Blockade of both $\alpha_{1A}$- and $\alpha_{1B}$-adrenergic receptor subtype signaling is required to inhibit neointimal formation in the mouse femoral artery

Chihiro Hosoda,1,2* Masami Hiroyama,1,* Atsushi Sanbe,† Jun-ichi Birumachi,† Tadaichi Kitamura,2 Susanna Cotecchia,3 Paul C. Simpson,4 Gozoh Tsujimoto,5 and Akito Tanoue†

1Department of Pharmacology, National Research Institute for Child Health and Development, Tokyo, Japan; 2Department of Urology, Faculty of Medicine, University of Tokyo, Tokyo, Japan; 3Institut de Pharmacologie et Toxicologie, Université de Lausanne, Lausanne, Switzerland; 4Cardiology Division, San Francisco Veterans Affairs Medical Center, and the Cardiovascular Research Institute and Department of Medicine, University of California San Francisco, San Francisco, California; and 5Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

Submitted 13 June 2006; accepted in final form 22 March 2007

Hosoda C, Hiroyama M, Sanbe A, Birumachi J, Kitamura T, Cotecchia S, Simpson PC, Tsujimoto G, Tanoue A. Blockade of both $\alpha_{1A}$- and $\alpha_{1B}$-adrenergic receptor subtype signaling is required to inhibit neointimal formation in the mouse femoral artery. Am J Physiol Heart Circ Physiol 293: H514–H519, 2007. First published March 23, 2007; doi:10.1152/ajpheart.00626.2006.—Attenuation of early restenosis after percutaneous coronary intervention (PCI) is important for the successful treatment of coronary artery disease. Some clinical studies have shown that hypertension is a risk factor for early restenosis after PCI. These findings suggest that $\alpha_1$-adrenergic receptors ($\alpha_1$-ARs) may facilitate restenosis after PCI because of $\alpha_1$-AR’s remarkable contribution to the onset of hypertension. In this study, we examined the neointimal formation after vascular injury in the femoral artery of $\alpha_1$-arginine (1A-KO), $\alpha_1$-arginine (1B-KO), $\alpha_1$-arginine (1D-KO), $\alpha_1$-arginine (1AB-KO), and wild-type mice to investigate the functional role of each $\alpha_1$-AR subtype in neointimal formation, which is known to promote restenosis. Neointimal formation was significantly smaller in those before it. These results suggest that lack of both $\alpha_1A$- and $\alpha_1B$-ARs could be necessary to inhibit neointimal formation in the mouse femoral artery.

$\alpha_1$-adrenergic receptor; vascular injury; smooth muscle cell; femoral artery of mouse; wire injury

Since the introduction of catheter-based coronary revascularization for the treatment of coronary artery disease, the procedure, termed percutaneous coronary intervention (PCI), has been improved with the development of newer devices, and its indication has been expanded with excellent success rates (>97%) (5). In approximately 20% of these patients, however, recurrent ischemia due to restenosis of the dilated segment developed within 6 mo (5). Because endothelial denudation following PCI stimulates smooth muscle cells to form neointima, neointimal formation is considered to be one of the most important mechanisms contributing to restenosis (9, 12, 17). Therefore, the elucidation of the precise mechanism of neointimal formation is important for the treatment and prevention of coronary artery disease. To elucidate the mechanisms of neointimal formation, a wire injury model of the mouse femoral artery was developed (6, 19, 23, 28). Furthermore, the introduction of genetically engineered mice into the vascular injury model enabled us to examine the role of specific molecules in neointimal formation (21, 25, 32, 33, 40).

$\alpha_1$-Adrenergic receptors ($\alpha_1A$, $\alpha_1B$, and $\alpha_1D$-AR), which activate vascular smooth muscle contraction, leading to vasconstriction and blood pressure regulation (7, 24, 34), are involved in the contraction of human coronary smooth muscle cells (31) and the decrease in coronary blood flow after PCI (13). Moreover, hypertension, to which the onset $\alpha_1$-AR markedly contributes (1, 8, 22, 38), is a risk factor for early restenosis after PCI (2, 29). These findings suggest that $\alpha_1$-AR may be involved in restenosis after PCI. In fact, $\alpha_1$-AR-mediated norepinephrine stimulation induces neointimal formation (12). In addition, recent studies using rat vessels with $\alpha_1$-antagonists revealed that the $\alpha_1A$-AR subtype, but not the $\alpha_1D$-AR subtype, was certainly involved in catecholamine-stimulated neointimal formation (10, 12). However, the exact functional role of $\alpha_1B$-AR subtypes in neointimal formation remained unclear (10, 36) because of the lack of appropriate $\alpha_1B$-selective antagonists and satisfactory methods of quantitative $\alpha_1$-AR-subtype mRNA expression analysis.

