β_2-Adrenergic Receptor Gene Delivery to the Endothelium Corrects Impaired Adrenergic Vasorelaxation in Hypertension

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Background—Impaired β-adrenergic receptor (AR)–mediated vasorelaxation in hypertension plays a role in increased peripheral vascular resistance and blood pressure. Because the β_2AR is the most abundant vascular AR subtype, we sought to enhance βAR vasorelaxation by overexpressing β_2ARs via adenoviral-mediated gene transfer (ADβ_2AR) to the vascular endothelium of the carotid artery.

Methods and Results—In normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats, we exposed the right common carotid artery to ADβ_2AR in situ for 15 minutes by injection into the lumen while the blood flow was interrupted. Control carotids received an empty vector (ADempty). Three days later, transgene expression and selective endothelial localization were confirmed in infected vessels. Vasoregulation after β_2AR overexpression (2-fold) was studied in isolated organ baths. ADβ_2AR carotid responses to α_1AR and α_2AR agonists were not affected, whereas responses to epinephrine were altered and βAR-mediated vasorelaxation was enhanced after β_2AR overexpression. As expected, βAR-mediated vasodilatation in control carotids of SHR rats was significantly less than in similar control WKY carotid arteries. ADβ_2AR treatment enhanced βAR vasorelaxation in SHR to levels similar to those seen in ADβ_2AR WKY carotids.

Conclusions—Our results demonstrate a critical role for the endothelium in βAR-mediated vasorelaxation and suggest that impaired βAR signaling may account for dysfunctional βAR vasorelaxation in hypertension rather than impaired endothelium-dependent nitric oxide metabolism. **(*Circulation*. 2002;106:349-355.)**

Key Words: endothelium ■ gene therapy ■ hypertension ■ signal transduction

Adrenergic receptors (ARs) represent major regulators of the cardiovascular system. At the vascular level, α and βARs play a pivotal role in balancing vascular tone and blood pressure homeostasis. Vascular βARs mediate adrenergic vasorelaxation through direct activation of vascular smooth muscle cells. However, recent data indicate that βAR-dependent vasorelaxation is mediated, at least in part, by endothelium- and NO-dependent processes.[1,2] Indeed, both β_1ARs and β_2ARs are expressed on endothelial cells,[3] and stimulation of endothelial β_2ARs causes endothelial nitric oxide synthase (eNOS) activation and NO release in human umbilical vein endothelium.[4]

In hypertension, βAR control of vasorelaxation is impaired, and this impairment seems to be involved in high blood pressure.[5] There are two alternative hypotheses to explain this alteration. The first is that attenuated βAR vasorelaxation is the result of the general impairment of endothelial function observed in hypertension. Accordingly, changes in NO synthesis and availability affect proper vasorelaxation in response to several stimuli, including βAR stimulation. The second hypothesis involves the possibility that impaired vasorelaxation after βAR stimulation results directly from dysfunctional βAR signaling. Indeed, in hypertensive conditions, several reports indicate a reduction in βAR signaling and regulation.[5–10] If these premises hold true, improving βAR signaling should result in the restoration of βAR vasorelaxation. In fact, some interventions have been effective in correcting βAR signaling in hypertension, such as dietary salt restriction[7] or pharmacological treatment.[11] Recently, a novel tool to modulate βAR signaling in a selective manner has been provided by adenoviral-mediated gene transfer of the human β_2AR cDNA. Indeed, in cardiac myocytes from both normal[12] and failing hearts,[13,14] adenoviral-mediated delivery and overexpression of the β_2AR enhanced signaling and physiological responses to βAR agonists.

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In this study, we sought to correct impaired βAR vasorelaxation in hypertension by adenoviral-mediated gene transfer of β_2ARs to the endothelium. First, in vitro in endothelial cells, we tested the effect of β_2AR stimulation on NO production. Then we evaluated in normotensive Wistar-Kyoto (WKY) rats the feasibility of in vivo gene transfer to the endothelium of the common carotid and whether β_2AR gene transfer can increase βAR vorelaxation. Finally, we tested whether β_2AR gene transfer can correct impaired βAR vasorelaxation in the spontaneously hypertensive rat (SHR) model.

Methods

Adenoviral Constructs

We used a previously described adenovirus encoding for the human β_2AR (ADβ_2AR) and an empty viral vector (ADempty).[12–14] The viruses were suspended in PBS at $\approx 1\times10^{10}$ plaque-forming units (pfu) per mL.

