Expression of $\alpha_2$-Adrenergic Receptors in Normal and Atherosclerotic Rabbit Aorta

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Abstract—$\alpha_2$-Adrenergic receptors ($\alpha_2$-ARs) in vascular smooth muscle cells are known to mediate vasoconstriction; however, it is unknown which of the 3 subtypes of $\alpha_2$-AR ($\alpha_{2A}$, $\alpha_{2B}$, or $\alpha_{2C}$) is expressed in vascular tissue. We have used subtype-specific probes in in situ hybridization and RNase protection assays to analyze the expression of $\alpha_2$-AR in the thoracic aorta of New Zealand White (NZW) and Watanabe heritable hyperlipidemic (WHHL) rabbits, a model for atherosclerosis. We found that the $\alpha_{2A}$-AR mRNA was in endothelial and smooth muscle cells in both NZW and WHHL aorta. In addition, the shoulders and subendothelial regions of the atherosclerotic lesions in WHHL aorta showed abundant expression of $\alpha_{2A}$-AR mRNA. Antibodies to macrophage (RAM-11) and smooth muscle cell (HHF-35) antigens were used to localize macrophage and smooth muscle cells in aortic sections from WHHL rabbits. The expression of $\alpha_{2C}$-AR mRNA within the lesions of WHHL rabbits correlated with the presence of infiltrating macrophages. We discuss the potential role of $\alpha_{2A}$-ARs in macrophage function and in promoting atherosclerosis. (Hypertension. 1998;32:311-317.)

Key Words: receptors, adrenergic ■ aorta ■ atherosclerosis ■ rabbits

Catecholamines mediate vasoconstriction through stimulation of $\alpha$-ARs. In vitro experiments on isolated vessels in the presence and absence of the endothelium have linked stimulation of $\alpha_2$-AR in endothelial cells with the release of endothelium-derived relaxing factor. In contrast, $\alpha_2$-ARs in vascular SMCs from coronary, arterial, or venous sources mediate vasoconstriction. $\alpha_2$-ARs can be divided into 3 subtypes ($\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$-AR), and it is unknown which subtype(s) of $\alpha_2$-AR is expressed in normal and atherosclerotic vascular tissue.

Atherosclerotic human coronary arteries show increased vascular reactivity in vivo that has been attributed to altered sensitivity to catecholamines or defects related to $\alpha_2$-AR functions. Other studies also suggest that $\alpha_2$-AR-mediated functions differ in normal and atherosclerotic arteries.

The earliest visible change in atherosclerosis is the fatty streak, which in experimental models such as the WHHL or cholesterol-fed rabbit involves the accumulation of macrophages in the subendothelial space. Later lesions include macrophages as well as proliferating SMCs that are phenotypically distinct from the SMCs in the media. In this report, we analyze the expression of $\alpha_2$-AR mRNA in aortas from NZW rabbits and WHHL rabbits, a model of atherosclerosis, by in situ hybridization with $\alpha_2$-AR probes.

Methods

Subtype-Specific Probes for In Situ Hybridization

Specific $\alpha_{2A}$-AR and $\alpha_{2C}$-AR probes for in situ hybridization have been described. To produce a probe specific for $\alpha_{2C}$-AR transcripts, a 369-bp XmnI/NaeI fragment was subcloned from the RB$\alpha_{2C}$ cDNA into the Smal site of psP65 (Promega).

Tissue Preparation and In Situ Hybridization

Homozygous male WHHL rabbits were obtained from the Boston University Medical Center Colony. Six NZW rabbits weighing 3 to 4 kg were obtained from Millbrook Farm, Amherst, Mass. All rabbits were housed in separate cages on a 12-hour light/dark cycle, fed Agway Prolab normal-fiber laboratory rabbit diet, and provided unlimited access to water. After overnight fasting, blood was collected from the ear vein in EDTA tubes for measuring total cholesterol with a kit (Sigma Chemical Co) or in EGTA/reduced glutathione tubes for measuring catecholamines with the Catecholamine Biotrak Research Assay System (Amersham). Additional rabbits were bled (3 mL) for catecholamine measurement. After blood collection, animals were killed by injection of 150 mg/kg sodium pentobarbital in the marginal ear vein. The thoracic aorta was rapidly removed, rinsed in cold (4°C) PBS, and fixed for at least 24 hours in freshly prepared 4% paraformaldehyde in PBS. Tissues were embedded in paraffin, sectioned (5 μm), and mounted on silanized slides.

