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Inhibition of Rho-kinase stimulates nitric oxide-independent vasorelaxation

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Abstract

Vasoconstrictor factors, like urotensin, angiotensin and catecholamines, activate Rho-dependent serine–threonine kinase (Rho-kinase) and inhibition of this pathway represents a novel therapy for cardiovascular diseases with hypertensive syndrome. The disbalance of relaxing endothelial nitric oxide (NO)-producing and vasoconstrictive pathways can be especially important in diseases where hypertension is accompanied by endothelial dysfunction that compromises NO generation. However, a recent study reported that the efficacy of the Rho-kinase inhibitor (*R*)-(+)-*trans*-*N*-(4-Pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide (Y27632) is dramatically attenuated upon removal of endothelium or inhibition of endothelial NO synthase (eNOS). This raises the question whether Rho-kinase inhibition would be an effective treatment in case of hypertension associated with endothelial dysfunction. The purpose of the present study was to determine whether the vasorelaxing effect of Rho-kinase inhibition is mediated through eNOS-dependent mechanisms. We show here that in the models of genetically reduced endothelial NO production ($eNOS^{-/-}$ mice and spontaneous hypertensive rats (SHR)) or in models of pharmacologically reduced endogenous NO production (*N*(omega)-nitro-L-arginine methyl ester (LNAME) treatment), Rho-kinase inhibition induced a strong vasodilation and reduction of blood pressure indicating independence of Rho-kinase pathway from eNOS. An additional important finding of our study is that Rho-kinase inhibitors induce a strong vasorelaxation and blood pressure reduction upon intravenous injection not only in hypertensive but in normotensive animals, as well. Inhibition of Rho-kinase represents a promising possibility to treat hypertension that is accompanied by endothelial dysfunction.

Keywords: Rho-kinase; eNOS; Hypertension; Endothelial function; Nitric oxide; Y27632

1. Introduction

Vascular hypercontractility, a major pathophysiological mechanism leading to hypertension, is associated with dysfunction of vascular smooth muscle cells and endothelial cells. The endothelium provides the vascular smooth muscle cell with a variety of active mediators, such as relaxing nitric oxide (NO), and contracting prostaglandins and endothelin. A major feature of endothelial dysfunction is reduced generation of the vasorelaxing NO through different mechanisms (Frisbee and Lombard, 1998; Mukundan and Kanagy, 2001; Puddu et al., 2000). Concomitantly developing alterations in vascular smooth muscle contractile mechanisms, e.g. hyperactivation of protein kinases involved in contraction, result in vascular smooth muscle hypercontractility. In animal models of hypertension, where endothelial dysfunction is characterized by reduced sensitivity of vessels to acetylcholine, overexpression of Rho-associated protein kinase (Rho-kinase) was described (Mukai et al., 2001; Seasholtz et al., 2001). Activation of a small GTPase RhoA upon agonist stimulation results in conversion of RhoA from the inactive GDP-bound form to the active GTP-bound form with a subsequent binding to

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and activation of Rho-kinase. Two isoforms, Rho-kinase 1 and Rho-kinase 2, are known. Rho-kinase 2 is expressed in vascular smooth muscle cells and endothelial cells (Barandier et al., 2003; Buyukafsar et al., 2004; Sauzeau et al., 2003; Seasholtz et al., 2001). Activation of Rho-kinase 2 by the active GTP-bound RhoA leads to calcium sensitization of smooth muscle cells through phosphorylation-mediated inhibition of the myosin light chain phosphatase activity and thereby up-regulation of the activity of myosin regulatory light chain (Uehata et al., 1997). Multiple mechanisms were recently described to link Rho-kinase pathways and NO-generating systems (Begum et al., 2002; Chitaley and Webb, 2002; Kataoka et al., 2002). Recent studies reported that stimulation of the cyclic GMP-dependent protein kinase leads to phosphorylation of RhoA and its inhibition. Therefore, inhibition of RhoA and its downstream target Rho-kinase by NO would contribute to the vasodilator action of NO (Begum et al., 2002; Chitaley and Webb, 2002; Sauzeau et al., 2000). According to Chitaley and Webb (2002), removal of endothelium leads to reduction of the vasorelaxing effect of the Rho-kinase inhibitors. From this point of view, it is expected that in animal models with endothelial dysfunction where the NO-producing system is compromised, inhibition of Rho-kinase pathway should have much less prominent vasorelaxant effect. On contrary, Uehata et al. (1997) reported that Rho-kinase inhibition exerts its relaxing effect only in hypertensive but not in normotensive animal models. We speculated that the most direct evidence, whether the Rho-kinase effects were endothelial NO synthase (eNOS)-dependent or not, could be addressed on models with the NO-producing system either genetically inactivated ($eNOS^{-/-}$ mice or spontaneous hypertensive rats (SHR)) or pharmacologically inhibited (*N*(omega)-nitro-L-arginine methyl ester (LNAME)).

