Identification of the Muscarinic Acetylcholine Receptor Subtype Mediating Cholinergic Vasodilation in Murine Retinal Arterioles

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PURPOSE. To identify the muscarinic acetylcholine receptor subtype that mediates cholinergic vasodilation in murine retinal arterioles.

METHODS. Muscarinic receptor gene expression was determined in murine retinal arterioles using real-time PCR. To assess the functional relevance of muscarinic receptors for mediating vascular responses, retinal vascular preparations from muscarinic receptor-deficient mice were studied in vitro. Changes in luminal arteriole diameter in response to muscarinic and nonmuscarinic vasoactive substances were measured by video microscopy.

RESULTS. Only mRNA for the M₃ receptor was detected in retinal arterioles. Thus, M₃ receptor-deficient mice (M3R^{-/-}) and respective wild-type controls were used for functional studies. Acetylcholine concentration-dependently dilated retinal arterioles from wild-type mice. In contrast, vasodilation to acetylcholine was almost completely abolished in retinal arterioles from M3R^{-/-} mice, whereas responses to the nitric oxide (NO) donor nitroprusside were retained. Carbachol, an acetylcholinesterase-resistant analog of acetylcholine, also evoked dilation in retinal arterioles from wild-type, but not from M3R^{-/-} mice. Vasodilation responses from wild-type mice to acetylcholine were negligible after incubation with the non-subtype-selective muscarinic receptor blocker atropine or the NO synthase inhibitor N^G-nitro-L-arginine methyl ester, and were even reversed to contraction after endothelial damage with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

CONCLUSIONS. These findings provide evidence that endothelial M₃ receptors mediate cholinergic vasodilation in murine retinal arterioles via activation of NO synthase. (*Invest Ophthalmol Vis Sci.* 2011;52:7479–7484) DOI:10.1167/iovs.11-7370

Muscarinic acetylcholine receptors are involved in various physiologic actions in the eye, such as regulation of intraocular pressure, pupil size, and ocular growth.¹ Moreover, muscarinic receptors were shown to mediate acetylcholine-induced vasodilation in retinal blood vessels.^{2,3} The contribution of individual muscarinic receptor subtypes to vasodilation responses in retinal arterioles, however, remains unknown at present.

Five muscarinic receptor subtypes, M₁–M₅, have been identified.⁴ These receptors are generally grouped according to their preferential signaling pathways, either to the mobilization of intracellular calcium via activation of phospholipase Cβ (M₁, M₃, M₅) or to the inhibition of adenylyl cyclase (M₂, M₄).⁵ In most vascular beds, activation of muscarinic receptors induces powerful vasodilation via the release of vasorelaxing agents from the endothelium.^{6–8} Remarkably, the expression pattern of muscarinic receptor subtypes and their role in mediating vascular responses differ substantially between individual vascular beds.^{9–14} Previous studies aimed at identifying the muscarinic receptor subtypes involved in regulation of ocular perfusion suggested that the M₃ receptor mediates cholinergic vasodilation in the choroid of pigeons and in ophthalmic arteries of mice.^{15,16} Thus, from a clinical point of view, the M₃ receptor may represent a pharmacologic target to modulate ocular vascular tone. However, before the potential clinical usefulness of this approach can be pursued further, it is important to identify the muscarinic receptor subtype mediating cholinergic responses in retinal arterioles. Thus, we used real-time PCR to determine mRNA expression of all five muscarinic receptor subtypes in murine retinal arterioles. Given the lack of highly selective agonists and antagonists for individual muscarinic receptor subtypes, we used muscarinic receptor knockout mice to perform functional studies in vascular preparations from the retina.

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.¹⁷ Briefly, the M₃ receptor gene was inactivated using mouse embryonic stem cells derived from 129SvEv mice. The resulting chi-

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meric mice were then 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 ranging in age from 4 to 6 months were used.

