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Vasodilatory effects of cholinergic agonists are greatly diminished in aorta from $M_3R^{-/-}$ mice

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Abstract

Acetylcholine interacts with endothelial muscarinic receptors to enhance nitric oxide (NO) release and thereby cause vasodilation. The present study was designed to determine if this effect of acetylcholine is mediated by muscarinic M_3 receptors. Thoracic aortae were isolated from wild-type (WT) and M_3 receptor knock out ($M_3R^{-/-}$) male mice, and endothelium-intact (I) and -denuded (D) aortic rings were bathed in physiological buffer. Preparations were utilized to examine the contractile response to phenylephrine (1×10^{-8}–3×10^{-4} M added cumulatively) and the vasodilatory actions of acetylcholine (10^{-8}–10^{-4} M), carbachol (10^{-9}–10^{-4} M), ATP (3×10^{-5} M) and the NO donor SIN-1 (10^{-4} M), each added in the presence of phenylephrine. Endothelium-dependent vasodilatory effects of acetylcholine and carbachol were obvious in aortae isolated from WT mice (56.3 ± 9.8% and 49.1 ± 4.1% reductions, respectively, in phenylephrine-induced contraction; $p < 0.05$), while acetylcholine and carbachol-associated relaxations observed in endothelium-intact $M_3R^{-/-}$ preparations (17.9 ± 2.6% and 13.5 ± 4.2% reductions, respectively) did not differ significantly from time-control values. ATP-induced, endothelium-dependent vasodilation was similar in preparations from $M_3R^{-/-}$ and WT mice, and SIN-1 elicited similar dilatory effects in intact and denuded WT and $M_3R^{-/-}$ segments. Phenylephrine concentration–response curves were shifted leftwards by removal of the endothelium in both groups (EC_{50} values: WT-I/D—$25.59 \pm 6.86/3.13 \pm 1.01 \times 10^{-7}$ M; $M_3R^{-/-}$I/D—$13.92 \pm 4.21/1.52 \pm 0.46 \times 10^{-7}$ M; both $p < 0.05$); however, the phenylephrine response did not differ significantly when compared between the WT and $M_3R^{-/-}$ groups. These results indicate that the attenuated vasodilatory effect of acetylcholine in endothelium-intact aortae from $M_3R^{-/-}$ mice is due to the absence of muscarinic M_3 receptors, and thus suggest that in mouse aorta, muscarinic M_3 receptors play a major role in the endothelium-dependent acetylcholine-induced vasodilation.

Keywords: Receptor subtype; Vascular relaxation; Cholinergic agonist; SIN-1; ATP

1. Introduction

In 1980, Furchgott and Zawadzki described a mediator that is released by the vascular endothelium in response to acetylcholine and causes vascular smooth muscle relaxation. This mediator, originally called endothelium derived relaxation factor (EDRF), was later shown to be nitric oxide (NO) (Palmer et al., 1987). NO synthesis and release by the endothelium is enhanced as a result of acetylcholine binding to and activation of muscarinic receptors present on the endothelial cell membrane. The NO enters neighboring vascular smooth muscle cells where it activates soluble guanylate cyclase, increasing cGMP levels and thereby causing smooth muscle relaxation (Ignarro, 1991; Ignarro et al., 1986).

Five molecularly distinct muscarinic receptor subtypes have been identified and designated M_1–M_5 (Wess, 1996). Previous studies using pharmacological approaches with muscarinic receptor agonists and antagonists suggest that

