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On the *in vitro* vasoactivity of bile acids

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1 We compared the vasorelaxant action of nine different bile acids and correlated their vasorelaxant activity with their individual indices for hydrophobicity or lipophilicity.

2 Vasorelaxant activity correlated with the relative lipid solubility of bile acids with lipophilic bile acids exhibiting the greatest vasorelaxant activity with modest to no vasorelaxant activity exhibited by hydrophilic bile acids.

3 We also investigated whether bile acid-induced vasorelaxation is mediated by antagonism of a prototypal contractile receptor, the α_1-adrenoceptor, by stimulation of a bile acid surface membrane receptor, by the release of endothelium-derived relaxant factors, by promoting the generation of reactive oxygen species and increasing the extent of lipid peroxidation, or by modifying membrane fluidity.

4 Lipophilic bile acids induce vasorelaxation possibly by antagonizing α_1-adrenoceptors, a phenomenon that manifests itself as a lowering of the affinity of vascular α_1-adrenoceptors. Bile acid-induced vasorelaxation was not dependent upon stimulation of a bile acid surface membrane receptor or the release of endothelium-derived relaxant factors.

5 Lipophilic bile acids can also increase the extent of lipid peroxidation with a subtle reduction in the fluidity of rat vascular smooth muscle membranes not associated with loss of membrane cholesterol or phospholipid.

6 We have concluded that lipophilic bile acids are non-selective vasorelaxants whose mechanism of action is a multifaceted process involving antagonism of contractile surface membrane receptors possibly effected by an increased extent of lipid peroxidation and/or membrane fluidity but occurs independent of the release of endothelial-derived relaxant factors or stimulation of a surface membrane bile acid binding site.

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Abbreviations: A_2C, 2-(2-methoxyethoxy) ethyl 8-(cis-2-n-octylcyclopropyl)ocatnoate; ANOVA, analysis of variance; AP, n-(9-anthroyloxy)- palmitic acid; AS, n-(9-anthroyloxy)-stearic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; EC_{50}, the concentration required to develop half maximal tension: GDCA, glycodeoxycholic acid; L-NAME, N^G-nitro-L-arginine-methyl-ester; MDA, malondialdehyde; NE, norepinephrine; R_{max}, maximal tension; ROC, receptor-operated calcium channels; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid

Introduction

Bile acids are naturally occurring amphiphilic steroids derived from cholesterol whose relative solubility in aqueous and lipid media is commonly referred to as the 'hydrophobic–hydrophilic balance'. This balance is determined by the state of ionization, the orientation, position and number of hydroxyl groups, and by the presence of the side chain ester (Heuman, 1989). Conjugation and the presence of hydroxyl groups increase hydrophilicity (Heuman, 1989). Heuman (1989) and Roda *et al.* (1990) independently derived indices of hydrophobicity and lipophilicity of bile acids, respectively, in order to quantify and predict the structure–activity relationships of an individual bile acid.

Bile acids are vasorelaxants (Bomzon & Ljubuncic, 1995). The evidence supporting this action was derived from two separate studies. In 1984 Bomzon and his colleagues (Bomzon *et al.*, 1984) demonstrated that the bile acids, deoxycholic acid (DCA), cholic acid (CA) and taurocholic acid (TCA) could attenuate the contractile response to norepinephrine (NE). Furthermore, they noted that DCA was more potent than either CA or TCA. Ten years later, Lee and his associates (Pak *et al.*, 1994) reported that incremental doses of the bile acids, tauroursodeoxycholic acid (TUDCA), taurochenodeoxycholic acid (TCDCA) and taurodeoxycholic acid (TDCA) caused dose-dependent vasorelaxation in the isolated perfused rat mesentery precontracted with the selective α_1-adrenoceptor agonist, cirazoline with an order of vasorelaxant potency TDCA > TCDCA > TUDCA. The indices of hydrophobicity or lipophilicity have not yet been applied to quantify and compare the vasorelaxant effects of bile acids. As DCA is considered a lipohilic bile acid whereas TUDCA is considered a hydrophilic bile acid (Heuman, 1989; Roda *et al.*, 1990), one could hypothesize that the vasorelaxant action of bile acids may be connected to their individual lipophilicity.

