Role of the M₃ Muscarinic Acetylcholine Receptor Subtype in Murine Ophthalmic Arteries After Endothelial Removal

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PURPOSE. We tested the hypothesis that the M₃ muscarinic acetylcholine receptor subtype mediates cholinergic responses in murine ophthalmic arteries after endothelial removal.

METHODS. Muscarinic receptor gene expression was determined in ophthalmic arteries with intact and with removed endothelium using real-time PCR. To examine the role of the M₃ receptor in mediating vascular responses, ophthalmic arteries from M₃ receptor-deficient mice (M3R^{-/-}) and respective wild-type controls were studied in vitro. Functional studies were performed in nonprecontracted arteries with either intact or removed endothelium using video microscopy.

RESULTS. In endothelium-intact ophthalmic arteries, mRNA for all five muscarinic receptor subtypes was detected, but M₃ receptor mRNA was most abundant. In endothelium-removed ophthalmic arteries, M₁, M₂, and M₃ receptors displayed similar mRNA expression levels, which were higher than those for M₄ and M₅ receptors. In functional studies, acetylcholine evoked vasoconstriction in endothelium-removed arteries from wild-type mice that was virtually abolished after incubation with the muscarinic receptor blocker atropine, indicative of the involvement of muscarinic receptors. In concentration-response experiments, acetylcholine and carbachol concentration-dependently constricted endothelium-removed ophthalmic arteries from wild-type mice, but produced only negligible responses in arteries from M3R^{-/-} mice. In contrast, acetylcholine concentration-dependently dilated ophthalmic arteries with intact endothelium from wild-type mice, but not from M3R^{-/-} mice. Responses to the nitric oxide donor nitroprusside and to KCl did not differ between ophthalmic arteries from wild-type and M3R^{-/-} mice, neither in endothelium-intact nor in endothelium-removed arteries.

CONCLUSIONS. These findings provide evidence that in murine ophthalmic arteries the muscarinic M₃ receptor subtype mediates cholinergic endothelium-dependent vasodilation and endothelium-independent vasoconstriction.

Keywords: acetylcholine, muscarinic receptors, vasoconstriction, ophthalmic arteries, mice

The muscarinic acetylcholine receptor family is composed of five subtypes denoted M₁ to M₅.¹ In most vascular beds, activation of muscarinic receptors induces powerful vasodilation via the release of vasorelaxing agents from the endothelium.^{2,3} Previous studies reported that the M₃ receptor subtype mediates cholinergic vasodilation in the choroid of pigeons and in ocular blood vessels of mice.^{4–6} Based on these findings, the M₃ receptor may represent a potential pharmacologic target to modulate blood flow in diseases associated with disturbances of ocular perfusion, such as diabetic retinopathy, age-dependent macular degeneration, nonarteritic anterior ischemic optic neuropathy (NAION), and glaucoma.^{7–10} However, in various nonocular vascular beds, cholinergic agonists were shown to exert only limited vasodilation effects or even to induce vasoconstriction when applied in pathologic conditions associated with endothelial dysfunction.^{11–17} These effects have been attributed to activation of muscarinic acetylcholine receptors localized on vascular smooth muscle and to an

influence of vasoconstrictor agents released from vascular endothelium.^{18,19} Because several diseases associated with impaired ocular perfusion also have been associated with endothelial dysfunction,^{20,21} one may argue that nonsubtype-selective muscarinic receptor agonists may exert a limited vasodilation effect or even to cause vasoconstriction when applied in these diseases. Subtype-selective muscarinic receptor ligands might be useful to circumvent this problem. Thus, it is important to define the functional role of individual muscarinic receptor subtypes in conditions of endothelial damage or dysfunction. Although it has been shown that the M₃ acetylcholine receptor subtype mediates vasodilation in endothelium-intact ocular blood vessels,^{4–6} to our knowledge there are at present no studies reporting on its functional role in ocular blood vessels with damaged or dysfunctional endothelium. Hence, the goal of the present study was to test the hypothesis that the M₃ muscarinic acetylcholine receptor subtype mediates responses in ophthalmic arteries after

endothelial removal. We used real-time PCR to determine mRNA expression of all five muscarinic receptor subtypes in murine ophthalmic arteries with and without endothelium. Because the selectivity of agonists and antagonists for individual muscarinic receptor subtypes was shown to be limited,^{1,22} we used muscarinic receptor knockout mice to perform functional studies on isolated ophthalmic arteries.