2. Materials and methods

2.1. In vitro vascular function

Adult male Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR) weighting 300 to 400 g (21 months old, Harlan Winkelmann, Borchen, Germany) and $eNOS^{+/+}$ and $eNOS^{-/-}$ mice (25 to 35 g, 12–16 weeks old, Jackson Laboratory, Maine, USA) were sacrificed by decapitation, and the thoracic aortas or mesenteric arteries were excised. Arteries were then quickly transferred to cold (4 °C) oxygenated with carbogen (95% O_2 and 5% CO_2) physiological salt solution, and dissected into 5 mm rings as described previously (Löhn et al., 2002), whereby perivascular fat and connective tissue were carefully removed. In some experiments, the endothelial cell layer was also removed. Rings of rat aortas were set at 1000 mg and rings of mouse aortas were set at 800 mg passive tension. After 1-h equilibration, aortic ring contractile force was measured isometrically using standard bath procedures as described earlier (Drab et al., 2001). For the measurement of force generation by mesenteric arteries of SHR and WKY, arteries were set at 5 mN passive tension on a myograph (Danish Myo Technology, Aarhus, Denmark). The composition of the bath solution (in mM) was 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11 glucose, 1.6 $CaCl_2$. Bath solutions were continuously gassed with carbogen to provide oxygenation and pH of 7.4. The temperature was maintained at 37 °C. Cumulative concentration–response curves were obtained for acetylcholine and for the specific Rho-kinase inhibitor (Y27632) (Uehata et al., 1997; Davies et al., 2000). If not otherwise indicated, the developed tension was expressed as a percentage of the steady-state tension induced by the preceding phenylephrine application. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). If not otherwise indicated, chemicals were obtained from Sigma (Deisenhofen, Germany). Y27632 was purchased from Calbiochem (Bad Soden, Germany).

2.2. Blood pressure measurement

Adult male WKY and SHR with the same age and weight as used for the in vitro vascular function studies were used for the invasive blood pressure measurements. Animals were anaesthetized with an intraperitoneal injection of trapanal (0.1 g/ml). Then the common carotid artery was catheterized with a heparinized microcatheter (diameter 1.6 mm, Vycon, France). Y27632 was infused into the tail vein. Blood pressure and heart rate were measured with a Hugo Sachs hemodynamic system (March-Hugstetten, Germany) using the software Hemodyn.

2.3. Western blot

The tissues were homogenized and lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membrane (Minigel system, Biometra). The Western blots were probed with a primary anti-Rho-kinase 2-antibody (SC-1851, Santa Cruz, USA) followed by anti-goat antibody coupled to Alexa Fluor (Molecular Probes, Germany) and visualized by fluorescence on an Odyssee system (Licor, MA, USA). Rho-kinase 2-expression was calculated relatively to the expression of α-actin, detected with a goat anti-α-actin (C11, SC-1615, Santa Cruz).

2.4. Statistical analysis

All values are given as mean and standard error of mean (S.E.M.). For group comparisons, paired and unpaired Student's *t*-tests or analysis of variance (ANOVA) were used as appropriate. A value of $P<0.05$ was considered

statistically significant; *n* represents the number of arteries tested.

3. Results

3.1. Endothelium dependent responses in aortas of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice

First, to confirm functional consequences of eNOS-deficiency, endothelium and eNOS-dependent relaxation was compared in aortas of eNOS$^{-/-}$ and eNOS$^{+/+}$ mice. For this purpose, thoracic aortic rings were pretreated with phenylephrine (5×10^{-7} M). It was tested whether vessels of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice were different with respect to the endothelium-dependent relaxation induced by acetylcholine, a known inducer of NO-generation by eNOS. A cumulative application of acetylcholine (10^{-7}, 5×10^{-7}, 10^{-6} and 10^{-5} M) to aortas of eNOS$^{+/+}$ mice induced a significant relaxation with an $EC_{50}<10^{-7}$ M (Fig. 1A). However, in vessels of eNOS$^{-/-}$ mice, the application of acetylcholine evoked a vasoconstriction in a concentration-dependent manner (Fig. 1B). These findings are consistent with those measurements performed by Huang et al. (1995). Fig. 1C summarizes the acetylcholine-induced endothelium-dependent responses in aortas of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice. At an acetylcholine concentration of 10^{-7} M, aortas of eNOS$^{+/+}$ mice were relaxed to 58±12% (n=8), whereas aortas of eNOS$^{-/-}$ mice were constricted by 8.5±5% (n=6). The acetylcholine-induced responses in aortic rings of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice were significantly different ($P<0.05$). These results clearly demonstrate that vaso-relaxation induced by acetylcholine was completely eNOS-dependent. In the absence of eNOS, acetylcholine stimulation of muscarinic receptors in vascular smooth muscle leads to vasoconstriction.

