Muscarinic (M) Receptors in Coronary Circulation
Gene-Targeted Mice Define the Role of M₂ and M₃ Receptors in Response to Acetylcholine

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Objective—Determining the role of specific muscarinic (M) receptor subtypes mediating responses to acetylcholine (ACh) has been limited by the specificity of pharmacological agents. Deletion of the gene for M₅ receptors abolished response to ACh in cerebral blood vessels but did not affect dilation of coronary arteries. The goal of this study was to determine the M receptors mediating responses to ACh in coronary circulation using mice deficient in M₂ or M₃ receptors (M₂⁻/⁻, M₃⁻/⁻, respectively).

Methods and Results—Coronary arteries from respective wild-type, M₂⁻/⁻, or M₃⁻/⁻ mice were isolated, cannulated, and pressurized. Diameter was measured with video microscopy. After preconstriction with U46619, ACh produced dose-dependent dilation of coronary arteries that was similar in wild-type and M₂⁻/⁻ mice. In contrast, dilation of coronary arteries from M₃⁻/⁻ mice to ACh was reduced by ≈80% compared with wild type. The residual response to ACh was atropine insensitive. Relaxation of coronary arteries to other stimuli was similar in M₂⁻/⁻ and M₃⁻/⁻ mice. Similar results were obtained in aorta rings.

Conclusion—These findings provide the first direct evidence that relaxation to ACh in coronary circulation is mediated predominantly by activation of M₃ receptors. (Arterioscler Thromb Vasc Biol. 2004;24:1253-1258.)

Key Words: acetylcholine  ■  muscarinic receptors  ■  genetically altered mice

Acetylcholine (ACh) is an important mediator of neurogenic vasodilation in coronary circulation1,2 as well as a major investigative tool for studies of endothelial function in experimental animals and in patients.3–5 Endothelial dysfunction, defined as impaired vasodilation to ACh, has been observed in many studies of cardiovascular diseases and may be associated with risk factors for coronary artery disease.3–5 In some cases, vascular responses to ACh are selectively altered,3–5 whereas responses to other endothelium-dependent dilators are minimally affected. To understand these mechanisms, including changes that occur with vascular dysfunction, it is important to define muscarinic (M) signaling at the molecular level. Although M receptor antagonists prevent ACh-induced relaxation,6–7 identification of specific M receptor subtypes mediating vascular relaxation to ACh has long been hampered by the lack of M antagonists with high selectivity for a single receptor subtype.8 In addition, overlapping expression patterns of different M receptors and identification of multiple M receptors within a given tissue contribute to the difficulty in linking specific M receptors with a specific physiological or pathophysiological response.9 Five major classes of M receptors, denoted M₁ through M₅, have been identified using pharmacological and molecular approaches.8 Depending on the specific receptor subtype, a wide variety of phenotypes has been observed in M receptor-deficient mice.10 These phenotypes have been reviewed recently in detail.10 Depending on the vascular bed and animal species, vascular relaxation or contraction to ACh has been suggested to be mediated by activation of different M receptors on the basis of pharmacological data.6,11–21 In cerebral circulation, we demonstrated that relaxation in response to ACh is mediated by activation of M₃ receptors using mice deficient in M₃ receptors.22 However, M₁ receptor activation did not mediate responses to ACh in coronary circulation.22 Previous studies using pharmacological approaches suggest that M₂ and M₃ receptor activation may mediate responses to ACh in coronary circulation.6,20 Genes for both receptors are expressed in vascular tissue.23 However, the relative importance of M₂ and M₃ receptors in coronary circulation is not clear. Conclusions regarding the physiological role of the individual M receptors are hampered by the limited specificity of the pharmacological agents tested.
because even “selective” M2 and M3 antagonists show high affinity for M1 and M4 receptors, respectively. Moreover, the pharmacological properties of the M1 receptor are very similar to those of the M3 subtype, raising the possibility that responses thought previously to be mediated by M1 receptors may in fact involve the activation of M3 receptors. Thus, the goal of this study was to determine the role of M2 and M3 receptors in mediating ACh-dependent vasodilation in coronary circulation using mice deficient in the expression of either receptor subtype. For comparison, we conducted analogous studies with mouse aorta, by far the most commonly used blood vessel for studies of vascular biology in mice.

Materials and Methods

M2−/− and M3−/− Mice

The generation of M2−/− and M3−/− mice has been described previously. The M2−/− mice and the corresponding wild-type mice had the following genetic background: 129SvEv (50%) × C57BL/6J (50%). The M2−/− mice and the corresponding wild-type mice had a slightly different genetic background: 229J (50%) × C57BL/6J (50%). For all experiments, adult male M2−/− and M3−/− mice and wild-type mice with the same respective genetic backgrounds (14 to 23 weeks of age) were used. Animal procedures were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee at the University of Iowa and the Veterans Affairs Medical Center.

