Loss of \(\alpha_{2B}\)-adrenoceptors increases magnitude of hypertension following nitric oxide synthase inhibition

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Duling, Laura C., Tom W. Cherng, Jason R. Griego, Michael F. Perrine, and Nancy L. Kanagy. Loss of \(\alpha_{2B}\)-adrenoceptors increases magnitude of hypertension following nitric oxide synthase inhibition. *Am J Physiol Heart Circ Physiol* 291: H2403–H2408, 2006. First published June 30, 2006; doi:10.1152/ajpheart.01066.2005.—Vascular \(\alpha_{2B}\)-adrenoceptors (\(\alpha_{2B}\)-AR) may mediate vasoconstriction and contribute to the development of hypertension. Therefore, we hypothesized that blood pressure would not increase as much in mice with mutated \(\alpha_{2B}\)-AR as in wild-type (WT) mice following nitric oxide (NO) synthase (NOS) inhibition with \(N^\omega\)-nitro-L-arginine (L-NNA, 250 mg/kg in drinking water). Mean arterial pressure (MAP) was recorded in heterozygous (HET) \(\alpha_{2B}\)-AR knockout mice and WT littermates using telemetry devices for 7 control and 14 L-NNA treatment days. MAP in HET mice was increased significantly on treatment days 0 and 4 to 14, whereas MAP did not change in WT mice (days 0 and 14 = 113 ± 3 and 114 ± 4 mm Hg in WT, 108 ± 0.3 and 135 ± 13 mm Hg in HET, *P < 0.05*). MAP was significantly higher in HET than in WT mice days 10 through 14 (*P < 0.05*). Thus blood pressure increased more rather than less in mice with decreased \(\alpha_{2B}\)-AR expression. We therefore examined constrictor responses to phenylephrine (PE, 10 \(^{-6}\) to 10 \(^{-4}\) M) with and without NOS inhibition to determine basal NO contributions to arterial tone. In small pressurized mesenteric arteries (inner diameter = 177 ± 5 \(\mu\)m), PE constriction was decreased in untreated HET arteries compared with WT (*P < 0.05*). L-NNA (100 \(\mu\)M) augmented PE constriction more in HET arteries than in WT arteries, and responses were not different between groups in the presence of L-NNA. Acetylcholine dilated preconstricted arteries from WT mice more than arteries from WT mice. Endothelial NOS expression was increased in HET compared with WT mesenteric arteries by Western analysis. Griess assay showed increased NO concentrations in HET plasma compared with those in WT plasma. These data demonstrate that diminished \(\alpha_{2B}\)-AR expression increases the dependence of arterial pressure and vascular tone on NO production and that vascular \(\alpha_{2B}\)-AR either directly or indirectly regulates vascular endothelial NOS function.

\(\alpha_{2B}\)-ADRENOGERIC RECEPTORS (\(\alpha_{2B}\)-ARs) have been postulated to play an important role in the regulation of blood pressure and vascular tone (19, 27). For example, \(\alpha_{2B}\)-AR antiserum delivered into the lateral cerebral ventricle lowers blood pressure in hypertensive rats (17), whereas intravenous administration of \(\alpha_{2B}\)-ARs agonists causes vasoconstriction (24) and salt retention (2). Thus activation of \(\alpha_{2B}\)-ARs may contribute to the development of some forms of hypertension (17). However, activation of postganglionic \(\alpha_{2B}\)-ARs can inhibit sympathetic nerve activity (35) and cause vasodilation (38), indicating that activation of \(\alpha_{2B}\)-ARs may either limit or promote the development of hypertension.

Recent studies suggest vascular \(\alpha_{2B}\)-ARs contribute to vasoconstriction under some conditions (14, 15). For example, our previous work demonstrates that nitric oxide (NO) synthase (NOS) inhibition hypertension increases vasoconstrictor sensitivity to \(\alpha_{2B}\)-AR activation, whereas studies by Flavahan et al. (1) suggest \(\alpha_{2B}\)-ARs contribute to cold-induced constriction. However, not all studies observe vasoconstriction in response to \(\alpha_{2B}\)-AR agonist activation (1, 13, 25) so that environmental and genetic influences may modulate the role of \(\alpha_{2B}\)-ARs in regulating vascular tone.