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M_3 receptors mediate the vasodilatory actions of acetylcholine in several vascular beds, including rat aorta (Boulanger et al., 1994), rabbit aorta (Jaiswal et al., 1991), feline middle cerebral artery (Dauphin and Hamel, 1990), rat renal artery (Eltze et al., 1993), guinea pig ileum submucosal artery (Bungardt et al., 1992), rabbit pial artery (Garcia-Villalon et al., 1991) and rat pulmonary artery (McCormack et al., 1988). However, the subtype selectivity of muscarinic receptor agonists and antagonists is often marginal, raising concern about conclusions drawn from these earlier studies. In addition, investigators have demonstrated that other muscarinic receptor subtypes, including M_1, M_2 and M_5, are expressed in various vascular beds (Elhusseiny et al., 1999; Phillips et al., 1997) and may be involved in the vasodilatory response. For example, it was suggested that M_1 receptors mediate cholinergic dilation in human pulmonary vasculature (Walch et al., 2001), and a recent report by Yamada et al. (2001a) showed that vasodilation elicited by acetylcholine is abolished in cerebral arteries isolated from muscarinic M_5 receptor-deficient mice, while effects of acetylcholine on coronary and carotid artery remain intact. Thus, it would appear that further studies are required to elucidate the muscarinic receptor subtypes involved in acetylcholine-induced, endothelium-dependent vascular relaxation among the various vascular beds. The present study was designed to determine if the endothelium-dependent vasodilatory actions of acetylcholine are present in thoracic aorta isolated from muscarinic M_3 receptor knock out ($M_3R^{-/-}$) mice.

2. Materials and methods

2.1. Muscarinic M_3 receptor knockout and wild-type mice

All experiments were performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. The M_3 receptor gene was inactivated using mouse embryonic stem cells derived from 129SvEv mice, as described previously (Yamada et al., 2001b). The resulting chimeric mice were then mated with CF-1 mice to generate $M_3R^{-/-}$ and wild-type (WT) 129SvEv/CF-1 hybrid mice (genetic contribution: 50% each). Male mice, 9–12 weeks of age, were used in all experiments. Body weight in the $M_3R^{-/-}$ mice was significantly lower than that in the WT group (33.8 ± 0.9 and 28.3 ± 1.2 g in WT and $M_3R^{-/-}$, respectively; $n = 13$/group). Previous reports indicate that this lower body weight in the knock out group is paralleled by hypophagia and lower leptin and insulin levels with no significant differences in serum glucose or total protein (Yamada et al., 2001b). Mean arterial blood pressure does not differ significantly when compared between WT and $M_3R^{-/-}$ mice (Fisher et al., 2004).

2.2. Isolated aortic preparations

Thoracic aortae were isolated from anesthetized mice and immediately bathed in Krebs-Henseleit (KH) solution (37 °C) containing (in mM): 118.0 NaCl, 25.0 $NaHC0_3$, 3.7 KCl, 1.0 KH_2P0_4, 1.4 $CaCl_2$, 1.2 $MgC1_2$, and 11.0 dextrose. The solution was saturated with 95% 0_2/5% $C0_2$ gas (pH—7.4). After careful removal of adventitia, each vessel was dissected into two ring segments (2–3 mm in length). The endothelial lining was removed from one segment by gentle rubbing. Each ring was suspended vertically between two 27-gauge stainless steel hooks, and the top hook was connected to a force–displacement transducer (Type FT03, Grass Instrument, Quincy, MA) for continuous recording of isometric tension (Grass Model 7 D Polygraph). The vessels were maintained at a resting tension of 250 mg and equilibrated for 90 min. A stable contractile response was acquired during equilibration by repeatedly increasing the buffer KCl concentration to 80 mM with subsequent washout after each steady-state contraction was obtained.

After equilibration, concentration–response curves for phenylephrine (1×10^{-8}–3×10^{-4} M) were obtained by cumulative addition. Each concentration of agonist was added to the medium only after the tissues reached a steady-state response at the previous level. Vasodilatory effects of acetylcholine (10^{-8}–10^{-4} M, added cumulatively), carbachol (10^{-9}–10^{-4} M, added cumulatively), ATP (3×10^{-5} M) and 3-Morpholinosydnonimine hydrochloride (SIN-1, 10^{-4} M, which liberates NO) were examined in segments pretreated with phenylephrine (1 or 3×10^{-4} M). The response to acetylcholine was compared in the absence or presence of L-nitro arginine methyl ester (L-NAME, 10^{-4} M), an inhibitor of NO synthase.