In situ hybridization experiments were performed as previously described based on the methods of Sassoon et al. RNA probes were synthesized from linearized psP65 plasmids containing sense or antisense $\alpha_2$-AR subtype-specific inserts by using Sp6 polymerase in the presence of 35S-dUTP. Sections were hybridized with 35 000 cpm/μL 35S-labeled RNA probes. NTR2 autoradiography emulsion was used to detect hybridization signal. Slides were developed in Kodak D19 at 14°C and fixed in Kodak Rapid Fix A after a 10-day exposure at 4°C. All sections were examined under bright- and dark-field illumination. Photomicrographs were taken with a Nikon microscope and Kodak Ektachrome 64T film.

Immunocytochemical Analysis of Lesions

Aortic sections were treated with xylene to remove paraffin and then rehydrated; endogenous peroxidase activity was blocked with...
0.6% H$_2$O$_2$ for 20 minutes. Slides were then pretreated with 0.1% protease type XXIV and 1.5% normal horse serum in PBS. Antibodies against macrophage cells (RAM-11) and SMC actin (HHF-35) were used at 1:100 dilutions for 1 hour at room temperature. After primary antibody incubation, slides were washed in PBS and incubated with 1:100 biotinylated horse anti-mouse antibody for 30 minutes, followed by avidin-biotin amplification with the Vectastain Elite ABC kit (Vector Laboratories Inc). Slides were washed in water and counterstained in diluted hematoxylin.

**PCR of Rabbit $\alpha_2$-AR Gene Fragment**

Rabbit genomic DNA was isolated from NZW liver. PCR was used to isolate a rabbit $\alpha_2$-AR gene fragment for use in RNase protection assays. The forward (GGGAAATTCGCGCCCCAAAACCTCTCTGGTG) and reverse (GGGAAATTCTGGCGTGCGCTTCAGGTGTACTC) primer sequences were chosen from a region of high homology among known $\alpha_2$-ARs from various species. EcoRI sites (underlined) were added for cloning of the PCR fragment into psP65 at the EcoRI site. The $\alpha_2$-AR gene fragment was amplified from 0.5-μg genomic DNA using the GeneAmp PCR core reagents kit (Perkin-Elmer), optimized with 0.5 μmol/L each primer, 20 μmol/L each dNTP, and 4 mmol/L magnesium. After 4 minutes at 96°C, 2.5 U of Taq DNA polymerase was added, and the amplification profile was run in a PE-9600 thermal cycler for 30 cycles: 15 seconds at 96°C, 10 seconds at 65°C, and 1 minute at 72°C, with a final elongation for 10 minutes at 75°C. The DNA sequence was determined with Sequenase.

**RNA Preparation and RNase Protection Assay**

Total RNA was prepared from the tissue of two retired breeder WHHL rabbits over 24 months of age and two 12-month-old NZW rabbits using Trizol (Gibco/BRL). Unlabeled sense RNA and 32P-labeled antisense probe RNA were prepared using the MAXIscript in vitro transcription kit (Ambion). RNase protection was performed with the RPA II kit (Ambion). Protected bands were separated on a denaturing 6% polyacrylamide gel and visualized by autoradiography.

**Quantitative Analysis of In Situ Hybridization**

Slides were viewed under ×600 magnification with a Nikon motor microscope and camera for analysis with the computerized BioQuant system (R&M Biometrics Inc). After the threshold function was adjusted to distinguish silver grains, the number of pixels within a sampled area was automatically counted. Pixel counts were converted to grain counts using empiric data to determine the average number of pixels in silver grains. Multiple areas were sampled in either lesions or media of each aortic ring, and average grain counts were determined per area (in square millimeters).

**Results**

**Expression of $\alpha_2$-AR Subtypes in Aorta of NZW Rabbits**

To investigate the expression of $\alpha_2$-AR in normal rabbit aortic samples, aorta from NZW rabbits were used in situ