Therefore, in this study, we examine the mRNA expression of each $\alpha_1$-AR subtype in the mouse femoral artery using an RT-PCR assay and further investigate the functional role of each $\alpha_1$-AR subtype in neointimal formation using a vascular injury model of mouse femoral artery in mice lacking the $\alpha_1$-AR subtype gene.

Materials and Methods

Mice lacking $\alpha_1$-AR subtypes. $\alpha_1A$-/ $\alpha_1B$-AR double-knockout (1AAB-KO), 1AAB-/-, $\alpha_1A$--/ $\alpha_1B$-- mice, whose genetic background was C57Bl6J (20), were bred with 129Sv mice to produce F1 heterozygous ($\alpha_1A$+/--/ $\alpha_1B$+/-- mice, whose genetic background was a mixture of 129Sv mice and C57Bl6J mice. For this study, wild-type (WT) mice, which were produced as $\alpha_1A$-/ $\alpha_1B$-AR positive mice during 1AAB-KO mice generation, were used as controls and maintained on the genetic background of 129Sv mice and C57Bl6J mice. F1 mice were subsequently intercrossed to generate 1AAB-KO and $\alpha_1A$-KO ($\alpha_1A$-- mice, $\alpha_1B$-KO ($\alpha_1B$-- and $\alpha_1D$-KO ($\alpha_1D$--)}
mice had already been generated and their viability confirmed (7, 34). The genetic background of both α1A-KO mice and α1D-KO mice was the same (a mixture of 129Sv and C57Bl6/J). Thus the genetic background of WT, the same (a mixture of 129Sv and C57Bl6/J). Thus the genetic background of both mice had already been generated and their viability confirmed (7, 34).

The expression levels of α1-AR subtypes were compared in all knockout (KO) mice by RT-PCR. In all types of mutant mice, the expression levels of other types of α1-AR were increased.

mRNA expression of α1-AR subtypes in the femoral artery of wild-type (WT) mice. Total RNA was isolated from the mouse tails (7, 24, 34). Six groups of 16- to 24-wk-old male WT, determined with DNA isolated from the mouse tails (7, 24, 34). Six groups of 16- to 24-wk-old male WT, determined with DNA isolated from the mouse tails (7, 24, 34). Six groups of 16- to 24-wk-old male WT, determined with DNA isolated from the mouse tails (7, 24, 34). Six groups of 16- to 24-wk-old male WT, determined with DNA isolated from the mouse tails (7, 24, 34). Six groups of 16- to 24-wk-old male WT, determined with DNA isolated from the mouse tails (7, 24, 34). Six groups of 16- to 24-wk-old male WT, determined with DNA isolated from the mouse tails (7, 24, 34). Six groups of 16- to 24-wk-old male WT, determined with DNA isolated from the mouse tails (7, 24, 34).

RNA isolation and RT-PCR. The femoral artery was isolated and dissected free of excess fat and connective tissue. These materials were then immediately pooled in the RNAlater RNA stabilization solution (Ambion, Austin, TX) for 1 day at room temperature so that the RNA volume would remain as large as possible. After that, the total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) and reverse-transcribed. The expression levels of femoral artery of wild-type (WT) mice. Total RNA was isolated from the femoral artery and then reverse transcribed. The expression levels of α1-AR subtypes were compared in all knockout (KO) mice by RT-PCR. In all types of mutant mice, the expression levels of other types of α1-AR were increased.

GAPDH was used as a control. The presented result is representative of three separate experiments.

Fig. 1. mRNA expression of α1-AR subtypes in the femoral artery. RT-PCR was carried out to investigate the expression of each receptor in the femoral artery of KO mice as well as of 5'-AACCTTGGGATTTGAGTCG-3' and 5'-TGCCACTGTCTAC-CAGAGAG-3'; and α1D receptor, 5'-GCTGGGAGACTGCC-TCAGCAT-3' and 5'-AGTGGTGCCTCAAGTCAGAATG-3'. The products were detected under UV illumination after agarose gel electrophoresis.

