EXPRESSION LEVEL OF RHO-KINASE AND THE INFLUENCE OF ITS INHIBITORS ON THE REACTIVITY OF THE DIABETIC MICE AORTA

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SUMMARY

Expression level of Rho-kinase (ROCK-2) protein and alteration in the relaxant responses to two Rho-kinase inhibitors, Y-27632 and fasudil were examined in the isolated aortic rings from streptozotocin (STZ, 100 mg kg-1 day-1, i.p. for two consecutive days)-induced diabetic mice. Acetylcholine (ACh, 10-9-10-4 M)-evoked relaxation was markedly suppressed in diabetic aorta; however, relaxant responses to Y-27632 (10-8-10-5 M) and fasudil (10-8-10-4 M) were not changed. pEC50 values for Y-27632 were 6.22±0.07 M in diabetic and 6.24±0.1 M in control aorta (P>0.05), respectively. pEC50 values for fasudil were 5.48±0.087 M in the diabetic and 5.57±0.08 M in control aorta (P>0.05), respectively. Based on the pEC50 values (6.24±0.1 M), Y-27632 is much more potent than fasudil (5.57±0.08 M, P<0.0001) in relaxing non-diabetic rings. Neither papaverine nor sodium nitroprusside-induced relaxation was modified in diabetic aorta. Expression of ROCK-2 protein levels was not different in aortic smooth muscle homogenates from control and diabetic mice. These results may suggest that relaxations to Rho kinase inhibitors are not altered in the isolated aorta from STZ-diabetic mouse, although it has been reported that activation of Rho kinase is enhanced and RhoA is upregulated in diabetes. Conclusively, the Rho-kinase inhibitors may have potential in the treatment of diabetes-associated vascular disorders.

Key Words: *Aorta, diabetes, fasudil, nitric oxide, rho-kinase, Y-27632*

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ÖZET

DİABETİK FARE AORTASINDA RHO-KİNAZ ENZİM EKSPRESYONLARI VE İNHİBİTÖRLERİNİN AORTA REAKTİVİTESİ ÜZERİNE ETKİLERİ

Streptozotosin (STZ, 100 mg/kg/gün, i.p. iki gün üst üste) ile indüklenen diyabetik farelerin aortalarında Rho-kinaz (ROCK-2) ekspresyon düzeyleri ve bu enzim inhibitörleri olan Y-27632 ve fasudilin aorta reaktivitesi üzerine etkileri araştırıldı. STZ, farelerde başarılı bir şekilde diyabet oluşturdu. Kan şeker düzeyleri kontrol grubunda 94.8±4.1 mg/dl iken, diyabetik grupta 428.0±26.2 mg/dl idi (P<0.001). Asetilkolin (10-9-10-4 M) ile oluşturulan gevşemeler diyabetik aortada önemli ölçüde baskılanmış bulundu buna karşılık, Y-27632 (10-8-10-5 M) ve fasudile (10-8-10-4 M) karşı oluşturulan gevşemeler değişmedi. Y-27632'nin pEC50 değeri, diyabetik aortada 6.22±0.07 M iken, kontrol aortasında 6.24±0.1 M idi (P>0.05). Fasudile ait pEC50 değerleri ise, diyabetik aortada 5.48±0.087 M, kontrol aortasında ise 5.57±0.08 M olarak bulundu (P>0.05). Bu verilere dayalı olarak, Y-27632'nin fasudilden çok daha güçlü (potent) olduğu ortaya çıkmaktadır. Diğer taraftan, papaverin ve sodyum nitroprussiyat gevşemeleri de diyabetik aortada değişmedi. Ayrıca, kontrol ve diyabetik aorta Rho-kinaz protein düzeylerinde anlamlı bir değişiklik saptanmadı. Bu sonuçlar, STZ ile indüklenen diyabetik farelerin aortalarında ROCK-2 enzim düzeylerinin değişmediğini, ayrıca bu enzim inhibitörlerine verilen vazodilatör yanıtlarda bir değişiklik olmadığını göstermektedir. Sonuç olarak, Rho-kinaz enzim inhibitörleri diyabetik vasküler disfonksiyonda faydalı olabilir.