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One of the mechanisms whereby bile acids exert their vasorelaxant effects has been attributed to their ability to restrict calcium entry through voltage-dependent calcium channels (Bomzon & Ljubuncic, 1995). However, a direct action on vascular contractile receptor systems with consequent restriction of calcium entry through receptor-operated calcium channels (ROC) has not yet been excluded. As bile acids attenuated the contractile vascular response to α_1-adrenoceptor agonists (Bomzon *et al*., 1984; Pak *et al*., 1994), it is possible that calcium entry through ROC was impeded due to bile acids behaving as a receptor antagonist.

Vasorelaxation can also be effected by stimulation of surface membrane receptors linked either to adenyl or guanyl cyclase. Previous studies did not consider the possibility that bile acids may be agonists whose vasorelaxant actions are mediated by stimulation of a bile acid surface membrane receptor linked to either adenyl or guanyl cyclase.

Vasorelaxation can also be effected by stimulation of release of the endothelium-derived relaxant factors, nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor. In 1994, Lee and his co-workers (Pak *et al*., 1994) reported that bile acid-induced vasorelaxation was endothelium and nitric oxide independent. Although this latter observation contributed to our present understanding of the mechanism of bile acid-induced vasorelaxation, we felt that additional experiments were needed in order to verify their observations.

Other studies into the actions of bile acids in non-vascular tissues have shown that lipophilic bile acids can promote the generation of reactive oxygen species (ROS) to increase the extent of lipid peroxidation (DeRubertis & Craven, 1987; Sokol *et al*., 1993). ROS are vasorelaxants, acting either directly on vascular smooth muscle or indirectly by attenuating NE-induced vasoconstriction (Gao *et al*., 1994; Katusic, 1993; Kontos, 1988; Wei *et al*., 1996). ROS can also modify adrenoceptor function and the signal transduction process (Kaneko *et al*., 1991; Meij *et al*., 1994; Persad *et al*., 1997; 1998a,b). Hence, it would not be unreasonable to assume that stimulation of the generation of ROS and initiation of the lipid peroxidation process by lipophilic bile acids in vascular smooth muscle represents another mechanism whereby bile acids induce vasorelaxation by modifying surface membrane contractile receptors and their signal transduction pathways.

It is well known that intact membrane integrity is a determinant of proper receptor function (Hirata & Axelrod, 1980; Hollenberg, 1990). Bile acids can disrupt cell membranes by initial solubilization with subsequent loss of membrane cholesterol and phospholipids (Güldütuna *et al*., 1993; Sagawa *et al*., 1993; Schubert & Schmidt, 1988). It is also known that ROS can increase membrane rigidity (Bagchi *et al*., 1989; Chen & Yu, 1994). Again, it would not be unreasonable to assume that the mechanism whereby bile acids induce vasorelaxation is mediated by alterations in membrane fluidity.

Against this background, we hypothesized that the vasorelaxant action of bile acids, as measured by their ability to attenuate vasocontraction or bring about vasorelaxation, is a function of the lipophilicity of an individual bile acid. At the same time, we also propose that the mechanism whereby lipophilic bile acids exert their vasorelaxant action is a multifaceted and possible interrelated process that occurs independent of the release of endothelial-derived relaxant factors involving (1) stimulation of a surface membrane bile acid binding site, (2) the generation of ROS to increase the extent of membrane lipid peroxidation, and (3) modification of membrane fluidity.

In this communication, we report our findings on the abilities of nine different bile acids of varying lipophilicity to attenuate the contractile responsiveness of isolated arterial rings to either NE or KCl and to relax arterial rings precontracted by NE or KCl. At the same time, we report on (i) the action of the lipophilic bile acid. DCA, on a prototypal surface membrane contractile receptor, the α_1-adrenoceptor, by assessing *in vitro* vascular contraction using the selective α_1-adrenoceptor agonists, phenylephrine and methoxamine in an isolated rat aortic ring preparation; (ii) the effects of DCA and TUDCA on the affinity and number of vascular α_1-adrenoceptors using a competitive antagonist radioligand binding assay; (iii) our attempt to identify a specific binding site for bile acids in a vascular smooth muscle cell membrane preparation using ^{14}C-DCA; (iv) the role of the endothelium in bile acid-induced vasorelaxation using precontracted aortic rings denuded of its endothelium or pretreated with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine-methyl-ester (L-NAME), and the cyclo-oxygenase inhibitor, indomethacin; (v) the effect of increasing concentrations of DCA on extent of lipid peroxidation in rat vascular smooth muscle microsomal membrane fractions using the thiobarbituric acid reactive substances (TBARS) assay; and (vi) the effect of different concentrations of DCA on the fluidity of rat vascular smooth muscle membranes using fluorescence anisotropy and by determining the cholesterol : phospholipid molar ratio.