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**Figure 1.** In situ hybridization to detect of $\alpha_2$-AR mRNA in NZW aorta. Bright-field micrographs are shown at ×4000 magnification. Sections of aorta are hybridized to the various $\alpha_2$-AR probes as follows: A, $\alpha_2$A-AR antisense; B, $\alpha_2$A-AR sense; C, $\alpha_2$B-AR antisense; and D, $\alpha_2$C-AR antisense. The conditions used for each of these probes are known to result in positive hybridization signals in brain, a tissue that expresses all 3 subtypes of $\alpha_2$-AR (D.E.H., C.J., H.G., unpublished observations, 1997). 12 Examples of endothelial cells (EC) and SMCs are labeled in each panel.
hybridization using antisense and sense probes specific for α2A-AR, α2B-AR, and α2C-AR transcripts. In the bright-field micrographs shown in Figure 1, clusters of silver grains indicative of positive hybridization signal were detected throughout the aorta, over both endothelial and SMC layers, when the α2A-AR antisense probe (Figure 1A) was used. The α2B-AR sense probe (Figure 1B) shows the background level of hybridization with a probe that does not hybridize to the RNA. The signals obtained with the α2B-AR (Figure 1C) and α2C-AR (Figure 1D) antisense probes were similar to that obtained with the α2A-AR sense probe. The α2B-AR and α2C-AR sense probes also appeared identical to the α2A-AR sense probe (data not shown). Similar patterns of hybridization were found in aortic sections from 5 other NZW rabbits ranging in age from 6 to 14 months.

Expression of α2A-AR in Aorta of WHHL Rabbits
To confirm the presence of α2A-AR mRNA in the aorta, a genomic fragment of the rabbit α2A-AR was cloned by PCR to use in RNase protection assays. The rabbit sequence was highly similar to the corresponding α2A-AR sequences from mouse,16 rat,17 pig,18 and humans19 (Figure 2, top). RNase protection showed a strong positive band with rabbit spleen RNA and a weak band with rabbit aortic RNA, consistent with high levels of expression in spleen and low levels of expression in aorta (Figure 2, bottom).

To determine whether the pattern of α2A-AR mRNA expression was altered in atherosclerosis, aortic sections from WHHL rabbits were hybridized to the α2A-AR probe (Figure 3). In addition to the presence of α2A-AR mRNA in the medial and endothelial cells, there appeared to be abundant α2A-AR mRNA in atherosclerotic lesions in each of the 6 WHHL rabbits analyzed. To correlate α2A-AR expression with the presence of macrophages or SMCs in the lesions, parallel sections were analyzed with in situ hybridization and immunohistochemistry using RAM-11 (macrophage) or HHF-35 (SMC actin–specific) antibodies. Figure 4 shows a representative comparison of in situ hybridization (panels A, C, E, and G) with immunodetection of macrophage (panels B, D, and F) from 2 different 16-month-old WHHL rabbits. Macrophage staining was strongest in the subendothelial space and along the shoulders of the lesions (Figure 4B and 4F). These are the same areas of the lesion that showed abundant expression of α2A-AR mRNA by in situ hybridization (Figure 3A and 3C, Figure 4A, 4E, and 4G). In comparison, in regions of the aortic section that showed no apparent lesion (Figure 4C and 4D), there was no macrophage staining (Figure 4D), and there appeared to be less α2A-AR mRNA.
than in the lesion areas (compare Figure 4C with Figure 4A). SMCs were rarely found in the lesions, which consisted mostly of rounded macrophage cells. Figure 4H shows an example of an SMC within a lesion.

Quantitative Analysis of In Situ Hybridization Signal

To determine whether the lesions of the WHHL rabbits had increased expression of α2A-AR mRNA over that found in the media, silver grains were counted over areas of the lesion and media using the BioQuant system. In each of the 6 WHHL samples, there was a significant increase in silver grains over the lesion area compared with the media within the same aortic section (Table). On average, there was a 3.39 ± 1.17-fold (P < .01) increase in silver grains in the lesion versus the media.

Measurements of Plasma Catecholamines and Cholesterol

To determine whether catecholamine levels differ in the WHHL and NZW rabbits, plasma norepinephrine and epinephrine levels were measured from 5 WHHL (average age, 23 months) and 5 NZW (average age, 11 months) rabbits. Norepinephrine levels were nearly 3-fold higher in WHHL than in NZW rabbits (4.28 ± 1.05 versus 1.50 ± 0.027 nmol/L, P < .001), whereas the difference in mean epinephrine levels was not statistically significant (0.475 ± 0.196 versus 0.328 ± 0.115 nmol/L). As expected, the total cholesterol level in WHHL rabbits was about 18-fold higher than the levels in NZW rabbits (12.42 ± 1.36 versus 0.678 ± 0.206 mmol/L, P < .001).