2.3. M_3 receptor genotyping

M_3 receptor knock out status was confirmed by polymerase chain reaction (PCR) analysis using genomic DNA extracted from the tails of $M_3R^{-/-}$ and WT mice. PCR primers used were: M3-A3 (5′ -aagaccacagtagcagtg), M3-B (5′ -ctctctacatccatagtccc) and M3-NEO9 (5′ -tggatgtggaatgtgtgcgagg). The M3-A3 and M3-B primer pairs generated a 226-bp fragment with DNA isolated from WT mice, whereas the M3-NEO9 generated a 170-bp fragment only with DNA from $M_3R^{-/-}$ mice (Fig. 1). PCR conditions were 94 °C for 10 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min.

2.4. Statistical analysis

EC_{50} values for the response to phenylephrine were obtained by graphical evaluation of individual concentration–response curves. Results are presented as means ± S.E.M. Data were compared by analysis of variance (ANOVA) and repeated measures ANOVA with Student–Newman Keuls post hoc test using SigmaStat (SPSS,

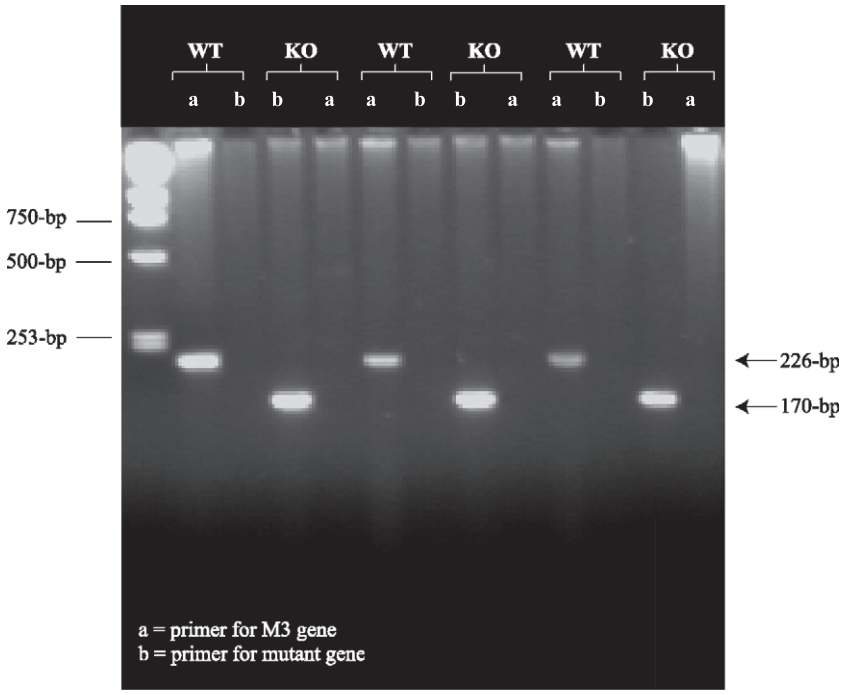

Fig. 1. Genotyping by PCR to confirm the knock out status of $M_3R^{-/-}$ mice. The lengths of the PCR products are indicated to the right. The lengths of the molecular weight markers in base pair are indicated to the left. The 170-bp band generated by PCR from the DNA extracted from $M_3R^{-/-}$ mice demonstrates the presence of the mutant muscarinic M_3 receptor allele, whereas the 226-bp band from the WT mice indicates the presence of the WT muscarinic M_3 receptor allele (see Materials and methods).

Chicago). The criterion for significance was a P value <0.05.

3. Results

3.1. Effects of phenylephrine

As shown in Fig. 2, the α-adrenoceptor agonist phenylephrine elicited a concentration-dependent vasoconstrictor effect in all groups. As reported previously (Stewart and Kennedy, 1999), removal of the endothelium from WT aortae resulted in the concentration–response curve being shifted to the left. EC_{50} values for phenylephrine in WT intact and WT denuded aortae were 25.59 ± 6.86 and $3.13 \pm 1.01 \times 10^{-7}$ M ($p<0.05$), respectively. The maximum response to phenylephrine tended to be greater in WT denuded as compared to WT intact preparations; however, this was not statistically significant. Similarly, removal of the endothelium from $M_3R^{-/-}$ aortae did not affect maximum phenylephrine-induced tension but did result in a leftward shift in the concentration–response curve (EC_{50} values -13.92 ± 4.21 and $1.52 \pm 0.46 \times 10^{-7}$ M in $M_3R^{-/-}$ intact and denuded aortae, respectively; $p<0.05$). The phenylephrine response did not differ significantly when compared between the WT and $M_3R^{-/-}$ groups in spite of the tendency for the potency of the agonist to be greater in the $M_3R^{-/-}$ as compared to the WT groups.

