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Rho-kinase expression and its contribution to the control of perfusion pressure in the isolated rat mesenteric vascular bed

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Abstract

Rho-kinase expression was investigated in the rat mesenteric artery and the effects of its inhibitors, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) and fasudil (HA-1077), were examined on the increase in perfusion pressure induced by two different receptor agonists, namely the α-adrenoceptor agonist, phenylephrine and, the endothelin ET_A and ET_B receptor agonist, endothelin-1. Y-27632 and fasudil produced a concentration-dependent decrease in perfusion pressure. There was no difference between the concentration–response lines of these two inhibitors. The maximum decrease in the perfusion pressure induced by 10^{-5} M Y-27632 was 85.8 ± 3.7% when the tone was increased by phenylephrine. However, it was 48.1 ± 5.4% ($P < 0.001$) when the perfusion pressure was elevated by endothelin-1. Saponin perfusion (100 mg l^{-1}, for 10 min), which abolished acetylcholine-induced relaxation, did not significantly modify the Y-27632-elicited relaxation. Western blot analysis revealed that rat mesenteric artery expresses Rho-kinase protein with a molecular weight of approximately 160 kDa. These results show that Rho-kinase enzyme is expressed in rat mesenteric artery and that it contributes to the control of vascular resistance. Moreover, endothelium removal had no marked effect on the vasodilatation induced by Y-27632. In addition, the endothelin-1-induced vasoconstriction was more resistant to the Rho-kinase inhibitors than was that induced by phenylephrine, probably because excitatory endothelin receptors are associated with this signal transduction pathway at a different level from that of α-adrenoceptors.

Keywords: Endothelin-1; Mesenteric artery; Rho-kinase; Saponin; Y-27632

1. Introduction

Increased vascular resistance is the major determinant of the pathogenesis of hypertension. Although many pathways are reported to be involved in the pathophysiology of hypertension, a common molecular mechanism leading to increased resistance has not been defined. It has been recently demonstrated that Rho/Rho-kinase signalling, which is the modulator mechanism for changing Ca^{2+} sensitisation, may have a substantial role in regulating vascular smooth muscle tone, and that inhibition of this pathway may be a novel therapeutic target in the treatment of hypertension (Mukai et al., 2001).

Various vasoconstrictor agents, including phenylephrine, angiotensin-II and endothelin-1, stimulate receptors which are coupled with both $G_{q/11}$-phospholipase C β-mediated Ca^{2+}-dependent myosin light chain kinase regulation and Rho/Rho-kinase-mediated myosin phosphatase regulation through the activation of $G_{12/13}$ proteins (Gohla et al., 2000). Rho is a small GTPase and stimulates one of its downstream effectors, Rho-kinase (ROKα, ROKβ), a serine/threonine-kinase, the molecular weight of which is approximately 160 kDa. Phosphorylation of myosin phosphatase, which dephosphorylates the phosphorylated myosin light chain to induce smooth muscle relaxation (Fukata et al., 2001), by Rho-kinase inhibits the enzyme. Therefore, inhibition of Rho-kinase may result in smooth muscle relaxation. Several Rho-kinase inhibitors have been identified, including Y-27632 and fasudil (Uehata et al., 1997). With these tools, as well as *Clostridium botulinum* C3 exoenyzyme, which specifically inhibits RhoA protein, it has been recognized that the Rho/Rho-kinase pathway could be involved in numerous biological events, for instance, cell motility and migration, focal adhesion formation, membrane

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ruffling, cytokinesis, smooth muscle proliferation and neurite contraction (Braga et al., 1997; Charzanowska-Wodnicka and Burridge, 1996; Gebbink et al., 1997; Gong et al., 1997; Loirand et al., 1999). Moreover, antagonism of this pathway may cause vasodilatation (Cario-Toumaniatz et al., 2002; Shirao et al., 2002), spasmolytic effects (Nakamura et al., 2001; Batchelor et al., 2001), penis erection (Chitaley et al., 2001; Büyükafşar and Ün, 2003), inhibition of neurotransmitter release (Büyükafş ar and Levent, 2003), and suppression of myogenic and electrical activity of the vas deferens (Büyükafşar et al., 2003).