One such influence appears to be NO synthesis. In coronary arteries, \(\alpha_{2B}\)-AR-mediated vasoconstriction was only observed when NOS was inhibited (13). Our studies demonstrate significant \(\alpha_{2B}\)-AR-mediated constriction only in arteries with the endothelium removed (5), a response most likely mediated by the \(\beta_{2}\) subtype (21). Thus we hypothesized that NOS inhibition in vivo would uncover endogenous \(\alpha_{2B}\)-AR-mediated vasoconstriction that would be diminished in mice heterozygous for a mutated, nonfunctional \(\alpha_{2B}\)-AR. In addition, previous studies demonstrated that blood pressure does not increase in heterozygous \(\alpha_{2B}\)-AR knockout (HET) mice following salt loading but does increase in wild-type (WT) mice with full \(\alpha_{2B}\)-AR expression (22). These studies suggest salt intake influences the contribution of \(\alpha_{2B}\)-AR to blood pressure regulation. However, it is unclear whether the protection from developing hypertension in the HET mice is mediated by loss of salt-dependent central nervous system activation of the sympathetic nervous system or by loss of \(\alpha_{2B}\)-AR-mediated vasoconstriction following salt-mediated sympathetic activation. To determine whether there was a vascular component to the protective effect of the HET genotype, we made mice hypertensive by inhibiting NOS, a model independent of the central “salt-sensing” pathway. We hypothesized that because \(\alpha_{2B}\)-AR-mediated vasoconstriction is augmented following NOS inhibition, \(\alpha_{2B}\)-AR-deficient HET mice would not have as great of an increase in arterial pressure as WT mice following NOS inhibition. To test this hypothesis, blood pressure was recorded in HET and WT mice at baseline and after NOS inhibition. Contractile responses to phenylephrine (PE) in the presence and absence of NOS inhibition, and genetic influences may modulate the role of \(\alpha_{2B}\)-ARs in regulating vascular tone.

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affect in vivo NOS expression and NO production, respectively.

**METHODS**

**Animals.** Mice with a mutated, nonfunctional α_{2B}-AR originally developed in Kobikà’s laboratory (21) were used in these studies. They were derived from a 129SvJ times C57BL/6J cross. The deletion mutation produces a receptor that does not bind ligand or couple to G proteins (21). Heterozygous crosses maintain the colony of mice in the University of New Mexico animal resources facility. Although other laboratories have found that homozygous knock-out mice (−/−) from this cross are viable (12, 21), no knock-out pups have ever been born in our facility and studies were conducted comparing heterozygous HET mice and their WT littermates. Mice were genotyped by PCR using three primers: WT+ (CTCTTCTGC-TACCTCTCCCAT), WT− (CATAGATTGCGAGGTAGACG) and KO− (TGGATGTGGAATGTGTGCGCA). Breeding produced viable, healthy HET and WT mice in a 2:1 ratio. There is a slight decrease in adult body weight and blood pressure in the HET mice (Table 1).

**Surgical procedures.** Telemeters were implanted using a modification of the procedure described by Butz and Davison (7). Mice (20–28 g) were given buprenex (0.5 mg/kg sc) 20 min before surgery and anesthetized using ketamine-acepromazine (100:1 mg−1kg−1 im). The catheter tip was inserted and secured in the carotid artery, and the transmitter (PA-C20, Data Sciences International) was secured subcutaneously above the right flank. Tobramycin (3 mg/kg im) and warmed sterile 0.9% NaCl solution (0.5 ml sc) were given postsurgery, and mice recovered 5–7 days before recording was started. Mean arterial pressure (MAP) and heart rate (HR) were recorded daily for 7 control days and for 14 NOS inhibition days. During the NOS inhibition period, mice were given NOS inhibitor 15 min before and then throughout exposure to PE. Vasoconstriction to L-NNA and PE is expressed as percent constriction (i.e., diameter in Ca^{2+} free buffer used as 0% constriction).

