by cromakalim (10^{-7} to 10^{-3}M) and HA 1077 (10^{-6} to 10^{-4}M) were significantly less in diabetic rings compared with control rings (p <0.01). The decrease in the relaxant effect of cromakalim was more in endothelium-denuded rings compared to the endothelium-intact rings (p <0.05). There were no significant differences between endothelium intact and non-intact rings in the presence of HA 1077. When two drugs were administered together, relaxation was significantly less than with seperate administration of each drug in the diabetic group (p <0.01). Pre-treatment with N omega-nitro-L-arginine methylester (L-NAME) (10^{-6} to 10^{-4} M), an NO synthase inhibitor, significantly decreased the relaxant response to cromakalime and HA 1077 in both the control and diabetic groups (p <0.05).
CONCLUSIONS: These results suggest that the impaired relaxant effects were further decreased depending on K_{ATP} channel activity but the effects of Rho-kinase enzyme inhibitors on relaxation responses were not significantly changed in diabetes mellitus.

Key words: *aorta; cromakalim; fasudil; K_{ATP} channels; Rho-kinase*

Introduction

Diabetes mellitus (DM) is a primary risk factor for cardiovascular disorders [1]. Diabetic patients suffer from vascular complications, which include atherosclerosis, nephropathy, retinopathy and neuropathy, and there is a strong correlation between the incidence of cardiovascular disease and mortality rates in diabetic patients [2].
Although the mechanisms by which diabetes increases cardiovascular complications are not completely understood, the change in the functions of the vascular smooth muscle is one of the mechanisms, among with several others, providing a basis for vascular problems in diabetes. Impaired function of the vascular endothelium, enhanced calcium mobilisation and inhibition of Na^+, K^+-ATPase activity appear to be the other contributing factors in vascular reactivity changes [3].
Potassium channel activity is one of the most determining factors of the vascular tone. ATP-sensitive K^+ (K_{ATP}) channels are mostly closed under resting physiological conditions and open in respond to changes in cellular metabolic states, such as ischaemia and hypoxia [4]. K_{ATP} channel expression or functions are thought to be decreased and altered regulation of K_{ATP} channel activity may participate in the abnormal vascular responses in diabetes mellitus [5, 6]. Though the mechanisms are not completely clear yet, some investigations show that the relaxation responses to K_{ATP} channel openers are decreased in diabetes mellitus [7, 8].
On the other hand, a role for RhoA/Rho-kinase activity has been demonstrated in the contraction of vascular smooth muscle. This contraction can occur independently of intracellular Ca^{2+} changes and is known as Ca^{2+} sensitisation. Rho-kinase has been shown to phosphorylate the myosin-binding sub-unit of myosin light chain (MLC) phosphatase, leading to the inhibition of phosphatase activity. The Rho-kinase mediated inhibition of MLC phosphatase leads to the maintenance of the phosphorylated state of MLC, promoting vascular smooth muscle contraction. It was also reported that the inhibition of RhoA/Rho-kinase–mediated Ca^{2+} sensitisation induces the relaxation of vascular smooth muscle [9–11].
The Rho– Rho-kinase pathway has attracted a great deal of attention, and cardiovascular research in particular. Recent studies have indicated that Rho-kinase inhibitors might be useful for treating cardiovascular diseases including angina pectoris, hypertension, pulmonary hypertension, cerebral vasospasm and stroke in humans [12–14]. Kikuchi et al. [29] showed that long-term treatment with fasudil not only improved insulin resistance but also might prevent diabetic nephropathy [15]. Furthermore, the inhibition of the Rho-Kinase (ROCK) pathway has a widespread prospect in treating diabetic retinopathy and diabetes-related endothelial dysfunction [16]. In diabetic patients,

hyperglycemia-induced oxidative stress promoted endothelial plasminogen activator inhibitor-1 (PAI-1) expression via Rho-kinase which might enhance the risk of cardiovascular events [17].

