Functional Role of $\alpha_1$-Adrenoceptor Subtypes in Murine Ophthalmic Arteries

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PURPOSE. To identify the $\alpha_1$-adrenoceptor ($\alpha_1$-AR) subtypes mediating vascular adrenergic responses in murine ophthalmic arteries.

METHODS. Expression of mRNA was quantified for individual $\alpha_1$-AR subtypes in murine ophthalmic arteries using real-time PCR. To assess the functional relevance of $\alpha_1$-ARs for mediating vascular responses, ophthalmic arteries from mice deficient in one of the three $\alpha_1$-AR subtypes ($\alpha_{1A}$-AR$^{-/-}$, $\alpha_{1B}$-AR$^{-/-}$, and $\alpha_{1D}$-AR$^{-/-}$ respectively) and wild-type controls were isolated, cannulated with micropipettes, and pressurized. Changes in luminal artery diameter in response to the $\alpha_1$-AR agonist phenylephrine, the sympathetic transmitter noradrenaline, and to phenylephrine, the sympathetic transmitter noradrenaline, were measured by video microscopy.

RESULTS. Using real-time PCR, mRNA for all three $\alpha_1$-AR subtypes was detected in ophthalmic arteries from wild-type mice. In functional studies, phenylephrine and noradrenaline produced dose-dependent constriction of ophthalmic arteries that was similar in wild-type, $\alpha_{1B}$-AR$^{-/-}$, and $\alpha_{1D}$-AR$^{-/-}$ mice. Strikingly, responses to phenylephrine and noradrenaline were almost completely abolished in $\alpha_{1A}$-AR$^{-/-}$ mice. In contrast, the nonadrenergic agonist AVP produced dose-dependent vasoconstrictor responses that did not differ between any of the mouse genotypes tested.

CONCLUSIONS. These findings provide evidence that the $\alpha_{1A}$-AR subtype mediates adrenergic vasoconstriction in murine ophthalmic arteries. (Invest Ophthalmol Vis Sci. 2011;52:4795–4799) DOI:10.1167/iovs.11-7516

Disturbances in ocular perfusion have been implicated in the pathophysiology of various eye diseases, including diabetic retinopathy, nonarteritic anterior ischemic optic neuropathy, and glaucoma.1–6 The $\alpha_1$-adrenoceptor ($\alpha_1$-AR) family plays a critical role in regulating ocular vascular tone and blood flow by mediating vasoconstrictor responses of catecholamines in the ocular circulation.7–11 Pharmacologic studies and molecular cloning techniques have revealed the existence of three $\alpha_1$-AR subtypes: $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$.12–16 All three receptor subtypes are expressed in blood vessels and can mediate vasoconstriction via G$_{\alpha_1}$ protein-mediated increases of inositol phosphates and intracellular calcium in vascular smooth muscle cells.17 However, the expression pattern of individual $\alpha_1$-AR subtypes and their role in mediating vascular responses to catecholamines differs considerably between individual vascular beds.18,19 Based on these findings, selective pharmacologic activation or blockade of individual $\alpha_1$-AR subtypes may provide a useful tool to selectively modulate perfusion of various organs, including the eye. Thus, we designed this study to identify the $\alpha_1$-AR subtypes that mediate adrenergic vascular responses in ophthalmic arteries. Since the expression pattern of $\alpha_1$-ARs is unknown in ocular vessels, we used real-time (RT) PCR to quantify mRNA expression of individual $\alpha_1$-AR subtypes in murine ophthalmic arteries. Due to the lack of highly selective agonists and antagonists for individual $\alpha_1$-AR subtypes, we used gene-targeted mice deficient in one of the three $\alpha_1$-ARs ($\alpha_{1A}$-AR$^{-/-}$, $\alpha_{1B}$-AR$^{-/-}$, or $\alpha_{1D}$-AR$^{-/-}$ respectively) to determine the role of each receptor subtype in mediating adrenergic vasoconstriction in ophthalmic arteries.

MATERIALS AND METHODS

Animals

All studies were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local government. The generation of $\alpha_{1A}$-AR$^{-/-}$, $\alpha_{1B}$-AR$^{-/-}$, and $\alpha_{1D}$-AR$^{-/-}$ mice has been described previously.20–22 Each genotype has been backcrossed with C57BL/6J mice for more than eight times and maintained on a C57BL/6J genetic background. For experiments, male mice at the age of 7 to 9 months were used. Mice were fed with standard mouse chow and allowed free access to tap water.

