Rho Kinase Inhibitors Prevent Endothelium-Dependent Contractions in the Rat Aorta

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ABSTRACT

Rho kinase is involved in the pathogenesis of hypertension, which favors the occurrence of endothelium-dependent contractions. The present study was designed to determine the effects of two Rho kinase inhibitors, HA1077 [(1S,3R,6S,8S,9R,11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-[(1H-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolcarboxylic acid (A23187). The Rho kinase inhibitors did not significantly affect prostacyclin production measured as 6-keto prostaglandin F₁α. They nearly abolished endothelium-independent contractions to (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid (U46619), prostaglandin F₂α, and phenylephrine. Western blotting revealed a comparable expression of Rho kinase in the aortae of the two strains. The reduction by Rho kinase inhibitors of endothelium-dependent contractions is mainly because of their direct effect on the vascular smooth muscle cells.

In the aorta of the spontaneously hypertensive rat (SHR), vasoconstrictor prostanoids act as an endothelium-dependent contracting factor (EDCF). They induce contraction by activating thromboxane-prostanoid (TP) receptors on vascular smooth muscle cells (Lüscher and Vanhoutte, 1986; Auch-Schwelk et al., 1990; Huang et al., 2004; Yang et al., 2004; Vanhoutte et al., 2005; Gluais et al., 2006). One of the signaling molecules activated by TP receptor in smooth muscle is Rho kinase (Somlyo and Somlyo, 2003). The activation of Rho kinase causes inhibition of myosin light chain phosphorylation, which decreases dephosphorylation of the regulatory myosin light chain. The altered balance of myosin light chain phosphorylation induces contraction of the vascular smooth muscle cells (Somlyo and Somlyo, 2000). Rho kinase is also important in regulating other cellular pathways, including calcium entry and sensitization (Boz et al., 2003; Ghisdal et al., 2003; Somlyo and Somlyo, 2003; Wilson et al., 2005) and the production of NO in endothelial cells (Ming et al., 2002; Büssemaeker et al., 2007).

Rho kinase contributes to the pathogenesis of diseases related to oxidative stress and inflammation, in particular hypertension and atherosclerosis (Shimokawa and Takeshita, 2005; Schulz et al., 2008; Terzuoli et al., 2008). Inhibitors of Rho kinase have been proposed as therapeutic agents for the treatment of hypertension in humans (Mukai et al., 2001). Animal studies showed that short-term treatment with Rho kinase inhibitors reduces reperfusion injury in the rat and the dog (Shimokawa and Takeshita, 2005), whereas long-term treatment with a low dose of Rho kinase inhibitor did not affect blood pressure but diminished lesion formation in small coronary arteries of the SHR (Mukai et al., 2001). In clinical studies, the intra-arterial infusion of HA1077 (fasudil) lowers blood pressure, and this decrement is higher in hypertensive patients than in normotensive subjects (Masumoto et al., 2001). How-

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ABBREVIATIONS: SHR, spontaneously hypertensive rat; EC, endothelial cells; EDCF, endothelium-dependent contracting factor; TP, thromboxane-prostanoid; HA1077 (fasudil), 1-(5-isooquinolinesulfonyl)-homopiperazine; WKY, Wistar Kyoto rat(s); 6-keto prostaglandin F₁α; A23187, (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid; U46619, (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid; N-(4-pyridyl) cyclohexane carboxamide dihydrochloride; L-NAME, Nω-nitro-L-arginine methyl ester, 6-keto prostaglandin F₁α, 6-keto prostaglandin F₂α, prostaglandin F₂α, phenylephrine.

820
ever it is not known whether Rho kinase contributes to endothelium-dependent contractions. The present study was designed to determine the effect of Rho kinase inhibitors on such contractions.

Materials and Methods
The present study was approved by the Institutional Animal Care Committee of the University of Hong Kong.