The involvement of Rho/Rho-kinase signalling in the regulation of vascular tone has been recently investigated in mesenteric artery rings from mineralocorticoid hypertensive rats (Weber and Webb, 2001) as well as Wistar Kyoto and spontaneously hypertensive rats (Asano and Nomura, 2003). However, the effects of Rho-kinase inhibitors have yet to be examined in the mesenteric vascular bed, which is composed of resistance arteries, which is a better model to study the effects of drugs on hypertension (D'Orleans-Juste et al., 1996). Moreover, Rho-kinase enzyme expression has not been detected in the rat mesenteric artery. Therefore, in the present study we examined the effect of two Rho-kinase inhibitors, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) and fasudil, on the perfusion pressure elevated by two distinct receptor agonists, namely the α-adrenoceptor agonist, phenylephrine, as well as the endothelin ET_A and ET_B receptor agonist, endothelin-1, in the rat mesenteric vascular bed, as a resistance arterial network involved in the development of hypertensive states. Additionally, whether endothelium removal had any effect on the vasodilatation by Y-27632 was also investigated. Furthermore, we detected the expression of Rho-kinase (ROKα, ROCK-2) protein by Western blotting.

2. Materials and methods

2.1. Perfusion experiments

Female Wistar rats (140–170 g) were used in the experiments. Animals were killed by a blow to the head and exsanguinated. The superior mesenteric artery was immediately cannulated and perfused with Krebs solution (in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, Na_2EDTA 0.01). Thereafter, all connections of the mesenteric vascular bed to the small intestine were carefully dissected and the vascular bed with omentum was removed and transferred to a jacketed chamber that was kept at 37 °C. The vascular bed was perfused in situ with aerated (95% O_2 and 5% CO_2 gas mixture) Krebs solution at a constant flow rate of 5–5.5 ml min^{-1}, using a peristaltic pump (Peristar, WPI, Berlin, Germany). Some vasodilator drugs, such as acetylcholine and sodium nitroprusside, were injected in a volume of 10 μl into the perfusate in the silicone rubber close to the vascular bed. Other drugs and agents were added to a scaled reservoir that was continuously gassed with 95% O_2 and 5% CO_2 and kept at 37 °C, and from which Krebs solution was continuously perfused to the mesenteric vascular bed. Alterations in perfusion pressure were recorded with a perfusion pressure transducer (COMMAT, Ankara Turkey) and displayed on a Biopac acquisition system (Biopac systems, California, USA).

After equilibration for 1 h, the perfusion pressure of the mesenteric vascular bed was increased by either phenylephrine (10^{-7}–10^{-4} M) or endothelin-1 (10^{-10}–10^{-7} M) added cumulatively. Thereafter, one concentration was chosen to induce submaximal vasoconstriction. It was generally 5×10^{-6} M for phenylephrine, and 10^{-8} M for endothelin-1. Following a steady-state increase in perfusion pressure, a Rho-kinase inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632, 10^{-8}–10^{-4} M), or fasudil (10^{-9}–10^{-5} M) was added cumulatively to the reservoir to allow a maximum decrease in perfusion pressure for each concentration. In some experiments acetylcholine (0.01 μg) and sodium nitroprusside (0.1 μg) were applied in a bolus manner to check endothelial and smooth muscle function. For endothelial removal, saponin was perfused at the concentration of 100 mg l^{-1} for 10 min. Thereafter, the vascular bed was immediately perfused with Krebs solution without saponin for 1 h.

2.2. Western blotting for ROKα

The superior mesenteric artery was freed along 3–4 cm and removed after dissecting out connective and fat tissue. The tissue was homogenized with lysis buffer (composition in mM: Tris–HCl (pH = 7.4) 50 mM, NaCl 400 mM, EGTA 2 mM, EDTA 1 mM, dithiothreitol 1 mM, phenylmethylsulfonyl fluoride 10 μM, leupeptin 10 μg ml^{-1}, pepstatin 1 μg ml^{-1}, benzamidine 1 mM). The homogenate was centrifuged at 900 × *g* for 10 min at 4 °C to remove nuclei and