MATERIALS AND METHODS

Animals

All experiments 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. Experiments were performed in M₃ receptor knockout mice (M3R^{-/-}) and in respective wild-type controls. The generation of M3R^{-/-} mice has been described previously.²³ Briefly, the M₃ receptor gene was inactivated using mouse embryonic stem cells derived from 129SvEv mice. The resulting chimeric mice then were mated with CF-1 mice to generate M3R^{-/-} and wild-type mice with the following genetic contribution: 129SvEv (50%) × CF-1 (50%). The genotype of each mouse was determined by PCR of DNA isolated from tail biopsies. In all experiments, male mice at the age of 4 to 6 months were used.

Real-Time PCR Analysis

Since commercially available muscarinic receptor antibodies lack subtype-specificity in mice,^{24,25} we used real-time PCR to determine expression of individual muscarinic receptor subtypes in murine ophthalmic arteries. Muscarinic receptor gene expression was determined in isolated ophthalmic arteries from wild-type mice with either intact or removed endothelium. After mice had been killed by CO₂ inhalation, the eyes were removed immediately together with the retrobulbar tissue and placed in ice-cold PBS (Invitrogen, Karlsruhe, Germany). Then, ophthalmic arteries were isolated by using fine-point tweezers under a dissecting microscope. To remove the endothelium, a human hair was inserted into the artery and the luminal surface rubbed. Histologic and functional studies have confirmed that the endothelium of small arteries is removed successfully by this method.^{26,27} Following this procedure, each artery was cannulated with a micropipette at one end and flushed with cold PBS to remove residual endothelial cells from the lumen. In arteries in which the endothelium was left intact, the preparation time was adjusted to be similar as for arteries with removed endothelium. Next, arteries were transferred into a 1.5-ml tube and immediately snap frozen. Per mouse, the ophthalmic artery tree from one eye was used for the endothelium-removed group and from the other eye for the endothelium-intact group. No tissue samples from different animals were pooled. Subsequently, vessels were homogenized in lysis buffer using a homogenizing device (FastPrep; MP Biomedicals, Illkirch, France) and total RNA was isolated with the RNeasy Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. After isolation, mRNA was reverse transcribed with M-MLV reverse transcriptase and random hexamers (Promega, Mannheim, Germany). Quantitative PCR analysis was performed with the ViiA 7 system (Applied Biosystems, Darmstadt, Germany). Then, SYBR green was used for the fluorescent detection of DNA generated during PCR. The PCR reaction was performed in a total volume of 10 μ L and $\times 2$ SYBR Green master mix (QIAGEN); 2 μ L cDNA corresponding to 13 ng RNA was used as template. Published sequences for mouse M₁ (NM_007698), M₂ (NM_203491), M₃ (NM_033269),

M₄ (NM_007699), and M₅ (NM_205783) were used to design primers for PCR amplification. Primer sequences were M₁ sense 5'-TGA CAG GCA ACC TGC TGG TGC T-3' and antisense 5'-AAT CAT CAG AGC TGC CCT GCG G-3'; M₂ sense 5'-CGG ACC ACA AAA ATG GCA GGC AT-3' and antisense 5'-CCA TCA CCA CCA GGC ATG TTG TTG T-3'; M₃ sense 5'-CCT CTT GAA GTG CTG CGT TCT GAC C-3' and antisense 5'-TGC CAG GAA GCC AGT CAA GAA TGC-3'; M₄ sense 5'-TGT GGT GAG CAA TGC CTC TGT CAT G-3' and antisense 5'-GGC TTC ATC AGA GGG CTC TTG AGG A-3'; M₅ sense 5'-ACC ACT GAC ATA CCG AGC CAA GCG-3' and antisense 5'-TTC CCG TTG TTG AGG TGC TTC TAC G-3'; β -actin sense 5'-CAC CCG CGA GCA CAG CTT CTT T-3' and antisense 5'-AAT ACA GCC CGG GGA GCA TC-3'. Standard negative controls were used for the PCR. A control lacking reverse transcriptase was used to check for DNA contamination of the isolated RNA. We also used a reverse transcription control without RNA to check for contamination of the chemicals used. In both controls, no PCR product was detected, indicating that genomic DNA was absent and the purity of the chemicals high. For positive control, we used RNA isolated from mouse brain. The expression levels of muscarinic receptor subtype mRNA were normalized to β -actin using the Δ Ct method. Parallelism of standard curves was confirmed.