Multiple mechanisms have been linked to the Rho-kinase pathways and nitric oxide (NO) systems. Accumulating evidence indicates that the expression and activity of eNOS is regulated by the rho/rho kinase pathway, and inhibition of Rho A and its downstream target of Rho-kinase by NO would contribute to the vasodilator action of NO [18–20]. The role of Rho-kinase in mediating contractile responses is well known, but to our knowledge in diabetes mellitus the role of this pathway has not been clarified yet. Therefore the present study was designed to investigate the effect of K_{ATP} channel opener, cromakalim, and the Rho-kinase enzyme inhibitor, HA 1077, on vasorelaxation in diabetic rat aorta.

Materials and methods

Animals and experimental design

All experimental procedures were approved by the Celal Bayar University of Animal Experimentation Ethics Committee. Adult male Wistar rats weighing (250–300 g), were studied (n = 40). They were housed under controlled temperature conditions of 21 ± 3 °C. Diabetes mellitus was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ), 55 mg/kg dissolved in 0.9% saline solution [21]. Experiments were performed 8 weeks after STZ injection. Group 1 served as a control (n = 20), and Group 2 had diabetes mellitus (n = 20).

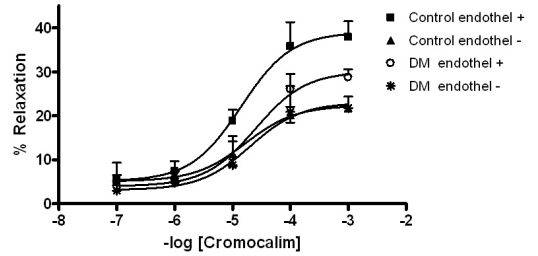

Figure 1

The relaxing effect of cromakalim (10^{-3} to 10^{-7}M) on phenylephrine-induced ($1x10^{-6}$M) contraction in presence (endothel +) or absence (endothel –) of endothelium, in control and streptozotocin-induced diabetic rats. All values represent mean ± S.E.M.

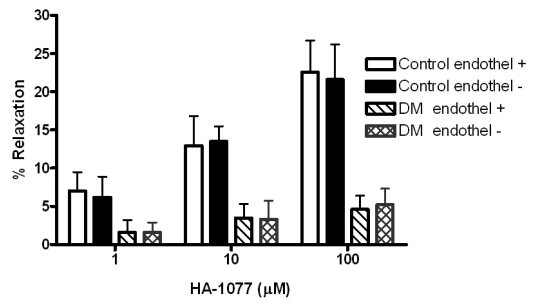

Figure 2

The relaxing effect of HA 1077 (10^{-6} to 10^{-4}M) on phenylephrine-induced ($1x10^{-6}$ M) contraction in the endothel + or endothel – of endothelium in control and streptozotocin-induced diabetic rats. All values represent mean ± S.E.M.

Bodyweight and serum glucose

Serum glucose levels and bodyweight were monitored at the end of the experiment. Blood samples for plasma glucose levels were collected after a 12 h fasting period from the thoracic aorta just before killing the rats and measurements were performed using a glucometer (IME-DC). Diabetes was verified by a serum glucose level higher than 250 mg/dl [21].

Preparation of tissues

Animals were anesthetised with sodium pentobarbital (50 mg/kg, ip). The aorta was cleaned of fat and connective tissue. Aortic rings 2–3 mm in length were prepared and mounted in 20 ml organ baths containing the Krebs–Henseleit solution. The solution consisted of (mM): NaCl 118. 0, KCl 4. 7, $NaHCO_3$ 25. 0, $CaCl_2$ 1.8, NaH_2PO_4 1.2, $MgSO_4$ 1.2, dextrose 11.0, bubbled with 95% O_2 and 5% CO_2 at 37 °C. The preparations were allowed to equilibrate for at least 1 h under 2 g resting tension. Tension was continuously recorded by connection to a data acquisition system (MP 150 Biopac Systems, Ankara, Turkey). After 60 min equilibration, cumulative concentration–response curves were generated for Phenylephrine (Phe (10^{-3}–10^{-5} M)) on each ring. Phe was added in a cumulative manner until a maximum response was achieved. After the addition of each dose of Phe, a plateau response was obtained before the addition of the next dose of Phe. Each ring was sequentially washed and re-equilibrated and was allowed to relax to baseline. The rings were contracted with a submaximal concentration of phenylephrine. The endothelium was removed by gently rubbing the intimal surface with a stainless steel rod. The functional integrity of the endothelium was evaluated by the presence of relaxation induced by acetylcholine (ACh, 10^{-5} M). The effectiveness of endothelium removal was confirmed by the absence of the relaxation induced by ACh. Sustained relaxation (70% of precontracted tone) in response to acetylcholine confirmed the presence of functional endothelium.