Methods

Bile salts and chemicals

Nine different bile salts purchased from two different sources were used in this investigation. DCA, TDCA, glycodeoxycholic acid (GDCA), chenodeoxycholic acid (CDCA), TCDCA, CA, and TCA were purchased as sodium salts of the highest purity from Sigma Chemical Corporation (St. Louis, MO, U.S.A.). The sodium salts of ursodeoxycholic acid (UDCA) and TUDCA were purchased from Calbiochem Corporation, (La Jolla, CA, U.S.A.). For the binding studies, ^{14}C-DCA (40 – 60 Ci/mmol, Amersham, (Buckinghamshire, U.K.) and ^{3}H-prazosin ([7-methoxy-^{3}H]-prazosin, 70-87 Ci/mmol, New England Nuclear, Mass, U.S.A.) were purchased. The selective α_1-adrenoceptor agonists, phenylephrine (phenylephrine hydrochloride) and methoxamine (methoxamine hydrochloride), the muscarinic agonist, methacholine (methacholine chloride), the nitric oxide synthase inhibitor, N^G-nitro-L-arginine-methyl-ester (L-NAME), and the cyclo-oxygenase inhibitor, indomethacin, were all purchased from Sigma Chemical Corporation (St. Louis, MO, U.S.A.). The fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and the n-(9-anthroyloxy)-stearic [AS] or palmitic [AP] fatty acid were purchased from Aldrich Chemical Co, Milwaukee, WI, U.S.A. and Molecular Probes, Junction City, Oregon, U.S.A., respectively.

Animals

Sprague-Dawley male rats, weighing between 300 – 350 g, were used in this study. The rats were housed in a temperature and humidity controlled holding facility with a 12/12 h light – dark cycle. The rats had free access to rat chow and water. On the day of the experiment, the rats were killed by rapid decapitation. The chest and abdominal cavities were opened and the entire aorta from its exit from the heart to its femoral bifurcation was removed. For those experiments involving isolated vascular rings, the vessels were placed and washed in

ice-cold Krebs' solution (see later for composition). For those experiments involving vascular smooth muscle membranes, the vessels were placed and washed in ice-cold 0.15 M saline. Consistent with the internationally accepted principle that a minimum number of animals be used to achieve the objectives of a study involving experimental animals, a minimalist approach was adopted, in particular with the number of repeat experiments involving membranes. Large numbers of rats were required in order to obtain a sufficient quantity of membranes for a particular determination because the membrane yield from the rat aorta is small. This was the case when purified membranes were needed for the measurement of membrane fluidity using fluorescence anistropy. In these experiments, the number of observations made for the determination of membrane fluidity using this methodology was two, and in one instance, only one measurement was possible in the untreated membranes. The use of the rats was approved by the Technion Committee for Supervision of Animal Experiments, Technion – Israel Institute of Technology, Haifa, Israel.

Experimental techniques

Isolated abdominal aortic ring preparation The aortic ring preparation was used to assess the ability of the different bile acids to attenuate the contractile responses to NE, the selective α_1-adrenoceptor agonists, phenylephrine and methoxamine, and KCl, as well as to relax precontracted vessels. For these experiments, abdominal aortae were removed from freshly killed rats and cleaned of all extraneous tissue from the adventitia under magnification, as previously described (Bomzon *et al*., 1985). The vessel was then cut into 0.5 cm circular rings and suspended on two triangular spring steel metal clips in an oxygenated [5% CO_2 95% O_2] warmed (37°C) organ bath containing Krebs' solution. The lower clip was fixed at one end and the other clip was attached to a UC2 Statham force transducer whose output was recorded on a Brush-Gould 2200S pen recorder. The endothelium was not denuded from the arterial rings. The composition of the Krebs' solution in mmol l^{-1} was: NaCl 118; KCl 4.7; $MgCl_2$ 1.05; NaH_2PO_4 1.33; $CaCl_2$ 2.7; $NaHCO_3$ 25; glucose 5.6. The preparation was allowed to equilibrate for at least 1 h until a stable resting tension of 1500 mg was reached. Prior to the construction of the concentration response curves, each vessel underwent a simple protocol to determine the optimal tension at which to measure vascular reactivity, as suggested by Price *et al*., (1981). This involved giving a near maximal dose of NE at four different tensions ranging from 1.25 to 2.5 g and measuring the height of the response. In all instances, maximal responses were observed between 1.5 and 2.0 g tension and the vessels were maintained within this tension range for all experiments involving this preparation.

