Original Article

Role of Rho/Rho-Kinase and NO/cGMP Signaling Pathways in Vascular Function Prior to Atherosclerosis

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Aim: Atherosclerosis is a cardiovascular disease; however, there is little information on signal transduction for vascular function in the early stage of atherosclerosis. In this work, we investigated the role of Rho/Rho-kinase and nitrogen oxide (NO)/cyclic GMP (cGMP) signaling pathways in the aorta prior to atherosclerosis.

Methods: Tension, the expression of RhoA protein, Rho-kinase activity and the cGMP level were measured using endothelium-intact or -denuded aorta prepared from apolipoprotein E-deficient (apoE-KO) and C57BL/6 wild-type (WT) mice at 2 months of age.

Results: Phenylephrine (PE) induced less maximal contraction in the endothelium-denuded aorta from apoE-KO than from WT mice. A Rho-kinase inhibitor (Y-27632) reduced more effectively the contraction of apoE-KO than WT mice, but their RhoA proteins and Rho-kinase activities were not so different. Acetylcholine caused larger relaxation of the PE-stimulated, endothelium-intact aorta in apoE-KO due to endothelial NO release than WT mice. The basal cGMP level in the endothelium-intact aorta of apoE-KO mice was higher than that of WT.

Conclusions: Smooth muscle contraction via α1-adrenergic receptor shows higher dependency on Rho-kinase activity, suggesting down-regulation of the mechanism different from Rho/Rho kinase signaling in the aorta prior to atherosclerosis. Endothelium-dependent relaxation is also intensified through the NO/cGMP pathway.


Key words: Apolipoprotein E-deficient (apoE-KO) mouse, Endothelium, Aorta, Atherosclerosis

Introduction

Atherosclerosis is a disease affecting arterial blood vessels, diminishing the function of smooth muscle contraction and/or endothelium-dependent relaxation. Aging and intake of cholesterol-rich foods cause fibrous plaque and atheroma, inducing dysfunction of endothelium to regulate the contraction/relaxation of smooth muscle because of decreased

endothelial cells. Atherosclerosis is a chronic inflammatory response in the artery walls due to the accumulation of cholesterol- and triglyceride-rich low-density lipoprotein (LDL); however, it is unclear whether the intracellular response influencing vascular function occurs in the smooth muscle or endothelium in the early stage of atherosclerosis to show hypercholesterolemia but not atheroma.

The contraction of smooth muscle is primarily regulated by calcium-dependent and -independent intracellular signal transduction. Calcium/calmodulin-dependent myosin light chain kinase (MLCK) is involved in the former calcium-dependent mechanism. The latter calcium-independent mechanism is known as calcium sensitization associated with the inhibition of myosin light chain phosphatase (MLCP)
activity. The Rho/Rho-kinase signal pathway plays an important role in the mechanism through phosphorylation of the myosin-targeting subunit of MLCP. Protein kinase C (PKC)/phosphatase inhibitor protein of the 17 kDa (CPI-17) signal pathway is also involved in the latter mechanism. On the other hand, the relaxation of vascular smooth muscle is considerably regulated by an endothelium-dependent mechanism through NO/cGMP and endothelium-derived hyperpolarizing factor (EDHF) -mediated signal transduction. It has been reported that an increase in calcium sensitivity, a decrease in endothelium-dependent relaxation and down-regulation of cGMP production were observed in the later phase of atherosclerosis.

In the current study, we examined the changes of intracellular signal pathways affecting aortic contraction and relaxation in aortic smooth muscle and endothelium in the early stage of atherosclerosis using apo-lipoprotein E-deficient (apoE-KO) mice as a model for atherosclerosis. We compared the contraction in apoE-KO mouse smooth muscle stimulated with agonists such as phenylephrine (PE) and 5-hydroxytryptamine (5-HT) with that in C57BL/6 wild-type (WT) mouse smooth muscle in relation to the activation of Rho-kinase. We also studied the relaxation induced by acetylcholine (ACh) and nitroglycerin (NTG), and cGMP production in both kinds of aorta.

Methods

Reagents

We used PE, 5-HT, ACh, N-nitro-L-arginine (LNA) (Sigma Chemical Co., St. Louis, MO), sodium cholate, papaverine hydrochloride (Wako Pure Chemical, Osaka, Japan), NTG (Nippon Kayaku, Tokyo) and (+)-(R)-trans-4-(1-Aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride, monohydrate (Y-27632; Calbiochem, Dermstadt, Germany). Monoclonal anti-RhoA antibody (Santa Cruz), monoclonal anti-smooth muscle actin antibody (PROGEN, Heidelberg, Germany) and peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz, CA, U.S.A.) were purchased. All other chemicals were of the highest reagent grade available.