Real-Time PCR Analysis

Muscarinic receptor gene expression was determined in isolated retinal arterioles from wild-type mice using real-time PCR. Since all five muscarinic receptor subtypes are known to be expressed in the mouse brain, we used RNA from the whole brain of wild-type mice as positive control.¹⁸ After mice had been killed by CO₂ inhalation, a midline abdominal and thoracic incision was made. Then, the inferior vena cava was cut and the left cardiac ventricle cannulated with a needle that was connected to a silicon tube, and perfused with 10 mL of phosphate-buffered solution (PBS; Invitrogen, Karlsruhe, Germany) followed by an injection of iron oxide suspension (1% in 20 mL of PBS). The injected iron oxide micro-particles are trapped in retinal arterioles, thereby enabling their visualization and differentiation from venules. Former studies in rabbit renal microvessels revealed no evidence of morphologic vascular damage by injection of iron oxide particles.¹⁹ After this procedure, the eyes were immediately removed and placed in ice-cold PBS. Subsequently, the retina was removed from the eye under a dissecting microscope and retinal arterioles were carefully isolated by using fine-point tweezers, transferred into a 1.5-mL tube, and immediately snap frozen. To increase RNA yield, arterioles from five different mice were pooled. Subsequently, vessels were homogenized in lysis buffer using a homogenizing device (FastPrep; MP Biomedicals, Illkirch, France). After homogenization, total RNA was extracted with a commercial kit (Absolutely RNA Nanoprep; Stratagene, La Jolla, CA) according to the manufacturer's protocol.

After complete DNA digestion, the RNA was reverse transcribed with the use of a reverse-transcription kit (Superscript; Invitrogen, Karlsruhe, Germany) and random hexamers. Quantitative PCR analysis was performed (GeneAmp StepOne Plus; Applied Biosystems, Darmstadt, Germany). Nucleic acid stain (SYBR Green; Biotline, 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 μ L with 0.4 pmol/ μ L of each primer and 2 $\times$ ready-to-use reaction mix (ImmoMix; Biotline); 2 μ L cDNA corresponding to 10 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'. 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

Due to the small size of murine retinal arterioles (inner diameter < 30 μ m), we established a method that allowed us to measure vascular reactivity in the mouse retina without the need of isolation and cannulation of individual vessels. A similar preparation has been used previously by Kulkarni et al.²⁰ to measure vascular reactivity in the bovine retina. After mice had been killed by CO₂ inhalation, the eyes were immediately removed, 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, and 11 glucose (Carl Roth GmbH, Karlsruhe, Germany). Then, the retrobulbar tissue was removed under a dissecting microscope using fine-point tweezers and microsurgical scissors, taking care that the ophthalmic artery was not damaged. Once the surrounding tissue was removed, the ophthalmic artery and the orbital branches were ligated at the open ends with a 10-0 nylon monofilament suture. To exclude the possibility that responses of the ophthalmic artery to vasoactive substances applied during the experiment might affect retinal arteriolar diameter, we deactivated the retrobulbar vasculature by immersing the intact eye globe for 10 seconds into 70% ethanol followed by an intensive wash in cold Krebs buffer. In previous tests, we observed that proximal and distal segments of the ophthalmic artery lost the ability to contract in response to KCl (10⁻¹ M) and the thromboxane mimetic 9,11-dideoxy-9 α ,11 α -methanoeperoxy prostaglandin F₂ α (U-46619, 10⁻⁶ M) following this procedure. Moreover, intracellular Ca²⁺ transients in response to acetylcholine (10⁻⁴ M) and U-46619 (10⁻⁶ M) were absent in ethanol-treated segments of the ophthalmic artery loaded with the Ca²⁺-sensitive dye fura-2. Thus, it is very unlikely that the ophthalmic artery exerted a significant effect on the retinal vasculature during the experiments.

Subsequently, the globe was opened and the cornea, iris, sclera, and lens were removed. A piece of sclera around the optic nerve was left to prevent injury and torsion of the very delicate distal part of the ophthalmic artery. Then, the preparation was placed into an organ chamber filled with cold Krebs buffer and the ophthalmic artery was cannulated with a glass micropipette and secured with 10-0 nylon monofilament suture. After this procedure, the retina was placed onto a transparent plastic platform, which had an indentation to fit the optic nerve and the retrobulbar blood vessels (Fig. 1), and four radial incisions were made, which enabled us to spread out the retina. Then, a stainless steel ring was placed onto the retina to fix it to the bottom (Fig. 1). Afterward, retinal vessels were pressurized via the micropipette located in the ophthalmic artery using a reservoir filled with Krebs solution to a level corresponding to 50 mm Hg and imaged

FIGURE 1. (A) Sketch of the experimental setup for videomicroscopic measurements of retinal arteriole responses. An isolated retina with the optic nerve and the ophthalmic artery left attached was mounted onto a transparent plastic platform and fixed with a steel ring. To pressurize vessels, the ophthalmic artery was cannulated with a micropipette that was connected to a reservoir filled with Krebs solution. (B) Photograph from above showing the experimental setup. At the 6 o'clock position, a micropipette is visible that was used to pressurize retinal arterioles via the ophthalmic artery. (C) First-order retinal arteriole with a red blood cell inside.