Measurements of Vascular Reactivity

Mice were killed by CO₂ inhalation, and the eyes were removed immediately together with the retrobulbar tissue, and were 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, 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 micropipettes, as reported previously.^{5,28} 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, and video sequences were captured to a personal computer for off-line analysis. The organ chamber was perfused continuously with oxygenated and carbonated Krebs buffer at 37°C and pH 7.4. Viability of vessels was assessed as satisfactory when at least 50% constriction from resting diameter in response to high KCl solution (100 mM) was achieved. In experiments with endothelium-removed arteries, functional endothelial damage was confirmed by absence of dilation in response to the endothelium-dependent vasodilator bradykinin (10⁻⁵ M; Sigma-Aldrich, St. Louis, MO) in phenylephrine-precontracted arteries.

Arteries were allowed to equilibrate for 60 minutes before study. During this time, ophthalmic arteries from both mouse genotypes developed similar spontaneous myogenic tone. In a first series of experiments, the endothelium of ophthalmic arteries from wild-type mice was removed by rubbing the luminal surface with a human hair. To test whether acetylcholine evoked muscarinic receptor-mediated responses in endothelium-denuded vessels, responses to acetylcholine (10⁻⁴ M) were examined in nonprecontracted ophthalmic arteries before and after incubation with the nonsubtype-selective muscarinic receptor blocker atropine (10⁻⁵ M; Sigma-Aldrich).

To test whether the M₃ receptor is involved in mediating acetylcholine-induced vasoconstriction in endothelium-denuded ophthalmic arteries, concentration-response curves were obtained in nonprecontracted endothelium-denuded ophthalmic arteries from M3R^{-/-} and wild-type mice to acetylcholine (10⁻⁹–10⁻⁴ M) and to the nitric oxide (NO) donor nitroprusside (10⁻⁹–10⁻⁴ M). Moreover, responses to acetylcholine

(10^{-9} – 10^{-4} M) and to the NO donor nitroprusside (10^{-9} – 10^{-4} M) were examined in nonprecontracted endothelium-intact ophthalmic arteries. To exclude the possibility that potential differences in acetylcholinesterase activity in the vascular wall between wild-type and M₃R^{-/-} mice affected acetylcholine-induced vascular reactivity in endothelium-denuded arteries, we additionally tested responses to carbachol (10^{-4} M; Sigma-Aldrich), an acetylcholinesterase-resistant analog of acetylcholine. In dose-response experiments, acetylcholine and nitroprusside were applied cumulatively into the bath solution. All reported concentrations are final molar concentrations in the organ chamber bath.

Statistical Analysis

Data are presented as mean $\pm$ SE, and *n* represents the number of mice per group. For statistical analysis of muscarinic receptor subtype mRNA expression levels, the Kruskal-Wallis 1-way ANOVA followed by the Dunn's multiple comparison test was used. Vascular responses are presented as percentage of change in luminal artery diameter from resting diameter. Statistical significance among concentration-responses was calculated using the Brunner test for nonparametric analysis of longitudinal data. For comparison of responses to acetylcholine before and after incubation with atropine, the Wilcoxon signed-rank test was used. Baseline luminal artery diameters and responses to KCl were compared using the Mann-Whitney *U* test. A value of *P* < 0.05 was defined as significant.