Acetylcholine-induced dilation was recorded in arteries pressurized to 60 mmHg without flow through the lumen to remove the influence of flow-induced dilation. Arteries were constricted with PE (10^{−6} M) and then exposed to increasing concentrations of acetylcholine (10^{−9} to 10^{−4} M) in the continued presence of PE (all drugs administered in superfusate). Dilation was recorded as percent reversal of the PE constriction (i.e., 100% = baseline diameter) as measured with IonWizard edge-detection software (IonOptix).

**Animal activity.** Daily activity was recorded from 0700 to 1900 and averaged using Telemetry Analyzer (JCL Consultants). Data are reported as means ± SE.

**Western analysis.** Mesenteric arteries (200 to 50 μm diameter) were cleaned and frozen in liquid nitrogen. Frozen tissues were homogenized in cold Tris-HCl homogenization buffer containing dithiothreitol (1.5 mg/ml), EDTA (3.7 mg/ml), benzamidine (1.57 mg/ml), Complete (Roche protease inhibitor cocktail, 30 μl/ml), and phenylmethylsulfonyl fluoride (25 μl/ml) using a ground glass homogenizer. Homogenates were sonicated then spun at 4,000 g for 4 min at 4°C. The supernatant was analyzed for protein concentration using the bichinchoninic acid method (Pierce). Samples were separated in a 4–20% Tris-HCl polyacrylamide gradient gel (Bio-Rad) containing samples (50 μg protein/lane) and molecular weight markers. After transfer to Immobilon membrane (Bio-Rad), blots were blocked overnight in Tris-buffered saline (TBS, with 0.5% Triton X-100, 3% BSA, and 5% milk) and then washed with TBS and incubated with monoclonal antibodies specific for eNOS (1:2,500, Transduction Labs). Developed blots were exposed to X-Omat film and analyzed using SigmaGel software (SyStat).

**Griess assay.** Plasma samples collected from untreated WT and HET mice were analyzed for NOx using a microplate Griess assay as described previously (32). Samples and NaNO_{3} standards were loaded into a 96-well plate in duplicate. Samples were reduced with nitrate reductase and NADPH at room temperature for 3 h. After reduction, Griess reagent (1% sulfanilamide, 0.1% naphthylethenediamine dihydrochloride, and 2.5% phosphoric acid) was added to each sample. After 10 min of incubation, absorption was read at 595 nM, and concentration was determined using the standard curve.

**RESULTS**

**Hemodynamic studies.** MAP and HR were not different between groups at baseline, although body weight was lower in the HET mice (Table 1). MAP increased significantly in the HET mice compared with that of the control period on days 1 and 4–14 of l-NNA treatment (Fig. 1). MAP in WT mice did not change from baseline pressure (Fig. 1). MAP was significantly higher in HET mice compared with MAP in WT mice on days 12–14 (Fig. 1).

**Contractile studies.** Vasoconstriction in response to increasing concentrations of PE demonstrate that HET arteries constrict less to PE than WT arteries (Fig. 2). However, PE constriction was not different between groups treated with l-NNA (Fig. 2). Starting diameters were not different between groups (174 ± 4 and 172 ± 4 μm in WT and HET, respectively; Table 2) but in the presence of l-NNA, diameter was less in HET arteries than in WT arteries (166 ± 12 and 163 ± 15 μm, respectively, P < 0.05), and l-NNA treatment caused a greater shift in the PE concentration response curve from the HET than from the WT mice (Fig. 3). In summary, although

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**Table 1. Baseline values in two genotypes**

<table>
<thead>
<tr>
<th></th>
<th>Body Weight, g</th>
<th>Blood Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>27 ± 1.1</td>
<td>110 ± 1</td>
<td>595 ± 7</td>
</tr>
<tr>
<td>HET</td>
<td>22 ± 0.6</td>
<td>107 ± 0.3</td>
<td>617 ± 11</td>
</tr>
</tbody>
</table>

Data are means ± SE, WT, wild-type mice; HET, heterozygous mice.

*Different from WT for P < 0.05.
L-NNA constricted all arteries, the constriction was greater in the HET arteries than in the WT arteries.

Dilation study. In arteries constricted with PE (10^(-6) M), acetylcholine caused a concentration-dependent dilation that was greater in the HET compared with WT arteries (Fig. 4). The dilation was reversed to a constriction at concentrations greater than 10^(-5) M. Diameters in Ca^2+ -free physiological saline solution were not different between groups (WT 163 ± 9 and HET 174 ± 6 μm).