FIGURE 2. Relative mRNA expression of individual muscarinic receptor subtypes (M_1 – M_5) normalized to β -actin transcripts in whole brain (positive control) (A) and retinal arterioles (B) from wild-type mice. As expected, mRNA of all five muscarinic receptor subtypes was detected in the brain. However, mRNA of the M_3 receptor subtype only was found to be expressed in retinal arterioles. The values are averages of triplicate measurements and expressed as mean $\pm$ SE.

under brightfield conditions using a video camera (TK-C1381; JVC Deutschland GmbH, Friedberg, Germany) mounted on an upright microscope (Olympus Vanox-T AH-2; Olympus Deutschland GmbH, Hamburg, Germany). The magnification resulted from a $\times 100$ water-immersion objective lens (LUMPlanFL, 1.0 numerical aperture; Olympus Deutschland GmbH) and $\times 2.5$ projection on a 0.5-inch chip digital camera (TK-C1381; JVC Deutschland GmbH). The spatial resolution of the system was 11 pixels/ μ m. Video sequences were captured to a personal computer for analysis. The organ chamber was continuously circulated with oxygenated and carbonated Krebs buffer at 37°C and pH 7.4. For our studies, we used retinal arterioles of the first order. Differentiation between arterioles and venules was made by morphology (arterioles were of smaller diameter than that of venules) and by the flow direction of the remaining red blood cells (centrifugal in arterioles and centripetal in venules) directly after pressurizing the vessels.

After 30 minutes of equilibration, arterioles were precontracted with the thromboxane mimetic U-46619 (5×10^{-9} to 10^{-7} M; Cayman Chemical, Ann Arbor, MI) to 50–70% of the initial vessel diameter. Then, responses of retinal arterioles from wild-type and $M3R^{-/-}$ mice to acetylcholine (10^{-8} to 10^{-5} M; Sigma-Aldrich, Munich, Germany) and to the nitric oxide (NO) donor sodium nitroprusside (10^{-8} to 10^{-5} M; Sigma-Aldrich) were tested by cumulative application of the respective substances into the organ chamber. To exclude the possibility that potential differences in retinal acetylcholinesterase activity between wild-type and $M3R^{-/-}$ mice affected acetylcholine-induced vascular reactivity, we additionally examined responses to carbachol (10^{-5} M; Sigma-Aldrich), an acetylcholinesterase-resistant analog of acetylcholine. To further examine the underlying mechanisms of cholinergic vasodilation, we tested responses in retinal arterioles from wild-type mice to acetylcholine (10^{-5} M) before and after incubation with the nonselective muscarinic receptor blocker atropine (10^{-5} M; Sigma-Aldrich) and the NO synthase inhibitor N_ω -nitro-L-arginine methyl ester (L-NAME, 10^{-4} M; Sigma-Aldrich). To test whether responses to acetylcholine were endothelium dependent, we damaged the endothelium of retinal arterioles from wild-type mice by intravascular perfusion with Krebs buffer containing 0.3% of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma-Aldrich) applied via the ophthalmic artery for approximately 1 minute, and examined responses to acetylcholine (10^{-5} M) and nitroprusside (10^{-5} M). As controls, we used retinal arterioles that had been perfused with Krebs buffer (vehicle) only.

Statistical Analysis

Data are presented as means $\pm$ SE. Vascular responses are presented as percentage of change in luminal diameter, and n represents the num-

ber of mice per group. Statistical significance among concentration-responses was calculated using the Brunner test for nonparametric analysis of longitudinal data. The Mann-Whitney test was applied for comparison of single-dose responses of retinal arterioles from wild-type and $M3R^{-/-}$ mice to carbachol and to compare responses of CHAPS-treated with vehicle-treated retinal arterioles to acetylcholine and nitroprusside. For comparison of responses to acetylcholine before and after incubation with atropine or L-NAME, the Wilcoxon signed-rank test was used. A value of $P < 0.05$ was defined as significant.

RESULTS

Muscarinic Receptor mRNA Expression in Retinal Arterioles

By the use of real-time PCR, we recently demonstrated that murine ophthalmic arteries expressed mRNA of all five muscarinic receptor subtypes.¹⁶ Using the same primers, we conducted analogous studies in isolated retinal arterioles from five wild-type mice of the same genetic background as that used in the previous study. Since all five muscarinic receptor subtypes are known to be expressed in mouse brain, we used mRNA from the whole brain as a positive control. As expected, all five muscarinic receptor subtypes were expressed in the brain. Remarkably, we detected mRNA for the M_3 receptor only in retinal arterioles (Fig. 2).