3.2. Endothelium and eNOS-dependency of relaxation mediated by Rho-kinase inhibition

It is well known that an important symptom of endothelial dysfunction is the partial or complete insufficiency of the NO-producing system in endothelial cells. To determine the impact of eNOS-derived nitric oxide on Rho-kinase mediated contraction, aortas of eNOS$^{-/-}$ mice were precontracted with 5×10^{-7} M phenylephrine and relaxed with Y27632. The cumulative application of Y27632 (10^{-7}, 5×10^{-7}, 10^{-6}, 2×10^{-6}, 3×10^{-6} and 5×10^{-6} M) induced a concentration-dependent relaxation in aortas of eNOS$^{+/+}$ (n=8, Fig. 2A) and in aortas of eNOS$^{-/-}$ mice (n=9, Fig. 2B). The relaxation induced by Y27632 was comparable and the difference was not statistically significant ($P>0.9$, Fig. 2C). We concluded that vasoconstriction induced by α-adrenoceptor agonist is Rho-kinase dependent and eNOS-independent. To determine whether relaxation induced by inhibition of Rho-kinase is dependent on the endothelium, we analysed the effects of Rho-kinase inhibition on denuded aortas and on aortas with an intact endothelium of WKY. Both, intact and denuded vessels were precontracted with 10^{-7} M phenylephrine and were relaxed with Y27632 (Fig. 3A and B). Completeness of endothelial denudation of the vessels was approved by acetylcholine application. Acetyl-

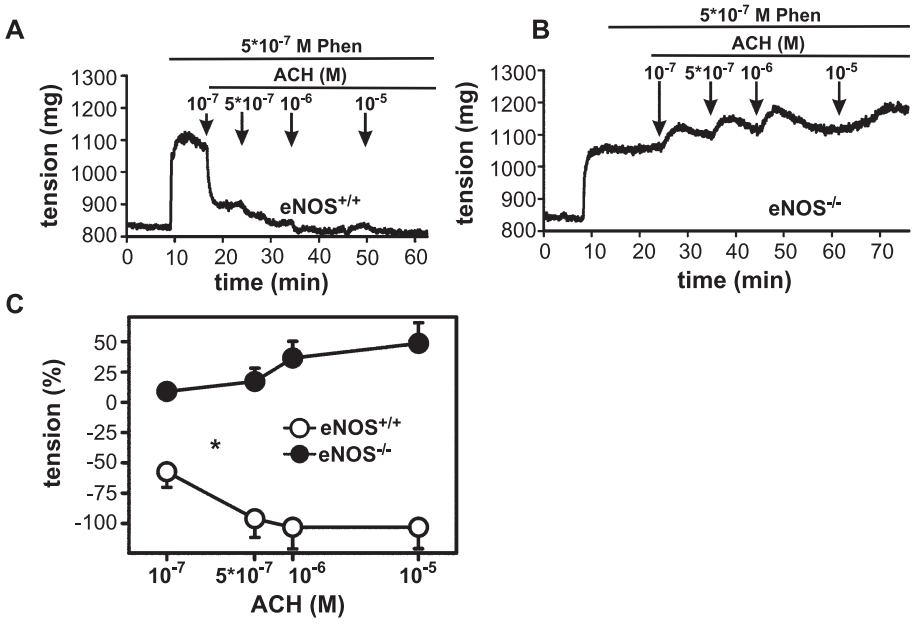

Fig. 1. Endothelium-dependent relaxation in aortas of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice. Thoracic aortic rings of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice were precontracted with 5×10^{-7} M phenylephrine (Phen). (A) In aortas of eNOS$^{+/+}$ mice, application of acetylcholine (ACH, 10^{-7}, 5×10^{-7}, 10^{-6} and 10^{-5} M) induced significant relaxation with an $EC_{50}<10^{-7}$ M. (B) In aortas of eNOS$^{-/-}$ mice, application of acetylcholine evoked a vasoconstriction in a concentration-dependent manner. (C) Summary of acetylcholine-induced endothelium-dependent responses in aortas of eNOS$^{+/+}$ and of eNOS$^{-/-}$ mice. The acetylcholine-induced responses in aortic rings of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice were significantly different (* $P<0.05$).