Tissue Preparation. One-year-old and 36-week-old male SHRs (370–460 g) and Wistar Kyoto rats (WKY; 360–490 g) were purchased from the Chinese University of Hong Kong (Hong Kong, China) and kept at 21 ± 1°C under a 12-h light/12-h dark cycle. They were fed with standard laboratory chow (Labdiet, St. Louis, MO) and water ad libitum. The rats were anesthetized with pentobarbital sodium (70 mg/ml/kg; Ganes Chemicals Inc., Pennsville, NJ). The thoracic aorta was excised and immersed in Krebs-Ringer-bicarbonate buffer of the following composition: 120 mM NaCl, 25 mM NaHCO3, 5.5 mM glucose, 4.76 mM KCl, 1.18 mM MgSO4, 1.18 mM NaH2PO4, and 1.25 mM CaCl2·2H2O (control solution). The fat and connective tissue of the adventitia were removed, and the aorta was cut into rings (approximately 3 mm in length) for the recording of isometric tension and the release of 6-keto prostaglandin F1α (6-keto PGF1α) measured by immunoassay. In some preparations, the endothelial cells were removed mechanically (Furchgott and Zawadzki, 1980; Tang et al., 2005). For immunoblotting, the cleaned aorta was cut into two pieces, and the endothelial cells were removed from one of the two pieces. They were then frozen immediately in liquid nitrogen and stored at −80°C until protein extraction.

Isometric Tension Recording. Rings of SHR and WKY aorta were equilibrated for 1 h in organ chambers containing control solution aerated with 95% oxygen and 5% carbon dioxide and main-
tained for 1 h in organ chambers containing control solution aerated with 95% oxygen and 5% carbon dioxide and main-
tained at 37°C at the optimal basal tension of approximately 2.5 g (determined in preliminary experiments; data not shown). They were connected to force transducers (ADInstruments Pty Ltd., Sydney, Australia) for isometric tension recording (PowerLab; ADInstruments Pty Ltd.). The rings were exposed to 60 mM potassium chloride twice before the actual experiment. The resulting increase in tension was used as reference contraction. To elicit endothelium-dependent contractions, rings with endothelial cells were incubated with Nω-nitro- l-arginine methyl ester (l-NAME; 10−4 M) (Auch-Schwelk et al., 1990; Yang et al., 2004) and exposed to either 2-(acetyloxy)-N,N,N-trimethylthanolamin chloride (acetylcholine) or the calcium ionophore A23187 in a cumulative manner. The role of Rho kinase was determined by comparing rings in control solution or in the presence of Rho kinase inhibitors. Several Rho kinase inhibitors are available with varying degree of selectivity (Davies et al., 2000). Therefore, the present study investigated the effects of two chemically distinct Rho kinase inhibitors: fasudil or HA1077 (10−5 M) and Y27632 (10−5 M).

The effect of the two Rho kinase inhibitors was also examined on endothelium-independent contractions in rings without endothelium exposed to increasing concentrations of either U46619, 9α,11α,15S-trihydroxy-prosta-5,13E-dien-1-oi acid (prostaglandin F2α), or (R)-(-)-1-(3-hydroxyphenyl)-2-methylaminoethanol hydrochloride (phenyl-
ephrine). To confirm the selectivity of TP receptor activation by U46619 and prostaglandin F2α, certain rings without endothelium were incubated with S18886 (terutroban, Triplion; 10−7 M), a selective TP recep-
tor antagonist (Simonet et al., 1997).

Prostacyclin Immunoassay. Rings of SHR were incubated in wells containing control solution for 40 min with either 1-(4-chloro-
benzoyl)-5-methoxy-2-methyl-1H-indole (indomethacin; 10−5 M), Y27632 (10−5 M), or HA1077 (10−5 M). A single dose of acetylcholine (3 × 10−6 M) or A23187 (10−6 M) was added to the wells. After 15 min, 20 μl of the incubation solution was sampled from each well and diluted 200 times with the enzyme immunoassay buffer for immu-
noassay. The samples were assayed in triplicates for the level of the prostacyclin metabolite (6-keto PGI2) using enzyme immunoassay assay kits (Cayman Chemical, Ann Arbor, MI).

Protein Extraction and Immunoblotting. The aortae of 1-year-old and 36-week-old SHRs and WKY (with or without endo-
thelium) were cut into small pieces and homogenized in lysis buffer (20 mM Tris-Cl, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophos-
phate, and 1 mM sodium orthovanadate) with a mix of protease inhibitors (100 mM phenylmethylsulfonyl fluoride, 10 μg/ml trypsin inhibitors, 1 mg/ml leupeptin, and 2 μg/ml peptatin A). The mixture was sonicated and then centrifuged at 5000 rpm for 3 min at 4°C, and the supernatant was kept at −80°C until use. The protein concentration was determined spectrophotometrically using the Bradford protein assay reagent with bovine serum albumin as a standard. Fifty micrograms of tissue homogenate protein was used. The samples were mixed with sample buffer and reducing agent and heated for 5 min at 95°C and subsequently separated by SDS-polyacryl-
amide gel electrophoresis (10%) at 200 V, 500 mA for 50 min. The proteins were transferred electrophoretically onto nitrocellulose membranes. The blotting was performed at maximal voltage, and a current of 300 mA for 45 min. The membranes were then blocked in Tris-buffered saline with 5% dry milk at room temperature for 1 h, washed in Tris-buffered saline with 0.5% Tween 20, and then incu-
bated with primary antibody anti-ROCK2 antibody (1:5000; Sigma-
Aldrich, St. Louis, MO) or A23187 (c and d) was added in a cumulatively manner to obtain concent-
tration-responses curves. The contractions are expressed as a percentage of the reference contraction to 60 mM KCl. Data are shown as means ± S.E.M.; n = 5 to 8. For statistical analysis, see Fig. 2.