RESULTS

Muscarinic Receptor mRNA Expression in Ophthalmic Arteries

We quantified muscarinic receptor mRNA expression in ophthalmic arteries with intact and with removed endothelium to examine whether the expression levels of individual muscarinic receptor subtypes depend on the presence of endothelium. We detected mRNA for all five muscarinic receptor subtypes in endothelium-intact ophthalmic arteries, and M₃ receptor mRNA was most abundant (Fig. 1A). In ophthalmic arteries with removed endothelium, still mRNA for all five muscarinic receptor subtypes was found to be expressed. However, M₁, M₂, and M₄ receptor mRNA expression increased, whereas the expression levels of M₃ and M₅ receptor mRNA remained unchanged (Fig. 1B).

Responses of Ophthalmic Arteries

To examine whether cholinergic responses in ophthalmic arteries with damaged endothelium were mediated by muscarinic receptors, we stimulated arteries from wild-type mice with acetylcholine (10^{-4} M) before and after addition of atropine (10^{-5} M), a nonselective muscarinic receptor blocker. Acetylcholine induced vasoconstriction responses in ophthalmic arteries that were virtually abolished following atropine treatment ($18 \pm 3\%$ vs. $2 \pm 3\%$, $^{*}P < 0.05$, nontreated versus treated, *n* = 6, Fig. 2), indicative of the involvement of muscarinic receptors.

To test whether the M₃ receptor is involved in mediating responses of ophthalmic arteries with damaged endothelium, concentration-response experiments to acetylcholine (10^{-9} – 10^{-4} M) were conducted in endothelium-removed ophthalmic arteries from M₃R^{-/-} and wild-type mice (*n* = 6 per genotype). After 60 minutes of equilibration, arteries from both mouse genotypes developed similar spontaneous myogenic tone (16

FIGURE 1. Relative mRNA expression of individual muscarinic receptor subtypes (M₁–M₅) normalized to β-actin transcripts in ophthalmic arteries from wild-type mice. (A) In arteries with endothelium, mRNA for all receptor subtypes was expressed, but M₃ receptor mRNA was most abundant (M₃ vs. M₄ and M₅, $^{***}P < 0.001$; M₃ vs. M₂, $^{*}P < 0.05$; *n* = 10). (B) In arteries without endothelium, also muscarinic receptor mRNA for all five subtypes was detected; however, M₁, M₂, and M₄ receptor mRNA expression increased, whereas the expression levels of M₃ and M₅ receptor mRNA remained unchanged. Values are expressed as means $\pm$ SE (M₁ vs. M₅, $^{*}P < 0.01$; M₂ vs. M₅, $^{+}P < 0.05$; M₃ vs. M₅, $^{***}P < 0.001$; M₃ vs. M₄, $^{**}P < 0.01$; *n* = 10).

$\pm 4\%$ vs. $19 \pm 5\%$ reduction from initial luminal diameter measured 5 minutes after pressurization in M₃R^{-/-} versus wild-type mice, respectively). After development of stable myogenic tone, baseline luminal diameter was $123 \pm 14 \mu\text{m}$ in M₃R^{-/-} mice and $113 \pm 12 \mu\text{m}$ in wild-type mice, and did not differ between arteries of both genotypes. Acetylcholine evoked concentration-dependent vasoconstriction in arteries from wild-type mice. Maximal reduction in luminal diameter was $24 \pm 5\%$ at 10^{-4} M. In contrast, only a negligible reduction in luminal diameter of $2 \pm 3\%$ was observed in arteries from M₃R^{-/-} mice ($^{***}P < 0.001$, M₃R^{-/-} versus wild-type mice, Fig. 3A). The NO donor nitroprusside elicited dose-dependent vasodilation in ophthalmic arteries with damaged endothelium from both genotypes. Increase in luminal diameter to 10^{-4} M nitroprusside was $28 \pm 7\%$ and $23 \pm 6\%$ in wild-type and M₃R^{-/-} mice, respectively (Fig. 3B), and did not differ between both genotypes. Reduction in luminal artery diameter in response to high KCl solution (10^{-1} M) also was similar in endothelium-removed arteries from both mouse genotypes ($77 \pm 4\%$ and $73 \pm 5\%$ in wild-type and M₃R^{-/-} mice, Fig. 3C).