Vascular smooth muscle membrane preparation Fresh vascular smooth muscle membranes were used for determination of the effects of bile acids on vascular α_1-adrenoceptors, on the extent of membrane lipid peroxidation, and membrane fluidity, as well as for attempting to identify a specific bile acid binding site. The specific details for the isolation and preparation of membranes for each study parameter are given individually. For all these studies, vascular smooth muscle membranes were prepared according to the methods described by Kwan *et al*., (1984) and Bylund (1987). The preparation of vascular smooth muscle membranes involved immediate harvesting of the entire aorta from a freshly killed rat and removal of all extraneous fat tissue. The vessel was cut along its entire length and the endothelium was removed by gentle rubbing on wet blotting paper. The vessel was then cut into small pieces using a McIlwain tissue chopper (Mickle Laboratory Engineering Company, U.K.) and then homogenized in ice-cold 0.25 M sucrose – 10 mM imidazole buffer, pH 7.5, using a tissue homogenizer (Ystral, Göttingen, Germany). The crude tissue homogenate was filtered through gauze and the supernatant centrifuged at $1000 \times g$ for 10 min at 4°C to remove additional cell debris, collagen fibres and nuclei. The post-nuclear supernatant was then centrifuged at $10{,}000 \times g$ for 10 min to sediment mitochondria. The resultant supernatant was centrifuged at $105{,}000 \times g$ for 30 min and the supernatant discarded.

For the binding studies, the pellet was re-suspended in cold 50 mM Tris buffer, pH 7.7, as previously described (Dabagh *et al*., 1999). For the determination of the extent of lipid peroxidation, the pellet was re-suspended in cold 0.1 M sodium phosphate buffer solution, pH 7.4 and re-centrifuged at $10{,}000 \times g$ for 10 min further to remove fragmented mitochondria and the pellet was discarded. For the determination of membrane fluidity by fluorescence anistropy where highly purified membranes were needed, the pellet was re-suspended in the sucrose – imidazole buffer and centrifuged again at $10{,}000 \times g$ for 10 min to further remove fragmented mitochondria. The resultant supernatant was then layered on a discontinuous sucrose density gradient containing 15, 30 and 60% sucrose. The loaded sucrose gradient tube was centrifuged at $113{,}000 \times g$ for 2 h. The fraction from 15 – 30% interface was collected and used for the fluidity studies. Membrane purity was monitored by the activity of the membrane specific marker enzyme, 5′-nucleotidase (Gerlach & Hiby, 1984) using a commercially available kit (Sigma Chemical Co., St. Louis, MO, U.S.A.). The activity of enzyme was 3 – 4 times greater in the purified membranes than that measured in the crude homogenate. For assessing membrane fluidity by determination of the cholesterol: phospholipid molar ratio, the membrane preparation involved homogenizing pieces of vessel in ice-cold Tris-HCl buffer, pH 7.7 followed by centrifugation at $60{,}000 \times g$ for 15 min. The resultant pellet was re-suspended in ice-cold Tris – HCl buffer, pH 7.7 and again centrifuged at $60{,}000 \times g$ for 15 min. The resultant pellet was re-suspended in Krebs' solution. The protein contents of the final membrane preparations were determined by the method of Lowry *et al*. (1951).

Description of the experiments

The choice of the concentrations of the bile acids (10^{-7} – 10^{-4} M) was influenced by the fasting total bile acid levels in the plasma of normal and cholestatic individuals and animals (Ostrow, 1993; Schaffner *et al*., 1971; Bomzon & Yousef, unpublished data). Before commencing the experiments, we performed some preliminary experiments to eliminate the possibility that the bile acids did not effect the pH and osmolality of the various buffers used in this investigation. The pH of the buffers was measured using a standard laboratory pH meter. Osmolality was measured using a model OS osmometer (Finke Associates, Burlington, Mass, U.S.A.). The pH and osmolality of the buffers were unaffected by the addition of the bile acids at all test concentrations.

Study One: Structure – Activity relationships The vasoactivity of the nine different bile acids was evaluated using two vascular bioassays. The first bioassay evaluated the ability of different bile acids over the concentration range, 10^{-6} – 10^{-4} M to attenuate the *in vitro* contractile responses of rat abdominal aortic rings to NE and KCl.