Primary Isolated Aortic Endothelial Cells and Arginine to Citrulline Conversion

Aortic endothelial cells were isolated from WKY rats (Charles River, Milan, Italy) and grown up to 6 passages as previously described.[15] Two days before the experiments, cells were incubated 30 minutes at 37°C with serum-free medium containing the virus at a multiplicity of infection of 100:1. NOS activity was assessed by the conversion of L-arginine into L-citrulline, which has a 1:1 stoichiometry to NO. Twenty-four hours after infection, equal numbers of cells were plated on 6-well plates and serum-starved overnight. The next day, cells were stimulated with isoproterenol (ISO) (10^{-4} mol/L), ionomycin (2×10^{-3} mol/L in DMSO), or vehicle at 37°C for 30 minutes. Cells were homogenized in 25 mmol/L Tris HCl, ph7.4, 1 mmol/L EDTA, and 1 mmol/L EGTA; the pellet was collected after centrifugation; and 20 μg of protein was incubated in 25 mmol/L Tris HCl, 3 μmol/L tetrahydrobiopterin, 1 μmol/L flavin adenine dinucleotide and 1 μmol/L flavin adenine mononucleotide, 25 μmol/L NADPH, 10 μmol/L CaCl, and 2 nCi/μL of [^{3}H] arginine for 60 minutes at 37°C. The reaction was stopped with equal volume of 50 mmol/L HEPES and 5 mmol/L EDTA and chromatographed on Dowex AG50WX-8 columns. Flow-throughs were counted by liquid scintillation. Citrulline production is expressed in pmol/mg of pellet protein/min.

Animals and Surgical Procedure

Twelve-week-old normotensive WKY and age-matched SHR rats were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (0.5 mg/kg), and the right external carotid was isolated and permanently closed with a nonreabsorbable suture placed as distally as possible. Common and internal carotids were clamped, and through an incision on the external carotid made proximal to the suture, a plastic cannula was advanced into the common carotid in a retrograde fashion. The virus (10^9 pfu in 100 μL PBS) was then injected in the common carotid and allowed to incubate for 15 minutes. Afterward, the virus solution was removed, the external carotid closed proximally to the incision, and the blood flow restored through the common and internal carotid. A group of carotids received only PBS and represent the sham-operated control. After 3 days, the common carotids were harvested and used for histological, biochemical, or functional assessments. We chose this time course because it represents the earliest occurrence of overexpression of the viral vector.[14] The study was performed in accordance to the National Institutes of Health guidelines for animal studies.

β_2AR Immunocytochemistry

Carotids of euthanized animals were immediately dissected out and frozen in isopentane chilled by liquid nitrogen. Cryostat sections 6 μm thick were cut and mounted on poly-L-lysine–coated slides. Sections were either kept frozen until use or fixed in cool acetone and dried. Nonspecific protein-binding sites on the tissue section were blocked by incubation with normal goat serum. This was followed, without additional washing, by incubation with 1:25 rabbit anti-β_2AR (Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C. An enzyme-labeled immunoreaction was carried out with a biotinylated secondary antibody followed by an avidin-conjugated alkaline phosphatase complex (Dako). Alkaline phosphatase was developed to give a red reaction product with naphthol AS-MX phosphate and new fuchsin in 0.1 mol/L Tris/HCl buffer, pH 8.2. Immunostaining controls consisted of substituting nonimmune serum for the primary antibody. Digital microphotographs were analyzed with ImageQuant 5.2 (Molecular Dynamics), and red staining intensity is expressed in arbitrary densitometric units.

βAR Binding Assay

The rat carotid endothelium expresses both β_1ARs and β_2ARs[16]; therefore, we measured the total number of βAR binding sites in carotid artery segments. Receptor binding was performed, partially modifying a previously described technique.[17] Common carotid segments were cut in 6 pieces of equal weight (100 to 200 μg) to calculate B_{max} and the nonspecific binding in triplicate. We used the nonselective βAR antagonist [^{125}I]-cyanopindolol as the ligand. Nonspecific binding was determined in the presence of 20 μmol/L of the nonselective antagonist alprenolol. Reactions were conducted in 500 μL of binding buffer (75 mmol/L Tris-Cl, pH 7.4, 12.5 mmol/L $MgCl_2$, 2 mmol/L EDTA) at 37°C for 1 hour and then terminated by 3 washes in ice-cold binding buffer. Receptor density (fmol) was normalized to milligram of carotid weight. In a subset of carotids, endothelium was removed with a needle to verify the relevance of endothelium in the total number of βAR binding sites in whole carotid segments.