FIGURE 2. Responses of ophthalmic arteries from wild-type mice with removed endothelium to acetylcholine (10^{-4} M) before and after incubation with the nonsubtype-selective muscarinic receptor blocker atropine (10^{-5} M). Remarkably, atropine virtually abolished acetylcholine-induced vasoconstriction. Values are expressed as mean $\pm$ SE ($^{*}P < 0.05$, treated versus nontreated, *n* = 6).

FIGURE 3. Responses of ophthalmic arteries from wild-type and M3R^{-/-} mice to acetylcholine, nitroprusside, and KCl. Values are expressed as means $\pm$ SE ($n = 6$ per drug, concentration and genotype). (A) After endothelial removal, acetylcholine evoked concentration-dependent constriction responses in arteries from wild-type mice, but only negligible responses in arteries from M3R^{-/-} mice ($***P < 0.001$, M3R^{-/-} versus wild type). (B) In contrast, nitroprusside produced similar vasodilation responses in endothelium-denuded arteries from wild-type and M3R^{-/-} mice. (C) Constriction responses to KCl also were similar in ophthalmic arteries from wild-type and M3R^{-/-} mice. (D) In arteries with intact endothelium, acetylcholine induced pronounced vasodilation in wild-type mice, but almost no responses in M3R^{-/-} mice ($***P < 0.001$, M3R^{-/-} versus wild type). (E) Vasodilation to nitroprusside was retained in endothelium-intact arteries from M3R^{-/-} mice. (F) Vasoconstriction to KCl also was similar in endothelium-intact ophthalmic arteries from wild-type and M3R^{-/-} mice.

We previously reported that in phenylephrine-preconstricted endothelium-intact ophthalmic arteries from wild-type mice of the same genetic background as used in the present study, acetylcholine and carbachol elicited pronounced vasodilation, whereas almost no reactivity was seen in M3R^{-/-} mice.⁵ Because responses to acetylcholine have been reported to differ depending on the level of preconstriction,^{18,29} we tested vascular responses to acetylcholine in endothelium-intact ophthalmic arteries without pharmacologic preconstriction in the present study. After 60 minutes of equilibration, endothelium-intact arteries from both mouse genotypes developed similar spontaneous myogenic tone ($13 \pm 4\%$ vs. $15 \pm 5\%$ in M3R^{-/-} versus wild-type mice). Baseline luminal diameter was $125 \pm 15 \mu\text{m}$ in M3R^{-/-} mice and $117 \pm 13 \mu\text{m}$ in wild-type mice after development of stable myogenic tone, and did not differ between both genotypes. Acetylcholine (10^{-9} – 10^{-4} M) elicited concentration-dependent dilation in ophthalmic arteries from wild-type mice. Increase in luminal diameter in response to 10^{-4} M acetylcholine was $17 \pm 5\%$ (Fig. 3D). In contrast, ophthalmic arteries from M3R^{-/-} mice showed almost no reactivity to acetylcholine. A negligible constriction of $3 \pm 2\%$ was observed at 10^{-4} M ($***P < 0.001$, M3R^{-/-} versus wild-type mice, Fig. 3D). In contrast, nitroprusside (10^{-9} – 10^{-4} M) induced concentration-dependent vasodi-

lation in wild-type and M3R^{-/-} mice (Fig. 3E) that did not differ between both genotypes. Increase in luminal artery diameter to 10^{-4} M nitroprusside was $23 \pm 4\%$ and $17 \pm 4\%$ in wild-type and M3R^{-/-} mice, respectively (Fig. 3E). Reduction in luminal artery diameter in response to high KCl solution (10^{-1} M) also was similar in endothelium-intact arteries from both mouse genotypes ($81 \pm 4\%$ and $80 \pm 4\%$ in wild-type and M3R^{-/-} mice, Fig. 3F).

To exclude the possibility that differences in acetylcholinesterase activity in the vascular wall between wild-type and M3R^{-/-} mice affected vascular responses to acetylcholine, we examined responses of ophthalmic arteries with damaged endothelium to carbachol, an acetylcholine analog, which is resistant to degradation by acetylcholinesterase. Similar to acetylcholine, carbachol induced pronounced reduction in luminal diameter in arteries from wild-type mice ($21 \pm 6\%$), but only a negligible vasoconstriction of $3 \pm 3\%$ in arteries from M3R^{-/-} mice ($**P < 0.01$, M3R^{-/-} versus wild-type mice, Fig. 4).