Anahtar Kelimeler: *Aorta, diyabet, fasudil, nitrik oksit, rho-kinaz, Y-27632*

INTRODUCTION

It has been demonstrated that there could be an enhanced sensitivity to vasoconstrictors in vascular smooth muscles from diabetic animals although decreased or unchanged contractile responses have also been reported (1). This augmented contractility may be attributed to an increased Ca2+ sensitivity rather than an elevation in cytoplasmic free Ca2+ (Ca2+i (2, 3, 4). A small GTPase, Rho and its dowstream effector, Rho kinase (ROCK-II) have been reported to take a role in the Ca2+-sensitization described that the phosphorylation of myosin light chain (MLC) and the subsequent regulation of contractile force are independent of changes in Ca2+i (5). In diabetic status, various signal transduction pathways such as Rho and mitogen-activated protein (MAP) kinase may be altered, and this alteration might take a role in the increased contractility, cellular proliferation, cytokine production as well as permeability (6, 7). Recently, it has been reported that Rho was increased at the transcription level and was activated in the basilar arteries from streptozotocin (STZ)-induced diabetic rats (8). Furthermore, in the vascular smooth muscle from aorta of Goto-Kakizaki rats, representing a type II diabetes model, Rho-kinase activity is increased but myosin phosphatase activity decreased (9), leading to an enhanced contractile response to vasoconstrictors in diabetic status. Consequently, Rho/Rho-kinase signalling could

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be associated with an increased risk of hypertension in diabetes.

Given that this pathway is upregulated in diabetic condition, we investigated, in the present study, how relaxant responses to two Rho-kinase inhibitors, Y-27632 and fasudil (HA-1077) (10) will be altered in the isolated aorta from STZ-induced diabetic mice.

MATERIALS AND METHODS

Diabetic protocol:

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Mersin University Centre for Experimental Medicine, and the protocol was approved by the local ethic committee. Male balb/c mice initially weighing 25-35 g were caged separately with a 12-h light-12-h dark photoperiod and received standard mice chow ad libitum throughout the experiment. The mice were randomly divided into two main groups. In the non-diabetic control groups (n=12), 0.1 M citrate buffer (pH=4.5), the vehicle of streptozotocin was injected (0.3 ml 40 g-1 mouse weight) intraperitoneally twice to all animals at a 24-h interval (for 2 days). In diabetic group, the mice (n=13) received intraperitoneal streptozotocin (100 mg kg-1) at a 24-h interval for 2 days. During 30 days two animals died in this diabetic group. One control and one diabetic mouse were used for daily experiment. Glucose level was detected from the tail blood of each animal on the 30th day by use glucose strips (Boehringer Mannheim, Germany).

Organ bath experiments:

The animal was sacrificed by cervical dislocation. The aorta was immediately removed and placed in a Petri dish containing Krebs-bicarbonate solution (in mM: NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, glucose 11, Na2EDTA 0.01). Four or five rings (about 3 mm width) from each aorta was obtained, and mounted on two steel wires inserted through the lumen under an initial tension of 0.5 g. The rings were equilibrated for 1 h in 10 ml organ chambers maintained at 37o C, filled with the Krebs-bicarbonate solution gassed with 95 % O2 and 5 % CO2 (pH= 7.4). Tension was recorded isometrically with a force displacement transducer (COMMAT, Ankara Turkey), and displayed on a Biopac acquisition system (Bipoac systems, California, USA).

After the equilibration, the rings were precontracted submaximally with 10-6 M phenylephrine. Once the steady state of contraction was obtained the relaxant agents were applied cumulatively on the rings from the diabetic or non-diabetic mice.

Western blotting for ROCK-2:

Following sacrification of the rat, the aorta was isolated along 4-5 cm and removed after clearly dissecting out connective and fat tissue. The tissues were homogenized with the lyses buffer solution (composition in mM; Tris-HCl (pH=7.4) 50 mM, NaCl 400 mM, EGTA 2mM, EDTA 1mM, dithiothreitol (DTT) 1 mM, phenylmethylsulfonyl fluoride (PMSF) 10 _M, leupeptine 10 _g/ml, pepstatin 1 _g/ml, benzamidine 1mM). The homogenate was centrifuged at 900 g for 10 min at 4o C to remove nuclei and unlysed cells, and the supernatant was removed. It was then used for protein analysis (with Lowry method) and Western blotting. The homogenate was loaded in wells, electrophoresed on 8 % polyacrilamide-SDS gels for 90 min and then transferred to a nitrocellulose membrane for 3 h. The membrane was blocked with the blocking agent of ECL advance kit (Amersham Biosciences, Freiburg, Germany) in Tris-buffered solution containing 0.05 % tween-20 (TBS-T) for 1 h. It was then probed with a primary antibody raised against ROK_ (ROCK-II, Polyclonal IgG, sc-1851, Santa Cruz Biotechnology Inc:, CA, USA) at 1:500