In order to assess the effect of the bile acids on the contractile response to NE and KCl, bile acids over the concentration range from $10^{-6}-10^{-4}$ M dissolved in Krebs' solution were added to organ bath 5 min prior to construction of the concentration response curve. The protocol for each arterial ring was construction of cumulative concentration response curves to either NE (4×10^{-10} to 27×10^{-6} M) or KCl (10–310 mM) (final bath concentrations). Upon reaching maximal tension, the vessel was repeatedly washed with Krebs' solution until the tension returned to baseline. Once the tension had reached the baseline level, it was allowed to equilibrate for a further 10 min. Then, the lowest concentration of bile acid dissolved in Krebs' solution was added to the organ bath and, after 5 min, a repeat concentration response curve was constructed. Upon completion of the construction of this concentration response curve, the ring was washed repeatedly until the tension returned to baseline, as already described. Then, a higher concentration of bile acid was added. This procedure was repeated until the concentration response curve to NE or KCl preincubated with the maximum concentration (10^{-4} M) of bile acid was performed. Upon completion of the experiments with the bile acids, a repeat control concentration ([bile acid] = 0) response curve to NE or KCl was performed to determine the effect of time on the response curve and the reversibility of any observed effect. As no differences between the two curves were observed, the average of the two control curves was considered to be the baseline for a single experiment and used as the reference point for a single experiment. The duration of each experiment involving a single aortic ring lasted between 5–6 h. For each bile acid, the experiment was performed between 5–7 times in aortic rings prepared and isolated from different rats.

In the second bioassay, the ability of the individual bile acids to relax aortic rings precontracted with either 30 nM phenylephrine or 30 mM KCl was evaluated. These concentrations were chosen since they approximately equal to the EC_{60}'s of the control concentration response curves. The aortic rings were prepared and mounted in the identical manner. After the 1 h equilibration period, 30 mM KCl or 20 nM NE, was added to the organ bath. Upon reaching a steady-state contraction, bile acids over the concentration range, $10^{-6}-10^{-4}$ M in 100 μl volumes, were added every 2–3 min and the vasorelaxant response monitored. Each experiment lasted between 3–4 h. For each bile acid, the experiment was repeated at least five times and only one vessel was used to test each bile acid. The vasorelaxant responses to 100 μl volumes of Krebs' solution in the precontracted vessels were also evaluated and these data were considered the control experiment.

Study Two: The effect of bile acids on a prototypal vascular contractile receptor In this study, we evaluated the action of bile acids on a prototypal vascular contractile receptor, the α_1-adrenoceptor by evaluating the effect of the lipophilic bile acid, DCA, over the concentration range, $10^{-6}-10^{-4}$ M to attenuate the *in vitro* contractile responses to the selective α_1-adrenoceptor agonists, phenylephrine and methoxamine using rat abdominal aortic rings. In these experiments, the abdominal aortae were mounted in the organ bath, as already described. Increasing concentrations of phenylephrine ranging from 4×10^{-10} to 27×10^{-6} M (final concentrations) and methoxamine (4×10^{-10} to 27×10^{-6} M; final concentrations) were added cumulatively to the bath and the contractions of the suspended aortic ring recorded, as previously described (Jacob *et al*., 1993). The experimental protocol to determine the effect of DCA ($10^{-6}-10^{-4}$ M; final concentrations) on the contractile response to the two α_1-adrenoceptor agonists was identical to that described in Study One when NE or KCl were used as the contractile agonists.

Study Three: The effect of bile acids on aortic α_1-adrenoceptors In this study, we evaluated the effect of DCA and TUDCA on the binding characteristics of aortic α_1-adrenoceptors using a competitive antagonist radioligand binding assay with ^{3}H-prazosin in vascular smooth muscle membranes prepared from rat aortae. The receptor binding assay was performed over the concentration range 0.125–16 nM of ^{3}H-prazosin ([7-methoxy-^{3}H]-prazosin, 70–87 Ci mmol^{-1}, New England Nuclear, Mass, U.S.A.) in an incubation volume of 1 ml. We have previously shown that that binding over this concentration range was competitive and saturable with specific binding varying between 50–60% (Dabagh *et al*., 1999).