3.2. Effects of acetylcholine on phenylephrine-constricted aortae

The endothelium-dependent vasodilator effect of acetylcholine was depressed in $M_3R^{-/-}$ as compared to WT aortae. As shown in Fig. 3, in endothelium-intact WT preparations acetylcholine elicited a dose-dependent vasorelaxation that was observed at concentrations as low as

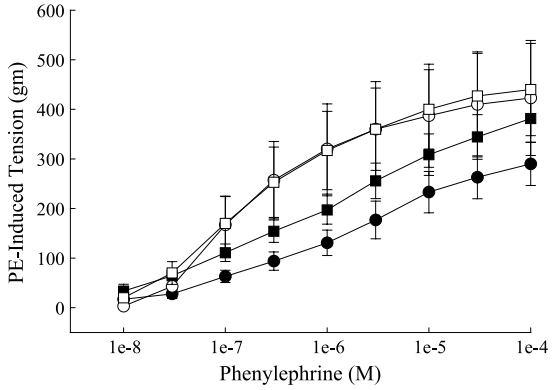

Fig. 2. Concentration-dependent vasoconstrictor effects of phenylephrine in thoracic aortae. Endothelium-intact (closed symbols) and endothelium-denuded (open symbols) preparations were obtained from WT (circles; $n=13$) and $M_3R^{-/-}$ (squares; $n=13$) mice, and bathed in oxygenated Krebs-Henseleit solution at 37 °C. After tissues were equilibrated at 250 mg resting tension, increasing concentrations of phenylephrine were added cumulatively to the buffer solution. Values for tension are reported as increases above resting tension. Vertical bars represent S.E.M.

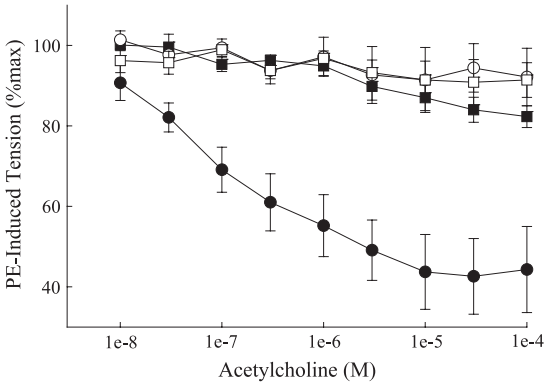

Fig. 3. Concentration-dependent effects of acetylcholine in phenylephrine-constricted thoracic aortae. Endothelium-intact preparations obtained from WT (circles; $n=5$/group) and $M_3R^{-/-}$ (squares; $n=6$/group) mice were bathed in oxygenated Krebs-Henseleit solution at 37 °C in the presence (open symbols) and absence (closed symbols) of 10^{-4} M L-NAME. After obtaining a steady-state response to 3×10^{-4} M phenylephrine, increasing concentrations of acetylcholine were added cumulatively to the buffer solution. Values for tension are reported as a percentage of the 3×10^{-4} M phenylephrine-induced tension. Vertical bars represent S.E.M.

3×10^{-8} M and was maximal at $1–3\times10^{-5}$ M. The maximum observed vasodilation was $56.3\pm9.8\%$ of the phenylephrine-induced contraction. In endothelium-intact aortae isolated from $M_3R^{-/-}$ mice, acetylcholine elicited no detectable effect at concentrations up to 1×10^{-6} M, but concentrations of 3×10^{-6} M and greater tended to cause a slight relaxation (maximum vasodilator response was $17.9\pm2.6\%$ of the phenylephrine-induced contraction). This relaxation, however, was not significantly different than that observed in time-control preparations that were not exposed to acetylcholine ($8.9\pm6.5\%$ of the phenylephrine-induced contraction at a time point equivalent to the end of the acetylcholine exposure, $n=10$). Similarly, the responses to acetylcholine in endothelium-denuded preparations isolated from WT and $M_3R^{-/-}$ mice (data not shown) and in endothelium-intact WT and $M_3R^{-/-}$ aortae that were pretreated with 10^{-4} M L-NAME (Fig. 3) were not different from values observed in time-controls.