dilution followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1:1000, Santa Cruz Biotechnology Inc., CA, USA). The blots were then detected with the ECL Advance kit (Amersham Biosciences, Freiburg, Germany).

Drugs and chemicals:

Phenylephrine hydrochloride, acetylcholine chloride, papaverine hydrochloride, sodium nitroprusside and streptozotocin were all obtained from Sigma Co. (St Lois, MO, USA). Y-27632 and fasudil (HA-1077) were purchased from Tocris Cookson Ltd (Bristol, UK). All chemicals except streptozotocin were dissolved in distilled water, and stored at -20o C. Streptozotocin was dissolved in 0.1 M citrate buffer (pH=4.5).

Analysis of results:

Relaxations were expressed as percent reductions of phenylephrine-induced tone, and shown as mean±S.E.M. Vasoconstrictor response to phenylephrine was expressed as mN of tension. For comparison, one way of analysis of variance (ANOVA), followed by the Dunnet post hoc test or student's t test, if appropriate, was used. P values less than 0.05 were considered as significant. The density of protein bands was evaluated by a computer program (Scion image, USA).

RESULTS

Plasma glucose level and body weight alterations in control and diabetic mice:

Streptozotocin (100 mg kg-1 day-1, for two days)-administered mice exhibited a remarkable diabetic status. Blood glucose levels and body weight alterations were shown in Table 1.

Effects of phenylephrine, acetylcholine, papaverine, sodium nitroprusside, Y-27632 and fasudil on the aorta from diabetic and non-diabetic mice:

Phenylephrine (10-6 M)-induced contraction was 3.43±0.20 mN in control; however, it was 4.22 ±0.29 mN in diabetic aorta (P<0.05). Acetylcholine (ACh, 10-8-10-4 M, Fig. 1), Y-27632 (10-8-10-5 M, Fig. 1), fasudil (10-8-3x10-5 M, Fig. 1), papaverine (10-8-3x10-5 M) and sodium nitroprusside (SNP, 10-9-3x10-7 M) relaxed the aortic rings in a concentration dependent manner. pEC50 values for papaverine were 5.26 ±0.09 M and 5.29±0.1 M (P>0.05) in diabetic and non-diabetic mice, respectively. The values for SNP were, respectively, 7.90±0.08 M and 7.99±0.1 M in diabetic and non-diabetic mice (P>0.05). ACh-induced relaxation was conspicuously suppressed in the aorta from diabetic mice. However, relaxations to Y-27632 and fasudil were not altered in the aortic rings from diabetic mice (Fig. 1).

Table 1: Alterations of body weight and plasma glucose level in diabetic (streptozocin-induced, 100 mg/kg/day/for two days) and non-diabetic mice.

	Body weight (g)		Plasma glucose levels (mg/dl)	
	0th day	30th day	0th day	30th day
Non-diabetic mice (n=12)	30.5±1.2	35.9±1.4**	93.3±6.5	96.4±6.0
Diabetic mice (n=11)	35.3±1.0	28.2±1.5***	94.8±4.1	428.0±26.2†

*Data represent mean±S.E.M. **: P<0.01, ***:P<0.001 different from that at the beginning of streptozocin or vehicle application, §: P<0.001 different from non-diabetic group. Student t test (paired and unpaired) was used for comparison.*

Expression level of Rho-kinase (ROCK-2) in the aortic homogenates from diabetic and non-diabetic rats:

Western blot analysis showed that ROCK-2 protein level did not differ in control and diabetic aortic smooth muscle homogenates (Fig. 2).