Measurements of Vascular Reactivity

Responses of coronary arteries and aorta were measured using methods published previously. Mice were anesthetized with ketamine (100 mg/kg IP). The heart and thoracic aorta were removed rapidly and placed in ice-cold Krebs buffer. The anterior descending and circumflex arteries were isolated from the left ventricle under a dissecting microscope, cannulated and sutured onto micropipettes filled with Krebs buffer in an organ bath, and pressurized to 40 mm Hg. The vessel was imaged using a microscope and video camera, and lumen diameter measured from the video image with an electronic dimension analyzer. Arteries were allowed to equilibrate for 60 minutes before study. Isolated arteries were preconstricted submaximally with potassium acetate (3 to 4 mm in length), mounted on wires, and connected to a force transducer in an organ bath. Tension was increased over 60 minutes to 0.75 g. Vessels were allowed to equilibrate 60 minutes before study. Rings were precontracted with U46619 (3 to 5 μmol/L) to maintain a stable precontraction of 50% to 60% of maximal KCl response before dose-response curves to ACh, calcium ionophore A23187, or papavarine were measured. Responses to ACh and sodium nitroprusside in aorta from wild-type and M2−/− mice were also compared before and after atropine (30 μmol/L). Relaxation responses were expressed as percentage decrease in tension from preconstriction values.

RT-PCR Analysis

Total RNA was extracted from aorta and brain using the total RNA isolation kit from Invitrogen. Extracted RNA samples were treated with 4 U of RNase-free DNase (Ambion) at 37°C for 1 hour to remove residual genomic DNA. The RNA was then reverse transcribed with an oligo-dT16 primer and murine leukemia virus reverse transcriptase using the GeneAmp RNA PCR kit, as described by the manufacturer (Applied Biosystems). The RT step was omitted in control samples to test for the presence of contaminating genomic DNA. The reverse-transcribed products were screened for the presence of M1 through M5 cDNA by PCR using the GeneAmp RNA PCR kit (Applied Biosystems) and an Eppendorf Mastercycler thermal cycler (40 cycles of 94°C at 1 minute, 56°C at 2 minutes, and 72°C at 3 minutes). PCRs were performed in a final volume of 50 μL containing 10 μL of the RT reaction product (corresponding to ~0.2 μg RNA), 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L MgCl2, 1 mmol/L of each 2′-deoxynucleoside 5′-triphosphate, 100 ng each of the sense and the corresponding antisense primers, and 1.25 U of AmpliTaq DNA polymerase. The identity of the PCR products was confirmed by restriction analysis (data not shown). The RT-PCR products were separated on 1.5% agarose gels containing ethidium bromide and photographed under UV illumination.

Statistical Analysis

Data are presented as mean±SEM. Constrictions are presented as percentage of change in diameter from baseline diameter. Responses of vessels from the same mouse were averaged, and n represents the number of mice per group. Comparisons were made using a 1-way ANOVA with repeated measures followed by Student-Newman–
Keuls test to detect individual differences. \( P < 0.05 \) was defined as significant.

**Results**

**Responses of Coronary Arteries from \( \text{M}_2{-/-} \) and \( \text{M}_3{-/-} \) Mice**

To determine whether \( \text{M}_2 \) receptors are involved in responses to ACh, we compared responses of coronary arteries from wild-type mice and \( \text{M}_2{-/-} \) mice. ACh produced relaxation of coronary arteries from \( \text{M}_2{-/-} \) mice to 10 \( \mu \text{mol/L} \) ACh was 36±9\% (n=7) and 36±6\% (n=6), respectively. Nitroprusside also produced dose-dependent dilation of coronary arteries not different in wild-type and \( \text{M}_2{-/-} \) mice (data not shown). Thus, activation of \( \text{M}_2 \) receptors is not involved in mediating dilator responses to ACh in coronary circulation of the mouse.

To determine the role of \( \text{M}_3 \) receptors in mediating responses to ACh in coronary circulation, we compared responses of coronary arteries from wild-type mice and \( \text{M}_3{-/-} \) mice in wild-type mice, ACh produced dose-dependent vasodilation that was maximal at 10 \( \mu \text{mol/L} \) (31±5\%; n=18; Figure 1, right). Dilation of coronary arteries from \( \text{M}_3{-/-} \) mice to ACh was reduced by \( \approx 80\% \) compared with responses in wild-type mice (percentage of change diameter of 6±3\% at 10 \( \mu \text{mol/L} \), not statistically different from 0; n=17; Figure 1, right). Thus, activation of the \( \text{M}_3 \) receptor is the primary mechanism that mediates dilation to ACh in coronary arteries.