Vascular Responses

To examine whether M_3 receptors play a role in mediating cholinergic vasodilation of retinal arterioles, we conducted functional studies in retinal vascular preparations from $M3R^{-/-}$ and wild-type mice. Baseline luminal diameters of first-order retinal arterioles (before precontraction) were $16.2 \pm 1.3 \mu\text{m}$ ($n = 6$) and $17.1 \pm 1.4 \mu\text{m}$ ($n = 6$) in $M3R^{-/-}$ and wild-type mice, and did not differ between the two genotypes. Acetylcholine (10^{-8} to 10^{-5} M) elicited dose-dependent dilation in retinal arterioles from wild-type mice. The maximal increase in luminal arteriole diameter to 10^{-5} M acetylcholine was $26 \pm 4\%$ ($n = 6$) (Fig. 3A). In contrast, retinal arterioles from $M3R^{-/-}$ mice showed almost no reactivity to acetylcholine. A negligible dilation of $1 \pm 2\%$ ($n = 6$) was observed at a concentration of 10^{-5} M (** $P < 0.01$, $M3R^{-/-}$ vs. wild-type mice; Fig. 3A). To examine whether deletion of the M_3 receptor gene affected responsiveness of vascular smooth muscle to NO, we examined responses of retinal arterioles to the NO

FIGURE 3. Responses of retinal arterioles from wild-type and $M3R^{-/-}$ mice to acetylcholine and nitroprusside. (A) Vasodilation to acetylcholine was virtually abolished in retinal arterioles from $M3R^{-/-}$ mice. (B) In contrast, responses of retinal arterioles to the NO donor nitroprusside did not differ between wild-type and $M3R^{-/-}$ mice. Values are expressed as mean $\pm$ SE ($n = 6$ per concentration and genotype; ** $P < 0.01$, $M3R^{-/-}$ vs. wild-type mice).

donor nitroprusside. Nitroprusside (10^{-8} to 10^{-5} M) produced concentration-dependent dilation of retinal arterioles that did not differ between wild-type and M3R^{-/-} mice (Fig. 3B). Maximal vasodilation to 10^{-5} M nitroprusside was $47 \pm 6\%$ ($n = 6$) and $42 \pm 6\%$ ($n = 6$) in wild-type and M3R^{-/-} mice, respectively.

To exclude the possibility that differences in retinal acetylcholinesterase activity between wild-type and M3R^{-/-} mice affected vascular responses to acetylcholine, we examined responses of retinal arterioles to carbachol, an acetylcholine analog, which is resistant to degradation by acetylcholinesterase. Similar to acetylcholine, carbachol (10^{-5} M) induced pronounced vasodilation in arterioles from wild-type mice ($23 \pm 5\%$, $n = 6$), but only an insignificant increase in retinal arteriole diameter in M3R^{-/-} mice ($3 \pm 3\%$, $n = 6$; $**P < 0.01$, M3R^{-/-} vs. wild-type mice; Fig. 4).

To confirm that cholinergic responses in the retinal circulation were mediated by muscarinic receptors, we tested responses of retinal arterioles from wild-type mice to acetylcholine before and after addition of atropine (10^{-5} M), a non-subtype-selective muscarinic receptor blocker. After atropine treatment, responses to acetylcholine were negligible ($29 \pm 6\%$ vs. $4 \pm 3\%$, $n = 5$; $*P < 0.05$, nontreated versus treated; Fig. 5A). To identify the postreceptor mechanisms by which acetylcholine mediates vasodilation in the retina, we tested responses of retinal arterioles from wild-type mice to acetylcholine before and after incubation with the NO synthase inhibitor L-NAME (10^{-4} M). Remarkably, L-NAME almost completely abolished acetylcholine-induced vasodilation in retinal arterioles. Changes in luminal diameter were $27 \pm 5\%$ and $-2 \pm 6\%$ in nontreated and treated arterioles, respectively ($n = 5$; $*P < 0.05$, nontreated versus treated; Fig. 5B). To test whether cholinergic vasodilation was endothelium dependent in the retina, we tested responses to acetylcholine and to the NO donor nitroprusside in retinal arterioles from wild-type mice that had been perfused with CHAPS, to damage the endothelium, and compared them with responses of vehicle-treated arterioles. Remarkably, retinal arterioles that had been treated with CHAPS did not dilate but contracted in response to acetylcholine. Changes in luminal diameter were $28 \pm 8\%$ and $-13 \pm 6\%$ in vehicle-treated and CHAPS-treated arterioles, respectively ($n = 5$ per group; $**P < 0.01$, vehicle-treated versus CHAPS-treated; Fig. 6A). In contrast, nitroprusside elicited pronounced vasodilation that was similar in vehicle-treated and CHAPS-treated arterioles ($36 \pm 7\%$ vs. $31 \pm 5\%$, $n = 5$; $P > 0.05$, vehicle-treated versus CHAPS-treated; Fig. 6B).