DISCUSSION

There are several major new findings in this study. First, muscarinic receptor mRNA of all five subtypes was expressed

FIGURE 4. Responses of ophthalmic arteries from wild-type and M3R^{-/-} mice with damaged endothelium to carbachol. Vasoconstriction in response to carbachol was almost completely abolished in ophthalmic arteries from M3R^{-/-} mice. Values are expressed as mean $\pm$ SE (** P < 0.01, M3R^{-/-} versus wild-type, n = 5 per concentration and genotype).

in both endothelium-intact and endothelium-denuded ophthalmic arteries. In arteries with intact endothelium, M₃ receptor mRNA was most abundant. After endothelial removal, however, M₁, M₂, and M₄ receptor mRNA expression levels increased, whereas M₃ and M₅ receptor mRNA expression levels remained unchanged. These findings suggested that mRNA expression of M₁, M₂, and M₄ receptors is relatively higher in smooth muscle or adventitia than in the endothelium. Since no pronounced decrease of any receptor subtype occurred after endothelial removal, obviously none of the receptor subtypes was expressed exclusively in the endothelium. Second, acetylcholine evoked vasoconstrictor responses in endothelium-damaged arteries from wild-type mice that were primarily mediated by activation of muscarinic receptors, because responses were virtually abolished by the muscarinic receptor blocker atropine. As opposed to arteries from wild-type mice, endothelium-damaged ophthalmic arteries from M3R^{-/-} mice showed no significant reduction in luminal diameter either to acetylcholine or to the acetylcholinesterase-resistant analog of acetylcholine, carbachol, suggesting that activation of the M₃ receptor is critical for mediating cholinergic vasoconstriction after endothelial damage and excluding the possibility that an increased acetylcholinesterase activity in the vascular wall accounts for the negligible responses to acetylcholine in M3R^{-/-} mice. Furthermore, acetylcholine concentration-dependently dilated nonprecontracted ophthalmic arteries with intact endothelium from wild-type mice, but not from M3R^{-/-} mice, further confirming that cholinergic vasoconstriction in endothelium-damaged arteries from wild-type mice was endothelium-independent. Smooth muscle reactivity of ophthalmic arteries was neither affected by deletion of the M₃ receptor gene nor by endothelial damage, because both KCl and the endothelium-independent vasodilator nitroprusside evoked vasoconstrictor and vasodilator responses, respectively, that did not differ between arteries from both genotypes either with intact or with damaged endothelium. These data provided evidence that the M₃ receptor subtype mediates endothelium-dependent vasodilation and endothelium-independent vasoconstriction.

In various vascular beds, acetylcholine has been reported to evoke vasodilation in endothelium-intact and vasoconstriction in endothelium-damaged blood vessels.^{2,30} This dual action of acetylcholine also has been observed in ocular blood vessels, such as bovine and murine retinal vessels as well as in porcine ophthalmic arteries.^{6,29,31} The vasoconstrictor response in experimentally endothelium-damaged arteries, including those from the eye, has been attributed to muscarinic receptors localized on vascular smooth muscle cells.^{2,29} So far, the

contribution of individual muscarinic receptor subtypes to cholinergic vasoconstriction responses has not been studied in ocular blood vessels, but has been examined in arteries from some other vascular beds by using subtype-preferring antagonists. Some of these studies suggested that different muscarinic receptor subtypes mediate constriction and relaxation within the same blood vessel. For example, studies in isolated lamb coronary arteries found functional muscarinic receptor subtype heterogeneity between endothelial and smooth muscle cells by suggesting that vasoconstriction is mediated via activation of M₃ receptors while endothelial receptors of another subclass, possibly M₁, modulate these responses.³² Studies in cat cerebral blood vessels suggested that M₁ receptors mediate vascular smooth muscle constriction while M₃ receptors mediate endothelium-dependent vasodilation.^{33,34} In human pulmonary arteries, only M₃ receptors were suggested to mediate acetylcholine-induced vasoconstriction, whereas M₁ and M₃ receptors mediate endothelium-dependent acetylcholine-induced relaxation.³⁵