3.3. Effects of carbachol on phenylephrine-constricted aortae

As observed with acetylcholine, the endothelium-dependent vasodilator effect of carbachol was depressed in $M_3R^{-/-}$ as compared to WT aortae. As shown in Fig. 4, in endothelium-intact WT preparations carbachol elicited a dose-dependent vasorelaxation that was observed at concentrations as low as 3×10^{-7} M and was maximal at $3\times10^{-5}–1\times10^{-4}$ M. The maximum observed vasodilation was $49.1\pm4.1\%$ of the phenylephrine-induced contraction. In endothelium-intact aortae isolated from $M_3R^{-/-}$ mice, carbachol elicited no effect at concentrations up to 1×10^{-6} M, but concentrations of 3×10^{-6} M and greater tended to cause a slight relaxation (maximum vasodilator response was $13.5\pm4.2\%$ of the phenylephrine-induced contraction). As with acetylcholine, this relaxation was not significantly different than that observed in time-control preparations.

3.4. Effects of ATP and SIN-1 on phenylephrine-constricted aortae

As shown in Fig. 5, ATP (3×10^{-5} M) elicited similar dilatory responses in endothelium-intact preparations isolated from WT and $M_3R^{-/-}$ mice ($32.8\pm11.7\%$ and $34.5\pm14.3\%$ of phenylephrine-induced maximum, respectively). In endothelium-denuded preparations, there was no significant difference between ATP treatment and time-control (7.8 ± 2.7 and 13.5 ± 2.7, respectively).

The relaxation elicited by SIN-1 (10^{-4} M) did not differ when compared among endothelium-intact and -denuded preparations isolated from WT and $M_3R^{-/-}$ mice. The maximum dilation in endothelium-intact and -denuded preparations from WT mice was 75.4 ± 6.1 and $81.7\pm8.8\%$ of phenylephrine-induced maximum, respectively. Corresponding values in endothelium-intact and -denuded preparations from $M_3R^{-/-}$ mice were $73.1\pm7.1\%$ and $78.0\pm6.8\%$ (Fig. 5).

4. Discussion

Results of this study indicate that the endothelium-dependent vasodilation elicited by acetylcholine in aortae from WT mice is markedly depressed, if not absent, in preparations isolated from $M_3R^{-/-}$ mice. Previous studies using muscarinic receptor subtype selective agonists and antagonists suggested that muscarinic M_3 receptors are

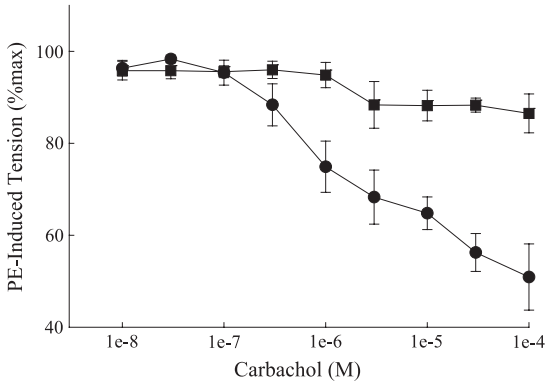

Fig. 4. Concentration-dependent effects of carbachol in phenylephrine-constricted thoracic aortae. Endothelium-intact preparations obtained from WT (circles; $n=4$) and $M_3R^{-/-}$ (squares; $n=4$) mice were bathed in oxygenated Krebs-Henseleit solution at 37 °C. After obtaining a steady-state response to 10^{-4} M phenylephrine, increasing concentrations of carbachol were added cumulatively to the buffer solution. Values for tension are reported as a percentage of the 10^{-4} M phenylephrine-induced tension. Vertical bars represent S.E.M.

