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Deoxycholyltaurine-induced vasodilation of rodent aorta is nitric oxide- and muscarinic M_3 receptor-dependent

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Abstract

Emerging evidence indicates that some secondary bile acids interact functionally with muscarinic cholinergic receptors. Using thoracic aortic rings prepared from rats and mice, we examined the mechanism of deoxycholyltaurine-induced vasorelaxation. Increasing concentrations of both acetylcholine (1 nM to 0.1 mM) and deoxycholyltaurine (0.1 μM to 1 mM) stimulated relaxation of phenylephrine-constricted rings prepared from rat thoracic aortae. These effects were reduced by endothelial denudation and by treatment with an inhibitor of nitric oxide formation and with a synthetic acetylcholine : bile acid hybrid that acts as a muscarinic receptor antagonist. Likewise, both acetylcholine (1 nM to 0.1 mM) and deoxycholyltaurine (0.1 μM to 0.1 mM) stimulated relaxation of phenylephrine-constricted rings prepared from mouse thoracic aortae. These effects were reduced by endothelial denudation, addition of an inhibitor of nitric oxide formation, and by muscarinic M_3 receptor knockout. We conclude that the systemic vasodilatory actions of deoxycholyltaurine are mediated in part by a nitric oxide-, muscarinic M_3 receptor-dependent mechanism. In advanced liver disease, interaction of serum bile acids with endothelial muscarinic receptors may explain nitric oxide overproduction in the systemic circulation and resulting peripheral arterial vasodilation.
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Keywords: Receptor subtype; Vascular relaxation; Cholinergic receptor; Deoxycholic acid; Cirrhosis

1. Introduction

Previous studies from our laboratory showed that bile acid-induced stimulation of gastric pepsinogen secretion and colon cancer cell proliferation is mediated by cholinergic mechanisms (Cheng et al., 2002a; Raufman et al., 1998). As discussed in a recent review (Raufman et al., 2003), activation of muscarinic receptors by bile acids may explain some actions of these agents in health and disease. For example, serum bile acids are increased in cirrhosis of the liver, a condition that is associated with systemic arterial vasodilation that is considered to be mediated, at least in part, by endothelial nitric oxide overproduction (Atucha et al., 1996; Pennington et al., 1977; Wiest and Groszmann, 2002). Nitric oxide activates soluble guanylate cyclase in vascular myocytes, thereby increasing cGMP levels and causing smooth muscle relaxation. The resulting arterial vasodilation plays a critical role in the development of complications of advanced liver disease, including a hyperdynamic circulation, decreased peripheral vascular resistance, and possibly renal failure (Atucha et al., 1996; Wiest and Groszmann, 2002). Because nitric oxide is a known mediator of cholinergic agonist-induced systemic vasodilation (Furchgott, 1996), we hypothesized that interaction of selected serum bile acids with endothelial muscarinic receptors might explain some hemodynamic consequences of cirrhosis. Bile acids have been implicated previously by

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several investigators as the cause of decreased vascular tone in advanced liver disease, but the mechanism of this effect and which particular bile acids are responsible remains uncertain (Bomzon and Ljubuncic, 1995).

Nitric oxide was originally discovered by Furchgott and colleagues as endothelium-derived relaxing factor (EDRF), an agent that mediated acetylcholine-induced vasodilation in dogs (Furchgott and Zawadzki, 1980). Using aortic rings prepared from wild-type and muscarinic M_3 receptor knockout ($M_3R^{-/-}$) mice, we showed recently that, of the five muscarinic receptor subtypes (Wess, 1996), M_3 receptors play a prominent role in activating endothelium-dependent, nitric oxide-mediated vasodilation (Khurana et al., 2004). Aortic preparations from rodents, and particularly muscarinic receptor knockout mice, are useful for studies of muscarinic receptor-dependent actions because apart from the absence of muscarinic M_3 receptors, acetylcholinesterase activity and the other underlying mechanisms that mediate vascular tone, including endothelial nitric oxide formation and vascular smooth muscle function, remain intact (Khurana et al., 2004; Wess, 2004). In thoracic aortic rings prepared from $M_3R^{-/-}$ mice, the vasodilatory actions of cholinergic agonists are selectively abolished (Khurana et al., 2004). Hence, thoracic aortic rings prepared from $M_3R^{-/-}$ mice provide an excellent model system to determine the potential role of muscarinic M_3 receptor-related mechanisms in mediating the actions of bile acids.

In the present study, we used thoracic aortic rings prepared from rats, and wild-type and $M_3R^{-/-}$ mice to examine the role of muscarinic mechanisms in mediating the vascular actions of bile acids. Specifically, experiments were designed to compare the actions of acetylcholine and physiologically relevant concentrations of a conjugated secondary bile acid, deoxycholyltaurine, and to determine whether the actions of the bile acid are nitric oxide- and muscarinic M_3 receptor-mediated. We focused our attention on deoxycholyltaurine because previous experiments in our laboratory demonstrated interactions between this secondary bile acid and muscarinic receptors (Raufman et al., 2002). Although deoxycholic acid derivatives have previously been reported to cause relaxation of rodent aortic rings, the mechanism of this action was uncertain (Ljubuncic et al., 2000). Moreover, deoxycholyltaurine is reported to stimulate a calcium-dependent increase in nitric oxide production in calf aortic endothelial cells (Nakajima et al., 2000) and plasma levels of deoxycholyltaurine are increased in advanced liver disease (Clain et al., 1977; Makino et al., 1969; Pennington et al., 1977).