FIGURE 4. Responses of retinal arterioles from wild-type and M3R^{-/-} mice to carbachol. Vasodilation in response to carbachol (10^{-5} M) was almost completely abolished in retinal arterioles from M3R^{-/-} mice. Values are expressed as mean $\pm$ SE ($n = 6$ per genotype; $**P < 0.01$, M3R^{-/-} vs. wild-type mice).

FIGURE 5. Responses of retinal arterioles from wild-type mice to acetylcholine (10^{-5} M) before and after incubation with the non-subtype-selective muscarinic receptor blocker atropine (10^{-5} M) (A) and the NO synthase inhibitor L-NAME (10^{-4} M) (B). Remarkably, vasodilation responses were negligible after incubation with either of the two blockers. Values are expressed as mean $\pm$ SE ($n = 5$ per group; $*P < 0.05$, treated versus nontreated).

DISCUSSION

The major goal of the present study was to identify the muscarinic receptor subtype that mediates vasodilation of murine retinal arterioles to acetylcholine. Since commercially available muscarinic receptor antibodies lack subtype specificity in mice and rats, we used real-time PCR to determine expression of individual muscarinic receptor subtypes in murine retinal arterioles.^{21–23} Remarkably, we detected mRNA of the M₃ receptor only in isolated retinal arterioles. Due to the lack of muscarinic receptor agonists and antagonists with pronounced subtype selectivity, we used M₃ receptor-deficient mice to assess the functional relevance of this receptor subtype in retinal arterioles. Although there are some subtype-preferring agonists and antagonists for individual muscarinic receptors (including the M₃ receptor) available, it has been shown that their selectivity is only moderate.⁴ In particular, the M₅ receptor shares very similar functional and ligand-binding properties with the M₃ receptor, which hampers the differentiation between these two receptor subtypes by classical pharmacologic approaches.²⁴

By modification of a method that has previously been used for measurements of vascular reactivity in the bovine retina, we were able to measure responses of murine retinal arterioles

FIGURE 6. Responses of retinal arterioles from wild-type mice to acetylcholine (10^{-5} M) (A) and to the NO donor nitroprusside (10^{-5} M) (B) that had been perfused either with Krebs buffer (vehicle) only or with Krebs buffer containing 0.3% of CHAPS before. Remarkably, acetylcholine produced vasoconstriction after perfusion with CHAPS, whereas vasodilation responses to nitroprusside were retained. Values are expressed as mean $\pm$ SE ($n = 5$ per group; $**P < 0.01$, CHAPS-treated versus vehicle-treated).

by video microscopy with high optic resolution *in vitro*.²⁰ Using this method, we observed that retinal arterioles from wild-type mice had the ability to dilate in response to acetylcholine. In contrast, deletion of the M_3 receptor gene almost completely abolished acetylcholine-induced vasodilation of retinal arterioles. However, responses to the NO donor nitroprusside did not differ between wild-type and $M3R^{-/-}$ mice, suggesting that the lack of M_3 receptors did not affect the ability of vascular smooth muscle to relax. The retina of mice and other species was shown to exert substantial acetylcholinesterase activity.^{25,26} To exclude the possibility that potential differences in retinal acetylcholinesterase activity between wild-type and $M3R^{-/-}$ mice were responsible for the different reactivities of retinal arterioles to acetylcholine, we tested responses of retinal arterioles to the acetylcholine analog carbachol, which is resistant to degradation by acetylcholinesterase. Remarkably, carbachol did not induce significant vasodilation in retinal arterioles from $M3R^{-/-}$ mice, thus excluding the possibility that changes in acetylcholinesterase activity might contribute to the different acetylcholine responses in wild-type and $M3R^{-/-}$ mice. An earlier study suggested that nicotinic acetylcholine receptors may participate in cholinergic vasodilation in canine retinal blood vessels.²⁷ However, in our vascular preparations, vasodilation to acetylcholine was negligible after treatment with the non-subtype-selective muscarinic receptor antagonist atropine, suggesting that acetylcholine-induced vasodilation in murine retinal arterioles is predominantly mediated by muscarinic receptors.