Animals

C57BL/6 WT mice were obtained from a local supplier, and C57BL/6 apoE-KO mice were purchased from Taconic/IBL (Germantown, NY/Fujioka, Japan). The experimental procedures were approved by the animal experiment and welfare committee of Nagoya City University Graduate School of Medical Sciences. Male apoE-KO and WT mice were received normal chow diet. Some apoE-KO mice were 2, 6 (Fig. 1) and 2 (Fig. 2–8) months of age when studied.

Histochemistry

After mice were anesthetized with sevoflurane (Maruishi Pharmaceutical Co., Osaka, Japan), aortas were dissected, immersed in paraformaldehyde fixative, dehydrated in ethanol and embedded in paraffin. Three-micron sections were cut and tissues were processed for staining with hematoxylin and eosin.

Tissue Preparation

Animal care and research protocols were conducted in accordance with the guidelines of our university. After mice were anesthetized with sevoflurane, and thoracic aortas were removed and dissected into ring strips (0.1 x 0.3 cm). In some experiments, endothelial cell layers were removed by rubbing the luminal side of the vessel with a cotton swab under a binocular microscope.

Measurement of Tension

Each strip of aorta was placed in a 5.0 mL organ bath containing normal Krebs solution (Na+ 137.4, K+ 5.9, Mg2+ 1.2, Ca2+ 2.6, HCO3− 15.5, H2PO4− 1.2, Cl− 134, glucose 11.5 mM, pH 7.4) bubbling with 95% O2− 5% CO2. After an equilibration period of 60 min, mechanical activity was measured by isometric recording under resting tension of 300 mg. PE or 5-HT was added in increments to study concentration-dependent effects. In some experiments, an inhibitor of Rho-kinase was added 20 min before exposure to the contracting agent. Aortic rings with endothelium were exposed to PE (10−5 M) to obtain submaximal contraction and then relaxed with increasing concentrations of ACh or NTG. These were pretreated with LNA (10−4 M) for 30 min before the injection of PE. At the end of the experiment, papaverine (10−4 M) was added to obtain the maximal relaxation. The relaxation response was evaluated as a percentage of ACh- or NTG-induced to papaverine-induced relaxation.

Western Blot Analysis of RhoA

Total proteins, including RhoA, were extracted and separated by SDS-PAGE, as reported previously. After transfer to a nitrocellulose membrane, the membrane was incubated with primary antibody for 3 h at room temperature. Blots were detected with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Tokyo, Japan) and quantified by Lumi-Imager F1 (Roche Diagnostics, Germany) using Lumi Ana-
lyst™ 3.0 software (Boehringer Mannheim Biochemicals, Mannheim, Germany). The relationship between the amount of protein loaded and the quantified blot values was linear. Protein concentrations were determined using the Bradford dye-binding assay (Bio-Rad, CA, U.S.A).
Measurement of Rho-Kinase Activity

The tissues were homogenized in buffer containing 50 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1 μg/mL pepstatin, 0.5 μg/mL leupeptin, 10 mM NaF, 2 mM NaVO₄, and 10 mM beta-mercaptoethanol. Rho-kinase activity was analyzed in the presence of 10⁻⁴ M adenosine triphosphate (ATP) using a Rho-kinase Assay Kit (Cyclex, Nagano, Japan).

Radioimmunoassay of cGMP

Radioimmunoassay techniques were used to
determine the levels of cGMP. Strips (0.2 × 0.3 cm) were immersed in control Krebs solution containing isobutylmethylxanthine (3 × 10⁻⁴ M), bubbled with a 95% O₂ and 5% CO₂ gas mixture and kept at 37°C, pH 7.4. The strips were incubated for 20 min in a Krebs solution containing PE (10⁻⁵ M) and isobutylmethylxanthine. Some strips were further incubated for 5 min after the addition of ACh (10⁻⁶ M). All strips were removed from the solution and quick-frozen in liquid nitrogen. The tissues were homogenized in 0.2 mL ice-cold 6% trichloroacetic acid. The homogenate was centrifuged at 3000 rpm at 4°C and the supernatant was extracted 3 times with 1 mL water-saturated ethylether. The cGMP level was determined with a radioimmunoassay kit (Yamasa Shoyu, Tokyo, Japan) and expressed as pmol/mg protein. Protein assay was performed using a DC Protein Assay Kit (Bio-Rad, CA, USA).