2. Materials and methods

2.1. Rats, wild-type and muscarinic M_3 receptor knockout 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. For rat experiments, male Sprague–Dawley animals, 16–20 weeks of age, were used. For mouse experiments, the muscarinic M_3 receptor gene was inactivated using mouse embryonic stem cells derived from 129SvEv mice, as described previously (Yamada et al., 2001). The resulting chimeric mice were then mated with CF-1 mice to generate $M_3R^{-/-}$ and wild-type 129SvEv/CF-1 hybrid mice (genetic contribution 50% each). Male mice, 9–12 weeks of age, were used in all experiments. As reported previously, body weight in the $M_3R^{-/-}$ animals was approximately 15% less than that in the wild-type animals (Khurana et al., 2004). Compared to wild-type mice, $M_3R^{-/-}$ mice are reported to be hypophagic and have decreased leptin and insulin levels (Yamada et al., 2001). However, there is no difference in mean arterial blood pressure when comparing wild-type to $M_3R^{-/-}$ mice (Fisher et al., 2004).

2.2. Isolated aortic preparations

Thoracic aortae were isolated from anesthetized rats and mice, and immediately bathed in Krebs–Henseleit solution (37 °C) containing (in mM): 118.0 NaCl, 25.0 $NaHCO_3$, 3.7 KCl, 1.0 KH_2PO_4, 1.4 $CaCl_2$, 1.2 $MgCl_2$, and 11.0 dextrose. The solution was saturated with 95% O_2/5% CO_2 gas (pH7.4). After careful removal of the adventitia, each vessel was dissected into two ring segments (each 2 to 3 mm in length). The endothelial lining was removed from one segment by gentle rubbing. Each ring was suspended vertically between two 27-guage 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 7D Polygraph). The vessels were maintained at a resting tension of 250 mg (mice) or 2.0 g (rats) 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, the rings were vasoconstricted to steady-state with phenylephrine (300 μM) before concentration–response curves for acetylcholine and deoxycholyltaurine were obtained by cumulative addition. Each concentration of test agent was added to the medium only after the tissues reached a steady-state response at the previous level. Vasodilatory effects of acetylcholine (0.1 nM–0.1 mM) and deoxycholyltaurine (0.1 μM–0.1 mM) were examined. Acetylcholine and deoxycholyltaurine were dissolved in water. Water alone did not alter vascular tension. The response to vasodilatory agents was compared in the absence and presence of L-nitro arginine methyl ester (L-NAME, 1 mM), an inhibitor of nitric oxide synthase, and lithocholylcholine (0.1 mM), a bile acid : acetylcholine hybrid molecule that is a muscarinic receptor antagonist (Cheng et al., 2002b).

2.3. Muscarinic M_3 receptor genotyping

Muscarinic M_3 receptor status was confirmed by polymerase chain reaction (PCR) analysis using genomic DNA extracted from the tails of mice. PCR primers used were: M_3-A3 (5′-AAGACCACAGTAGCAGTG), M_3-B (5′-CTCTCTACATCCA-TAGTCCC) and M_3-NEO9 (5′-TGGATGTGGAATGTGTGC-GAGG). PCR conditions were 94 °C for 10 min; 30 cycles

at 94 °C for 30 s; 55 °C for 320 s and 72 °C for 2 min. The M_3-A3 and M_3-B primer pairs generated a 226-bp fragment with DNA isolated from wild-type mice. The M_3-NEO9 and M_3-B primer pairs generated a 170-base pair fragment only with DNA from $M_3R^{-/-}$ mice (data not shown) (Khurana et al., 2004).

2.4. Statistical analysis

Results are presented as mean ± S.E.M. EC_{50} values were obtained by graphical evaluation of individual dose–response curves. Data were compared by analysis of variance (ANOVA) and repeated measures ANOVA with Student–Newman Keuls post hoc test using SigmaStat (SPSS, Chicago). The criterion for significance was a P value <0.05.

3. Results

3.1. Effects of acetylcholine and deoxycholyltaurine on phenylephrine-constricted vascular rings prepared from rat aortae

Phenylephrine, an α-adrenoceptor agonist, causes concentration-dependent vasoconstriction in intact and endothelium-denuded thoracic aortic ring preparations (Khurana et al., 2004). As shown in Fig. 1, in endothelium-intact rat aortic ring preparations that were pre-treated with 0.3 mM phenylephrine (a maximum effective concentration), acetylcholine and deoxycholyltaurine elicited dose-dependent vasorelaxation that was detected at concentrations as low as 1 nM for acetylcholine and 0.1 μM for deoxycholyltaurine. Maximal vasorelaxation was observed with 10 μM acetylcholine and 0.1 mM deoxycholyltaurine, the highest concentration tested. EC_{50} values for the responses to acetylcholine and deoxycholyltaurine were 6.6×10^{-8} and 3.4×10^{-5} M, respectively. With acetylcholine, endothelial denudation abolished agonist-induced vasodilation (Fig. 1A). Likewise, with micromolar concentrations of the bile acid, endothelial denudation abolished deoxycholine-induced vasodilation (Fig. 1B). However, with 0.1 mM deoxycholyltaurine, although endothelial denudation caused a significant reduction in deoxycholyltaurine-induced vasodilation, it was not abolished (Fig. 1B). These findings indicate that an intact aortic endothelium is necessary for full expression of acetylcholine- and deoxycholyltaurine-induced vasodilation of phenylephrine-constricted rat aortic rings.