In contrast, studies in coronary arteries from different species suggested that cholinergic constriction and relaxation are mediated by the same muscarinic receptor subtype within one vessel. For example, data obtained in isolated simian coronary arteries suggested that acetylcholine and carbachol mediate vasodilation in endothelium-intact vessels via activation of endothelial M₃ receptors and vasoconstriction in endothelium-denuded vessels via activation of M₃ receptors located on smooth muscle cells.³⁶ Similar findings were reported in equine coronary artery rings.³⁷ Our findings in murine ophthalmic arteries are in line with the findings in coronary arteries, suggesting that the M₃ receptor mediates vasodilation in endothelium-intact ophthalmic arteries and vasoconstriction after endothelial damage.

The pathophysiologic relevance of our findings must be established in models for endothelial dysfunction, such as animal models for diabetes or oxidative stress. It may be possible that vasoconstrictor responses to acetylcholine are even potentiated in the presence of a dysfunctional endothelium. Support for this concept comes from studies reporting that the vasoconstrictor action of various substances was even increased in the presence of endothelial cells compared to endothelium-denuded arteries.³⁸ Based on studies in the aorta of spontaneously hypertensive rats, it has been suggested that the M₃ receptor mediates the release of contracting and relaxing factors from endothelium.³⁹ Thus, changes in endothelial signaling mechanisms in states of endothelial dysfunction might further augment the contractile effects of acetylcholine. A study on human subjects undergoing cardiac catheterization reported that acetylcholine induced vasodilation in normal epicardial coronary arteries, but vasoconstriction in arteries with angiographically evident arteriosclerosis.¹⁷

Systemically and topically applied cholinergic agents were reported to increase ocular blood flow in experimental animals^{40,41} and humans,⁴² suggesting that acetylcholine may be involved in regulation of ocular perfusion by inducing vasodilation via activation of muscarinic receptors. However, as yet, no evidence for abnormal vasoconstriction to acetylcholine has been provided for the ocular circulation in a model of endothelial dysfunction. Such a study may be useful to understand the pathophysiologic relevance of acetylcholine or other transmitters in ocular diseases associated with endothelial dysfunction, such as NAION and glaucoma.

Whether our findings can be extrapolated to humans remains to be established. In several vascular beds, functional similarities between humans and mice regarding cholinergic endothelium-dependent vasodilation responsiveness have been reported. For example, the M₅ receptor was suggested to

mediate vasodilation in cerebral arteries of humans and mice.^{43,44} Moreover, in human forearm resistance arteries cholinergic vasodilation responses were reported to be mediated by the M₃ receptor, which corresponds to findings in mouse cutaneous and skeletal muscle arteries.^{45,46} However, as yet to our knowledge no such data are available for muscarinic receptor-mediated contractile responses in states of endothelial damage or dysfunction.

Our findings in murine ophthalmic arteries suggest that the M₃ receptor mediates endothelium-dependent vasodilation and endothelium-independent vasoconstriction. Thus, it appears that these two responses cannot be influenced selectively by classical subtype-selective muscarinic receptor ligands. On the other hand, M₃ receptors can couple to multiple signaling pathways, and a given compound can be a strong agonist for one, but a much weaker agonist or even antagonist for another signaling response, a phenomenon called functional selectivity, ligand-directed signaling, or biased agonism. Recent studies detected biased agonism at M₃ receptors for several ligands.^{47,48} An M₃ receptor ligand blocking vasoconstriction and simultaneously promoting vasodilation may prove interesting, but has not been tested in this regard.

In conclusion, the data of our study provided evidence that in murine ophthalmic arteries, the M₃ muscarinic acetylcholine receptor subtype mediates vasodilation in endothelium-intact arteries, whereas it evokes vasoconstriction in endothelium-damaged arteries. Experiments in gene-targeted mice may help to define clearly the role of these receptors in regulation of ocular vascular tone and blood flow, and to design specific drugs aimed at modulating local perfusion. To clarify the role of endothelium-dependent and -independent cholinergic responses in regulation of ocular vascular tone under pathophysiologic conditions, studies using in vitro and in vivo models of endothelial dysfunction are needed.

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