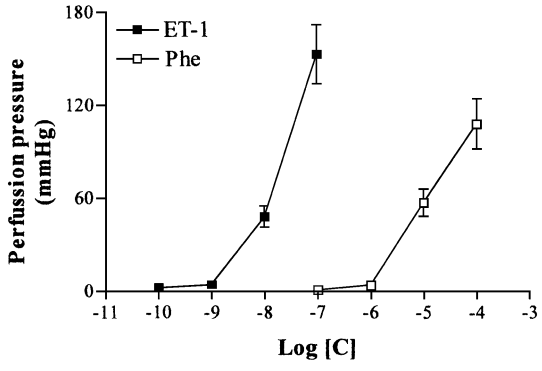

Fig. 1. Effects of endothelin-1 (10^{-10}–10^{-7} M, cumulatively) and phenylephrine (10^{-7}–10^{-4} M, cumulatively) on the perfusion pressure of the rat mesenteric arterial network. Data represent means ± S.E.M. of six to nine observations.

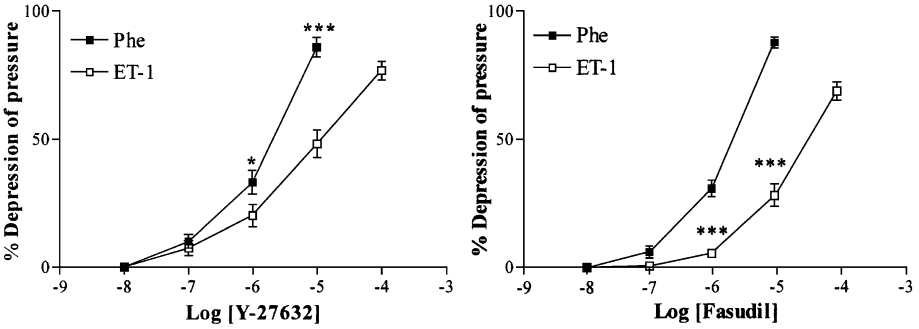

Fig. 2. Vasodilatation induced by Y-27632 (10^{-8}–10^{-4} M, cumulatively, left panel) and fasudil (10^{-8}–10^{-5} M, cumulatively, right panel) in the rat mesenteric vascular bed, and alteration of this vasodilatation when the perfusion pressure was increased by α-adrenoceptor agonist, phenylephrine, or by endothelin ET_A and ET_B receptor agonist, endothelin-1. Data represent means ± S.E.M. of five to seven observations. Note that Y-27632 and fasudil produced a smaller relaxation of the endothelin-1-induced contraction than of the phenylephrine-induced contraction. *$P<0.05$, ***$P<0.0001$, one-way of ANOVA followed by Bonferroni post hoc test was used for comparison.

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% polyacrylamide-sodium dodecyl sulfate gels for 90 min and then transferred to a nitrocellulose membrane for 3 h. The membrane was blocked with the blocking agent of the enhanced chemiluminescence advance kit (ECL advance, 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 ROKα (ROCK-2, Polyclonal Immunoglobulin G, sc-1851, Santa Cruz Biotechnology, California, USA) at 1:500 dilution followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1:1000, Santa Cruz Biotechnology). The blots were then detected with the ECL advance kit (Amersham Biosciences).

2.3. Drugs and chemicals

Phenylephrine hydrochloride, acetylcholine chloride and sodium nitroprusside were all obtained from Sigma (St Louis, MO, USA) and saponin from Fluka (Deisenhofen, Germany). Endothelin-1, Y-27632 and fasudil (HA-1077) were purchased from Tocris Cookson (Bristol, UK). All chemicals were dissolved in distilled water except for saponin, which was added and dissolved in Krebs solution so that the vascular network could be perfused for effective endothelium removal.

2.4. Analysis of results

The increase in perfusion pressure produced by the vasoconstrictors is expressed in mm Hg. The decrease in perfusion pressure is expressed as percent reduction of phenylephrine and endothelin-1-induced tone, and shown as means ± S.E.M. For comparison, one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test or Student's *t*-test, if appropriate, was used. *P* values less than 0.05 were considered as significant.