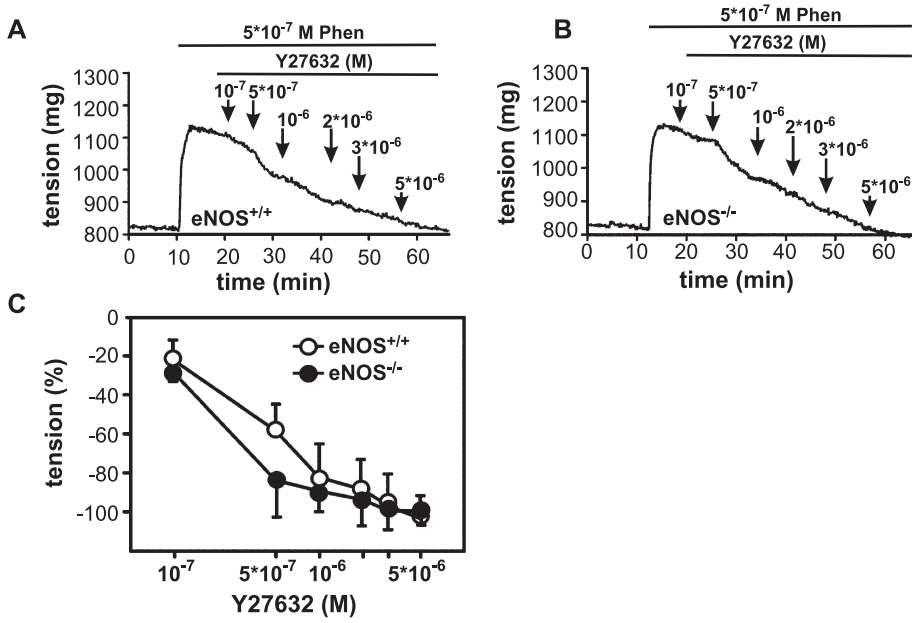

Fig. 2. Effects of Rho-kinase inhibition in aortas of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice. Thoracic aortic rings of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice were precontracted with 5×10^{-7} M phenylephrine. (A, B) The application of Y27632 (10^{-7}, 5×10^{-7}, 10^{-6}, 2×10^{-6}, 3×10^{-6} and 5×10^{-6} M) induced a concentration-dependent relaxation in aortas of eNOS$^{+/+}$ (n=8) and eNOS$^{-/-}$ mice (n=9). (C) Summary of the Y27632 induced relaxation of aortas of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice. There was no difference in Y27632-induced relaxation (P>0.9) between aortas of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice.

choline (10^{-8}, 10^{-7} and 10^{-6} M) evoked a concentration-dependent relaxation only in aortas with an intact endothelium (Fig. 3A). Denuded aortas showed no relaxation to acetylcholine (Fig. 3B). Then the aortas were washed, precontracted with phenylephrine (10^{-7} M), and relaxed with Y27632. The cumulative application of Y27632 (10^{-7}, 5×10^{-7}, 10^{-6} and 10^{-5} M) induced a concentration-dependent relaxation in intact aortas (n=6, Fig. 3A) and in

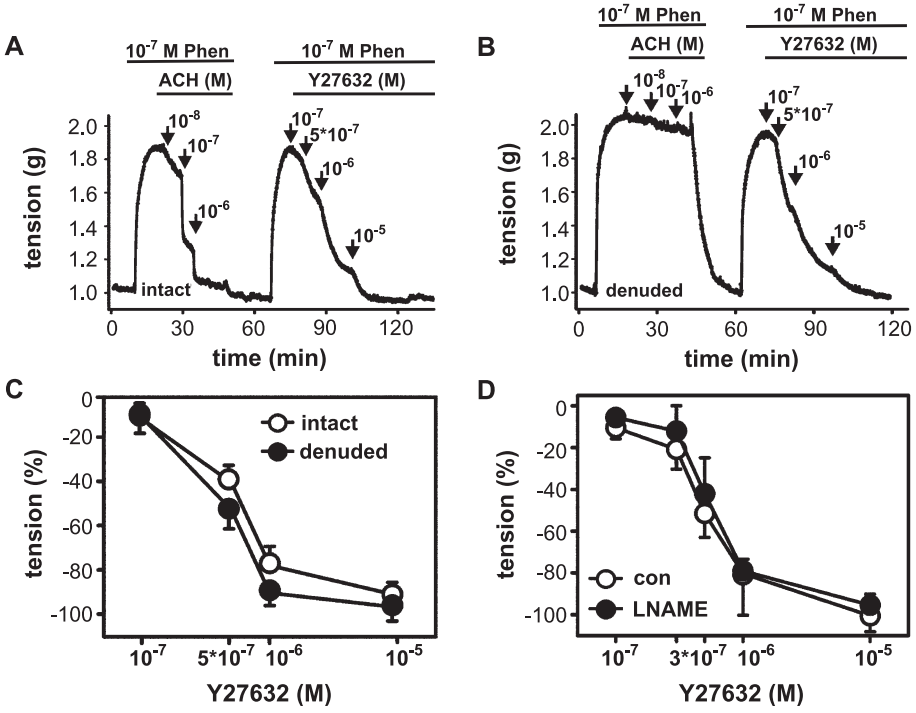

Fig. 3. Rho-kinase inhibition in endothelium-denuded or LNAME-treated aortas. Intact aortas (A) and denuded aortas (B) of WKY were precontracted with phenylephrine (10^{-7} M). Removal of endothelium was examined by the application of acetylcholine (10^{-8}, 10^{-7} and 10^{-6} M). Acetylcholine evoked a concentration-dependent relaxation only in intact vessels. Aortas were washed and precontracted with phenylephrine (10^{-7} M). The cumulative application of Y27632 (10^{-7}, 5×10^{-7}, 10^{-6} and 10^{-5} M, P=0.2) evoked a similar concentration-dependent relaxation in intact and in denuded aortas (C). Rho-kinase dependent vasorelaxation was not altered by LNAME treatment of the WKY aortas (D, P>0.2).