Real-Time PCR Analysis

Expression of $\alpha_1$-AR mRNA was quantified in isolated ophthalmic arteries from wild-type mice (C57BL/6J) using RT-PCR. After mice had been killed by CO$_2$ inhalation, the eyes were immediately removed together with the retrobulbar tissue and placed in ice-cold PBS (Invitrogen, Karlsruhe, Germany). Then, ophthalmic arteries were isolated by the use of fine-point tweezers under a dissecting microscope, transferred into a 1.5-mL tube, and immediately snap frozen. To increase the RNA yield, arteries were pooled from three mice. Subsequently, vessels were homogenized in lysis buffer using a homogenizing device (Schwingmühle MM 300; Retsch GmbH, Haan, Germany; Lysing Matrix D MP, MP Biomedicals, Illkirch, France). After homogenization, total RNA was extracted with a kit (Absolutely RNA Nano-prep; Stratagene, La Jolla, CA) according to the manufacturer’s protocol. After complete DNA digestion, the RNA was reverse transcribed.

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with the use of a reverse transcription kit and random hexamers (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Darmstadt, Germany). Quantitative PCR analysis was performed (GeneAmp StepOne Plus; Applied Biosystems). Nucleic acid stain (SYBR Green; Bioline, Luckenwalde, Germany) was used for the fluorescent detection of DNA generated during PCR. The PCR reaction was performed in a total volume of 12.5 μL with 0.4 pmol/μL of each primer and ready-to-use 2x reaction mix (ImmoMix; Bioline); 2 μL cDNA corresponding to 10 ng RNA was used as a template. Published sequences for mouse α1A-AR (NM_015341), α1B-AR (NM_007416), and α1D-AR (NM_015340) were used to design primers for PCR amplification. Primer sequences were α1A-AR sense 5'-TGC GAG GAC TTG GCC GCC GCT-3' and antisense 5'-CAT GGA CAT GGC TGG GCC GAT-3', α1B-AR sense 5'-TGC GAG GAA AAG AAA GCA GCC AA-3' and antisense 5'-GGG TAG ATG ATG GTG TAGG AAC-3'; α1D-AR sense 5'-TAA GCC TGG TCA AGT TTT CCC GC-3' and antisense 5'-TGA GCG GGT TCA GAC TAT TGA-3'; 8-actin sense 5'-CAC CCG GGA GCA CAG CT TTT-3' and antisense 5'-AAT ACA GCC CGG GGA GCA TC-3'. The expression levels of α1A-AR, α1B-AR, and α1D-AR mRNA were normalized to β-actin using the ΔCt method. Parallelism of standard curves was confirmed.

Measurements of Vascular Reactivity
Mice were killed by CO2 inhalation and the eyes were rapidly removed, together with the retrobulbar tissue, and placed in ice-cold Krebs buffer with the following ionic composition (in mM): 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 glucose (Carl Roth GmbH, Karlsruhe, Germany). Then, ophthalmic arteries were isolated under a dissecting microscope, placed in an organ chamber filled with cold Krebs solution, and cannulated and sutured onto microtipettes, as described previously.25 Vessels were pressurized via the micropipettes to 50 mm Hg under no-flow conditions using two reservoirs filled with Krebs solution and imaged using a video camera mounted on an inverted microscope; video sequences were captured to a personal computer for offline analysis. The organ chamber was continuously circulated with oxygenated and carbonated Krebs buffer at 37°C and pH 7.4. Arteries were allowed to equilibrate for 30 to 40 minutes before study. During this time, ophthalmic arteries of all groups developed spontaneous myogenic tone by constriction to 80% to 81% of initial diameter recorded immediately after pressurization. Reduction in luminal artery diameter during the equilibration period was similar in all four mouse genotypes. Viability of vessels was assessed as satisfactory when at least 50% constriction from stabilized resting diameter in response to high KCl solution (100 mM) was achieved. Then, cumulative concentration-response curves were obtained to phenylephrine (1 nM to 300 μM), a non-subtype-selective α1-AR agonist; to noradrenaline (1 nM to 300 μM), a major neurotransmitter of sympathetic nerves; and to arginine vasopressin (AVP, 1 pM to 300 nM), a nonadrenergic receptor agonist, which induces vasoconstriction via V1a-receptor-mediated increases of inositol phosphates and intracellular calcium in vascular smooth muscle. Responses to noradrenaline (10 μM) were also compared before and after addition of prazosin (100 nM), a competitive non-subtype-selective α1-AR antagonist.

Statistical Analysis
Data are presented as mean ± SE and n represents the number of mice per group. Vascular responses are presented as percentage of change in diameter from stabilized resting diameter. Comparisons of concentration–response curves were made using the Brunner test for non-parametric analysis of longitudinal data.24 The Bonferroni adjustment was used to correct for multiple comparisons. To compare vascular responses to noradrenaline before and after prazosin treatment, the Wilcoxon signed-rank test was used. Comparisons of α1-AR mRNA expression levels were made using the Kruskal–Wallis test. The level of significance was set at 0.05.