Animal activity. Although activity appears to be greater in HET mice compared with WT mice (Fig. 5), there were no significant differences either between groups (P = 0.165) or between control and treatment periods (P = 0.987).

Western analysis. Mesenteric artery homogenates were probed for eNOS using a monoclonal antibody (1:2,500, Transduction Labs), and the membranes were stained for total protein loading using Coomassie blue dye. The immunostained bands detected with X-OMAT film were scanned and densitized (Sigma Gel, SyStat Software) and then normalized for Coomassie staining intensity. The normalized values for the small mesenteric arteries from HET mice were significantly greater than those from the WT tissues (Fig. 6).

Griess assay. Plasma NOx concentrations were significantly elevated in samples from HET mice compared with samples from WT mice (Fig. 7).

### Table 2. Vascular parameters in two genotypes

<table>
<thead>
<tr>
<th>Vascular parameter</th>
<th>WT</th>
<th>Maximal Constriction, %</th>
<th>EC50 (-log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel Diameter, μM</td>
<td>174 ± 4</td>
<td>59 ± 4</td>
<td>6.42 ± 0.12</td>
</tr>
<tr>
<td>Maximal Constriction, %</td>
<td>172 ± 5</td>
<td>55 ± 4</td>
<td>6.01 ± 0.11*</td>
</tr>
<tr>
<td>EC50 (-log)</td>
<td>163 ± 15</td>
<td>74 ± 4</td>
<td>6.62 ± 0.16</td>
</tr>
<tr>
<td>WT</td>
<td>166 ± 12</td>
<td>79 ± 3</td>
<td>6.81 ± 0.14†</td>
</tr>
<tr>
<td>HET</td>
<td>172 ± 5</td>
<td>55 ± 4</td>
<td>6.01 ± 0.11*</td>
</tr>
<tr>
<td>HET</td>
<td>166 ± 12</td>
<td>79 ± 3</td>
<td>6.81 ± 0.14†</td>
</tr>
</tbody>
</table>

Values are means ± SE. L-NNA, N^ω-nitro-L-arginine. *Different from WT; †different from vehicle.
DISCUSSION

Conflicting reports on the role of \( \alpha_{2B} \)-ARs in cardiovascular control have left its involvement in blood pressure regulation unclear. Activation of central \( \alpha_{2B} \)-ARs has been reported to increase blood pressure (10), and ablation of \( \alpha_{2B} \)-ARs prevents both the initial pressor response to the \( \alpha_{2A} \)-AR agonist clonidine (21) and the hypertensive response to salt loading (22). Studies have also shown that a deletion polymorphism in the human \( \alpha_{2B} \)-AR gene, which prevents receptor desensitization, is associated with hypertension in some populations (37). Thus activation of \( \alpha_{2B} \)-ARs may increase blood pressure under certain conditions. One condition that is thought to increase arterial pressure by activating \( \alpha_{2B} \)-ARs is salt loading. This is supported by observations that uninephrectomized WT mice become hypertensive to salt loading but \( \alpha_{2B} \)-AR HET knockout mice do not (22). Furthermore, intracerebroventricular administration of \( \alpha_{2B} \)-AR antisense decreases blood pressure in hypertensive rats (16, 17) suggesting central \( \alpha_{2B} \)-ARs are at least partially responsible for this effect. Therefore, most studies suggest central \( \alpha_{2B} \)-ARs actively participate in the development and/or maintenance of salt-sensitive increases in blood pressure and are necessary for the development of some forms of hypertension.

However, our observation that HET mice became hypertensive following chronic NOS inhibition, whereas WT mice did not become hypertensive, suggests that very different mechanisms mediate the increases in arterial pressure between this model and salt-induced hypertension. In salt-induced hypertension, there is at least an initial volume expansion that contributes to the sustained increase in blood pressure (11). Salt loading is also thought to increase sympathetic activity to mediate the sustained increase in arterial pressure by a “salt sensor” system in the brain stem (4) so that the hypertension is maintained by elevated vasoconstriction. In contrast, NOS inhibition induces hypertension primarily through loss of a vasodilator (33). Because previous studies have observed that increased eNOS expression protects against vasoconstriction-dependent hypertension (26, 36), elevated eNOS expression in arteries of HET mice might have contributed to the resistance of these mice to developing hypertension in previous studies (3). Indeed, the greater increase in blood pressure following NOS inhibition in HET mice appears to have uncovered a unique role for \( \alpha_{2B} \)-ARs in regulating vascular tone through controlling eNOS expression and function.