Aortic membranes (100 μl) at a concentration of 0.1 mg ml^{-1} were incubated with eight different concentrations of 100 μl ^{3}H-prazosin, with and without 100 μl 10^{-4} M phentolamine, and in the presence and absence of either 100 μl 10^{-4} M DCA or 10^{-4} M TUDCA, made up with Tris buffer in a total volume of 1 ml. Each test point was done in triplicate. The reaction was performed for 30 min at 25°C. The reaction was stopped by transferring the tubes to an ice-cold water bath and performing rapid filtration under vacuum using GFB filters followed by washing twice with ice-cold buffer. The filters were air-dried overnight and then placed in vials to which 5 ml (2 : 1) xylene : Lumax scintillation cocktail fluid was added. Radioactivity was counted in a RackBeta liquid scintillation counter (LKB 1211, LKB Wallac, Turku, Finland) for 2 min with external standardization. Each binding assay was performed 4–5 times and for each assay, between 25–30 rat aortae were needed.

Study Four: Bile acid binding site in vascular smooth muscle membranes In this study, we attempted to identify a specific binding site for the bile acid in vascular smooth muscle membranes by incubating $10^{-7}-10^{-4}$ M ^{14}C-DCA with a purified rat vascular smooth muscle membrane preparation. In order to obtain the desired concentrations, radiolabelled DCA was mixed with unlabelled DCA. Vascular smooth muscle membranes (100 μg) were incubated with eight different concentrations of ^{14}C-DCA in Tris buffer in a total volume of 1 ml. The reaction was performed for 30 min at 25°C and stopped by placing the tubes in an ice-cold water bath. Thereafter, the experimental procedure and counting was identical to that described in Study Three. Each binding assay was performed four times and for each assay, between 25–30 rat aortae were needed.

Study Five: Role of the endothelium in bile acid-induced vasorelaxation In these experiments, the role of the vascular endothelium in bile acid-induced vasorelaxation was investigated. Four series of experiments were performed. Aortic rings were prepared and mounted in the identical manner, as described earlier. After the 1 h equilibration period, the integrity of endothelium was tested by the addition of 20 nM methacholine to the organ bath and observing significant vasorelaxation. Upon establishing the integrity of the endothelium, the arterial ring was washed several times and allowed to equilibrate for a further 30 min. Upon completion of the equilibration period, 30 nM phenylephrine was added to the organ bath. Upon reaching a steady-state contraction, nine different concentrations of DCA, over the concentration range, $10^{-6}-5\times10^{-4}$ M in 100 μl volumes, were added every 3–4 min and the vasorelaxant response monitored.

In addition to the above mentioned control series of experiments, the remaining three series of experiments were almost identical to that described in the control experiments, except for some specific preparatory procedures aimed at assessing the role of the endothelium in bile acid-induced vasorelaxation. In the first instance, the endothelium was removed prior to its mounting in the organ bath. The denudation of the endothelium was verified by the failure to demonstrate a vasorelaxant response to methacholine. Upon verification that the endothelium had been denuded from the aorta, DCA was added in the identical manner, as previously described. In the second instance, after confirming the integrity of the endothelium, the arterial ring was incubated with the nitric oxide synthase inhibitor, L-NAME (10^{-4} M final concentration) for 10 min before adding increasing concentrations of DCA. In the third instance, the arterial ring was incubated with cyclo-oxygenase inhibitor, indomethacin (10^{-5} M final concentration) for 10 min before adding DCA. Each experiment lasted between 3–4 h and was repeated at least five times. Each experiment was always performed in pairs with one organ bath containing an untreated aortic ring and the second bath contained the treated aortic ring.

Study Six: The effect of bile acids on the extent of lipid peroxidation by the TBARS assay The extent of lipid peroxidation in vascular smooth muscle membranes was measured by TBARS assay according to the method described by Beuge & Aust (1978) with modifications according to Draper & Hadley (1990). Membranes (100 μg) were incubated with 10^{-5} M or 10^{-4} M DCA (final concentrations) dissolved in 0.1 M phosphate buffer for 1 h at 37°C. Membranes incubated with buffer alone served as the control. Ice-cold 20% trichloracetic acid was added to the tubes to stop the reaction. Following centrifugation ($1000 \times g$ for 10 min) 500 μl supernatants were taken for analysis of TBARS. The TBARS results were expressed as malondialdehyde (MDA) equivalents using 1,1,3,3-tetraethoxypropane as the standard.