3. Results

3.1. Effects of phenylephrine, endothelin-1, Y-27632 and fasudil on the perfusion pressure

Both endothelin-1 (10^{-10}–10^{-7} M) and phenylephrine (10^{-7}–10^{-4} M) produced vasoconstriction in a concentra-

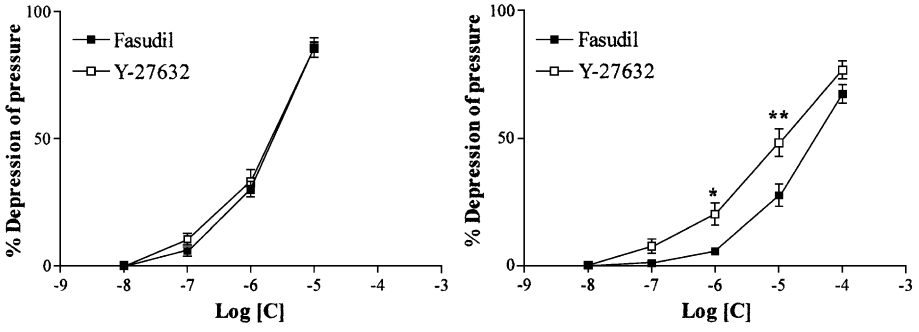

Fig. 3. Comparison of the vasodilatation induced by the Rho-kinase inhibitors, Y-27632 and fasudil, in the isolated perfused rat mesenteric vascular bed, the perfusion pressure of which was elevated by phenylephrine (left panel) and endothelin-1 (right panel). Data represent means ± S.E.M. of five to seven observations. *$P<0.05$, **$P<0.001$, one-way of ANOVA followed by Bonferroni post hoc test was used for comparison.

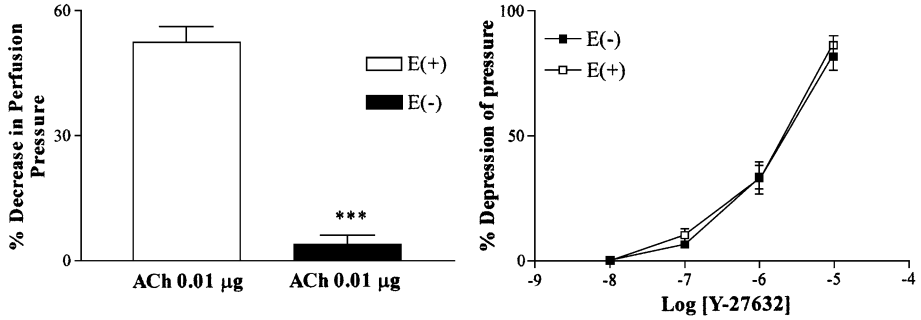

Fig. 4. Influence of saponin perfusion (100 mg l^{-1}, for 10 min) on the ability of acetylcholine (left panel, ACh, bolus injection, 0.01 μg) and Y-27632 (right panel, 10^{-8}–10^{-5} M) to induce vasodilatation in the isolated perfused rat mesenteric artery. Data represent means ± S.E.M. of four observations. ***$P<0.0001$. Student t-test was used for statistical comparison. Note that saponin removed functional endothelium because acetylcholine-induced vasodilatation was almost abolished, and it had no effect on Y-27632-induced vasodilatation.

tion-dependent manner, with endothelin-1 being more potent. At 10^{-7} M, phenylephrine produced almost no vasoconstriction (1.0 ± 0.3 mm Hg); however, endothelin-1 increased the perfusion pressure to 152.2 ± 19.2 mm Hg ($P<0.0001$, Fig. 1). The Rho-kinase inhibitors produced concentration-dependent vasodilatation (Figs. 2 and 3). There was no potency and efficacy difference between these two inhibitors in that the Y-27632 and fasudil concentration–response lines were contiguous. Maximum vasodilatation induced by 10^{-5} M Y-27632 was 85.8 ± 3.7%. Likewise, it was 86.2 ± 3.8% ($P>0.05$) with fasudil. Y-27632 and fasudil elicited a comparatively smaller relaxation when the perfusion pressure was increased by endothelin-1 (Figs. 2 and 3). Following elevation of perfusion pressure by phenylephrine, the maximum vasodilatation induced by 10^{-5} M Y-27632 was 85.8 ± 3.7%; however, it was 48.1 ± 5.4% ($P<0.0001$) when the perfusion pressure was increased by endothelin-1. Likewise, fasudil (10^{-5} M) induced a vasodilatation of 86.2 ± 3.8% and 27.6 ± 4.3% ($P<0.0001$) when the perfusion pressure was elevated with phenylephrine and endothelin-1, respectively.