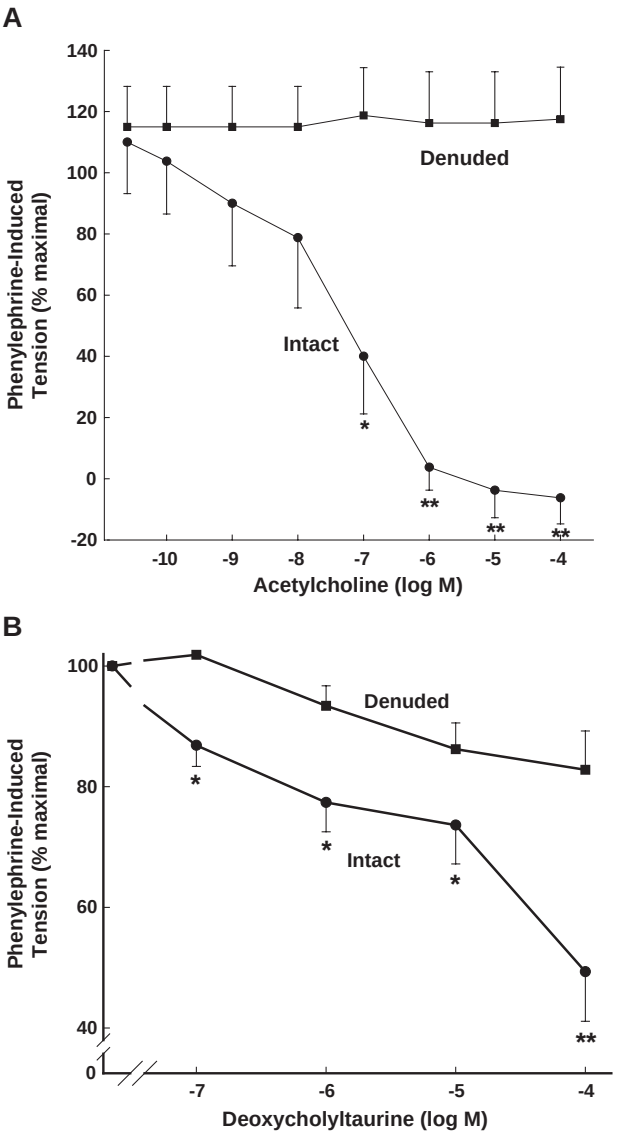

Fig. 1. Concentration-dependent effects of (A) acetylcholine ($n=4$) and (B) deoxycholyltaurine ($n=10$) in phenylephrine-constricted rings prepared from rat thoracic aortae. Endothelium-intact (closed circles) and endothelium-denuded (closed squares) aortic rings were obtained from rats and bathed in oxygenated Krebs–Henseleit solution at 37 °C. After tissues were equilibrated at 2.0 g resting tension, increasing concentrations of the agonist were added cumulatively to the buffer solution. Values for tension are reported as a percentage of phenylephrine (0.3 mM)-induced tension. Vertical bars represent S.E.M. *, ** indicate values that are significantly less than those observed with phenylephrine alone ($P<0.05$ and 0.001, respectively).

3.2. Reversibility of bile acid-induced relaxation of phenylephrine-constricted rings prepared from rat thoracic aortae

Compared to acetylcholine, bile acids are bulky molecules with a hydrophobic steroid nucleus that might cause a prolonged, or even persistent, interaction with the vascular endothelium (Raufman et al., 2002). Hence, to determine the reversibility of bile acid-induced vasorelaxation, we examined the effect of washing the deoxycholyltaurine-vasorelaxed preparation with fresh phenylephrine solution. As shown, in Fig. 2, following a wash with the phenylephrine solution even the vasodilatory response of a high bile acid concentration (1 mM deoxycholyltaurine) was completely reversed to values observed in endothelium-denuded preparations. These findings indicate that the effects of the bile acid are completely reversible.