RESULTS
α1-Adrenoceptor mRNA Expression in Ophthalmic Arteries
Expression of α1-AR mRNA was determined in ophthalmic arteries (five pooled samples) from wild-type mice (n = 15) by the use of quantitative RT-PCR. Remarkably, mRNA of all three α1-AR subtypes was found to be expressed at high levels, although there was no difference between mRNA expression levels of individual receptor subtypes (Fig. 1).

Responses of Ophthalmic Arteries
Baseline luminal diameters of ophthalmic artery segments (after development of stable myogenic tone) were (in μm) 118 ± 13, 130 ± 9, 125 ± 8, and 152 ± 10 in α1A-AR−/−, α1B-AR−/−, α1D-AR−/−, and wild-type mice and did not differ between individual mouse genotypes (P > 0.05, one-way ANOVA, n = 8–10 per genotype). To identify the α1-AR subtypes that mediate adrenergic vasoconstriction of ophthalmic arteries, we compared vascular responses from α1A-AR−/−, α1B-AR−/−, α1D-AR−/−, and wild-type mice to the non-subtype–selective α1-AR agonist phenylephrine. Phenylephrine elicited concentration-dependent vasoconstriction in arteries from wild-type, α1B-AR−/−, and α1D-AR−/− mice that was similar in the three groups (Fig. 2). Maximal reduction in luminal diameter in response to phenylephrine was 41% ± 6%, 43% ± 8%, and 38% ± 4% in wild-type (n = 10), α1B-AR−/− (n = 8), and α1D-AR−/− (n = 10) mice, respectively. The pD2 values (mean negative log of the vasoconstrictor concentration producing 50% of maximal response) were 5.79 ± 0.16, 5.75 ± 0.19, and 5.68 ± 0.19 in wild-type, α1B-AR−/−, and α1D-AR−/− mice, respectively. In contrast, phenylephrine-induced vasoconstriction was almost completely abolished in ophthalmic arteries from α1A-AR−/− mice (n = 8), differing significantly from responses of all other genotypes. Maximal reduction in luminal diameter to phenylephrine was only 6% ± 5% in this group (Fig. 2).

Moreover, we examined responses to the sympathetic transmitter noradrenaline that, apart from α1-ARs, can also activate α2-ARs and β-ARs. Noradrenaline also produced concentration-dependent vasoconstriction in ophthalmic arteries from wild-

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**Figure 1.** Relative mRNA expression of individual α1-AR subtypes (α1A, α1B, and α1D) normalized to β-actin transcripts in ophthalmic arteries from wild-type mice. Values are averages of five independent experiments and are expressed as mean ± SE.
and genotype).

type, α_{1A}-AR−/−, and α_{1D}-AR−/− mice that did not differ between the three genotypes. Maximal constriction to noradrenaline was 42% ± 7%, 52% ± 7%, and 44% ± 5% in wild-type (n = 10), α_{1B}-AR−/− (n = 8), and α_{1D}-AR−/− (n = 10) mice, respectively. The pD2 values were 5.97 ± 0.20, 5.75 ± 0.17, and 5.91 ± 0.17 in wild-type, α_{1B}-AR−/−, and α_{1D}-AR−/− mice, respectively. In α_{1A}-AR−/− mice (n = 8), however, maximal reduction in luminal artery diameter in response to noradrenaline was negligible and differed significantly from responses of all other genotypes in this maximal constriction to AVP was 48% ± 8%, 49% ± 8%, 52% ± 8%, and 45% ± 7% in wild-type (n = 10), α_{1A}-AR−/− (n = 8), α_{1B}-AR−/− (n = 8), and α_{1D}-AR−/− (n = 10) mice, respectively. The pD2 values were 9.28 ± 0.25, and 9.06 ± 0.27 in wild-type, α_{1A}-AR−/−, α_{1B}-AR−/−, and α_{1D}-AR−/− mice, respectively.

DISCUSSION

The purpose of the present study was to identify the α_{1}-AR subtypes that mediate adrenergic responses in ophthalmic arteries. Using RT-PCR, we found mRNA of all three α_{1}-AR subtypes that mediate adrenergic responses in ophthalmic arteries of wild-type mice. Since highly selective agonists and antagonists are not available for all three subtypes, we used mice with targeted disruption of single α_{1}-AR subtype genes to assess the functional relevance of each receptor subtype. Strikingly, oph-

![Figure 2](image2)

**Figure 2.** Responses of ophthalmic arteries from wild-type, α_{1A}-AR−/−, α_{1B}-AR−/−, and α_{1D}-AR−/− mice to the α_{1}-AR agonist phenylephrine. Vasconstriction to phenylephrine was almost completely abolished in ophthalmic arteries from α_{1A}-AR−/− mice. In contrast, deletion of α_{1B}-AR and α_{1D}-AR genes had no significant effect on vascular reactivity. Values are expressed as mean ± SE (n = 8 to 10 per concentration and genotype).