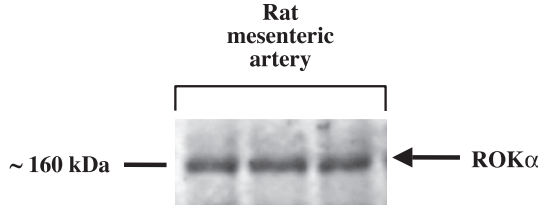

Fig. 5. Western blotting for Rho-kinase (ROKα, ROCK-2) in the rat mesenteric artery. Mesenteric artery homogenates were submitted to sodium dodecyl sulfate-PAGE with 8% polyacrylamide and then transferred to a nitrocellulose membrane. The membrane was blocked with the blocking agent of the enhanced chemiluminescence advance kit (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 ROKα (Polyclonal Immunoglobulin G, Santa Cruz Biotechnology) at 1:500 dilution followed by horseradish peroxidase-conjugated secondary antibody (donkey anti-goat, 1:1000). The blots were then detected with an Advance Chemiluminescence Detection Kit (Amersham Biosciences).

3.2. Effect of saponin perfusion on acetylcholine and Y-27632-induced vasodilatation

Saponin perfusion (100 mg l^{-1}, for 10 min) removed the vascular endothelium of the mesenteric bed because the acetylcholine-evoked relaxation was almost abolished. In control, the acetylcholine (0.01 μg)-induced relaxation was 52.4 ± 3.7%; however, it was 3.7 ± 2.2% after saponin perfusion ($P<0.0001$, Fig. 4). The sodium nitroprusside-induced vasodilatation was not changed after perfusion of saponin (data not shown). Saponin treatment had no significant effect on the vasodilatation produced by Y-27632 (Fig. 4).

3.3. ROKα expression in the mesenteric artery

Western blot analysis revealed that rat mesenteric artery expressed Rho-kinase protein with a molecular weight of approximately 160 kDa (Fig. 5).

4. Discussion

The main findings of the present study are that ROKα is expressed in the mesenteric artery and contributes to the control of perfusion pressure, that endothelium removal does not change the vasodilator effect of Y-27632 and finally that receptors for endothelin-1 and phenylephrine are associated with Rho/Rho-kinase signalling to a different extent.

It has been reported that alteration of Rho-kinase-mediated regulation of vascular tone is a possible cause of increased vascular resistance (Wettschureck and Offermanns, 2002). Therefore, manipulation of this pathway with specific pharmacological tools, including Y-27632, may control elevated blood pressure. In this study, both Rho-kinase inhibitors produced vasodilatation which was not dependent on an intact endothelium. There was no potency and efficacy difference between the two inhibitors. It has been reported that Y-27632 is more selective than fasudil in inhibiting Rho-kinase (Davies et al., 2000). However, at the same concen-