The effect of K_{ATP} channel opener cromakalim (10^{-7} to 10^{-3}M) on contractile responses was assessed by treating arterial segments for 30 min prior to addition of phenyleph-

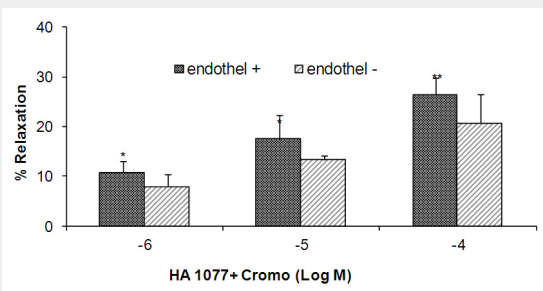

Figure 3

The relaxing effect of HA 1077 + cromakalim (10^{-6} to 10^{-4}M) on phenylephrine-induced ($1x10^{-6}$ M) contraction in the endothel + or endothel – of endothelium in streptozotocin-induced diabetic rats. All values represent mean ± S.E.M.

rine. Similarly, the effect of the Rho-kinase inhibitor, HA 1077 (10^{-6} to 10^{-4}M), on contractile responses was assessed by 30 min treatment prior to addition of phenylephrine. Since the elucidation of the biosynthesis of NO, L-arginine analogs (such as NG-nitro-L-arginine methylester; L-NAME) have been used as inhibitors of NOS to assess the contribution of NO in many diverse physiological and pathological processes. Therefore to investigate the influence of L-NAME on dilator responses, the strips were pre-incubated with L-NAME for 30 min, and then cromakalim and HA 1077 were added.

Drugs and chemicals

All chemicals were purchased from Sigma Chemical (St Louis, MO, USA). All drugs, except STZ, were dissolved in Krebs solution. All the testing compounds were dissolved in distilled deionised water to prepare stock solutions, and further dilutions were made in Krebs solution. Streptozotocin was freshly dissolved in 0.9% saline solution. The concentrations of drugs are expressed as final molar concentration of the base in the organ bath.

Data and statistical analysis

Data are expressed as the mean ± S.E.M. Relaxation responses for cromakalim and HA 1077 are expressed as a percentage decrease of the maximum contractile response induced by phenylephrine. Results were analysed using Student's t test and analyses of variance (ANOVA) followed by Tukey Kramer multiple comparisons test. Differences were considered to be statistically significant for $p < 0.05$.

Results

Relaxant responses to cromakalim

To investigate KATP channel-induced relaxation, cromakalim (10^{-7} to 10^{-3}M) was added cumulatively to the pre-contracted tissues by phenylephrine (fig. 1).

In rings from age-matched control rats, cromakalim induced concentration-dependent relaxation, with the maximum response at 10^{-3}M (1 mM). These relaxation responses were significantly less in rings of streptozotocin-induced diabetic rats ($p < 0.01$ vs. control group). Also relaxation responses attenuated significantly in aortas without endothelium compared to those with endothelium ($p < 0.05$) (fig. 1).