![Figure 3](image3)

**Figure 3.** Responses of ophthalmic arteries from wild-type, α_{1A}-AR−/−, α_{1B}-AR−/−, and α_{1D}-AR−/− mice to the sympathetic transmitter noradrenaline. (A) Vasconstriction to noradrenaline was almost completely abolished in ophthalmic arteries from α_{1A}-AR−/− mice. In contrast, deletion of α_{1B}-AR and α_{1D}-AR genes had no significant effect on vascular reactivity. Values are expressed as mean ± SE (n = 8 to 10 per concentration and genotype). (B) Vasconstrictor responses to noradrenaline (10⁻⁵ M) were virtually abolished after incubation with prazosin (10⁻⁷ M). Values are expressed as mean ± SE (n = 6 per genotype).

![Figure 4](image4)

**Figure 4.** Responses of ophthalmic arteries from wild-type, α_{1A}-AR−/−, α_{1B}-AR−/−, and α_{1D}-AR−/− mice to the nonadrenergic vasoconstrictor AVP. Deletion of α_{1A}-AR, α_{1B}-AR, or α_{1D}-AR genes did not affect responses to AVP. Values are expressed as mean ± SE (n = 8 to 10 per concentration and genotype).
thalamic arteries from mice deficient in the α1c-AR gene showed almost no reactivity to phenylephrine and noradrenaline. The α1B-AR antagonist prazosin almost completely abolished noradrenaline-induced responses, indicative of the predominant involvement of α1B-ARs in adrenergic vasoconstriction of ophthalmic arteries. Deletion of either receptor subtype did not affect vasoconstriction induced by AVP, suggesting that the lack of a single α1-AR subtype does not affect the downstream signaling cascades that ultimately mediate vasoconstriction.

Previous studies using electrical stimulation of sympathetic nerve pathways and intravenous application of α1-AR antagonists demonstrated that α1-ARs mediate neurogenic vasoconstriction in the anterior choroid of rats and in long posterior ciliary arteries of cats. Another study using transmural electrical stimulation in isolated vascular strips showed that α1-ARs contributed to neurogenic vasoconstriction in dog short posterior ciliary and ophthalmic arteries. The present study is the first to demonstrate that adrenergic vasoconstriction of murine ophthalmic arteries is mediated predominantly by the α1A-AR subtype.

Earlier pharmacologic studies making use of subtype-selective agents and functional studies in gene-targeted mice lacking one or more α1-AR subtypes revealed that the contribution of individual α1-AR subtypes to adrenergic vasoconstrictor responses differs considerably depending on the vascular bed. Based on these studies, the α1A-AR is significantly involved in adrenergic vasoconstriction of rat and mouse small mesenteric and tail arteries, but plays only a minor role in α1-AR-mediated contraction of large vessels, such as aorta, iliac, and carotid arteries.

In contrast, the α1D-AR was shown to play a major vasoconstrictor role in large vessels, but was also suggested to participate in α1A-AR-mediated vasoconstriction of some small arteries, such as coronary and femoral small arteries. The α1B-AR was shown to play only a minor role in adrenergic vasoconstriction. In vivo studies have demonstrated that blood pressure responses to phenylephrine and to noradrenaline are reduced in gene-targeted mice lacking the α1D-AR. However, no differences in resting blood pressure between α1D-AR knockout and respective wild-type mice were detected. Moreover, several in vitro studies using α1-AR subtype-selective antagonists and mice with targeted disruption of the α1A-AR gene revealed only a minor contribution of the α1A-AR subtype to vasoconstrictor responses in mouse aorta, carotid, mesenteric, and tail arteries.

Studies of mRNA and protein expression revealed diverse distribution of α1-AR receptor subtypes among vascular beds. Some of these studies reported that the mRNA expression levels of individual α1-AR subtypes were in fairly good agreement with their protein levels or their contribution to adrenergic vasoconstrictor responses. Other studies, however, demonstrated that the presence of mRNA or even protein for a particular α1-AR subtype does not ensure its expression as affected by cholinergic and nitroxidergic nerves in dog ciliary and ophthalmic arteries. From a clinical point of view, selective α1A-AR antagonists may become therapeutically useful to increase ocular perfusion in certain pathologic conditions, such as diabetic retinopathy and glaucoma.

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