trations both inhibitors produced vasodilatation when the perfusion pressure was elevated by phenylephrine. However, in the case of endothelin-1-induced vasoconstriction, Y-27632 produced a greater vasorelaxation than fasudil at the same concentration. Although both phenylephrine and endothelin-1 receptors are coupled with heterotrimeric $G_{q/11}$ proteins (Sah et al., 1996; Kuwahara et al., 1999), the Rho-kinase inhibitors induced vasodilatation to a different extent in the mesenteric vascular bed, in that when the perfusion pressure was elevated by endothelin-1, both inhibitors significantly produced a less pronounced relaxation. This might indicate that α-adrenoceptors are coupled to the Rho/Rho-kinase pathway in a different way than are excitatory endothelin receptors. It is possible that endothelin-1 causes excessive activation of Rho-kinase and thus Y-27632 could induce a smaller relaxation. However, one could argue that the greater relaxation induced by Y-27632 when the perfusion pressure was increased by phenylephrine reflects a greater contribution of Rho-kinase to the apparent vasoconstrictor response to phenylephrine. Two main types of endothelin receptors exist, ET_A and ET_B, both of which are, coupled to G-proteins (Wanecek et al., 2000). Apart from coupling to the phospholipase C cascade and other post-receptor events, excitatory endothelin receptors (ET_A and ET_{B2}) on smooth muscle are associated with the recently defined Rho/Rho-kinase signalling pathway (Sakurada et al., 2001; Cavarape et al., 2003). It has been recently reported that in vascular smooth muscle cells endothelin-1 itself and the endothelin receptor complex can be internalised and active for several hours, and thus mediate a long-lasting effect (Bkaily et al., 2003). This might lead the mesenteric vascular bed to be less responsive to Rho-kinase inhibitors. However, α-adrenoceptors are known to be coupled to the phospholipase C cascade, Ca^{2+} channels and inhibition of cAMP formation, which all result in smooth muscle contraction. It is recognized that α-adrenoceptors may also be associated with RhoA activation and subsequently Rho-kinase stimulation (Uehata et al., 1997; Carter et al., 2002). However, it remains unknown whether endothelin-1 utilizes this signalling pathway more than does the α-adrenoceptor agonist, phenylephrine. In order to examine this hypothesis, further studies of the activation or expression of Rho-kinase in the presence of endothelin-1 and phenylephrine are needed.

The involvement of Rho/Rho-kinase signalling in the physiological control of vascular smooth muscle contraction has been widely investigated, and it has been found that this pathway may have a role in pulmonary vasoconstriction (Boer et al., 2002; Wang et al., 2001), increased vascular reactivity in mineralocorticoid-induced hypertension in rats (Weber and Webb, 2001), and cerebral vasospasm (Shirao et al., 2002; Nakamura et al., 2001; Batchelor et al., 2001). Furthermore, in arteries from rats made hypertensive by chronic inhibition of nitric oxide (NO) synthase, contractile activity in response to α_2-agonist was enhanced through RhoA activation (Carter et al., 2002).

Saponin treatment, which almost abolished the acetylcholine-induced vasodilatation, did not affect the Y-27632-elicited relaxation, suggesting that this vasodilatation was not dependent on the presence of endothelium. In agreement with this finding, in the rat tail small artery Y-27632-induced relaxation was not modified by endothelium removal (Schubert et al., 2002). However, it was previously demonstrated that Y-27632-induced relaxation was attenuated by endothelial denudation in the rat aorta (Chitaley and Webb, 2002). The unchanged relaxation to Y-27632 in the absence and presence of endothelium may point out different characteristics of the vessels used. In the rat aorta, Y-27632 has been proposed to release NO from the endothelium (Chitaley and Webb, 2002). NO is main paracrine mediator that regulates vascular smooth muscle tone in large arteries such as aorta. However, the role of other vasodilator mediators, namely endothelium-derived hyperpolarizing factor (EDHF), seems to be much more important in resistance arteries such as mesenteric vessels (Nagao et al., 1992). Therefore, Y-27632 could not cause sufficient release of NO from the endothelium to enhance its vasodilator effect, because NO is less important as mediator in the mesenteric vascular bed. Another finding of the present study is the expression of Rho-kinase protein in the rat mesenteric artery, which has not been shown before, although it has been proposed that ROKα is ubiquitously expressed.

In conclusion, Rho-kinase protein is expressed in the mesenteric artery and regulates agonist-induced smooth muscle contraction. Moreover, the vasodilator effect of the Rho-kinase inhibitor does not depend on an intact endothelium. In addition, excitatory endothelin receptors and α-adrenoceptors are associated with Rho-signalling at different levels. Consequently, the results of this study give support to the previous findings that Rho/Rho-kinase signalling may be involved in the control of vascular resistance, and that inhibitors of Rho-kinase may have therapeutic potential in the treatment of some cardiovascular diseases including hypertension, vasospasm and arteriosclerosis (Batchelor et al., 2001; Kandabashi et al., 2002; Weber and Webb, 2001).

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