DISCUSSION

In the present study, we have investigated how relaxant responses can be altered in the isolated aortic rings from streptozocin-induced diabetic mice as there are no functional studies where Rho-kinase inhibitors have been tested in the vascular tissue obtained from diabetic animals although Rho proteins and Rho-kinase enzyme

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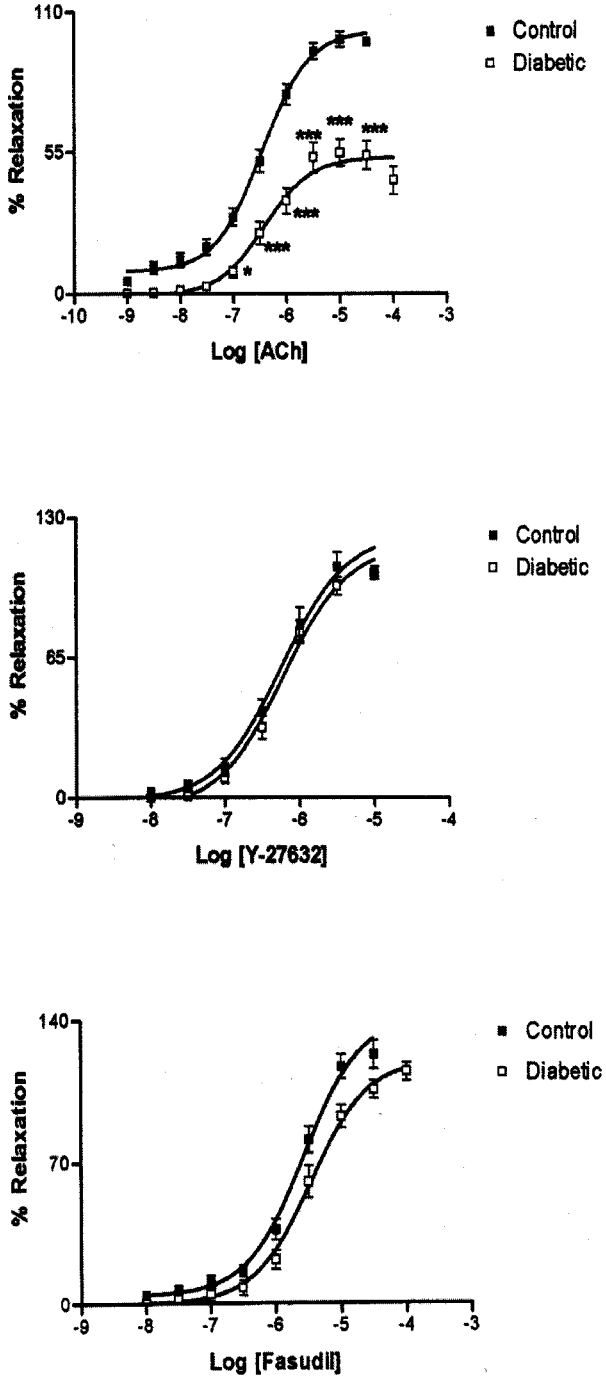
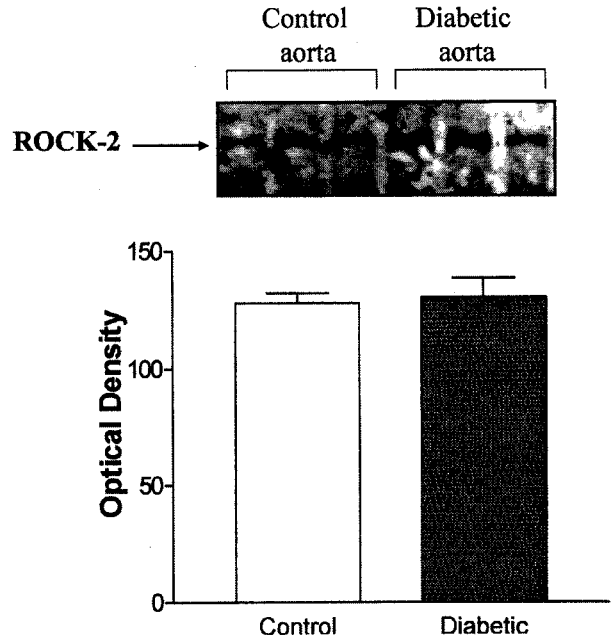
activation have been reported to be upregulated in diabetic conditions (8, 9).