Vascular Reactivity Determined on Common Carotid Rings

After isolation, common carotids were suspended in isolated tissue baths filled with 25 mL Krebs solution (in mmol/L: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 5.6) continuously bubbled with a mixture of 5% CO_2 and 95% O_2 (pH 7.37 to 7.42) at 37°C. One end of the vessel was secured to a tissue holder and the other to an isometric force transducer connected to a Gould signal processor. The signal was analyzed by a computerized data acquisition system (Power Lab, ADI Instruments). Carotid arteries (≈1 cm length) were pretensioned to 0.5 g. In pilot studies, we have found that this is the optimal preload for carotid responses. Vasoconstrictions to norepinephrine (NE) and epinephrine (EPI) were assessed by generating concentration response curves (10^{-9} to 10^{-6} mol/L and 10^{-9} to 10^{-5} mol/L, respectively). Vasorelaxation was assessed in vessel preconstricted with phenylephrine (PE) (10^{-6} mol/L) in response to the βAR agonist ISO (10^{-10} to 3×10^{-8}), EPI (10^{-9} to 10^{-5} mol/L), or the α_2AR agonist brimonidine, also known as UK14,304 (10^{-9} to 10^{-5} mol/L)[15,18] and sodium nitroprusside (10^{-9} to 10^{-5} mol/L). Drug concentrations are reported as the final molar concentration in the organ bath. Drugs were prepared daily in distilled water, except UK14,304, which was dissolved in DMSO and then diluted in water. The final DMSO-to-water ratio (>0.01%) does not exert any vasoactive effect.[15]

Statistical Analysis

Data are expressed as mean±SEM. Because no difference was observed between ADempty and sham-operated carotids, we pooled these data together to simplify the analysis and referred to this group as the control. ANOVA was used to compare densitometric data, βAR density, and vasoconstrictive responses to PE. Two-way ANOVA was applied to analyze concentration-dependent curves. A value of $P<0.05$ was considered statistically significant.

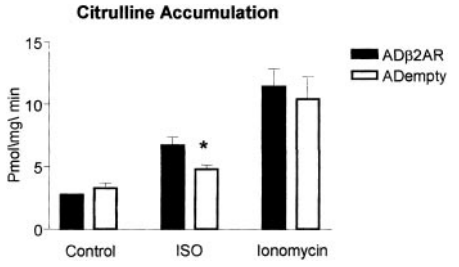

Figure 1. eNOS activity in primary cultured WKY rat aorta endothelial cells. ADβ_2AR enhanced ISO induced eNOS activity without affecting basal and ionomycin responses. The same result was obtained when the response to ISO was normalized either to the basal (ADβ_2AR, 2.4±0.2; Adempty, 1.5±0.1-fold of basal, $P<0.02$) or to the maximal activity induced by ionomycin (ADβ_2AR, 0.60±0.01; ADempty, 0.47±0.04-fold of ionomycin, $P<0.05$). *$P<0.05$. The histogram bars represent the average of 3 individual experiments performed in triplicate.

Results

eNOS Activity in Primary Isolated Aortic Endothelial Cells From WKY Rats

To determine eNOS activity after β_2AR overexpression, primary aortic endothelial cells were infected with either ADempty or ADβ_2AR. eNOS activity was measured by citrulline accumulation basally and after stimulation with the βAR agonist ISO or the calcium ionophore ionomycin. This latter, by increasing intracellular calcium levels, can maximally activate eNOS. ADβ_2AR increases ISO-induced citrulline production without affecting basal or ionomycin-stimulated NOS activity (Figure 1). Thus, under these in vitro conditions, β_2AR overexpression in endothelial cells results in apparent NO enhancement (Figure 1).

In Vivo Transgene Delivery and Expression

In the rat common carotid, transgene expression analysis was performed by immunocytochemistry (Figure 2A). Endogenous β_2AR distribution in control nontreated carotid arteries localizes at both endothelial cells and smooth muscle cells (Figure 2A, top). In ADβ_2AR-treated carotids, overexpression of the β_2AR transgene predominates in the endothelium (Figure 2A, bottom). Densitometry, performed on 5 sections from 3 carotids per group, revealed no difference in the expression of the βAR at the smooth muscle cell level between the ADβ_2AR and the control carotids (352±3 versus 331±3 densitometric units, respectively; not significant), whereas ADβ_2AR treatment almost doubled the βAR density at the endothelium when compared with control (498±2 versus 280±6 densitometric units, respectively; $P<0.01$). Similarly, using a βAR-binding assay, ADβ_2AR leads to an overall doubling of βAR receptor density when compared with control (either PBS or ADempty treatment) (Figure 2B). Moreover, this increase in βAR density was seen both in WKY and SHR carotids (Figure 2B). In endothelium-denuded WKY carotids, no differences could be noted in the total βAR binding sites between ADβ_2AR and control carotids (0.44±0.1 versus 0.46±0.1 fmol/mg of carotid, respectively; n=5 for each group; P=not significant).