Mouse femoral wire injury model. Surgery was carried out using a dissecting microscope (C-DSS 115, Nikon, Tokyo, Japan). Transluminal mechanical injury of the femoral arteries via arteriotomy in a small muscular branch using a straight spring wire (0.38 mm in diameter, no. C-SF-15-15, Cook, Bloomington, IN) was performed according to the method described previously (28). The wire was left in place for 1 min to denude and dilate the artery. The muscular branch was ligated, and the blood flow of the femoral artery was restored. One to five weeks later, the injured segment of the femoral artery was surgically exposed by intraperitoneal administration of an overdose of Nembutal. At death, the mice were perfused via the left ventricle with a 0.9% NaCl solution followed by perfusion fixation with 4% paraformaldehyde overnight at 4°C and embedded in parafin. Uninjured femoral arteries were also used as controls.

Tail-cuff method. Systolic blood pressure was measured in conscious mice with a computerized tail-cuff system (Muromachi Kikai, Tokyo, Japan) that determines systolic blood pressure using a photoelectric sensor, as described previously (14). Before the study was initiated, at least 3 days of training (that is, sessions of unrecorded measurements) were provided for the mice to become accustomed to the tail-cuff procedure. Sessions of recorded measurements were then made from 1 to 5 PM daily on 3 consecutive days. Each session included more than 10 tail-cuff measurements; thus 30–50 measurements were used for the determination of the blood pressure of each mouse. For the inclusion of each set of measurements for an individual mouse, we required that the computer successfully identify the blood pressure in at least 7 of the 10 trials within the set.

Morphometry. Five serial sections (3 μm thick) from each artery were stained with elastic van Gieson staining to visualize the elastic laminae or with hematoxylin and eosin staining. The image was digitized using a digital camera (Axio-Cam HRC, Zeiss, Oberkochen, Germany) on a microscope (ECLIPSE TE300, Nikon, Tokyo, Japan). An image-processing and analysis program (NIH image, NIH, Bethesda, MD) was used to measure the total cross-sectional medial area (between the external elastic lamina and the internal elastic lamina) and the intimal area (between the innermost side of the arterial wall and the internal elastic lamina). Subsequently, the intimal area-to-medial area ratio was calculated.

Data analysis. Numerical results were expressed as the mean ± SE. One-way ANOVA followed by a Bonferroni post hoc test was used for pairwise multiple comparison. A P value of <0.05 was considered a significant difference.

RESULTS

mRNA expression of α1-AR subtypes in the mouse femoral artery. RT-PCR was carried out to investigate the expression of each receptor in the femoral artery of KO mice as well as of...
the WT (control) mice. This assay revealed that the expression of each α1-AR subtype was detected in the femoral artery of the WT mouse (Fig. 1); that of α1A-AR was the most predominantly detected by the RT-PCR assay. This predominant expression pattern of α1A-AR in the mouse femoral artery was also observed by a quantitative analysis with a real-time PCR assay (data not shown), suggesting that α1A-AR was predominantly expressed among the α1-AR subtypes. This assay also revealed that the mRNA expressions of other types were increased in the KO mice. For instance, α1B-AR and α1D-AR were increased in α1A-KO mice, α1A-AR and α1D-AR were increased in α1B-KO mice, α1A-AR and α1B-AR were increased in α1D-KO mice, and α1D-AR was increased in α1AB-KO mice (Fig. 1).

**Blood pressure.** The influence of surgery on the blood pressure was examined using the tail-cuff method. The blood pressure in each mutant mouse after surgery was not significantly different from that before the surgery (Table 1). With regard to the basal blood pressure, the blood pressure in α1D-KO mice was significantly lower than that in WT (Table 1). These results indicated that the surgical operation did not affect the blood pressure.

**Neointimal formation after vascular injury.** The nonoperated normal femoral arteries of each group of mice were dissected

![Fig. 2](http://ajpheart.physiology.org/)

**A**

![Morphometric analysis of injured mouse femoral arteries](http://ajpheart.physiology.org/)

**B**

![Morphometric analysis of injured mouse femoral arteries](http://ajpheart.physiology.org/)

**C**

![Morphometric analysis of injured mouse femoral arteries](http://ajpheart.physiology.org/)

![Fig. 2. Morphometric analysis of injured mouse femoral arteries. The mean cross-sectional medial area (A), the mean cross-sectional intimal area (B), and the mean intimal area-to-medial area ratio (C) are shown. Values represent means ± SE of 10–12 independent experiments. *P < 0.05 vs. WT mice. D: representative cross sections of uninjured and 4 wk after wire injury of femoral arteries from WT and α1AB-KO mice with hematoxylin and eosin staining and elastica van Gieson staining. Neointimal formation after wire injury in α1A-KO, α1B-KO, and α1D-KO mice was similar to that in WT mice. Magnification ×100.**