**Statistical Analysis**

Results are expressed as the mean ± SE and as a percentage of maximal effect. All statistical comparisons were performed using Student’s t test or one-way analysis of variance (ANOVA) followed by Fisher’s PLSD where appropriate. Values were considered significantly different at p < 0.05.

**Results**

**Vascular Histology in Thoracic Aorta of ApoE-KO and WT Mice**

While the plasma cholesterol level of 2-month-old apoE-KO mice was higher than that of WT mice (data not shown), aortic sections from both apoE-KO and WT mice showed no morphological changes, as shown in Fig. 1. At 6 months of age, aortic sections from apoE-KO mice showed atherosclerotic plaque. Accordingly, we used the thoracic aorta of apoE-KO and WT mice at 2 months of age to study smooth muscle function prior to atherosclerosis.

**Agonist-Induced Contraction in Endothelium-Denuded Aortas from ApoE-KO and WT Mice**

Fig. 2A shows the time course of PE- or 5-HT-induced contraction in endothelium-denuded rings of aortic strips prepared from apoE-KO and WT mice at
2 months of age. The effect of the former stimulant 
was sustained at least for 10 min after stimulation, 
while the latter was transient. Maximal contraction 
induced by PE (10^{-5} M) but not 5-HT (10^{-5} M) was 
significantly worse in apoE-KO than WT mice 
(Fig. 2B). This finding implies that the mechanism of 
PE action to induce the contraction of aortic smooth 
muscle is different from that of 5-HT action.

**Effects of Y-27632 on Agonist-Induced Contraction 
in Endothelium-Denuded Aortas from ApoE-KO 
and WT Mice**

The contractions of aortic smooth muscles from 
WT and apoE-KO mouse, however, were enhanced 
by PE and 5-HT in the same concentration-depen-
dent manner at a concentration range of 10^{-9} - 10^{-4} 
M (Fig. 3, 4). A Rho-kinase inhibitor, Y-27632, at a 
concentration of 10^{-6} M reduced more effectively the 
effect of PE (10^{-6} - 10^{-4} M) on the aorta of apoE-KO 
mice than that of WT mice (Fig. 3). There was no sig-
nificant difference in the inhibition of 5-HT-induced 
aortic contraction by Y-27632 between apoE-KO 
and WT mice (Fig. 4). The PE-induced contraction 
of endothelium-denuded aorta from apoE-KO mice 
appears to depend on Rho-kinase more than that from 
WT mice.

**RhoA Level in Aortic Smooth Muscles of ApoE-KO 
and WT Mice**

RhoA protein levels in aortic smooth muscles 
from apoE-KO and WT mice at 2 months of age were 
compared by immunoblotting with anti-RhoA anti-
body. Similar levels of RhoA were observed in both 
strains of mice when these RhoA proteins were nor-
malized by the amount of smooth muscle α-actin 
(Fig. 5A, WT: 0.29 ± 0.07; apoE-KO: 0.26 ± 0.03).

**Rho-Kinase Activity During PE-Induced Contraction 
in ApoE-KO and WT Mice**

PE at the concentration of 10^{-5} M enhanced the 
Rho-kinase activity of aortic smooth muscles in both 
apoE-KO and WT mice, as measured by the phos-
phorylation of myosin light chain phosphatase target 
subunit (MYPT-1) (Fig. 5B). Y-27632 exhibited the
same degree of activity to suppress PE-stimulated Rho-kinase activity in the apoE-KO and WT mouse aorta, suggesting that PE-induced contraction depends on signaling other than the Rho/Rho-kinase pathway more strongly in WT mouse aorta than apoE-KO aorta.

**Agonist-induced Contraction in Endothelium-Intact Aortas from ApoE-KO and WT Mice**

An injection of PE ($10^{-9}$ to $10^{-4}$ M) or 5-HT ($10^{-8}$ to $10^{-5}$ M) also produced a contraction in endothelium-intact rings of aortic strips from apoE-KO and WT mice at 2 months of age. The maximal contraction of endothelium-intact aorta stimulated by PE ($10^{-5}$ M) was much less potent than that of endothelium-denuded preparations (Fig. 2B, 6A, $p < 0.05$) and significantly less in apoE-KO than WT mice (Fig. 6A, $p < 0.05$). The 5-HT ($10^{-5}$ M)-induced maximal contraction was more potent than PE-induced contraction and the effects on apoE-KO tended to be weaker than on WT unlike endothelium-denuded preparations. Both concentration response curves for these agents were similar in apoE-KO and WT mice (Fig. 6B).