A previous study using electrical stimulation of parasympathetic nerve pathways and intravenous administration of subtype-preferring muscarinic receptor antagonists suggested that endothelial M_3 receptors mediate cholinergic vasodilation in the choroid of pigeons.¹⁵ Using mice with targeted disruption of the M_3 receptor gene, we recently demonstrated that responses of ophthalmic arteries to acetylcholine are also mediated by M_3 receptors.¹⁶ Thus, the present study together with the previous findings in other ocular vascular preparations indicate that the M_3 receptor subtype is the predominant mediator of cholinergic vasodilation within the ocular circulation. Interestingly, murine retinal arterioles seem to differ with regard to their cholinergic vasodilation mechanisms from murine cerebral vessels, where acetylcholine-induced responses were shown to be mediated by the M_5 receptor subtype.¹³ Thus, retinal and cerebral vascular tone may be independently influenced by selective targeting of these two receptor subtypes. It remains to be established, however, whether this difference between cerebral and retinal vessels also exists in humans. Although the M_5 receptor has also been proposed to mediate vasodilation in the human brain, there are so far no data regarding the expression and functional role of individual muscarinic receptor subtypes in human retinal vessels.¹¹

Our data suggest that cholinergic vasodilation of murine retinal arterioles is primarily mediated by NO, since blockade of NO synthases by L-NAME almost completely abolished responses to acetylcholine. This finding is consistent with previous studies in bovine and rat retinal arterioles, where responses to acetylcholine were shown to be abolished or impaired after incubation with NO synthase blockers.^{20,28,29} After endothelial damage with CHAPS, no dilation but vasoconstriction of retinal arterioles to acetylcholine was observed, whereas dilation to the NO donor nitroprusside was retained. This finding indicates that the source of NO needed for cholinergic vasodilation in the retina is the endothelium. A possible explanation for the vasoconstriction responses of retinal arterioles after endothelial damage is that muscarinic receptors are also localized on vascular smooth muscle cells or pericytes and can induce contraction of these cells when activated. Support for this hypothesis comes from studies in isolated

bovine retinal arteries, where acetylcholine-induced vasodilation was reversed to contraction when the endothelium was removed, and from experiments in isolated pericytes of the rat retina demonstrating that the muscarinic receptor agonist oxotremorine was able to induce Ca^{2+} -dependent contractions.^{3,30} These findings suggest that a vasoconstrictor response of cholinergic agents is masked in retinal arterioles when the endothelium is intact.

The physiologic role of acetylcholine in the control of retinal perfusion is unknown at present. In contrast to most other blood vessels, the retinal vasculature lacks autonomic innervation.^{31,32} Thus, the vessels and the starburst amacrine cells have been proposed to be possible sources of acetylcholine according to their ability to synthesize this substance.³³⁻³⁵ However, whether stimulation of these cells may evoke acetylcholine release in conjunction with muscarinic receptor-mediated vascular responses must still be established. A series of *in vivo* studies in humans has shown that systemic administration of the non-subtype-selective muscarinic receptor antagonist atropine does not affect retinal and choroidal perfusion.³⁶⁻³⁸ Thus, either pharmacologic blockade of muscarinic receptors can be compensated by other vascular mechanisms to keep ocular blood flow constant or the receptors do not play a major role in regulation of retinal and choroidal perfusion. In contrast, activation of muscarinic receptors with pilocarpine was shown to increase pulsatile ocular blood flow in subjects with ocular hypertension.³⁹ Moreover, various cholinergic substances were reported to increase diameter or blood flow in retinal, choroidal, and retrobulbar vessels of different animal species *in vivo*.^{29,40,41} Thus, even if acetylcholine does not play a role in physiologic regulation of ocular blood flow, selective muscarinic receptor agonists may become therapeutically useful to modulate perfusion in the retina and choroid. However, before the M_3 receptor may be considered as a therapeutic target to increase retinal perfusion under pathophysiologic conditions, the mechanisms mediating vasoconstriction in retinal arterioles with dysfunctional endothelium need to be determined. This is particularly important because most diseases associated with impaired ocular perfusion, such as diabetic retinopathy and glaucoma, are also associated with endothelial dysfunction.^{42,43}

In conclusion, the data of the present study provide evidence that cholinergic vasodilation of murine retinal arterioles is mediated by endothelial M_3 muscarinic acetylcholine receptors, involving activation of NO synthase.