**Fig. 2.** Morphometric analysis of injured mouse femoral arteries. The mean cross-sectional medial area (A), the mean cross-sectional intimal area (B), and the mean intimal area-to-medial area ratio (C) are shown. Values represent means ± SE of 10–12 independent experiments. *P < 0.05 vs. WT mice. D: representative cross sections of uninjured and 4 wk after wire injury of femoral arteries from WT and α1AB-KO mice with hematoxylin and eosin staining and elastica van Gieson staining. Neointimal formation after wire injury in α1A-KO, α1B-KO, and α1D-KO mice was similar to that in WT mice. Magnification ×100.
and observed as controls. The gross and microscopic appearances of control femoral arteries in all groups of mice were similar and normal (Fig. 2D). We observed neointimal formation after wire injury of mouse femoral arteries. Quite consistently with a previous study (28), neointimal formation was observed in WT femoral arteries 1–5 wk after wire injury (data not shown). Neointimal formation, however, continued to grow for up to 4 wk after wire injury and did not advance further. Therefore, we subsequently examined the morphometric changes of the injured arteries 4 wk after denudation of the intima in each group of mice (Fig. 2, A–C, n = 10–12 mice/group). There was no significant difference in the medial area among all groups of mice (Fig. 2A, n = 10–12 mice/group). On the other hand, the neointimal area in α1AB-KO mice was significantly smaller than that in any other group of mice (P < 0.05) (Fig. 2B, n = 10–12 mice/group). Furthermore, the mean intimal area-to-medial area ratios in α1AB-KO mice were significantly smaller than those in any other group of mice (P < 0.05) (Fig. 2C, n = 10–12 mice/group). The neointimal area and mean intimal area-to-medial area ratios were 54 and 52% lower in α1AB-KO mice than in WT mice, respectively (Fig. 2, B and C). On the other hand, the neointimal area and mean intimal area-to-medial area ratios in other mice were not significantly different from those in WT mice (Fig. 2, A–C).

A comparison of the expression levels of each α1-AR subtype between noninjured (control) and injured arteries. RT-PCR was carried out to determine which subtypes of α1-AR are expressed in the injured femoral artery. Interestingly, all types of α1-AR were increased in the injured femoral artery in WT, α1A-KO, α1B-KO, and α1D-KO, and α1AB-KO mice (Fig. 3).

**DISCUSSION**

Since neointimal formation is considered to be one of the most important mechanisms that underlie restenosis (9, 12, 17), we analyzed the involvement of α1-AR in neointimal formation. For that purpose, we examined the mRNA expression level of each α1-AR subtype in the mouse femoral artery and investigated the functional role of those in neointimal formation 4 wk after wire injury in mice lacking the α1-AR subtype gene. An mRNA analysis of the α1-AR subtypes revealed that all subtypes of α1-AR were detected in mouse femoral arteries and that α1A-AR was most predominantly detected. Since mRNA abundance is not always reflected at the protein levels, we performed immunohistochemical studies using anti-mouse α1-AR antibodies to investigate the protein level of α1-AR in the femoral artery. No specific signal, however, was obtained by our immunohistochemical studies, probably due to insufficient specificity and/or sensitivity of the antibodies. In addition to the expression of the α1-AR subtypes in the femoral artery, the blood pressure was not altered after the wire injury. This indicated that the vascular injury model of the mouse femoral artery, with respect to α1-AR, was suitable to elucidate the mechanisms of neointimal formation. Both the neointimal area and the intimal area-to-medial area ratios in the femoral artery of α1AB-KO mice were significantly smaller than those in any other group of mice, while no difference was seen among α1A-KO, α1B-KO, α1D-KO, and WT mice. Other subtypes of α1-AR seemed to increase when one type of α1-AR expression was lost in the femoral artery. These results suggest that blockade of the signaling of both α1A-AR, which is predominant in the mouse femoral artery, and α1B-AR could be necessary to inhibit neointimal formation. Interestingly, the expression of all α1-AR types was increased in each mutant mouse after surgery, suggesting that those expressions were enhanced during intimal formation.