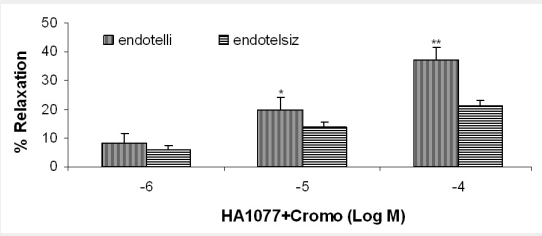

Figure 4

The relaxing effect of HA 1077+ cromakalim (10^{-6} to 10^{-4}M) on phenylephrine-induced ($1x10^{-6}$ M) contraction in the endothel + or endothel – of endothelium in control rats. All values represent mean ± S.E.M.

Relaxant responses to HA 1077

Relaxation responses to the Rho-kinase inhibitor, HA 1077 (10^{-6} to 10^{-4}M), were significantly less in diabetic rats compared to the control group ($p < 0.05$). However the dilator responses between aortas with and without endothelium were not changed (fig. 2).

Co-treatment with cromakalim and HA 1077 markedly decreased relaxation responses in diabetic rats (fig. 3), compared to the control group ($p < 0.01$) (fig. 4).

HA 1077 did not alter relaxation between the groups with or without endothelium.

Effect of L-NAME on the relaxant responses to cromakalim and HA 1077

Pre-incubation of the aorta with L-NAME resulted in a significant inhibition of relaxant responses to cromakalim + HA 1077 in both control and diabetic groups ($p < 0.05$), but its inhibitory effect was more significant in the diabetic group ($p < 0.01$) (fig. 5).

Discussion

In the present study, we investigated the functional role of the K_{ATP} channel opener cromakalime and the Rho-kinase enzyme inhibitor HA 1077 on the vascular responses in diabetic rat aorta. According to our findings, cromakalim caused significantly impaired relaxation responses in diabetic rats. Also a decrease in the relaxation response caused by cromakalim was monitored in both the control group and diabetic group due to endothelial removal.

There were no significant differences in the relaxation responses where K_{ATP} channel opener cromakalim and rho kinase enzyme inhibitor HA 1077 were applied together, compared to the single effect of cromakalim in both the diabetic and control groups. However the relaxation responses significantly increased in the endothelium-intact rings compared to endothelium-removed rings in both groups. These results show that the Rho-kinase enzyme inhibitor HA 1077 does not increase the cromakalim's vas-

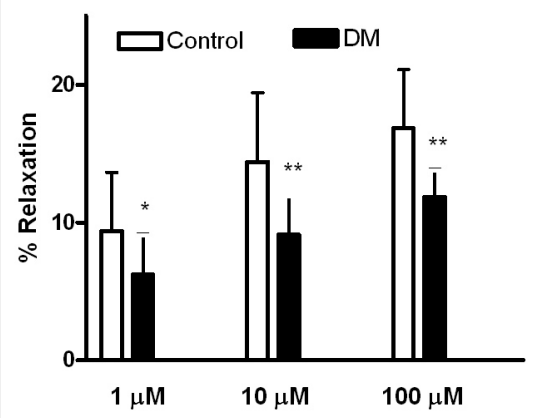

Figure 5

Effect of L-NAME on relaxation responses produced by cromakalim + HA 1077 in streptozotocin-induced diabetic rats. Aortic rings were pre-treated for 30 min with L-NAME (10^{-6} to 10^{-4}M) in the endothelium intact rings. All values represent mean ± S.E.M. Relaxation responses were statistically significant in the DM group compared to control group. * ($p < 0.05$, ** $p < 0.01$).

odilator effect. When used concomitantly, the relaxation responses were not different than when cromakalim was used alone. The relaxation responses to HA 1077 alone were significantly less in diabetic rats compared to the control group. Therefore the relaxation responses in the endothelial rings in this group were considered to be the effect of cromakalim.