3.3. Effects of L-nitro arginine methyl ester (L-nitro arginine methyl ester [L-NAME]) on bile acid-induced relaxation of phenylephrine-constricted rings prepared from rat thoracic aortae

Using L-nitro arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, our previous study confirmed that nitric oxide mediates the vasodilatory actions of cholinergic agonists (Khurana et al., 2004). To determine the role of nitric oxide in mediating the actions of bile acids, we examined the actions of L-

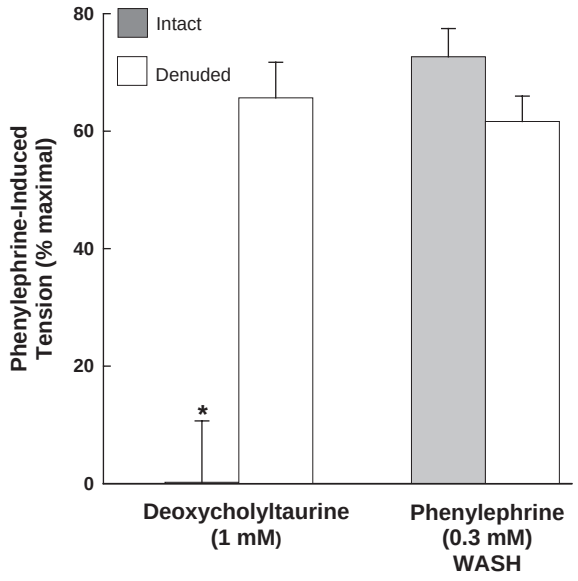

Fig. 2. Reversibility of bile acid effects on relaxation of phenylephrine-constricted rings prepared from rat thoracic aortae. Endothelium-intact (solid bars) and endothelium-denuded (white bars) aortic ring preparations ($n=4$) were obtained from rats and bathed in oxygenated Krebs–Henseleit solution at 37 °C. After obtaining a steady-state response to 0.3 mM phenylephrine, the indicated concentration of deoxycholyltaurine was added to the buffer solution (left bars). After obtaining a steady-state response to deoxycholyltaurine, it was washed from the preparations using buffer solutions containing 0.3 mM phenylephrine. Values for tension are reported as a percentage of initial phenylephrine (0.3 mM)-induced steady-state tension. Vertical bars represent S.E.M. * indicates that value is significantly less than that observed in endothelium-denuded rings ($P<0.05$).

NAME (1 mM) on the relaxation of phenylephrine-constricted, endothelium-intact rings from rat thoracic aortae that were treated with increasing concentrations of deoxycholyltaurine. As shown in Fig. 3, whereas 1 to 100 μM deoxycholyltaurine alone stimulated vasodilation, in the presence of the nitric oxide synthase inhibitor aortic tension was not significantly different than control. No difference in bile acid actions was observed in endothelium-denuded rat aortic ring preparations that were incubated with L-NAME (data not shown). These findings indicate that, as observed previously with acetylcholine in rings prepared from mouse thoracic aorta (Khurana et al., 2004), nitric oxide production is necessary for mediation of bile acid-induced relaxation of phenylephrine-constricted, endothelium-intact rings prepared from rat thoracic aortae.

3.4. Effects of lithocholylcholine on bile acid-induced relaxation of phenylephrine-constricted rings prepared from rat thoracic aortae

In a previous study that examined the ability of bile acids to compete with the binding of a muscarinic radioligand, we observed that deoxycholic acid conjugates, including deoxycholyltaurine, interact with rat muscarinic M_3 receptors (Raufman et al., 2002). To determine whether the vasodilatory actions of deoxycholyltaurine on phenylephrine-constricted rings prepared from rat thoracic aortae were mediated by interaction with muscarinic receptors, we examined the actions of adding muscarinic receptor antagonists. Atropine, in concentrations to 10 μM did not block the effects of DCT (not shown). Hence, we turned to a synthetic bile acid:-acetylcholine hybrid molecule, lithocholylcholine, that acts as a muscarinic receptor antagonist (Cheng et al., 2002b). Lithocholylcholine has proven to be a particularly useful agent in evaluating bile acid interactions with rat and human muscarinic M_3 receptors (Cheng et al., 2002b). As shown in Fig. 4, the addition of lithocholylcholine (0.1 mM) to endothelium-intact rings prepared from rat thoracic aorta antagonized both deoxycholyltaurine- and acetylcholine-induced vasodilation. These findings suggest that, as observed with acetylcholine, the bile acid interacts with muscarinic receptors. Moreover, the data support the concept that deoxycholyltaurine interacts with the muscarinic M_3 receptors that are expressed on aortic endothelium (Khurana et al., 2004). Conventional muscarinic receptor antagonists or inverse agonists, like atropine, have not been helpful in elucidating cholinergic actions of bile acids (Cheng et al., 2002b). Therefore, we employed a receptor knockout approach to confirm the observations with lithocholylcholine. To confirm the role of the muscarinic M_3 receptor in mediating the vasodilatory actions of bile acids and to validate our observations in another species, we switched experimental models from rats to wild-type and $M_3R^{-/-}$ mice.

3.5. Effects of deoxycholyltaurine on intact and endothelium-denuded phenylephrine-constricted vascular rings prepared from wild-type mouse thoracic aorta

Before considering the effects of the bile acid in this experimental model, it is important to review differences in the actions of acetylcholine on vascular rings prepared from rat and mouse thoracic aorta. In endothelium-intact, phenylephrine-constricted rings prepared from wild-type mouse thoracic aorta, acetylcholine causes dose-dependent vasorelaxation that is detected with 1 nM and maximal with 0.1 μM acetylcholine (see Fig. 3 in Khurana et al., 2004). Maximal acetylcholine-induced