Fig. 1. Effects of the Rho kinase inhibitor Y27632 on endothelium-dependent contraction. Aortic rings from SHRs (b and d) and WKY (a and c) with (+EC) or without (-EC) endothelium were incubated with 1-NAME (10−4 M) and with Y27632 (10−5 M). Acetylcholine (a and b) or A23187 (c and d) was added in a cumulatively manner to obtain concent-
tration-responses curves. The contractions are expressed as a percentage of the reference contraction to 60 mM KCl. Data are shown as means ± S.E.M.; n = 5 to 8. For statistical analysis, see Fig. 2.
Results

Endothelium-Dependent Contractions. In the presence of L-NAME (10^-4 M), acetylcholine caused endothelium-dependent contractions that were significantly larger in aortae of SHRs than in WKY (Fig. 1). The contractions induced by acetylcholine were abolished by either Y27632 (10^-5 M) (Fig. 1) or HA1077 (10^-5 M) (Fig. 2). The endothelium-dependent contractions induced by the calcium ionophore A23187 were comparable in aortae of SHRs and WKY (Fig. 1). They were reduced significantly but not abolished by the two Rho kinase inhibitors. The inhibitory effects of HA1077 and Y27632 were comparable on acetylcholine-induced contractions but not on those to the calcium ionophore (Fig. 2).

Endothelium-Independent Contractions. U46619, prostaglandin F_2alpha, and phenylephrine contracted SHR and WKY aortae without endothelium, and the responses were comparable in the arteries of the two strains (Fig. 3). In the absence of Rho kinase inhibitors, the contractions induced by the three agonists (U46619, prostaglandin F_2alpha, and phenylephrine) were not significantly different in SHR and WKY aortae. The two Rho kinase inhibitors reduced the contractions significantly and to a comparable extent in preparations of the two strains (Fig. 4).

Prostacyclin Release. The release of 6-keto prostaglandin F_2alpha, induced by either acetylcholine (3 × 10^-6 M) or A23187 (10^-5 M), was not significantly affected by Y27632 (10^-5 M) or HA1077 (10^-5 M). The production of the metabolite of prostacyclin was reduced significantly by indomethacin (10^-4 M) (Fig. 5).

Protein Expression. The Rho kinase expression was significantly larger in the aortae of 1-year-old SHRs and WKY than in those of the same strains at 36 weeks of age. The expression of Rho kinase was not significantly different between WKY and SHR aortae and was not affected significantly by the removal of the endothelium (Fig. 6).

Discussion

In the present study, response to acetylcholine was higher in the aorta of SHRs than in that of WKY, but these differences were absent in the response to the calcium ionophore A23187, which confirms results from a previous study (Lüs...
Both Y27632 and HA1077 were effective in reducing endothelium-dependent contractions, because the two inhibitors abolished the response to acetylcholine and reduced that to the calcium ionophore A23187. However, the present experiments suggest that this inhibition is because of an effect on vascular smooth muscle rather than on endothelial cells. This conclusion is based on the observation that the two Rho kinase inhibitors do not decrease the release of 6-keto prostaglandin F₃₀, the metabolite of prostacyclin. Prostacyclin is the main EDCF in the SHR aorta when stimulated by acetylcholine (Gluais et al., 2006). Therefore, the production of prostacyclin metabolite was