There have been different studies that have reported about the attenuation of the vasodilator effect of K_{ATP}-channel openers in the diabetic rat [6, 7]. In some studies, attenuated dilator responses have been obtained in the cerebral arterioles [8]. These results were consistent with our results. The role of the endothelium in K_{ATP} channel openers' vasodilator effect is controversial because increased, decreased or stable responses have been reported [22–24]. In another study it was reported that inhibition of vasodilator effects of basally released nitric oxide can reduce relaxation via ATP-sensitive K^+ channels in rat aortas [22].

The maximum relaxation responses obtained with HA 1077 did not show any difference between the endothelial and non-endothelial aortic rings in the control group. According to a previous study, removal of endothelium leads to reduction of the vasorelaxing effect of the Rho-kinase inhibitors [20]. However we found no significant difference in the relaxation responses between endothelium intact and removed rings. There were significant differences found in the maximum relaxation responses between the diabetes and control groups. In the diabetic arteries we found no relaxing effect of Rho-kinase enzyme inhibitors. Yet to the best of our knowledge there have been few studies investigating the role of Rho-kinase inhibitors on the vascular tonus in diabetes mellitus. However inhibitors of the RhoA/ROCK pathway are showing promise as potential regulators of vascular damage. ROCK inhibitors such as fasudil and Y-27632 protect against various cardiovascular diseases such as atherosclerosis, pulmonary and systemic hypertension and chronic heart failure in clinical and pre-clinical studies [26, 27].

Also in another study it was stated that the Rho kinase-pathway participated in the pathogenesis of diabetic nephropathy, and that fasudil could prevent development of this complication [28].

When the effect of NO synthase inhibitor L-NAME on the relaxation responses obtained with HA 1077 and cromakalim was researched, the relaxation responses were greatly decreased in both groups though it was more significant in the diabetes group. This is quite similar with research showing the decrease in the endothelial dependent relaxation responses because the EDRF/NO system in particular is seriously affected in diabetes. Diabetes-induced endothelial dysfunction is characterised by reduced bioavailability of nitric oxide (NO) in the vessel wall. Endothelial dysfunction reflects insufficient NO-mediated relaxation, which has been suggested to be an early event in diabetic atherosclerosis and is associated with vascular complications [29]. Therefore it is expected that in endothelial dysfunction where the NO-producing system is compromised, inhibition of Rho-kinase pathway should have much less vasorelaxant effect [30–32].

In conclusion, the present study shows that the impairment in relaxation responses in the diabetic rat aorta, mostly depends on the changes in the functioning of ATP-sensitive potassium channels, with less apparent role for Rho-kinase pathway. The results also suggested that inhibition of K_{ATP}-channels cause more impairment in vasodilator responses in endothelium-denuded rings than in endothelium-intact rings.

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Figures (large format)

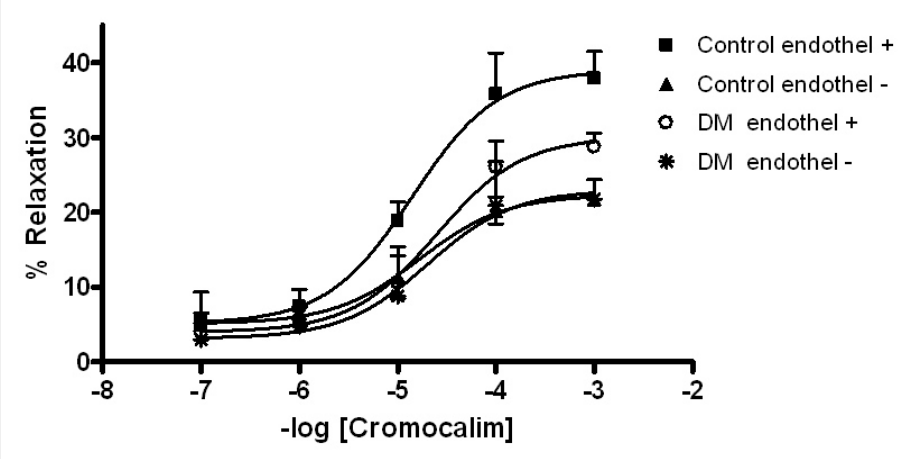

Figure 1

The relaxing effect of cromakalim (10^{-3} to 10^{-7}M) on phenylephrine-induced ($1x10^{-6}$M) contraction in presence (endothel +) or absence (endothel –) of endothelium, in control and streptozotocin-induced diabetic rats. All values represent mean ± S.E.M.