The mechanism whereby \( \alpha_{2B} \)-ARs might regulate eNOS expression is unknown. It has been demonstrated that \( \alpha_{2A} \)-ARs stimulate eNOS activity in endothelial cells (9, 18). Therefore, loss of endogenous \( \alpha_{2A} \)-AR activation of eNOS could potentially remove NO feedback inhibition to increase eNOS expression. However, endothelium-dependent dilation appears to be mediated through activation of \( \alpha_{2A} \)-ARs (28) and is unaltered in \( \alpha_{2B} \)-AR HET mice. Thus decreased NO feedback inhibition of eNOS expression is unlikely to explain the elevated eNOS expression in the HET mice.

Other receptor-dependent regulators of eNOS expression include PPAR activators that increase eNOS expression.

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**Fig. 4.** Percent vasodilation to acetylcholine in arteries constricted with phenylephrine (10^(-6) M). Acetylcholine caused greater dilation in arteries from HET mice than in arteries from WT mice (*different from WT for \( P < 0.05 \)).

**Fig. 5.** Daily activity of mice recorded as events per minute. HET mice appear to be more active in both the control and treatment period than WT mice. There is, however, not a significant difference between groups (\( P = 0.165 \)).

**Fig. 6.** Immunostaining of endothelial nitric oxide synthase (eNOS) in mesenteric artery homogenates from HET and WT mice. Densitized values of the immunoblot were normalized to the density of corresponding lanes in Coomassie blue-stained membrane (bottom) and are expressed as a ratio. *Significantly different from WT for \( P < 0.05 \).

**Fig. 7.** NO\(_x\) levels in plasma from untreated WT and HET mice. There was significantly more NO\(_x\) detected in the plasma from the HET mice compared with the plasma from the WT mice (\( P < 0.05 \)).
through a mitogen-activated protein kinase, cSrc pathway (6), and TNF-α, which activates Rho kinase (ROK)-dependent mRNA destabilization (34). Because we have previously demonstrated that α2B-ARs also activate ROK (5), it will be interesting to determine whether α2B-ARs directly control eNOS expression through this pathway in endothelial cells.

Alternatively, there may be increased blood flow in arteries of the α2B-AR HET mice caused by diminished α2B-AR constriction (21). It has been demonstrated that flow is a potent regulator of eNOS expression (20), and thus this may be an indirect mechanism leading to the upregulation of eNOS in the mesenteric circulation.

Several common genetic variants in the human α2B-ARs have been identified that are associated with cardiovascular pathology. One of the best characterized is a nine-base pair in-frame deletion that leads to the loss of three glutamine residues [Del(301–303)]. One recent study reported that the Del(301–303) polymorphism does not affect resting arterial pressure or the pressor response to yohimbine (8), but most previous studies have found an association between the Del(301–303) polymorphism and the incidence of hypertension (37), coronary disease (30, 31), and risk for cardiovascular mortality (23, 29). Therefore, if α2B-ARs regulate NOS expression in humans, altered α2B-AR function in the Del(301–303) polymorphism population may exacerbate vascular disease by reducing eNOS expression. One speculative conclusion would be that decreased α2B-AR expression protects from the development of cardiovascular diseases by upregulating eNOS, suggesting that α2B-AR selective antagonists should be cardioprotective.

Even though the mechanism underlying the upregulation of eNOS vasodilation is not defined, this study does demonstrate that diminished α2B-AR expression increases the dependence of blood pressure on NO synthesis. Thus α2B-ARs appear to regulate eNOS expression either directly or indirectly and to decrease the contribution of NO production to the control of arterial pressure and vascular tone. Determining the mechanisms underlying this regulation should increase our understanding of the interaction between the sympathetic nervous system and endothelial function in blood pressure control.

ACKNOWLEDGMENTS

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GRANTS

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