Discussion

The purpose of this study was to examine the vascular expression of α2-AR. Recent studies in mice suggest that both the α2A-AR and α2B-AR may play a role in vasoconstriction,20,21 since the immediate vasoconstrictive effect of α2-AR agonists was absent in both the α2B-AR21 knockout mice and the mice with a mutant α2A-AR substituted for the wild-type receptor.20 Our results suggest that only α2A-AR mRNA is expressed in SMCs from rabbit aorta. It is possible that in mice, both α2A-AR and α2B-AR are expressed in vascular tissue, whereas in rabbits only the α2A-AR is found in vascular tissue. Another possibility is that the aortic expression of α2A-AR does not reflect the expression patterns of other vascular beds.

In the WHHL rabbits, the levels of norepinephrine were nearly triple the levels of norepinephrine in NZW rabbits, whereas the epinephrine levels were similar in both groups. However, given that the WHHL were much older than the NZW rabbits, the effects of age on norepinephrine levels cannot be discounted. It has been established that atherosclerosis in WHHL rabbits is a result of an absence of functional
Figure 4. In situ hybridization of WHHL aorta colocalizes abundant $\alpha_2$-AR mRNA with macrophages. A through D are sections from one 16-month-old WHHL aorta. A and C, In situ hybridization with $\alpha_2$-AR antisense probe from the same slide and aortic ring; A shows a lesion area and C shows a region with no apparent lesion. B and D, Corresponding immunodetection of macrophages with RAM-11 in the lesion (B) and nonlesion areas (D). E through H are from a different 16-month-old WHHL aorta. E and G, In situ hybridization with $\alpha_2$-AR antisense probe in dark and bright fields, respectively. F, Immunodetection of macrophages with RAM-11. Note the abundant presence of rounded macrophage cells within the subendothelial space where abundant $\alpha_2$-AR mRNA can be found. H, Immunodetection of SMCs with HHF-35.
LDL receptors, which leads to hypercholesterolemia and hypertriglyceridemia. Further studies are necessary to determine the relationship (if any) between atherosclerosis and the elevation of norepinephrine in WHHL rabbits.

The contractile responses to norepinephrine and α2-AR agonists have been shown to be blunted in aortic or carotid arteries taken from rabbits made hypercholesterolemic by diet, whereas the responses to phenylephrine, an α1-AR agonist, are not impaired. Although the effect of α2-AR agonists on WHHL arteries has not been tested, norepinephrine-induced vasoconstriction was found to be diminished in WHHL compared with NZW controls. It is possible that excess catecholamines, as we found in WHHL rabbits, could promote receptor desensitization; however, several studies have shown a lack of agonist-induced downregulation of α2-AR.

It has been proposed that increases in sympathetic activation can enhance atherosclerosis. Administration of norepinephrine or epinephrine to rabbits or monkeys correlates with increased progression of atherogenic changes in these animal models. Sympatholytic agents, such as β-blockers and α1-blockers, have been shown to reduce atherosclerosis in some animal models of atherosclerosis; however, neither β- nor α1-blockers reduced atherosclerosis in WHHL rabbits (reviewed in Reference 30).

Many factors can affect the density of silver grains in situ hybridization, including hybridization and wash conditions, specific activity of the probe, and the time of exposure to emulsion. These conditions can be controlled within an experiment, but it is difficult to control for all factors that can contribute to tissue-to-tissue variation. Thus, in situ hybridization is usually used for qualitative comparisons and to localize mRNA transcripts within distinct anatomic structures. However, some comparisons can be made on any given slide where all of the above factors are controlled. Although the intensity of silver grains varies in the different samples, our results clearly show that the concentration of signal is significantly greater over areas of the lesion than over the media of the same vessel in each of the 6 WHHL rabbits analyzed (Table). This apparent abundance of α2A-AR mRNA correlates with the presence of macrophages. In addition, we found no evidence for significant expression of α2B-AR or α2C-AR mRNA in the macrophages (data not shown).

The presence of α2A-AR in macrophages is a novel and potentially important finding. Macrophages accumulate early in the atherogenic process and may promote vascular damage through the release of cytokines that stimulate SMC chemotaxis and proliferation, as well as migration of other monocyte/macrophage cells. Furthermore, in vitro studies suggest that catecholamines act through the α2-AR to activate macrophage function and augment the lipopolysaccharide-induced cytokine production. The presence of α2A-AR mRNA within macrophages suggests the need for further study of the role of α2A-AR stimulation on macrophage function.

Acknowledgments

These studies were supported by National Institutes of Health grants HL48181 and P50 HL55001. We thank Susan Hope for her assistance with the Watanabe rabbits.

References


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Hypertension. 1998;32:311-317
doi: 10.1161/01.HYP.32.2.311

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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