Regarding the functional role of α1-AR, recent studies have shown that all α1-AR subtypes in vascular smooth muscle cells mediate catecholamine-stimulated vasoconstriction (7, 15, 24, 34) and that α1-AR markedly contributes to the onset of hypertension (1, 8, 14, 22, 35, 38). Furthermore, other studies have revealed that the functional role of α1-ARS in the cardiovascular system is not only vasoconstriction but also postnatal cardiac development (20). In addition, previous studies using rat aorta, artery, and cultured cells with selective α1-antagonists have demonstrated that the activation of the α1-AR subtype (particularly, the α1A-AR subtype, but not the α1D-AR subtype) can induce neointimal formation (10, 12, 36), even though the α1B-AR and α1D-AR subtypes were mainly expressed in the rat aorta at both the mRNA and protein levels (11). On the other hand, the exact functional role of α1B-AR subtypes in neointimal formation still remains unclear because of lack of appropriate α1B-selective antagonists and satisfactory methods of quantitative α1-AR subtype mRNA expression analysis (10, 36, 41). As in a previous study using rat vessels (10, 12, 36), our study also showed that lack of the α1D-AR gene was not required to inhibit neointimal formation in the mouse femoral artery. The α1B-KO mice, however, cannot be directly compared with other α1-KO mice, because the blood pressure in α1D-KO mice was lower than that in other α1-KO mice. Regardless of low blood pressure, neointimal formation was normally observed in α1D-KO mice, emphasizing the fact that lack of the α1D-AR gene was not required to inhibit neointimal formation in the mouse femoral artery. Different from previous reports (10, 12, 36), on the other hand, our study showed that a single deletion of α1A-AR or α1B-AR subtype genes could not inhibit neointimal formation, but that deletion of both the α1A-AR and α1B-AR subtype genes could significantly inhibit it. These findings demonstrated that blockade of both α1A-AR and α1B-AR is required to inhibit neointimal formation.

With respect to the signaling pathway of α1-AR, previous studies have suggested that α1B-AR may control the receptor density and protein expression of α1A-AR by synergism and/or cross talk of the signaling pathways (16, 37). Although it is believed that all α1-AR subtypes are coupled to the Gq-protein (42), a study with α1AB-KO mice suggested the existence of distinct signaling pathways of extracellular signaling-regulated
kinase via α1A-AR and of protein kinase C via α1B-AR in postnatal cardiac hypertrophy (20). In fact, these molecules differentially regulate the α1-AR-mediated contraction of smooth muscle cells in arteries (39). Similarly, in neointimal formation in the vascular injury model, these molecules were implicated in the signaling pathway (4, 30). However, the implication and synergism of these molecules in α1-AR-mediated neointimal formation remain uncertain. Similar signaling pathways and synergism may be implicated in neointimal formation.

On the other hand, there is no direct clinical evidence that the α1-AR antagonist has a preventive effect on restenosis after PCI. As described in the Introduction, however, recent clinical studies have suggested that α1-AR might facilitate restenosis after PCI (1, 2, 13, 22). In addition to these previous studies, the present study may support the importance of the proper blockade of α1A- and α1B-AR stimulation by the α1-AR antagonist for the prevention of restenosis. On the other hand, the large Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial recently conducted has raised a serious concern about the long-term use of α1-AR antagonists in the treatment of hypertension (3, 18). Therefore, the short-term use of α1-AR antagonists may be effective for the prevention of restenosis after PCI without adverse effects on cardiac function, especially in patients with complications of hypertension.

In summary, we characterized the mRNA expression of each α1-AR subtype and the functional role of those in neointimal formation in the mouse femoral artery. Our results demonstrated that this vascular injury model of the mouse femoral artery is suitable for the investigation of the functional role of α1-AR in neointimal formation after vascular injury. In addition, our findings suggested that both α1A-AR and α1B-AR subtypes were important for developing neointimal formation after vascular injury. Although this vascular injury model using genetically altered mice is not exactly the same as models with blocking receptors by antagonists, this result may support the clinical outcome that proper blockade of α1-AR antagonists is needed for the prevention of restenosis after PCI without adverse effects on cardiac function, especially in patients with complications of hypertension. Further elucidation of the mechanisms underlying neointimal formation is to be expected.

ACKNOWLEDGMENTS

We are grateful to Masataka Sata (University of Tokyo) for the kind provision of a videotaped tutorial for the surgical procedure.

GRANTS

This work was supported in part by research grants from the Scientific Fund of the Ministry of Education, Science, and Culture of Japan; the Ministry of Human Health and Welfare of Japan; the Japan Health Sciences; the NOVARATIS Foundation; and the Takeda Science Foundation.

REFERENCES

21. Peng L, Bhatia N, Parker AC, Zhu Y, Fay WP. Endogenous vitronectin and plasminogen activator inhibitor-1 promote neointima formation in