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References

1. Duncan G, Collison DJ. Role of the non-neuronal cholinergic system in the eye: a review. *Life Sci*. 2003;72:2013-2019.
2. Benedito S, Prieto D, Nielsen PJ, Nyborg NC. Role of the endothelium in acetylcholine-induced relaxation and spontaneous tone of bovine isolated retinal small arteries. *Exp Eye Res*. 1991;52:575-579.
3. Hoste AM, Andries IJ. Contractile responses of isolated bovine retinal microarteries to acetylcholine. *Invest Ophthalmol Vis Sci*. 1991;32:1996-2005.
4. Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev*. 1998;50:279-290.
5. Wess J, Eglén RM, Gautam D. Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. *Nat Rev Drug Discov*. 2007;6:721-733.
6. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288:373-376.

7. Faraci FM, Sigmund CD. Vascular biology in genetically altered mice: smaller vessels, bigger insight. *Circ Res*. 1999;85:1214-1225.
8. Leung HS, Leung FP, Yao X, et al. Endothelial mediators of the acetylcholine-induced relaxation of the rat femoral artery. *Vascul Pharmacol*. 2006;44:299-308.
9. Phillips JK, Vidovic M, Hill CE. Variation in mRNA expression of alpha-adrenergic, neurokinin and muscarinic receptors amongst four arteries of the rat. *J Auton Nerv Syst*. 1997;62:85-93.
10. Bruning TA, Hendriks MG, Chang PC, Kuypers EA, van Zwieten PA. In vivo characterization of vasodilating muscarinic-receptor subtypes in humans. *Circ Res*. 1994;74:912-919.
11. Elhousseiny A, Hamel E. Muscarinic—but not nicotinic—acetylcholine receptors mediate a nitric oxide-dependent dilation in brain cortical arterioles: a possible role for the M5 receptor subtype. *J Cereb Blood Flow Metab*. 2000;20:298-305.
12. Pesic S, Jovanovic A, Grbovic L. Muscarinic receptor subtypes mediating vasorelaxation of the perforating branch of the human internal mammary artery. *Pharmacology*. 2001;63:185-190.
13. Yamada M, Lamping KG, Duttaroy A, et al. Cholinergic dilation of cerebral blood vessels is abolished in M(5) muscarinic acetylcholine receptor knockout mice. *Proc Natl Acad Sci U S A*. 2001;98:14096-14101.
14. Lamping KG, Wess J, Cui Y, Nuno DW, Faraci FM. Muscarinic (M) receptors in coronary circulation: gene-targeted mice define the role of M2 and M3 receptors in response to acetylcholine. *Arterioscler Thromb Vasc Biol*. 2004;24:1253-1258.
15. Zagvazdin Y, Fitzgerald ME, Reiner A. Role of muscarinic cholinergic transmission in Edinger-Westphal nucleus-induced choroidal vasodilation in pigeon. *Exp Eye Res*. 2000;70:315-327.
16. Gericke A, Mayer VG, Steege A, et al. Cholinergic responses of ophthalmic arteries in M3 and M5 muscarinic acetylcholine receptor knockout mice. *Invest Ophthalmol Vis Sci*. 2009;50:4822-4827.
17. Yamada M, Miyakawa T, Duttaroy A, et al. Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. *Nature*. 2001;410:207-212.
18. Wess J. Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. *Annu Rev Pharmacol Toxicol*. 2004;44:423-450.
19. Chaudhari A, Kirschenbaum MA. A rapid method for isolating rabbit renal microvessels. *Am J Physiol Renal Physiol*. 1988;254:F291-F296.
20. Kulkarni P, Joshua IG, Roberts AM, Barnes G. A novel method to assess reactivities of retinal microcirculation. *Microvasc Res*. 1994;48:39-49.
21. Jositsch G, Papadakis T, Haberberger RV, Wolff M, Wess J, Kummer W. Suitability of muscarinic acetylcholine receptor antibodies for immunohistochemistry evaluated on tissue sections of receptor gene-deficient mice. *Naunyn Schmiedebergs Arch Pharmacol*. 2009;379:389-395.
22. Pradidarcheep W, Labruyere WT, Dabhoiwala NF, Lamers WH. Lack of specificity of commercially available antisera: better specifications needed. *J Histochem Cytochem*. 2008;56:1099-1111.