Vasomotor Responses in WKY Rats

In carotid arteries from WKY rats, we tested the vascular responses to AR stimulations using PE, NE, and EPI as well as ISO, UK14,304, and the AR-independent vasodilator sodium nitroprusside. PE and NE vasoconstrictions were not affected (Figure 3, A and B), whereas EPI response was attenuated by ADβ_2AR (Figure 3C). Because vascular responses to EPI ($\beta_2>\alpha_2>\alpha_1$) result from the balance between α_1AR vasoconstriction and β_2AR vasorelaxation, impaired EPI vasoconstriction could result from the imbalance of these two opposing signals induced by the increased number of βARs. Therefore, we tested whether in the ADβ_2AR carotids, the vasorelaxation to EPI is enhanced. Indeed, a clear vasorelaxation to EPI was observed in the ADβ_2AR carotids,

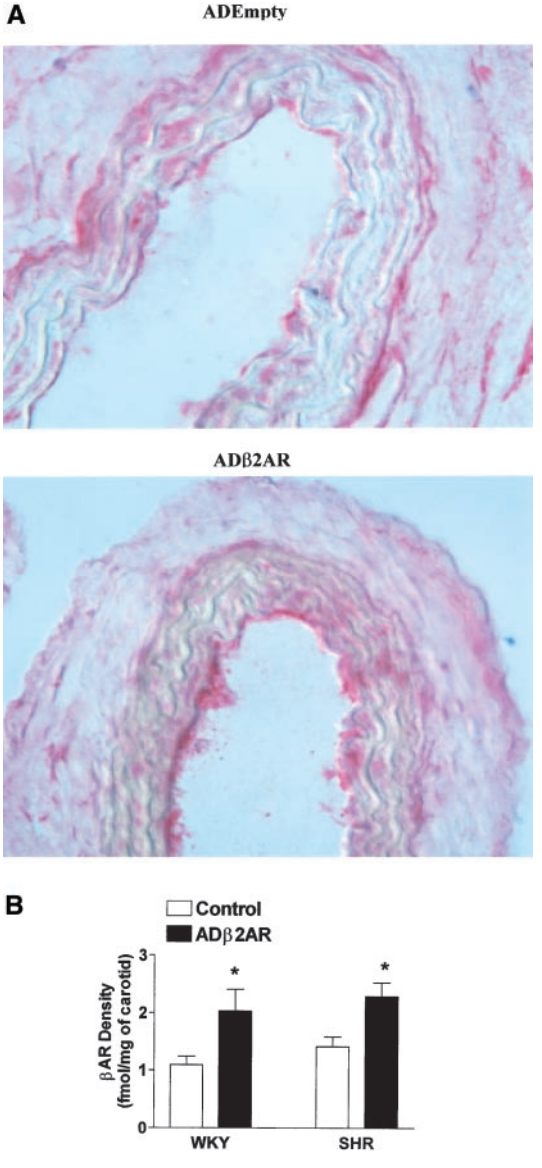

Figure 2. Vascular adenoviral-mediated transgene expression. A, Top, Immunostaining of β_2AR on 6-μm-thick common carotid sections revealed that the β_2AR is expressed endogenously in both the endothelium and vascular smooth muscle cells in ADempty-treated carotids. A, Bottom, Transgenic overexpression of the β_2AR localizes mainly to the endothelium in ADβ_2AR-treated carotids. These pictures are representative of 3 carotids per group. B, Expression of the ADβ_2AR transgene assessed by ^{125}I-CYP binding in common carotid arteries increased the βAR density to a similar extent both in WKY and SHR. n=6 to 9; *$P<0.05$.