Fig. 3. Concentration-response curves to endothelium-independent vasoconstrictors in SHR and WKY aortae. The endothelium was removed from all rings. Effects of the Rho kinase inhibitor, Y27632 (10⁻⁵ M) on the responses to U46619 (a), prostaglandin F₂₀ (b), and phenylephrine (c). Contractions are represented as a percentage of the reference contraction to 60 mM KCl obtained at the beginning of the experiment. Data shown as means ± S.E.M.; n = 5. For statistical analysis, see Fig. 4.
measured as an index of EDCF release in the presence of Rho kinase inhibition. The confirmed inhibitory effect of indomethacin (Shaul et al., 1991; Gluais et al., 2006) on the production of prostacyclin served as a positive control. Because the breakdown of prostacyclin into 6-keto prostaglandin $F_1\alpha$ is by nonenzymatic hydrolysis (Sun and Taylor, 1978; Rosenkranz et al., 1980), the present experiments indicate Rho kinase inhibitors expectedly do not affect it. Likewise, the unchanged levels of released 6-keto prostaglandin $F_1\alpha$ upon exposure to either acetylcholine (Gluais et al., 2005) or the calcium ionophore A23187 (Gluais et al., 2006) make an inhibitory effect of the Rho kinase inhibitors on either cyclooxygenase or prostacyclin synthase unlikely. The present findings thus indicate that Rho kinase, although it is present in endothelial cells (Ming et al., 2002; Büssemaker et al., 2007; Gien et al., 2008; van Nieuw Amerongen et al., 2008), does not play a significant role in EDCF production. This interpretation is strengthened by the immunoblotting data, which did not reveal an increased presence of Rho kinase in preparations with endothelium of SHR aorta. One could argue that the technique used has too low a sensitivity to accurately measure Rho kinase expression in the endothelium, in view of the overwhelming presence of smooth muscle cells that express Rho kinase, as also observed previously (Mukai et al., 2001; Cario-Toumaniantz et al., 2002; Bolz et al., 2003; Jernigan et al., 2008). In the present study, the expression of Rho kinase in vascular smooth muscle of SHR was comparable with that in the WKY. This observation contrasts with the findings that Rho kinase mRNA is augmented in the smooth muscle cells of the SHR carotid arteries (Mukai et al., 2001). The lack of differences in the present study of expression of Rho kinase of in the aorta of WKY and SHRs agrees with the functional studies on endothelium-independent contractions, in which the inhibitory effects of Rho kinase inhibitors were comparable in both strains. The difference between the current and previous findings (Mukai et al., 2001) could be because of either differences in age and/or the anatomical origin of the blood vessels used. The rats used in the functional studies reported in the present study were 1 year old, compared with the 16-week-old animals used by Mukai et al. (2001). The comparison of the Rho kinase expression of aortae of 36-week-old and 1-year-old rats permits the conclusion that indeed this expression increases with age.

The responses to U46619, prostaglandin $F_2\alpha$, and phenylephrine were not augmented in the SHR, consistent with results of previous work (Ge et al., 1995). Both U46619 and prostaglandin $F_2\alpha$ induce contraction of the aorta by the activation of TP receptors. This conclusion is prompted by the observation that S18886 abolishes the response to the two agonists, as demonstrated previously (Yang et al., 2004). Rho kinase inhibitors reduced endothelium-independent contractions of preparations, in confirmation of previous observations in isolated blood vessels (Mukai et al., 2001; Wilson et al., 2005). The present findings that the two inhibitors reduced contractions caused by both TP receptor and $\alpha_1$-receptor activation reinforce the conclusion that Rho kinase is the common pathway activated by those two cell membrane receptors in vascular smooth muscle (Somlyo and Somlyo, 2000). The inhibition of the contractions was comparable
in SHRs and WKY, to judge from the reduction in the area under the concentration-response curve. However, the relative inhibitory effect of the Rho kinase inhibitors varied depending on agonist used. Thus, the concentration-response curve to U46619 and prostaglandin F2α/H9251 were shifted to the right, whereas the contraction to phenylephrine was abolished. The results observed with phenylephrine could be explained if the α1-adrenergic agonist were less potent in terms of activation of Rho kinase in the concentration range used in the present experiments. Alternatively, activation of TP receptors, but not of α1-adrenoceptors, may involve other signaling pathways through the Rho kinase-dependent cascade, producing the contraction observed with higher concentrations of U46619 and prostaglandin F2α.

The present findings demonstrate that Rho kinase inhibition curtails EDCF-mediated responses. Although Rho kinase can regulate the production of nitric oxide (Ming et al., 2002; Büssemaker et al., 2007), it does not seem to do so for EDCF production, and the inhibitory effect of Rho kinase inhibitors on endothelium-dependent contractions is due solely to inhibition of the contractile process in the vascular smooth muscle cells. Because EDCF-mediated responses are exacerbated by hypertension (Vanhoutte et al., 2005), the inhibitory effect of Rho kinase inhibitors on these responses may contribute to the lowering of arterial blood pressure that they cause (Masumoto et al., 2001).

References


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