denuded aortas (n=6, Fig. 3B). The Y27632-induced relaxation was comparable and the difference was not statistically different (P=0.2, Fig. 3C). In another experimental setup, the NO-producing system was pharmacologically inhibited by the treatment of the aortas with the NOS-inhibitor LNAME. For that purpose, rings of thoracic aortas of WKY were preincubated with LNAME (3×10^{-4} M) for 20 min and thereafter precontracted with phenylephrine (10^{-7} M). The phenylephrine induced contraction was effectively reversed by the cumulative application of Y27632 (10^{-7}, 3×10^{-7}, 10^{-6}, 2×10^{-6}, and 10^{-5} M) in a concentration-dependent manner in aortas of WKY without LNAME (n=6) or with LNAME (n=6, P>0.2, Fig. 3D). These data indicate that vasorelaxation achieved through Rho-kinase inhibition is completely eNOS and endothelium-independent.

3.3. Inhibition of Rho-kinase as an alternative way to achieve vasorelaxation in animals with hypertension and endothelial dysfunction

3.3.1. In vitro vascular function studies

To test whether Y27632 can induce an effective vasorelaxation in other animal models of hypertension accompanied by endothelial dysfunction, the relaxant effects of this inhibitor were determined in mesenteric arteries of SHR and WKY (Fig. 4). The function of the endothelium in mesenteric arteries of WKY and SHR was evaluated by application of acetylcholine. For this purpose, mesenteric arteries were precontracted with phenylephrine (10^{-5} M). Then, acetylcholine was cumulatively applied (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) and induced in arteries of WKY a significantly (P<0.05) stronger relaxation (Fig. 4A and C) than in arteries of SHR (Fig. 4B and C). Arteries of SHR expressed a pronounced endothelial dysfunction and showed only a marginal vasodilation even at higher concentrations of acetylcholine (Fig. 4B and C). At a concentration of 10^{-5} M acetylcholine, control arteries of WKY were relaxed to 86±19% (n=12) and arteries of SHR were relaxed to 37±5% (n=12). Relaxation induced by Y27632 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) was not different between arteries of SHR (n=7) and arteries of WKY (n=6, P>0.05). These results indicate that inhibition of Rho-kinase induces an efficient vasorelaxation in an animal model with compromised endothelial function and thus, relaxation induced by Rho-kinase inhibition does not depend on endothelium function.

3.3.2. Blood pressure measurements

The in vitro vascular function studies showed that relaxation induced by Rho-kinase inhibition is eNOS and endothelium-independent. Therefore, the hypothesis was tested that Rho-kinase inhibition in vivo should result also in an effective reduction of blood pressure in animal models with normal or compromised endothelial function. For that purpose, blood pressure was measured invasively in normotensive WKY and in hypertensive SHR. Y27632 was infused intravenously through the tail vein (Fig. 5A–C). Fig. 5A and B shows the absolute values of the systolic (P_{SYS}) and mean arterial pressure (P_{MEAN}) in