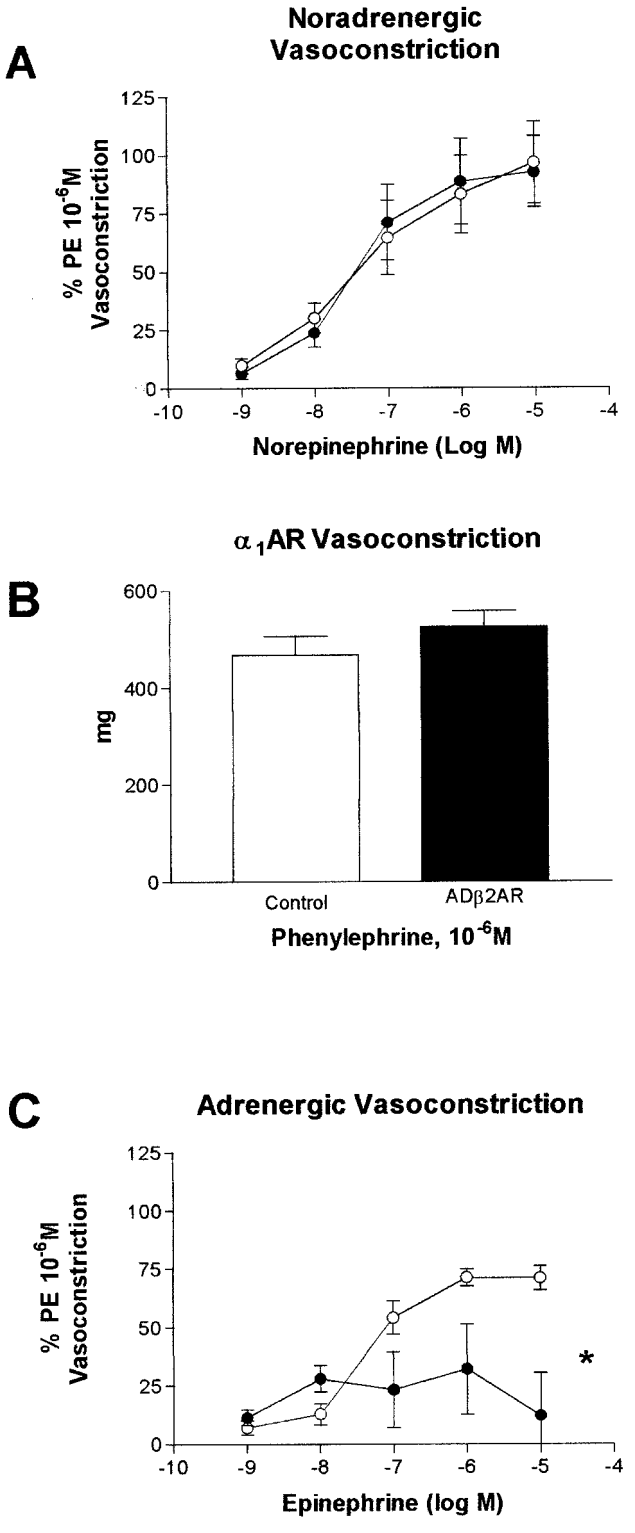

Figure 3. Effect of ADβ_2AR on the adrenergic vasoconstrictions of common carotid arteries from WKY rats. Vasoconstriction was tested in response to the α_1 AR agonist PE (A), the mixed α_1 and β_1 AR agonist NE (B), and the α and β_2 agonist EPI (C). ADβ_2AR treatment did not change vasoconstriction to NE and PE but largely attenuated the response to EPI. ○ indicates control carotids; ●, ADβ_2AR-treated carotids; n=8 to 12 per group. *F=3.088; $P<0.05$; 2-way ANOVA.

whereas EPI failed to induce any vasorelaxation in the control carotids (Figure 4A). The enhanced βAR vasorelaxation was also demonstrated by the observation that ISO-induced concentration-dependent vasorelaxation was doubled in the ADβ_2AR carotids (Figure 4B) compared with controls. It is possible to speculate that the βAR increased response could be attributable to β_2AR overexpression at the vascular smooth muscle level. This possibility is unlikely, because we used an intraluminal adenovirus delivery in absence of endothelial removal and basal lamina enzymatic digestion, which are needed for targeting vascular smooth muscle cells.[19] We performed two sets of experiments to ascertain the nature of ISO-induced vasorelaxation. As expected,[16] βAR vasorelaxation is largely endothelium-dependent, because the NOS inhibitor L NMMA (10^{-5} mol/L) inhibited vasorelaxation to ISO to a similar extent in both the control and ADβ_2AR vessels (Figure 4C). This result was confirmed in endothelium-denuded carotids (Figure 4D). In addition, no difference was observed between ADβ_2AR and control carotids in the vasorelaxation to the α_2AR agonist UK14,304, an endothelium-dependent vasodilator (Figure 4E), or sodium nitroprusside, an endothelium-independent vasodilator (Figure 4F). Therefore, ADβ_2AR selectively enhanced βAR-stimulated endothelium-dependent vasorelaxation.

Vasomotor Responses in Spontaneously Hypertensive Rats

Control PE and NE vasoconstrictions were not different in carotid arteries of SHR and WKY rats, and ADβ_2AR treatment did not alter the maximal vasoconstriction responses to PE and NE in SHR rat carotid arteries (Figure 5, A and B). In SHR control-treated carotids, βAR-induced vasorelaxation was significantly impaired compared with that observed in WKY (Figure 5C). However, ADβ_2AR treatment resulted in the enhancement of the ISO-induced vasorelaxation (Figure 5D), which was actually similar to that observed in ADβ_2AR-treated WKY carotid arteries (Figure 5E). This response was specific for βAR-mediated effects because sodium nitroprusside induced a concentration-dependent vasorelaxation that did not differ between ADempty and ADβ_2AR carotids (Figure 5F).