**ACh- and NTG-Induced Relaxation in Endothelium-Intact Aortas from ApoE-KO and WT Mice**

An injection of ACh ($10^{-9}$ to $10^{-5}$ M) induced the relaxation of endothelium-intact aortas from apoE-KO and WT mice, which had been pre-contracted with PE, in a concentration-dependent manner (Fig. 7A). There was no difference in the maximal relaxation induced by $10^{-4}$ M papaverine between apoE-KO and WT mice; however, the ACh-induced relaxation expressed as % of the maximal effect by papaverine was larger in apoE-KO mice than WT mice. The maximal relaxation in apoE-KO and WT mice was approx. 80% and 50%, respectively. Pretreatment with NO synthase (NOS) inhibitor LNA ($10^{-4}$ M) completely inhibited relaxation in both strains (data not shown). An NO donor, NTG, also relaxed aortas from apoE-KO and WT mice in a concentration-dependent manner (Fig. 7B). The concentration-dependent curves for relaxing effects in both strains was almost the same, and the maximal relaxation was almost the same as papaverine-induced maximal effects.

**Basal and PE-Stimulated cGMP Levels in Endothelium-Intact Aortas from ApoE-KO and WT Mice**

As shown in Fig. 8A, basal levels of cGMP in endothelium-denuded aorta of both strains were as low as approximately 1.5 pmol/mg protein; however, the basal cGMP level in endothelium-intact aorta of apoE-KO mice was over 2-fold higher than that of WT. PE increased the cGMP level in WT mice much more than apoE-KO mice. Pretreatment with LNA ($10^{-4}$ M) decreased the aortic cGMP level in apoE-KO and WT mice, which had been pre-treated with PE, in a concentration-dependent manner (Fig. 7A).
KO and WT mice (Fig. 8B). These findings suggest that the higher level of cGMP is maintained by endothelium-produced NO in the aorta of apoE-KO mice than WT mice even without stimulants in the early stage of atherosclerosis, and that PE induces NO release from the endothelium of WT mice.

**Discussion**

Histological changes and lesions in the aortic smooth muscle and endothelium are in progress in apoE-KO mice between 6–8 months of age. At this stage, atherosclerotic lesions mainly in the aortic arch and plaque formations are visible on the luminal surface of the thoracic aortas and aortic arches, however, altered gene expression has been detected in early atherosclerosis. We studied the signaling pathways for vascular function prior to atherosclerosis, using aortic strips from 2-month-old apoE-KO mice without aortic damage detectable by light microscopy but with a higher level of plasma cholesterol.

In this study, we investigated the functions of Rho/Rho-kinase and NO/cGMP pathways in aortic smooth muscle and endothelium to understand the change in signaling transduction incidental to contraction/relaxation of vascular smooth muscle in the early stage of atherosclerosis. The results are summarized as follows: 1) Endothelium-denuded aorta prepared from apoE-KO mice at 2 months of age exhibited weaker maximal contraction against PE but not 5-HT than that from WT mice. 2) A Rho-kinase inhibitor, Y-27632, reduced more effectively the PE-dependent contraction in the endothelium-denuded aorta from apoE-KO than WT mice. 3) The expression of RhoA protein and Rho-kinase activity in aortas stimulated with PE were not so different between apoE-KO and WT mice. 4) PE-induced contraction in endothelium-intact aorta was less active than in the endothelium-denuded preparation. 5) ACh-induced relaxation in endothelium-intact aorta of apoE-KO was more potent than that of WT mice. 6) The basal cGMP level in endothelium-intact aortas of apoE-KO was higher than that of WT mice.

There were no difference between apoE-KO and WT mice at 2 months of age in terms of body and heart weight and structures in the smooth muscle and endothelium, although plasma cholesterol levels of apoE-KO mice were higher than in WT mice (data not shown). We thus examined the effects of vasoactive agents, such as PE and 5-HT, on endothelium-denuded thoracic aortas from apoE-KO and WT mice at 2 months of age. Although no differences were observed in concentration-response curves for PE and 5-HT in both strains of mice (Fig. 3, 4), the endothelium-denuded aorta from apoE-KO mouse contracted against PE but not 5-HT less potently than that from
WT mice (Fig. 2B). Contractions by PE and 5-HT have been reported to be mediated through alpha1-adrenergic subtype and 5-HT3 receptor, respectively; therefore, down-regulation of the cellular level of alpha1-adrenergic receptor or its signaling pathway may already appear in the mechanism for smooth muscle contraction in the aortas of apoE-KO mice in the early stages of atherosclerosis, whereas the function of 5-HT2a-subtype receptor may be not damaged in the same stage.