23. Pradidarcheep W, Stallen J, Labruyere WT, Dabhoiwala NF, Michel MC, Lamers WH. Lack of specificity of commercially available antisera against muscarinic and adrenergic receptors. *Naunyn Schmiedebergs Arch Pharmacol*. 2009;379:397-402.
24. Eglen RM, Nahorski SR. The muscarinic M(5) receptor: a silent or emerging subtype? *Br J Pharmacol*. 2000;130:13-21.
25. Ross D, Cohen AI, McDougal DB Jr. Choline acetyltransferase and acetylcholine esterase activities in normal and biologically fractionated mouse retinas. *Invest Ophthalmol*. 1975;14:756-761.
26. Sastry BV. Cholinergic systems and multiple cholinergic receptors in ocular tissues. *J Ocul Pharmacol*. 1985;1:201-226.
27. Toda N, Zhang JX, Ayajiki K, Okamura T. Mechanisms underlying endothelium-independent relaxation by acetylcholine in canine retinal and cerebral arteries. *J Pharmacol Exp Ther*. 1995;274:1507-1512.
28. Kazama A, Ikomi F, Yashiro Y, Ohhashi T. Development of pressurized retinal resistance-sized arteriolar preparation with special reference to acetylcholine-induced nitric-oxide-mediated vasodilation. *Jpn J Physiol*. 2002;52:285-291.
29. Nakazawa T, Mori A, Saito M, Sakamoto K, Nakahara T, Ishii K. Vasodilator effects of adenosine on retinal arterioles in streptozotocin-induced diabetic rats. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;376:423-430.
30. Wu DM, Kawamura H, Sakagami K, Kobayashi M, Puro DG. Cholinergic regulation of pericyte-containing retinal microvessels. *Am J Physiol Heart Circ Physiol*. 2003;284:H2083-H2090.
31. Hogan MJ, Feeney L. The ultrastructure of the retinal blood vessels, I: the large vessels. *J Ultrastruct Res*. 1963;39:10-28.
32. Laties AM. Central retinal artery innervation. Absence of adrenergic innervation to the intraocular branches. *Arch Ophthalmol*. 1967;77:405-409.
33. Ferrari-Dileo G, Davis EB, Anderson DR. Biochemical evidence for cholinergic activity in retinal blood vessels. *Invest Ophthalmol Vis Sci*. 1989;30:473-477.
34. Masland RH, Mills JW, Hayden SA. Acetylcholine-synthesizing amacrine cells: identification and selective staining by using radioautography and fluorescent markers. *Proc R Soc Lond B Biol Sci*. 1984;223:79-100.
35. Tauchi M, Masland RH. The shape and arrangement of the cholinergic neurons in the rabbit retina. *Proc R Soc Lond B Biol Sci*. 1984;223:101-119.
36. Jandrasits K, Polak K, Luksch A, et al. Effects of atropine and propranolol on retinal vessel diameters during isometric exercise. *Ophthalmic Res*. 2001;33:185-190.
37. Polska E, Luksch A, Schering J, et al. Propranolol and atropine do not alter choroidal blood flow regulation during isometric exercise in healthy humans. *Microvasc Res*. 2003;65:39-44.
38. Fuchsjaeger-Mayrl G, Malec M, Amoako-Mensah T, Kolodjaschna J, Schmetterer L. Changes in choroidal blood flow during light/dark transitions are not altered by atropine or propranolol in healthy subjects. *Vision Res*. 2003;43:2185-2190.
39. Shaikh MH, Mars JS. The acute effect of pilocarpine on pulsatile ocular blood flow in ocular hypertension. *Eye*. 2001;15:63-66.
40. Mann RM, Riva CE, Stone RA, Barnes GE, Cranston SD. Nitric oxide and choroidal blood flow regulation. *Invest Ophthalmol Vis Sci*. 1995;36:925-930.
41. Okubo H, Gherezhiher T, Koss MC. Long posterior ciliary arterial blood flow and systemic blood pressure. *Invest Ophthalmol Vis Sci*. 1990;31:819-826.
42. Malecki MT, Osmenda G, Walus-Miarka M, et al. Retinopathy in type 2 diabetes mellitus is associated with increased intima-media thickness and endothelial dysfunction. *Eur J Clin Invest*. 2008;38:925-930.
43. Resch H, Garhofer G, Fuchsjaeger-Mayrl G, Hommer A, Schmetterer L. Endothelial dysfunction in glaucoma. *Acta Ophthalmol*. 2009;87:4-12.