Discussion

Our hypothesis is that by selectively enhancing signaling through one receptor system, it is possible to alter endothelial function and vascular responses. Indeed, a recent study[20] indicates that increasing intracellular signal transduction pathways can positively alter endothelial function. With this in mind, we speculated that by increasing βAR density we could increase vascular βAR responses. In umbilical vein endothelial cells, it has been demonstrated that β_2AR stimulates eNOS activation.[4] However, most studies in other preparations have failed to demonstrate an active release of NO in response to βAR agonists.[21] Therefore, we first confirmed that ISO in normotensive rat aorta endothelial cells can induce eNOS activation and also demonstrated that ADβ_2AR treatment can enhance this response. We then used adenoviral-mediated gene transfer of the human β_2AR to selectively target the endothelium in normotensive rat com-

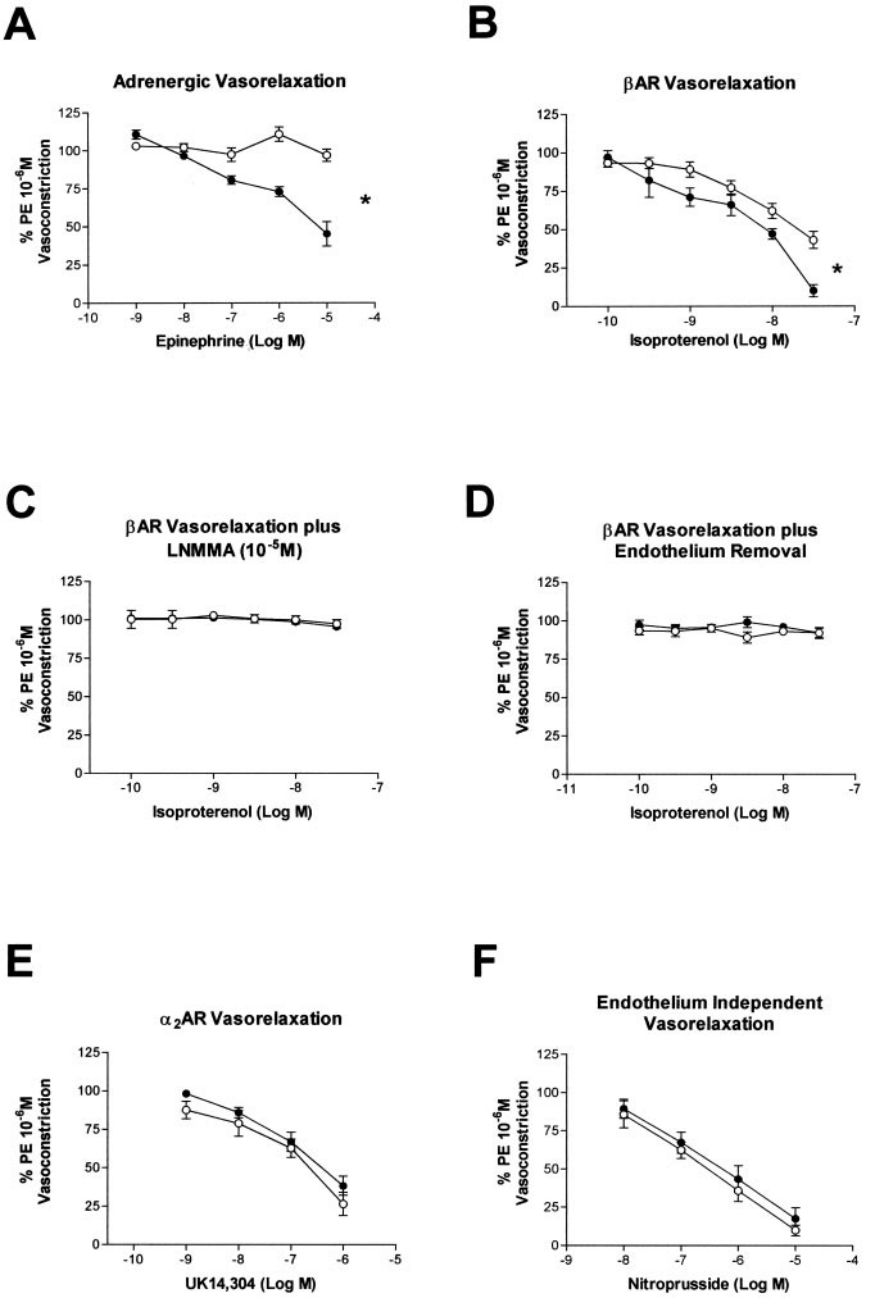

Figure 4. Effect of ADβ_2AR on the adrenergic vasorelaxations of common carotid arteries from WKY rats. ADβ_2AR enhances vasorelaxations to EPI (A) or ISO (B). In the presence of the eNOS-inhibitor LNMMA (10^{-5} mol/L) (C), there was no longer ISO vasorelaxation in both control and ADβ_2AR-treated carotids, suggesting that the βAR response is endothelium-dependent in both vessels. A similar result was observed when the endothelium was removed (D). The selective action of ADβ_2AR on the β-adrenergic responses is supported by the fact that the α_2 AR endothelium-dependent vasorelaxation (E) as well as the nitroprusside endothelium-independent vasorelaxation (F) were not affected by the ADβ_2AR treatment. ○ indicates control carotids; ●, ADβ_2AR-treated carotids; n=6 to 12 per group; §F=4.024; $P<0.02$. *F=5.719, $P<0.01$; 2-way ANOVA.