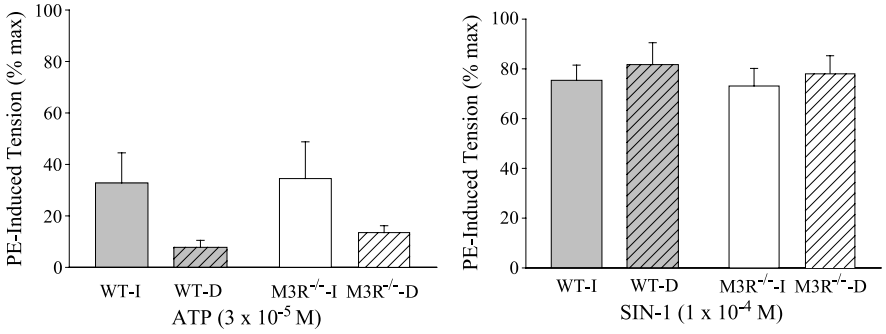

Fig. 5. Effects of ATP (3×10^{-5} M; left panel) and SIN-1 (10^{-4} M; right panel) on phenylephrine-constricted thoracic aortae. Endothelium-intact (solid bars) and -denuded (hatched bars) preparations from WT (gray bars) and $M_3R^{-/-}$ (white bars) mice were bathed in oxygenated Krebs-Henseleit solution at 37 °C. After obtaining a steady-state response to 10^{-4} M phenylephrine, ATP ($n=3-4$/group) or SIN-1 ($n=4-5$/group) was added to the buffer solution. Maximum values for the relaxation elicited by each agent are expressed as a percentage of the 10^{-4} M phenylephrine-induced tension. Vertical bars represent S.E.M.

involved in the cholinergic agonist-induced dilation observed in rat and rabbit aorta (Boulanger et al., 1994; Jaiswal et al., 1991) as well as numerous other vascular beds in several species including rat, cat, rabbit and guinea pig (Bungardt et al., 1992; Dauphin and Hamel, 1990; Eltze et al., 1993; Garcia-Villalon et al., 1991; McCormack et al., 1988). However, since the receptor subtype selectivity of pharmacological agents is sometimes marginal and investigators have demonstrated that other muscarinic receptor subtypes are expressed in various vascular beds (Elhusseiny et al., 1999; Phillips et al., 1997), these earlier studies left some doubt regarding the receptor subtypes involved. The current results, which were obtained using a muscarinic M_3 receptor-deficient mouse strain, indicate that muscarinic M_3 receptors are the primary vasodilatory muscarinic receptors in mouse aorta.

Experiments with carbachol, ATP and SIN-1 were used to support the conclusion that the altered response to acetylcholine is mediated solely by the absence of muscarinic M_3 receptors. The endothelium-dependent vasodilatory response to carbachol was depressed in aortae from $M_3R^{-/-}$ mice. Since carbachol is resistant to degradation by acetylcholinesterases, these results indicate that the diminished response to acetylcholine in endothelium-intact $M_3R^{-/-}$ aortae is not mediated by increases in cholinesterase activity. The response to ATP, an endothelium-dependent vasodilator that does not require muscarinic receptor activation, did not differ when compared in endothelium-intact preparations from the two groups. This indicates that post-receptor endothelial mechanisms are not altered in the knock-out model. In addition, since the NO donor SIN-1 elicited similar responses in endothelium-intact and -denuded preparations from both groups, it appears that vascular smooth muscle responsiveness to NO is not altered in $M_3R^{-/-}$ preparations. Finally, our data demonstrate that the antagonistic effect of the endothelium on the vasoconstrictor potency of phenylephrine, which has been observed previously in WT preparations (McCormack et al., 1988), is similar in WT and $M_3R^{-/-}$ groups. This suggests that phenylephrine-induced vasodilatory mechanisms in endothelium-intact WT preparations are preserved in the $M_3R^{-/-}$ model.

In summary, our results indicate that in mouse aorta, the muscarinic M_3 receptor plays a major role in the endothelium-dependent acetylcholine-induced vasodilation. Further work is required to elucidate the role of the muscarinic M_3 receptor in normal vascular physiology as well as its role in pathophysiological conditions.

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