To study whether deletion of the gene for \( \text{M}_3 \) receptors affects responses to non-M vasodilators, we measured responses of coronary arteries from wild-type and \( \text{M}_3{-/-} \) mice to nitroprusside and papavarine. Nitroprusside and papavarine produced dose-dependent dilation of coronary arteries from wild-type mice. Maximal dilation to papavarine (percentage of change diameter at 200 \( \mu \text{mol/L} \), 67±2\% n=13, 72±6\% n=10) was not different in wild-type and \( \text{M}_3{-/-} \) mice, respectively. Similar results were obtained with nitroprusside (data not shown). Thus, deletion of the \( \text{M}_3 \) receptor gene selectively decreased responses to ACh.

**Responses of Aorta from \( \text{M}_2{-/-} \) and \( \text{M}_3{-/-} \) Mice**

To determine whether activation of \( \text{M}_2 \) receptors is involved in relaxation of aorta to ACh, we compared responses from wild-type and \( \text{M}_2{-/-} \) mice. Relaxation of aorta in response to ACh (Figure 2, left), A23187 (Figure 3, left), and papavarine (maximal response at 10 \( \mu \text{mol/L} \), papavarine: wild type 103±3\%, n=4; \( \text{M}_3{-/-} \) 106±2\%, n=4) was not different in wild-type and \( \text{M}_3{-/-} \) mice. Thus, as observed in coronary circulation, activation of \( \text{M}_3 \) receptors is not involved in mediating relaxation of aorta in response to ACh.

To determine whether the role of \( \text{M}_1 \) receptors in mediating responses to ACh was specific for coronary circulation, we also compared responses of aorta from wild-type and \( \text{M}_2{-/-} \) mice. ACh produced marked relaxation of aorta from wild-type mice (n=12; Figure 2, right). Relaxation to ACh at 10 \( \mu \text{mol/L} \) was reduced by \( \approx 60\% \) in aorta from \( \text{M}_2{-/-} \) mice (n=11; Figure 2, right). In contrast, relaxation to papavarine (maximal response at 10 \( \mu \text{mol/L} \): wild type 103±2\%, n=7; \( \text{M}_2{-/-} \) 105±1\%, n=6) and the calcium ionophore A23187, which releases NO via non-M receptor mechanism.
(Figure 3, right) were not different in aorta from wild-type and M$_3^{-/-}$ mice. Thus, vascular responses to activation of a non-M receptor mechanism, including another endothelium-dependent agonist, were normal in M$_3^{-/-}$ mice.

**Effects of Atropine on Responses to ACh**

To confirm that the responses to ACh were because of activation of M receptors, we compared responses before and after atropine (30 μmol/L), a nonselective M receptor blocker. In coronary circulation (n=6) and aorta (n=5) of wild-type mice, atropine reduced the dilation to ACh by 60% to 70% in both vessels (Figure 4) but had no effect on the dilation to ACh in M$_3^{-/-}$ mice (coronary arteries n=7; aorta n=5). Atropine had no effect on responses to nitroprusside in either coronary arteries or aorta (data not shown). Thus, the responses to ACh in the aorta and coronary circulation appear to be mediated primarily by activation of M receptors.

**RT-PCR Analysis of M Receptor Expression in Mouse Aorta**

A recent RT-PCR study showed that coronary arteries not only express M$_2$ but also M$_2$ receptors. To examine which M receptor subtypes are expressed in the mouse vascular tissue, we conducted analogous studies using mouse aorta total RNA. Because all 5 M receptors are known to be expressed in the brain, mouse brain total RNA served as a positive control. cDNA was prepared from total RNA samples and subjected to PCR amplification by using primer pairs specific to the individual mouse M receptor genes, as described in Materials and Methods. The identity of all amplified PCR products was confirmed by restriction enzyme analysis (data not shown). As expected, all 5 M receptors were found to be expressed in the brain (Figure 5). In contrast, only M$_2$ and M$_3$ receptor cDNA could be detected consistently in samples from mouse aorta (Figure 5). Whereas the M$_2$ signal was always very strong, the M$_3$ signal was usually weaker. Occasionally, very faint bands were also observed with the M$_1$-, M$_4$-, or M$_5$-specific primers. However, these signals were not always reproducible, probably because of the very low expression levels of these receptors in the mouse aorta.