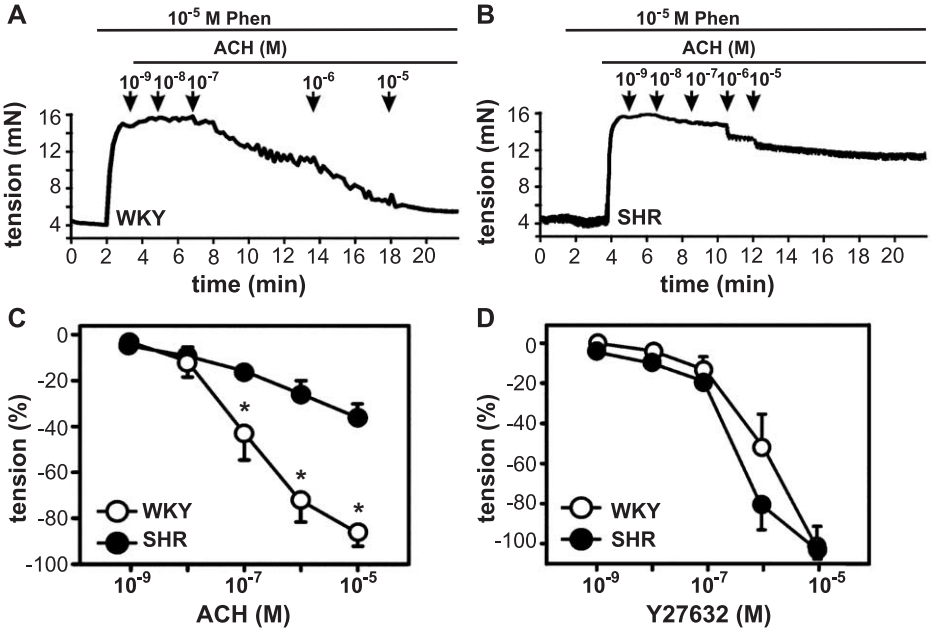

Fig. 4. Effects of Rho-kinase inhibition in arteries of SHR. Mesenteric arteries of SHR were precontracted by phenylephrine (10^{-5} M) and acetylcholine (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) was cumulatively applied to the vessels. Relaxation induced by acetylcholine was significantly stronger in arteries of WKY (A and C, *P<0.05) than in arteries of SHR (B and C). Arteries of SHR with compromised endothelial function showed only marginal relaxation even at high concentration of acetylcholine (B and C). Y27632 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) induced a similar relaxation in mesenteric arteries of SHR (n=7) and WKY (n=6, P>0.05, D).

WKY (Fig. 5A) and SHR (Fig. 5B). Fig. 5C depicts the delta-changes of mean arterial blood pressure induced by Y27632. The cumulative bolus injection of Y27632 induced a similar concentration-dependent reduction of blood pressure (P>0.05) in both normotensive WKY (n=5) and hypertensive SHR (n=6). We conclude that

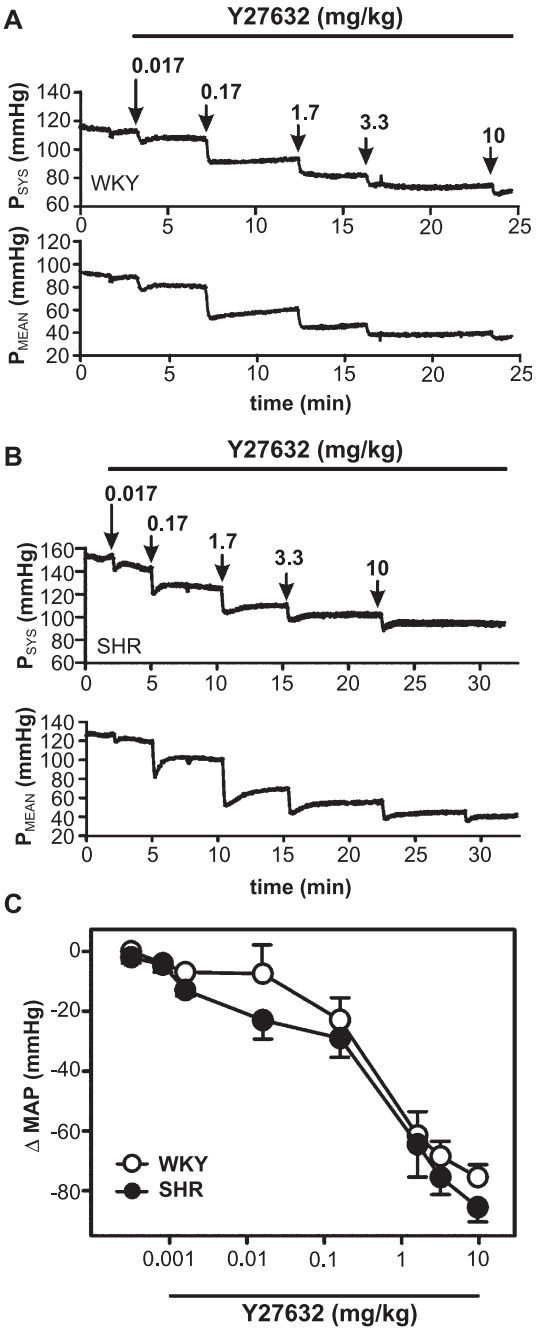

Fig. 5. Effect of Rho-kinase inhibition on blood pressure. Blood pressure measurements in catheterized SHR and WKY (A–C). A and B show the absolute values of the systolic (P_{SYS}) and mean arterial pressure (P_{MEAN}) in WKY (A) and SHR (B). C depicts the delta-changes of mean arterial blood pressure induced by Y27632. Infusion of Y27632 resulted in a similar concentration-dependent reduction of the mean arterial blood pressure (P>0.05) in normotensive WKY (n=5) and in SHR (n=6).