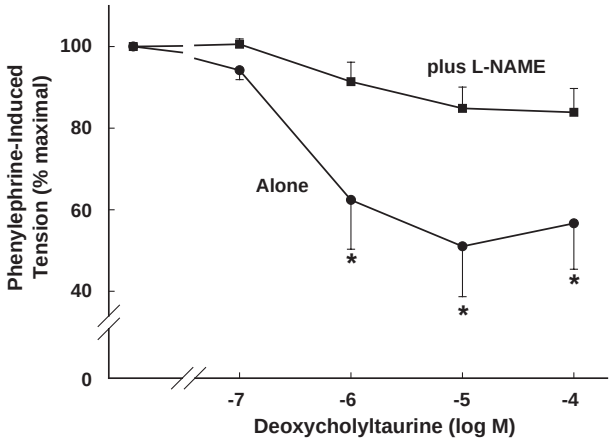

Fig. 3. Effect of L-nitro arginine methyl ester (L-NAME, 1 mM) on bile acid-induced relaxation of phenylephrine-constricted rings prepared from rat thoracic aortae. Endothelium-intact aortic ring preparations ($n=10$) were obtained from rats and bathed in oxygenated Krebs–Henseleit solution at 37 °C. After obtaining a steady-state response to phenylephrine, the indicated concentrations of deoxycholyltaurine, alone (closed circles) or in the presence of L-NAME (closed squares), were added to the buffer solution. Values for tension are reported as a percentage of phenylephrine (0.3 mM)-induced tension. Vertical bars represent S.E.M. * indicates values that are significantly less than corresponding value with L-NAME ($P<0.05$).

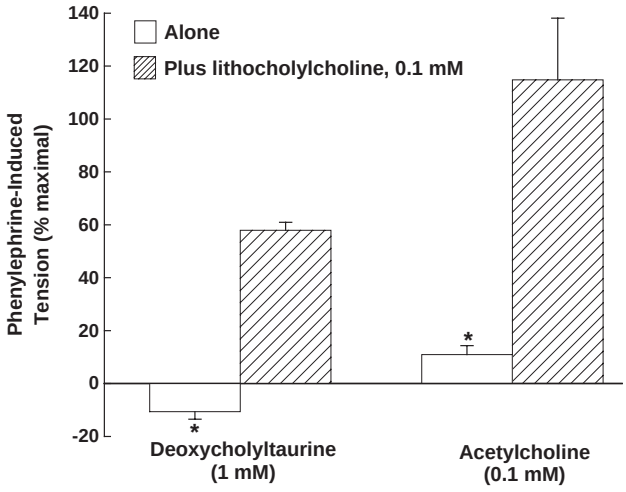

Fig. 4. Effects of lithocholylcholine (0.1 mM) on bile acid- and acetylcholine-induced relaxation of phenylephrine-constricted rings prepared from rat thoracic aortae. Endothelium-intact aortic ring preparations ($n=4$) were obtained from rats and bathed in oxygenated Krebs–Henseleit solution at 37 °C. After obtaining a steady-state response to phenylephrine, the indicated concentrations of deoxycholyltaurine and acetylcholine, alone (open bars) or in the presence of lithocholylcholine (hatched bars), were added to the buffer solution. Values for tension are reported as a percentage of phenylephrine (0.3 mM)-induced tension. Vertical bars represent S.E.M. * indicates values that are significantly less than those observed in the presence of lithocholylcholine ($P<0.05$).

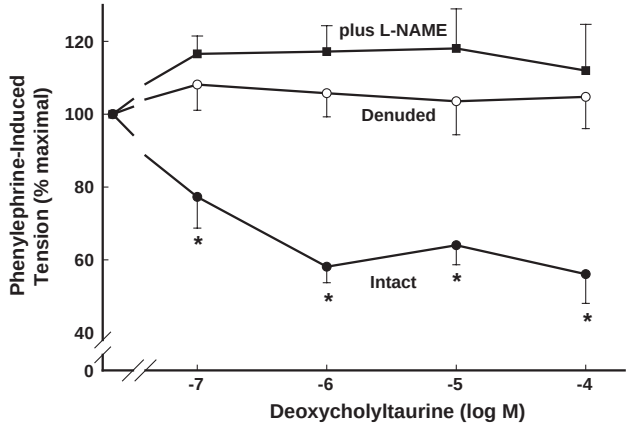

Fig. 5. Concentration-dependent effects of deoxycholyltaurine in phenylephrine-constricted rings prepared from mouse thoracic aortae. Endothelium-intact, alone (closed circles) or in the presence of 1 mM L-NAME (closed squares), and endothelium-denuded (open circles) aortic ring preparations ($n=6$) were obtained from wild-type mice and bathed in oxygenated Krebs–Henseleit solution at 37 °C. After tissues were equilibrated at 250 mg resting tension and a steady-state response to phenylephrine (0.3 mM) was obtained, increasing concentrations of the test agent were added cumulatively to the buffer solution. Values for tension are reported as a percentage of the phenylephrine (0.3 mM)-induced tension. Vertical bars represent S.E.M. * indicates values that are significantly less than those observed with phenylephrine alone ($P<0.05$).

vasodilation of phenylephrine-induced contraction in mouse rings was only 60% (see Fig. 3 in Khurana et al., 2004), compared to 100% in rat rings (Fig. 1). Hence, acetylcholine is equipotent in mouse and rat preparations, but somewhat less efficacious in the mouse preparation compared to that from rat. With both mouse and rat aortic rings, endothelial denudation abolishes acetylcholine-induced vasodilation (see Fig. 3 in Khurana et al., 2004 and Fig. 1 in the current paper, respectively).

