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

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Submitted: November 3, 2013
Accepted: December 26, 2013


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 nonpreconstricted 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,2,22 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,23,25 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, arteries from wild-type mice with either intact or removed gene expression was determined in isolated ophthalmic arteries. Functional studies have confirmed that the endothelium of the artery and the luminal surface rubbed. Histologic and To remove the endothelium, a human hair was inserted into the eye 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 test whether the M3 receptor is involved in mediating dilation in response to the endothelium-dependent vasodilator achieved. In experiments with endothelium-removed arteries, responses to acetylcholine (104 M) 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 subjected 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.

**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 subjected 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, responses to acetylcholine (10–4 M) were examined in nonpreconstricted 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 nonpreconstricted 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^{-12} - 10^{-4} \text{M}) and to the NO donor nitroprusside (10^{-6} - 10^{-4} \text{M}) were examined in nonpreconstricted 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} \text{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 ± 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 \( M_4 \) 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, \( M_1 \), \( M_2 \), and \( M_3 \) receptor mRNA expression increased, whereas the expression levels of \( M_4 \) and \( M_5 \) 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} \text{M}) before and after addition of atropine (10^{-5} \text{M}), a nonselective muscarinic receptor blocker. Acetylcholine induced vasodistraction responses in ophthalmic arteries that were virtually abolished following atropine treatment (18 ± 3\% vs. 2 ± 3\%, \( *P < 0.05 \), nontreated versus treated, \( n = 6 \)), indicative of the involvement of muscarinic receptors.

To test whether the \( M_4 \) receptor is involved in mediating responses of ophthalmic arteries with damaged endothelium, concentration-response experiments to acetylcholine (10^{-9} - 10^{-4} \text{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 ± 4\% vs. 19 ± 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 ± 14 \mu \text{m} in M3R^{−/−} mice and 113 ± 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 ± 5\% at 10^{-4} \text{M}. In contrast, only a negligible reduction in luminal diameter of 2 ± 5\% 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} \text{M} nitroprusside was 28 ± 7\% and 23 ± 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} \text{M}) also was similar in endothelium-removed arteries from both mouse genotypes (77 ± 4\% and 75 ± 5\% in wild-type and M3R^{−/−} mice, Fig. 3C).

**FIGURE 1.** Relative mRNA expression of individual muscarinic receptor subtypes (\( M_1 - M_5 \)) 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 \( M_1 \) receptor mRNA was most abundant (\( M_1 \) vs. \( M_4 \) and \( M_5 \), ***\( P < 0.001 \); \( M_3 \) vs. \( M_2 \), *\( P < 0.05 \) vs. \( M_5 \), +\( P < 0.05 \); \( M_3 \) vs. \( M_5 \), ***\( P < 0.001 \); \( M_1 \) vs. \( M_4 \), **\( P < 0.01 \); \( n = 10 \)). (B) In arteries without endothelium, also muscarinic receptor mRNA for all five subtypes was detected; however, \( M_1 \), \( M_3 \), and \( M_4 \) receptor mRNA expression increased, whereas the expression levels of \( M_2 \) and \( M_5 \) receptor mRNA remained unchanged. Values are expressed as means ± SE (\( M_1 \) vs. \( M_5 \), *\( P < 0.01 \); \( M_2 \) vs. \( M_5 \), ***\( P < 0.001 \); \( M_1 \) vs. \( M_5 \), **\( P < 0.01 \); \( n = 10 \)).

**FIGURE 2.** Responses of ophthalmic arteries from wild-type mice with removed endothelium to acetylcholine (10^{-4} \text{M}) before and after incubation with the nonsubtype-selective muscarinic receptor blocker atropine (10^{-3} \text{M}). Remarkably, atropine virtually abolished acetylcholine-induced vasoconstriction. Values are expressed as mean ± SE (\( P < 0.05 \), treated versus nontreated, \( n = 6 \)).
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 ± 4% vs. 15 ± 5% in M3R−/− versus wild-type mice). Baseline luminal diameter was 125 ± 15 μm in M3R−/− mice and 117 ± 13 μm in wild-type mice after development of stable myogenic tone, and did not differ between both genotypes. Acetylcholine (10−8–10−4 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 ± 5% (Fig. 3D). In contrast, ophthalmic arteries from M3R−/− mice showed almost no reactivity to acetylcholine. A negligible constriction of 3 ± 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 vasodilation 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 ± 6% and 17 ± 4% in wild-type and M3R−/− mice, respectively (Fig. 3E). Reduction in luminal artery diameter in response to high KCl solution (10−2 M) also was similar in endothelium-intact arteries from both mouse genotypes (81 ± 4% and 80 ± 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 ± 6%), but only a negligible vasoconstriction of 3 ± 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...
carbachol, suggesting that activation of the M3 receptor is critical for mediating cholinergic vasoconstriction after endothelial damage, because both KCl and the endothelium-denuded vessels via activation of M3 receptors located on smooth muscle cells.60 Similar findings were reported in equine coronary artery rings.77 Our findings in murine ophthalmic arteries are in line with the findings in coronary arteries, suggesting that the M3 receptor mediates vasoconstriction 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 vasoconstriction in normal epicardial coronary arteries, but vasoconstriction in arteries with angiographically evident arteriosclerosis.78

Systemically and topically applied cholinergic agents were reported to increase ocular blood flow in experimental animals40,41 and humans,42 suggesting that acetylcholine may be involved in regulation of ocular perfusion by inducing vasodilatation 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 vasodilatation responsiveness have been reported. For example, the M3 receptor was suggested to contribute 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-prefering antagonists. Some of these studies suggested that different muscarinic receptor subtypes mediate contraction 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.52 Studies in cat cerebral blood vessels suggested that M1 receptors mediate vascular smooth muscle constriction while M3 receptors mediate endothelium-dependent vasodilatation.53,54 In human pulmonary arteries, only M4 receptors were suggested to mediate acetylcholine-induced vasoconstriction, whereas M3 and M4 receptors mediate endothelium-dependent acetylcholine-induced relaxation.55
mediated vasodilation in cerebral arteries of humans and mice.\textsuperscript{43,44} Moreover, in human forearm resistance arteries cholinergic vasodilation responses were reported to be mediated by the M\textsubscript{3} receptor, which corresponds to findings in mouse cutaneous and skeletal muscle arteries.\textsuperscript{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 M\textsubscript{3} 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, M\textsubscript{3} 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 M\textsubscript{3} receptors for several ligands.\textsuperscript{47,48} An M\textsubscript{3} receptor ligand blocking vasoconstriction and simultaneously promoting vasodilatation 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 M\textsubscript{3} 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.

Acknowledgments

The authors thank Brigitte Ruhland, Department of Internal Medicine II, University Medical Center Regensburg, Germany, for expert technical assistance with PCR experiments. Supported by funds from the Intramural Research Program of the National Institutes of Health (NIH), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, Maryland. The authors alone are responsible for the content and writing of the paper.

Disclosure: A. Gericke, None; A. Steege, None; C. Manicam, None; T. Böhmcr, None; J. Wess, None; N. Pfeiffer, None

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