A Rho-kinase inhibitor, Y-27632, attenuated PE-induced contractions in aortas of apoE-KO more potently than WT mice (Fig. 3). This result indicates that the role of Rho kinase in the PE-induced contraction is greater in apoE-KO than WT mice, considering the smaller maximal contractions of PE in apoE-KO compared with those in WT mice. On the other hand, we observed that not only the expression of RhoA but also PE-stimulated activity of Rho-kinase in the aortic smooth muscle were not different between apoE-KO and WT mice (Fig. 4). Therefore, our result shows that the change of Rho-kinase activation does not appear in the early stages of atherosclerosis, although involvement of the kinase in the disease has been reported previously; however, we are not able to reject the alterations of other contraction-related signal pathways in the early stage of the syndrome. The decrease in PE-induced maximal contraction in apoE-KO mouse aorta suggests that the Rho/Rho-kinase-independent signal pathway is impaired in the early stage of atherosclerosis. The increase in intracellular Ca2+ and Ca/calmodulin-mediated MLCK activation is involved in agonist-induced contractions. Alternatively, it is possible that PKC/CPL-17-mediated MLCP inhibition, known as another Ca2+-independent mechanism, is impaired in the early stage of atherosclerosis. In contrast, Y-27632 was less effective in reducing 5-HT contractions than PE contractions and the degree of inhibition in the 5-HT contraction was similar in both strains of mice. These results suggest differences in the role of Rho/Rho-kinase signaling between PE- and 5-HT-mediated mechanisms and that the Rho/Rho-kinase-independent signaling during the early stages of atherosclerosis is disrupted in the mechanism of PE not 5-HT.

In endothelium-intact aorta, there is no significant difference in PE concentration-dependent contraction curves between apoE-KO and WT mice (Fig. 6B1); however, the absolute value of tension induced by 10^{-5} M PE markedly decreased in the presence of endothelium and was smaller in aortas from apoE-KO than WT mice (Fig. 2B, 6A). Previously, it has been reported that some agonists caused endothelium-dependent relaxation in arteries from rats, pigs and dogs and that the prolonged exposure of rat aorta to PE stimulated basal NOS activity, reducing alpha-adrenergic receptor-mediated contraction. Therefore, the results suggest that PE induces not only contraction but also relaxation due to endothelium-dependent NO release and that the effects of PE are more potent in apoE-KO than WT mouse at 2 months of age. Another agonist, 5-HT, caused less potent maximal contraction of endothelium-intact aorta compared with endothelium-denuded aorta in apoE-KO mice (Fig. 2B, 6A), but such effects were not observed significantly in aortas from WT mice.

ACh relaxed aortas from apoE-KO mice more potently than from WT mice (Fig. 7A). NOS inhibitor, LNA, completely blocked ACh-induced relaxation in both strains (data not shown), implying that ACh-induced relaxation is due to NO released from the endothelium. As NTG-induced relaxations are the same in both strains, the ability of NO-mediated relaxation is not considered to be different. Accordingly, ACh-induced relaxation due to endothelial NO release appears to be potentiated in apoE-KO mice aged 2 months. Further, the basal cGMP level in endothelium-intact aorta from apoE-KO mice was higher than WT (Fig. 8A). PE enhanced the cGMP level significantly in WT mice but not apoE-KO mice (Fig. 8B), suggesting that the NO/cGMP signaling mechanism compensatively increased in aortas from apoE-KO mice in early atherosclerosis, as reported previously. The reason why PE does not raise the cGMP level of aortas from apoE-KO mice so much is unclear.

In conclusion, this study suggests that alpha-mediated contraction in smooth muscle was reduced through Rho/Rho-kinase signaling-independent mechanisms, whereas basal and agonist-stimulated NO release from the endothelium increased in the early stage of atherosclerosis. Therefore, the increased release of NO may play an important role as an endogenous compensative effector against atherosclerosis to protect against smooth muscle stiffness in young mice.

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**References**

2) Harrison DG: Endothelial dysfunction in atherosclerosis.
4) Somlyo AP, Somlyo AV: Ca$^{2+}$ sensitivity of smooth muscle and nonmuscle myosin II; modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev, 2003; 83: 1325-1358
32) Ma Y, Malbon CC, Williams DL, Thorngate FE: Altered gene expression in early atherosclerosis is blocked by low
level of apolipoprotein E. PLOS ONE, 2008; 3: e2503