mon carotids. Our methods only allow a rough estimation of the relative density of endothelial versus vascular smooth muscle βARs on treated and control carotids. However, this strategy enhanced the vascular response to βAR stimulation. Both eNOS inhibition and endothelial removal showed that the enhancement of vasorelaxation to ISO in ADβ_2AR-treated carotids is endothelium-dependent. Therefore, we exclude the hypothesis of adenoviral-mediated β_2AR overexpression in smooth muscle cells. These functional experiments together with βAR binding and immunocytochemistry indicate the selective targeting of the endothelium in the rat carotid by our gene transfer technique. Accordingly, this is the first demonstration that gene-targeted overexpression of the human β_2AR causes eNOS activation and endothelium NO-dependent vasorelaxation in the rat carotid.

The physiological relevance of endothelial β_2ARs is supported by their distribution in the vasculature. Evidence is mounting that βAR vasorelaxation is largely endothelium-dependent in a wide range of vascular districts that actively participate in the determination of total peripheral resistance, including skeletal muscle[2,22] and mesenteric[23] and pulmonary vasculature systems.[24] Furthermore, in vivo studies in cat hind limb,[25] canine coronary artery,[26] and newborn pial arteries[27] suggest that the endothelium dependency of βAR vasorelaxant responses is generalized. Finally, recent studies in humans indicate that endothelial βARs are totally, or at least predominantly, of the β_2AR subtype.[4,22]

The experiments in normotensive rats suggest a novel approach to correct impaired endothelial function in cardiovascular conditions. We speculated that by using the gene