*Figür 1 Effect of acetylcholine (ACh, 10-8-10-4 M, upper panel), Y-27632 (10-8-10-5 M, central panel), fasudil (10-8-3x10-5 M, lower panel) on the aortic reactivity in control and diabetic mice. Data are expressed mean±S.E.M. of 12-16 observations. Comparison was made by use of one-way of ANOVA, followed by Bonferoni post hoc test. *: P<0.05, ***: P<0.001.*



Figür 2. Expression of Rho kinase protein in contol (saline-treated) and diabetic (STZ-treated) aortic smooth muscle homogenates. The homogenates were submitted to SDS-PAGE with 8 % polyacrilamide and then transferred to a nitrocellulose membrane. The membrane was blocked with the blocking agent of the ECL Advance kit in Tris-buffered solution containing 0.05 % tween-20 (TBS-T) for 1 h. It was then probed with a primary antibody raised against ROKa (Polyclonal IgG, Santa Cruz Biotechnology Inc., USA) at 1:500 dilution followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1:1000). The blots were then detected with an Advance Chemiluminescence Detection Kit (Amersham Biosciences, Germany). Data are expressed mean±S.E.M. of 3 observations. Comparison was made by use of one-way of ANOVA, followed by Dunnet post hoc test.



In diabetic animal models, there are a great number of studies, the results of which are controversial by the findings of decreased, unchanged or increased contractile responses to vasoconstrictors (1). These intriguing observations are also valid for vasodilator agents. In this study, acetylcholine-induced relaxation was significantly attenuated, which might be due to abnormal endothelial function. On the other hand, we observed an enhanced contractile response to phenylephrine in the aorta from streptozocin-diabetic mice. This may

be attributed different kind of mechanisms such as overproduction of superoxide anions (11), activation of protein kinase C (6) or upregulation of Rho/Rho-kinase signalling (8, 9).

Rho-dependent signalling has been reported to regulate a diverse array of cellular functions, including vascular smooth muscle contraction, cytokinesis, cell morphology, cell migration (12) and neurotransmitter release (13). On the other hand, in animal disease models Rho/Rho-kinase pathway has been hold responsible, at least in part, for hypertension, vascular spasm and atherosclerosis (12) as well as astma (14,) and malignant cell migration (15).

In streptozocin-induced diabetic rats it was demonstrated that there was an upregulation of RhoA in the basilar artery, suggesting that this small G protein might be involved in the cerebral vascular pathogenesis during diabetes mellitus (8). Likewise, in a type II diabetic model, Rho kinase activity is increased but myosin phosphatase activity decreased, an action favouring enhanced responses to vasoconstrictors. The failure of insulin release, which effectively inhibits Rho-kinase activity, has been hold responsible for this alteration in diabetes (9). Similarly, in our STZ-induced diabetic model, an increased response to phenylephrine might occur probably due to the activation of Rho/Rho-kinase signalling because of the impairment of insulin secretion. In this context, one would consider that the inhibition of Rho-kinase might cause more relaxation in precontracted diabetic aorta relative to the control one as the contribution of this pathway may be increased in the contractile ability of the vascular smooth muscle. In contrast, Y-27632 and fasudil-induced relaxation did not differ in diabetic aorta. On the other hand, Western blot analysis exhibited that ROCK-2 protein level was unchanged in diabetic aortic smooth muscle cell homogenates compared to control. This seems to be consistent with the data obtained from organ bath studies showing that relaxation to the Rho-kinase inhibitors did not differ in control and diabetic aorta. However, Sandu et al (9) has claimed that ROCK is upregulated in diabetic states. Perhaps, different experimental models or diabetic duration in that study might be responsible for the controversy. Endothelial dysfunction, which develops in diabetes may deteriorate the ability of the Rho-kinase inhibitors to relax the aortic rings since it has been suggested that in the presence of nitric oxide synthase (NOS) or guanylyl cyclase inhibitors, Y-27632 was less effective at inhibiting phenylephrine-induced contraction in endothelium-intact rat aorta (16), which seems to be the most plausible explanation for the discrepancy to us.

In conclusion, these results suggest that Rho-kinase expression stays unchanged in this diabetic status and its inhibitors, Y-27632 and fasudil, can produce indifferent relaxations in the isolated diabetic and non-diabetic mouse aorta.

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