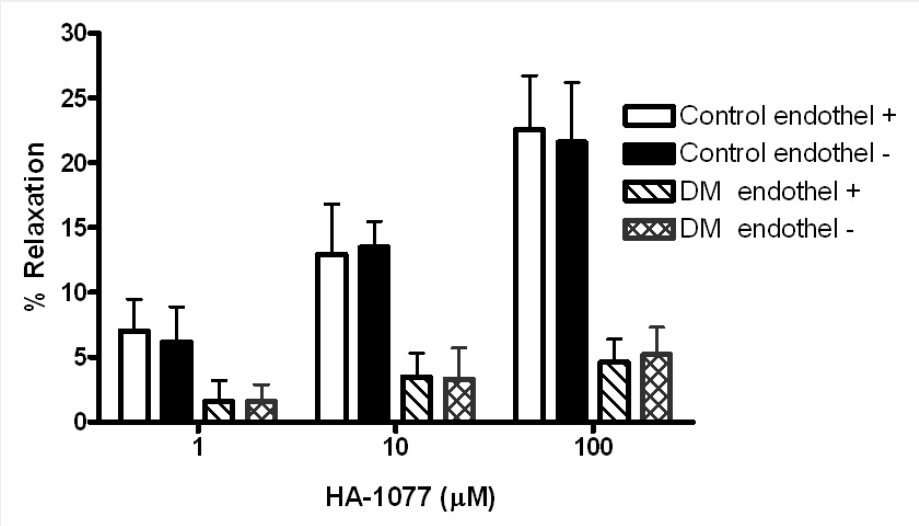

Figure 2

The relaxing effect of HA 1077 (10^{-6} to 10^{-4}M) on phenylephrine-induced ($1x10^{-6}$ M) contraction in the endothel + or endothel – of endothelium in control and streptozotocin-induced diabetic rats. All values represent mean ± S.E.M.

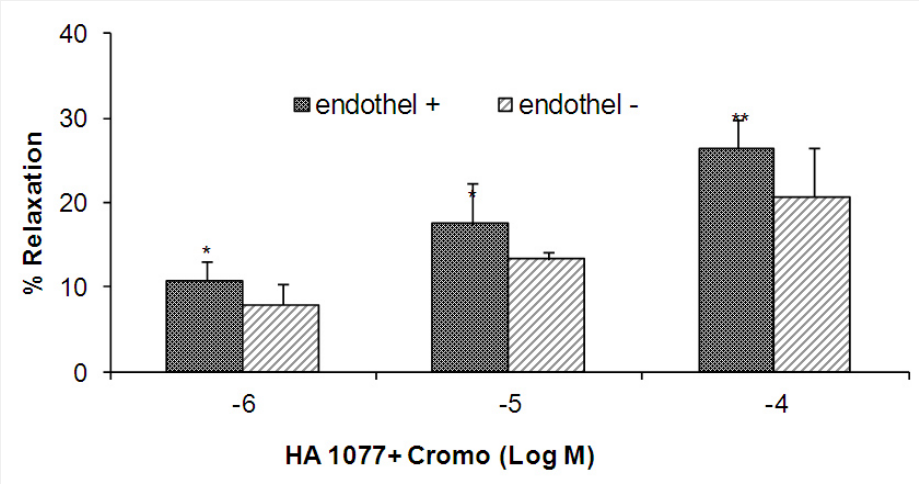

Figure 3

The relaxing effect of HA 1077 + cromakalim (10^{-6} to 10^{-4}M) on phenylephrine-induced ($1x10^{-6}$ M) contraction in the endothel + or endothel – of endothelium in streptozotocin-induced diabetic rats. All values represent mean ± S.E.M.