**Discussion**

The major goal of the present study was to use gene-targeted mice to identify the M receptor subtype that mediates responses of coronary circulation to ACh. Deletion of the M$_3$
receptor in the mouse reduced coronary vasodilatation to ACh by \( \approx 80\% \). The response to ACh was primarily mediated by activation of M receptors because blockade of M receptor activation with atropine decreased responses to levels similar to deletion of the gene expressing M receptors. Relaxation of the aorta in response to ACh was also reduced to a large extent in M\( \sim -/- \) mice. In contrast, deletion of M receptors had no effect on responses to ACh in either coronary circulation or the aorta. Deletion of either M or M receptor did not affect responses to non-M vasodilators, such as papaverine and nitroprusside, or the endothelium-dependent agonist A23187. These data provide the first direct evidence regarding the importance (or lack thereof) of activation of M receptors and M receptors in mediating vasodilatation to ACh.

In previous studies from our laboratory, we tested the role of M receptors in mediating dilation to ACh in cerebral and coronary circulation. Cerebral blood vessels from M\( \sim -/- \) mice failed to dilate in response to ACh. In contrast, responses to ACh in coronary circulation from M\( \sim -/- \) mice were intact. These data provided the first physiological evidence of a role for M receptors in the blood vessels but suggest that this receptor subtype is not functionally important in coronary arteries.

Pharmacological studies and studies of mRNA expression have suggested diverse expression of M receptor subtypes in vascular tissue. In coronary circulation, studies with subtype-preferring M antagonists suggested a role for M receptors in mediating endothelium-dependent relaxation. However, the proper interpretation of such classical pharmacological studies is complicated by the fact that the subtype selectivity of the M antagonists used in these studies is relatively small. Moreover, the pharmacological properties of the M receptor are very similar to those of the M subtype, raising the possibility that responses thought previously to be mediated by M receptors may in fact involve the activation of M receptors. Finally, it is generally very difficult to predict the simultaneous involvement of 2 or more M receptor subtypes in a specific functional response by using antagonists of limited subtype selectivity.

The recent development of gene-targeted mice lacking specific M receptor subtypes allows a more definitive approach to defining the physiological roles of M2 and M receptors. Recent studies have examined the role of these receptors in function of nonvascular smooth muscle. Using a similar approach, the present study clearly indicates that M receptors mediate the majority of the response to ACh in coronary circulation and in aorta. The findings in this study and our previous study, indicating that vascular responses to ACh are mediated by M receptors in brain and predominantly by M receptors in heart, highlight the importance of studies designed to define M signaling at the molecular level in different vascular beds. Identification of tissue-specific mechanisms that mediate vascular responses may be beneficial in designing organ-specific pharmacological approaches for investigative study or to treat vascular disease.

Although a major component of the vasodilator response to ACh was mediated by M receptors, a minor component was M receptor independent. Consistent with a previous RT-PCR study using RNA isolated from human coronary arteries, we found that mouse aorta does not only express M but also M receptors. However, as observed recently with M\( \sim -/- \) mice, ACh-induced vasodilation responses in aorta and in coronary circulation were not significantly affected by the absence of M receptors, suggesting that the residual vascular responses remaining in the M\( \sim -/- \) mice are probably not mediated by M or M receptors. However, given the apparent abundance of M receptor mRNA in vascular tissue, we cannot rule out the possibility that a potential involvement of M receptors in this response may have remained undetected in the M\( \sim -/- \) mice because of the presence of the predominant M receptor pathway. One possibility is that M receptors are expressed by vascular muscle rather than endothelial cells and play a role in modulating signal pathways in smooth muscle. However, in coronary circulation, our results indicate that an atropine-insensitive mechanism mediates a very small (statistically insignificant) portion of the ACh response in wild-type and M\( \sim -/- \) mice. Although this small residual response may be mediated by nicotinic receptors, we feel this is unlikely because previous studies did not find evidence for nicotinic receptor activation in responses of normal arteries to ACh.

Studies in knockout models for isoforms of nitric-oxide synthase (NOS), including our own, have demonstrated compensatory upregulation of other pathways in the absence of endothelial NOS. An analogous compensation does not appear to occur after deletion of a single M receptor. Because the majority of the response to ACh was absent in coronary arteries of M\( \sim -/- \) mice, any compensatory expression of other M receptors must be very modest and does not result in preservation of a functional response.

In conclusion, the results of the present study provide direct evidence that ACh-induced vasodilation in aorta and coronary circulation is mediated predominantly by M receptors. These findings highlight the usefulness of using M receptor mutant mice to study the complex mechanisms regulating responses in different vascular beds.

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