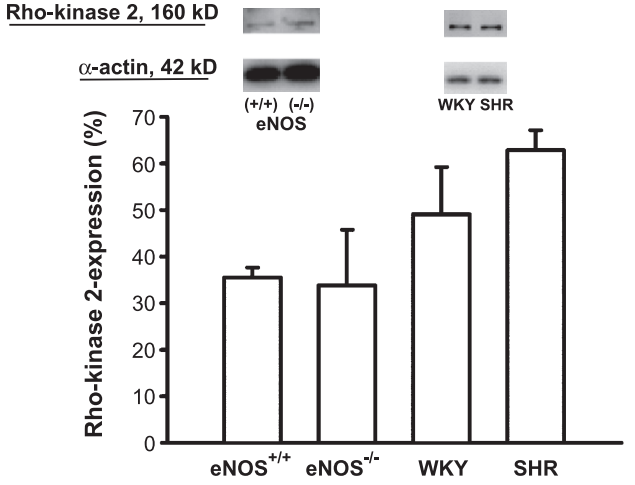

Fig. 6. Rho-kinase 2 expression in aortic tissues. Immunoblot analysis of Rho-kinase 2 expression in aortic tissues of WKY, SHR, eNOS$^{+/+}$ and eNOS$^{-/-}$ mice. For protein loading control, the same blots were re-probed with anti-actin antibodies. Normalized expression of Rho-kinase 2 showed no statistically significant differences.

Rho-kinase inhibition reduces blood pressure in normotensive and in hypertensive animal models.

3.3.3. Rho-kinase 2-expression in mouse and rat aortic tissues

Rho-kinase 2 protein expression was measured in aortic tissues from WKY, SHR, eNOS$^{+/+}$, and eNOS$^{-/-}$ mice by immunoblotting in four separate experiments. Amount of Rho-kinase 2 was similar in aortas from SHR and from eNOS$^{-/-}$ mice in comparison with the appropriate controls (Fig. 6).

4. Discussion

Physiological vasorelaxation is regulated by NO generated by the endothelial eNOS. The major effect of NO is mediated by activation of protein kinase G (PKG) and subsequent phosphorylation and activation of myosin light chain phosphatase (MYPT) and phosphorylation of Ca^{2+}- and K^{+}-channels (Hofmann et al., 2000; Nakamura et al., 1999). Multiple mechanisms were recently described to link Rho-kinase pathways and the NO-generating system. Inhibition of Rho-kinase has been proposed as one of mechanisms involved in NO-mediated vasorelaxation (Begum et al., 2002; Chitaley and Webb, 2002; Kataoka et al., 2002). According to the data of Chitaley and Webb (2002), the inhibition of the phenylephrine-induced contraction by Y27632 was dramatically attenuated upon removal of the endothelium or upon disruption of the NO-producing pathway (inhibition of eNOS by *N* omega-nitro-L-arginine (L-NNA)) or inhibition of the NO-sensing pathway (inhibition of soluble guanylate cyclase by 1H-(1,2,4)Oxadiazole(4,3-a)quinoxalin-1-one (ODQ). Authors came to conclusion that NO inhibits Rho-kinase activity and the endogenous NO-

mediated vasodilation occurs through the inhibition of Rho-kinase constrictor activity in the intact rat aorta. This data would pose the question whether Rho-kinase inhibition could be an effective treatment for hypertensive diseases accompanied by compromised endothelial function. Recently, it has been found that the chronic treatment with 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A (HMG-CoA) reductase inhibitors (statins) improve endothelial dependent relaxation of arteries in response to acetylcholine (Kobayashi et al., 2000; Tsunekawa et al., 2001; Wassmann et al., 2001). The effect of statins supposedly depends on the reduction of RhoA activity as statins inhibit the production of mevalonic acid which serves as a precursor for a number of isoprenoid metabolites (Budzyn et al., 2004; Chen et al., 2000; Dechend et al., 2002). In the presence of statins, RhoA is not geranylated and cannot attach to the inner membrane. If data regarding interdependence of Rho-kinase and eNOS are right then it would be impossible to evoke an acetylcholine-induced endothelial dependent relaxation in statin-treated arteries. However, in the study of Budzyn et al. (2004), who analysed the effect of statins (mevastatin) on smooth muscle contractility, a similar acetylcholine-induced relaxation between non-treated and mevastatin-treated animals was found. These facts contradict a hypothesis that the effect of NO is mediated through inhibition of Rho-kinase. Furthermore, we compared the effect of Rho-kinase inhibition in normotensive versus hypertensive animal models and found reduction of blood pressure by Y27632 in both models to the same extent. This is in a good agreement with a study of the blood pressure lowering effect of Y27632 in LNAME-treated WKY performed by Kataoka et al. (2002). Here, although the NO-producing system was inhibited by the chronic treatment of LNAME, Y27632 reduced the blood pressure significantly in a concentration-dependent manner. These data confirm the hypothesis that relaxation induced by Rho-kinase inhibition is a general mechanism and exerts its blood pressure reducing effect in normotensive as in hypertensive animal models. Endogenous NO may counterbalance Rho-kinase induced contractions by activation of PKG and activation of MYPT. However, in the absence of NO the relaxing effect of Rho-kinase inhibitors is preserved and leads to reduction of blood pressure in vivo, regardless whether endothelium is present or not. Our data contradict the data reported by Uehata et al. (1997) and Mukai et al. (2001) who found that Rho-kinase inhibition induces blood pressure reduction only in hypertensive but not in normotensive animal models, although Y27632 relaxed strips of rabbit aorta, pig coronary strips and guinea-pig trachea; but, in general, data by Uehata et al. (1997), Mukai et al. (2001), and our data agree in one important statement, that Rho-kinase inhibition efficiently reduces blood pressure in animals with endothelial dysfunction. These data contradict the hypothesis that the vasorelaxant effect of NO is mediated by Rho-kinase inhibition. To confirm that the effect of Rho-kinase inhibition is not species-dependent, we studied normotensive rabbits, pigs, and rats and found a decrease of blood pressure upon intravenous bolus infusion of Rho-kinase inhibitor originating from different chemical classes, e.g. isochinoline (fasudil, HA1077, 1-(5-Isoquinolinylsulfonyl) homopiperazine) or pyridine (Y27632, (*R*)-(+)-*trans*-*N*-(4-Pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide) (Löhn, unpublished observation). A possible explanation can be the different route of Rho-kinase inhibitor administration, intravenous in our study and oral in the studies by Uehata et al. (1997) and Mukai et al. (2001).