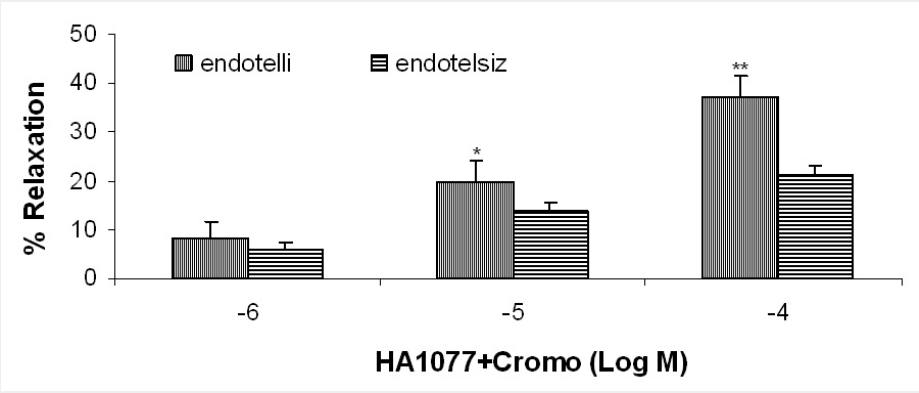

Figure 4

The relaxing effect of HA 1077+ cromakalim (10^{-6} to 10^{-4}M) on phenylephrine-induced ($1x10^{-6}$ M) contraction in the endothel + or endothel – of endothelium in control rats. All values represent mean ± S.E.M.

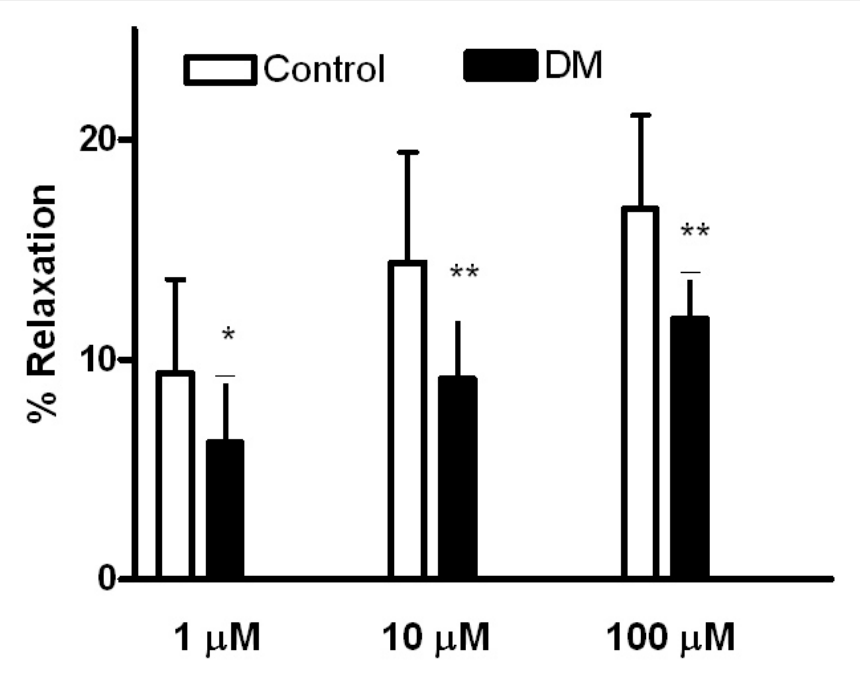

Figure 5

Effect of L-NAME on relexation responses produced by cromakalim + HA 1077 in streptozotocin-induced diabetic rats. Aortic rings were pre-treated for 30 min with L-NAME (10^{-6} to 10^{-4}M) in the endothelium intact rings. All values represent mean ± S.E.M. Relaxation responses were statistically significant in the DM group compared to control group. * (p <0.05, **p <0.01).