As shown in Fig. 5, in phenylephrine-constricted, endothelium-intact preparations, deoxycholyltaurine elicited dose-dependent vasorelaxation that was observed at concentrations as low as 0.1 μM. Maximal vasorelaxation was observed with 0.1 mM deoxycholyltaurine, the highest concentration tested. The calculated EC_{50} value for the effects of deoxycholyltaurine on intact phenylephrine-contracted mouse aortic rings was 7.1×10^{-6} M. Maximum observed deoxycholyltaurine-induced vasodilation of phenylephrine-induced contracted rings was approximately 40%. Hence, in the mouse model, deoxycholyltaurine was less potent but had similar efficacy when compared with acetylcholine. Moreover, endothelial denudation or the addition of L-NAME completely abolished deoxycholyltaurine-induced vasodilation (Fig. 5). These findings confirm the observation made in rings prepared from rat aorta that vasorelaxation with the bile acid is endothelium- and nitric oxide-dependent (Figs. 1B and 3).

3.6. Effects of ATP and SIN-1 on phenylephrine-constricted aortae from wild-type and muscarinic M_3 receptor knockout mice

To determine whether knockout of the muscarinic M_3 receptor alters the tissue response to vasodilators that do not interact with muscarinic receptors, we compared the actions of ATP that interacts with a different class of receptors. We also tested the effects of SIN-1 which liberates NO by a non-receptor mediated mechanism, thereby testing the ability of the vascular myocytes to respond to this signaling molecule. As shown in Table 1, ATP and SIN-1 elicited similar vasodilatory responses in intact and denuded preparations from WT and $M_3R^{-/-}$ mice. These findings indicate that, as shown previously (Khurana et al., 2004), differences in agonist-induced responses in $M_3R^{-/-}$ mice are mediated solely by the absence of muscarinic M_3 receptors.

3.7. Effects of acetylcholine and deoxycholyltaurine on phenylephrine-constricted vascular rings prepared from wild-type and $M_3R^{-/-}$ mouse thoracic aorta

To determine the role of the muscarinic M_3 receptor in mediating the actions of deoxycholyltaurine, we compared the actions of acetylcholine and the bile acid on relaxation of

Table 1
Comparison of the effects of non-muscarinic vasodilators on phenylephrine-constricted thoracic aortae from wild-type and $M_3R^{-/-}$ mice

Agonist	Phenylephrine-induced tension (% maximal)			
	Wild-type		$M_3R^{-/-}$	
	Intact	Denuded	Intact	Denuded
ATP, 30 μM	32.8±11.7	7.8±1.7	34.5±14.3	13.5±2.7[a]
SIN-1, 100 μM	47.8±15.4	51.7±13.4	59.2±13.5	48.4±17.1[b]

Endothelium-intact and denuded preparations from WT and $M_3R^{-/-}$ mice were bathed in oxygenated Krebs–Henseleit solution at 37 °C. After obtaining a steady-state response to 100 μM 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±S.E.M. of the phenylephrine-induced tension. [a] and [b] indicate that there were no significant differences for the effects of ATP and SIN-1, respectively, when comparing intact and denuded preparations from WT animals to those from $M_3R^{-/-}$ animals.

endothelium-intact aortic ring preparations from wild-type and $M_3R^{-/-}$ mice. As shown in Fig. 6, in phenylephrine-constricted, endothelium-intact preparations from wild-type mice, concentrations of acetylcholine and deoxycholyltaurine $\geq$100 nM elicited dose-dependent vasorelaxation. Maximal vasorelaxation was observed with 10 μM acetylcholine and 1 μM deoxycholyltaurine. EC_{50} values for the response to acetylcholine and deoxycholyltaurine were 1.4×10^{-7} and 7.1×10^{-6} M, respectively. The maximum observed vasodilation of the phenylephrine-induced contraction was approximately 50% for acetylcholine and 40% for deoxycholyltaurine. These findings indicate that, compared to the observations in rat aortic rings, the dose–response curves for acetylcholine- and deoxycholyltaurine-induced vasodilation of phenylephrine-contracted mouse aortic rings are more similar in terms of potency and efficacy (compare EC_{50} values and Figs. 1 and 6).