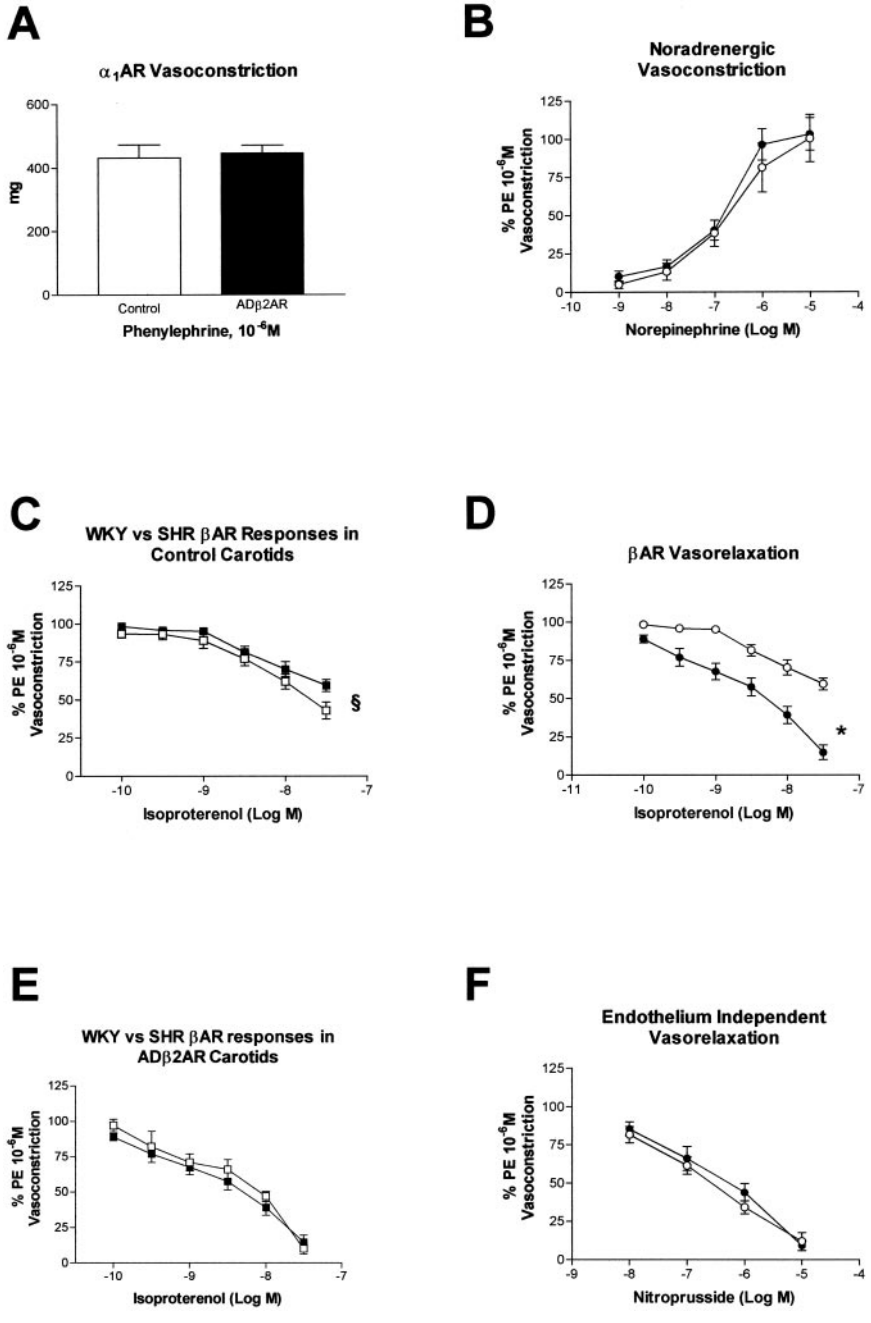

Figure 5. Effect of ADβ_2AR on the adrenergic vascular reactivity of common carotid arteries of SHR rats. As in normotensive rats, vasoconstriction to PE (A) and NE (B) were not affected by ADβ_2AR. In SHR, vasodilatation to ISO is attenuated compared with WKY (C). As in WKY, ADβ_2AR enhanced ISO vasorelaxation (D), which was not different from that observed in ADβ_2AR-treated WKY carotids (E). As in WKY, also in SHR, ADβ_2AR treatment did not affect the endothelium-independent vasorelaxation to nitroprusside (F). □ indicates WKY; ■, SHR; §F=5.756; $P<0.01$; ○, control carotids; ●, ADβ_2AR-treated carotids; n=6 to 10 per group. *F=14.038, $P<0.001$; 2-way ANOVA.

transfer of molecules that magnify intracellular signaling, it would be possible to correct abnormal vascular responses. We focused on βAR and hypertension because vascular βAR response is impaired in this condition and probably contributes to the progression of the disease.[28] Indeed, the combination of reduced βAR vasorelaxation and increased sympathetic nervous system activity is thought to participate in the increase of vascular resistance, vascular remodeling, and the increase of blood pressure levels.[5] Therefore, we aimed to increase βAR density by adenoviral-mediated gene transfer to the endothelium in hypertensive rats. A similar strategy in which the same virus was used has revealed efficacy to magnify βAR signaling and functional responses in vitro in cardiac myocytes from failing hearts.[13,14] It is important to note that this strategy does not correct the biochemical impairment of βAR signaling but rather circumvents it by increasing the receptor number over physiological levels. In SHR carotids, ADβ_2AR magnified the physiological response to βAR stimulation and increased vasorelaxation to ISO without affecting other adrenergic responses or the intrinsic ability of the vessel to vasodilate in response to NO donors. Moreover, in ADβ_2AR-treated carotid arteries, no difference was observed between SHR and WKY. Thus, it seems that impaired βAR vasorelaxation in hypertension is directly related to dysfunctional βAR signaling.

In conclusion, endothelial β_2ARs may represent a target for correcting adrenergic endothelial dysfunction in hypertension, and genetic manipulation of endothelial β_2ARs may be a novel therapeutic strategy for hypertension. An important study supporting our conclusion is the recent finding that selective β_2AR-mediated increase of endothelial NO produc-

tion is an additional therapeutic effect of the third-generation β-blocker nebivolol,[29] a β_1AR-selective antagonist with vasodilating properties.[30]

Acknowledgments

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