The main purpose of the present study was to elucidate the role of Rho-kinase pathway in animal models with compromised endothelial function. We demonstrate that relaxation of arteries induced by Rho-kinase inhibition is completely eNOS and endothelium-independent. Endothelial dysfunction was induced either genetically in $eNOS^{-/-}$ mice (Huang et al., 1995) or in SHR (Begum et al., 2002; Mukai et al., 2001) or pharmacologically in LNAME-treated vessels, and NO-production was additionally interrupted by mechanical endothelial denudation of the arteries. In accordance with recently reported results by Carter et al. (2002), we found that the removal of the endothelium did not affect the vasorelaxation induced by Rho-kinase inhibition. Additionally, we compared the relaxant effect of Y27632 in aortas of eNOS wild type and eNOS-deficient mice. Y27623 efficiently relaxed aortas from both strains indicating that Rho-kinase signaling operates independently from NO. During submission of this work, Budzyn et al. (2004) published data confirming our observations regarding efficiency of Rho-kinase inhibition in aorta of eNOS-deficient mice. Additional evidence that NO elicits Rho-kinase independent vasorelaxation can be drawn from endothelin-induced arterial contractions. Endothelin causes a strong contraction of arteries through Rho-kinase independent mechanisms as even high doses of Rho-kinase inhibitors show no vasorelaxing effect (Löhn, unpublished observations). However, endothelin-induced contractions are effectively reversed by NO. Altogether, our data and data by Carter et al. (2002) and Budzyn et al. (2004) contradict findings by Chitaley and Webb (2002) and bring more arguments to the statement that Rho-kinase inhibition operates independently from NO and eNOS. Immunoblot analysis showed that Rho-kinase expression was not elevated in aortas of eNOS deficient mice or of SHR in comparison to the appropriate controls. Similar findings were published by Budzyn et al. (2004) and Seasholtz et al. (2001). Several authors reported an increased sensitivity to Y27632 in coronary, carotid, mesenteric, basilar arteries and cerebral arteries from hypertensive SHR (Asano and Nomura, 2003; Chrissobolis and Sobey, 2001; Kitazono et al., 2002; Mukai et al., 2001). Mukai et al. (2001) reported that the contractile responses of isolated mesenteric arteries were preferentially and significantly inhibited by hydroxyfasudil in young SHR but not in WKY. The extent of inhibitory effect of hydroxyfasudil was greater in young SHR than in adult SHR. Our data comparing vasorelaxation induced by Y27632 in mesenteric arterial tissues of hyper-

tensive and normotensive animal models showed no differences of the Y27632 effect and a similar dose-dependence. We noticed a slightly higher sensitivity to the vasorelaxing effect of Y27632 in mesenteric arteries of SHR versus mesenteric arteries of WKY; but this difference was not statistically significant (Fig. 4D) and no difference has been found between non-treated and LNAME-treated vessels of WKY (Fig. 3D). A higher sensitivity to the relaxant effect of Y27632 has been found in vessels of eNOS$^{-/-}$ mice (Fig. 2C) but was also statistically insignificant.

In conclusion, inhibition of Rho-kinase causes vasorelaxation of vessels with endothelial dysfunction as well as vessels with an intact endothelium. We showed here that inhibition of Rho-kinase induces relaxation independently from eNOS. Inhibition of Rho-kinase resulted in an effective blood pressure reduction in both, normotensive WKY and hypertensive SHR. Therefore, inhibition of Rho-kinase can help to treat hypertension associated with endothelial dysfunction.

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