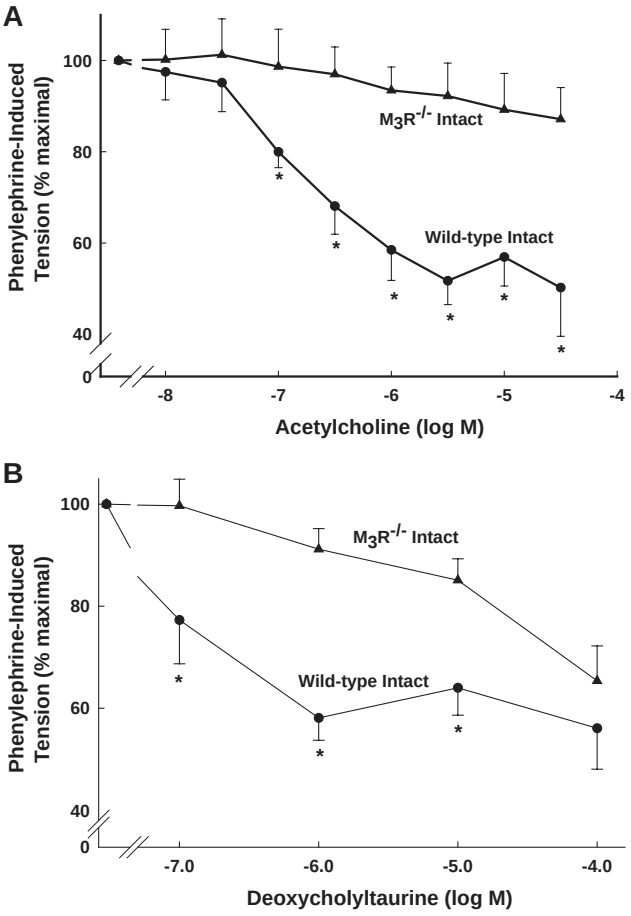

Fig. 6. Concentration-dependent effects of acetylcholine and deoxycholyltaurine in phenylephrine-constricted rings prepared from wild-type and $M_3R^{-/-}$ mouse thoracic aortae. (A) Effects of acetylcholine ($n=5$). (B) Representative tracing of the effects of deoxycholyltaurine on phenylephrine-constricted rings prepared from wild-type and $M_3R^{-/-}$ mouse thoracic aortae. (C) Effects of deoxycholyltaurine ($n=6$). Endothelium-intact aortic ring preparations were obtained from wild-type (closed circles) and $M_3R^{-/-}$ (closed triangles) mice and bathed in oxygenated Krebs–Henseleit solution at 37 °C. After tissues were equilibrated at 250 mg resting tension and a steady-state response to phenylephrine (0.3 mM) was obtained, increasing concentrations of the test agent were added cumulatively to the buffer solution. Values for tension are reported as a percentage of the phenylephrine (0.3 mM)-induced tension. Vertical bars represent S.E.M. * indicates values that are significantly less than those observed in rings prepared from $M_3R^{-/-}$ mice ($P<0.05$).

With both acetylcholine and the bile acid, the endothelium-dependent vasodilator effect was reduced in aortae from $M_3R^{-/-}$ as compared to wild-type mice. As shown in Fig. 6, in aortae prepared from muscarinic M_3 receptor knockout animals, acetylcholine and deoxycholyltaurine elicited no vasodilatory effect at concentrations up to approximately 10 μM. At higher concentrations, a modest relaxation was noted; the maximum observed vasodilatation in rings from $M_3R^{-/-}$ animals ranged from 10% to 20% with acetylcholine and deoxycholyltaurine, respectively. The magnitude of the responses in aortae prepared from muscarinic M_3 receptor knockout mice with these cholinergic agents are similar to those previously reported for carbachol-induced vasodilation (Khurana et al., 2004).

Overall, in concert with our findings in endothelium-intact versus endothelium-denuded rat aortic rings and those regarding the inhibitory actions of lithocholylcholine, the effects of deoxycholyltaurine in wild-type compared to knockout mice indicate that muscarinic receptors mediate the actions of the bile acid. More specifically, in endothelium-intact aortic rings, the findings shown in Fig. 6B indicate that expression of the muscarinic M_3 receptor is required for maximal bile acid-induced vascular relaxation.

4. Discussion

Vasodilation and reduced resistance in the peripheral vascular bed are observed consistently in experimental animal models of portal hypertension and in patients with cirrhosis (Bernardi et al., 1995; Bernardi and Trevisani, 1997; Groszmann, 1994; Moller et al., 1997; Rockey, 2003; Schrier et al., 1988). Several endogenous agents, including nitric oxide, prostaglandins, catecholamines, carbon monoxide, and others have been considered candidate molecules for mediating the abnormal regulation of vascular tone in cirrhosis (Rockey, 2003; Shah and Kamath, 2003). The evidence has been particularly compelling with regard to the role of nitric oxide (Battista et al., 1997; Matsumoto et al., 1995; Niederberger et al., 1995a,b; Sogni et al., 1995). This includes the observations that cirrhotics have elevated concentrations of plasma and exhaled nitric oxide, and that inhibition of endothelial nitric oxide formation normalizes peripheral vascular tone (Battista et al., 1997; Matsumoto et al., 1995; Niederberger et al., 1995a,b; Sogni et al., 1995). However, even in the absence of liver disease, elevated concentrations of circulating bile acids cause vasodilation and an impaired response to circulating vasoconstrictors (Bomzon and Ljubuncic, 1995; Bomzon et al., 1986).

Herein, we report evidence indicating that by interacting with muscarinic receptors, bile acids may play a role in regulating systemic vascular tone by a nitric oxide-mediated mechanism. Micromolar concentrations of deoxycholyltaurine stimulate reversible, endothelium-dependent, nitric oxide-mediated vascular smooth muscle dilation in rings prepared from rat and mouse thoracic aorta. Deoxycholyl-

taurine, a conjugated secondary bile acid, comprises a major proportion of the normal serum bile acid pool, and concentrations of this bile acid are elevated in patients with advanced liver disease. Specifically, reports indicate that in patients with cirrhosis or bile duct obstruction, plasma concentrations of secondary bile acids, primarily deoxycholyltaurine, may exceed 100 μM (Bogin et al., 1983; Bomzon and Ljubuncic, 1995; Clain et al., 1977; LaRusso et al., 1975; Makino et al., 1969; Pennington et al., 1977; Song et al., 1983). Hence, the concentrations of deoxycholyltaurine that stimulate vascular relaxation in our studies are well within the range of concentrations achieved in the circulation of patients with advanced liver disease or bile duct obstruction.

The results with vascular rings prepared from wild-type and $M_3R^{-/-}$ mice indicate that endothelium-dependent deoxycholyltaurine-induced vasodilation is mediated, at least to a major extent, by interaction of the bile acid with muscarinic M_3 receptors located on vascular endothelial cells. The use of thoracic aortic rings prepared from $M_3R^{-/-}$ mice was validated here and previously with experiments demonstrating that the altered response to test agents in this preparation is due solely to the absence of muscarinic M_3 receptors (Khurana et al., 2004). In particular, as shown herein, responses to ATP and SIN-1, vasodilators whose actions are mediated by non-muscarinic mechanisms, are not altered in tissue from $M_3R^{-/-}$ mice when compared to those in WT mice. Collectively, these data indicate a specific role for M_3R in mediating the actions of the bile acid, despite the failure of atropine to block its effects in rat aortic rings.

Although, by virtue of their detergent properties, bile acids may have non-specific effects on tissues, available evidence supports the specificity of the observations reported here. In their excellent review of the role of bile acids in mediating vasodilation, based on the observed vascular effects of these agents at concentrations much lower than critical micellar concentration (>1 mM), Bomzon and Ljubuncic argue convincingly that 'the vasorelaxant actions of bile acids cannot be attributed solely to a detergent action' (Bomzon and Ljubuncic, 1995). We have shown previously that interaction of bile acids with muscarinic receptors is limited to deoxycholic and lithocholic acids, and their conjugates (Raufman et al., 2002). Because lithocholic acid is a minor component of the bile acid pool, we did not explore its actions in the current study. We and others have shown that the bile acid concentrations used in this study do not cause cell injury (determined by trypan blue exclusion and lactate dehydrogenase release) (Nakajima et al., 2000; Raufman et al., 2002). In addition, we showed (Fig. 2) that the effects of even a high concentration of deoxycholyltaurine (1 mM) are entirely reversible. Finally, endothelial denudation reduced the vasodilatory effects of deoxycholyltaurine, indicating that the actions of the bile acid were specifically directed at endothelial cells with no direct effect on vascular myocytes (Figs. 1 and 5).

It is necessary to discuss discrepancies between our observations and those made previously by other investigators. Pak et al. reported that in isolated arterial mesenteric vessels from rat, the vasodilatory actions of deoxycholyltaurine were not altered by endothelium denudation or by addition of L-NAME (Pak et al., 1994). Likewise, using rat aortic rings, Ljubuncic et al. reported that vasorelaxation observed with deoxycholic acid, a more lipophilic bile acid, was not altered by endothelium denudation or treatment with L-NAME (Ljubuncic et al., 2000). Although these investigators showed that 0.1 mM deoxycholyltaurine reduced the contractile response of aortic rings to norepinephrine they did not study further the mechanism of action of the conjugated bile acid (Ljubuncic et al., 2000).

Species differences cannot explain these discrepant results, since, like us, both Pak et al. and Ljubuncic et al. used rat vascular preparations (Ljubuncic et al., 2000; Pak et al., 1994). However, a major strength of our investigation is that we also confirmed our observations in a second species, the mouse. It is most likely that differences in experimental approach account for the different results. Pak et al. studied mesenteric vessels that may respond differently to bile acids than systemic vessels like the aorta (Pak et al., 1994). Ljubuncic et al. studied deoxycholic acid, which is more lipophilic than the conjugated bile acid, deoxycholyltaurine, and is, therefore, more likely to have non-specific effects related to its detergent properties (Ljubuncic et al., 2000). Collectively, our findings in two rodent species using several biochemical and molecular approaches provide compelling evidence in support of a role for NO and muscarinic M_3 receptors in mediating the vasodilatory actions of deoxycholyltaurine.

Overall the findings reported here support our hypothesis that interaction of serum bile acids with endothelial muscarinic receptors may explain some hemodynamic consequences of conditions that result in elevated circulating bile acid concentrations. In patients with advanced liver disease, increased systemic concentrations of conjugated deoxycholic acid may play a role in the overproduction of nitric oxide in the systemic circulation and the hemodynamic changes in the peripheral vasculature. At present one can only speculate about potential clinical applications of our findings. Nevertheless, studies evaluating anti-muscarinic agents, nitric oxide inhibitors and other targeted approaches to blocking bile acid-induced vascular endothelial cell muscarinic receptor signaling in experimental animals and in patients with liver disease are likely to be of great interest.

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