Study Seven: The effect of bile acids on membrane fluidity The term 'lipid fluidity', as applied to biological membranes, refers to the relative motional freedom of the bilayer lipid molecules or substitutes thereof, combining in the one term concepts of both the rate of movement and the extent of movement of lipid molecules. Fluidity *per se* depends upon membrane composition with the cholesterol: phospholipid molar ratio wielding considerable import since membrane fluidity decreases with cholesterol enrichment and increases with phospholipid loading. Operatively, fluidity can be assessed by measuring the motional freedom of lipid-soluble membrane-inserted fluorescent probes – 1,6-diphenyl-1,3,5-hexatriene (DPH) and n-(9-anthroyloxy)-stearic [AS] or palmitic [AP] fatty acid where the values of *n* were 2, 6, 9, 12, and 16. A rise in anisotropy indicates a reduction in membrane fluidity. Fluidity can also be determined by calculating the membrane cholesterol : phospholipid molar ratio. We have used these three approaches to evaluate the effect of DCA on the fluidity of vascular smooth muscle membranes.

Determination of fluidity by fluorescence anisotropy

DPH (10^{-6} M final concentration) was incorporated into vascular smooth muscle membranes (50 μg ml^{-1}) solutions separately containing five different concentrations of DCA (0, 5×10^{-5}, 10^{-5}, 5×10^{-4}, and 10^{-4} M final concentrations). Fluidity measurements were performed after a 1 h incubation at 37°C with the bile acid by fluorescent spectrophotometry, where fluorescence was measured using a SPF-500C fluorometer (SLM-Amino, Urbana, III, U.S.A.). We also evaluated the effects of the membrane fluidizing compound 2-(2-methoxyethoxy) ethyl 8-(cis-2-n-octylcyclopropyl)ocatnoate (A_2C) (Sigma Chemical Co., St. Louis, MI, U.S.A.) as a positive control.

When the n-AS or n-AP fatty acid probes were used, the membranes (30 μg ml^{-1}) were incubated with 10^{-4} M DCA (final concentration) for 1 h at 37°C. The n-AS or AP probes were added 10 min before the end of the incubation. These probes are useful in determining fluidity gradients within the membrane (Meddings *et al*., 1990). 2-AS places the fluorescent group just underneath the phospholipid head group, while 12-AS and 16-AP localize the fluorescent group close to the core of the bilayer (Meddings *et al*., 1990). Fluorescence was again measured using a SPF-500C fluorometer (SLM-Amino, Urbana, III, U.S.A.).

Determination of fluidity by calculating the cholesterol: phospholipid molar ratio

The membrane pellet was re-suspended in Krebs' solution containing no DCA (control) or 10^{-4} M DCA (final concentration) and incubated for 1 h at 37°C. Upon completion of the incubation, the membrane lipids were extracted in hexane: isopropanol (3 : 2, v v^{-1}) and dried under nitrogen. Cholesterol content was determined by the method described by Chiamori & Henry (1959), and the phospholipid content was determined by the procedure described by Rouser *et al*. (1966).

Data analysis

Analysis of the concentration response curves For those experiments involving the rat abdominal aorta, the relationships between tension (*T*) and the concentration of agonist or bile acid (C) were analysed using the following equation plotting dose versus effect

$$T = \frac{\min + (\max - \min)}{1 + \left(\frac{x}{x50}\right)^{-p}}$$

where *T* represents the tension developed, min equals the minimal tension, max equals the maximal tension (R_{max}), *x* equals the concentration of the agonist, *x*50 equals the concentration required to develop half maximal tension EC_{50}, and *P* represents the slope of the response curve. For each concentration response curve, the data relating concentration and effect were fitted by nonlinear regression using the Figure Perfect statistical package (Fig.P®; Biosoft, Cambridge, U.K.) and analysed for the R_{max} and EC_{50}. The correlation between the model and the data of the individual concentration response curves was always greater than 0.93.

Correlation between vasoactivity and hydrophobicity or lipophilicity In order to correlate the degree of vasoactivity of an individual bile acid, the change in EC_{50} and R_{max} at the concentration of bile acid which had the greatest vasoactivity, 10^{-4} M, expressed as either (EC_{50} bile acid EC_{50} control^{-1}) or (R_{max} control R_{max} bile acid^{-1}) for both NE and KCl, was correlated with the hydrophobic index and the lipophilic capacity factor for the ionized forms of the bile acids, determined by Heuman (1989) and the Roda *et al*. (1990), respectively, using the Instat statistical package (Instat®, GraphPad Software Inc., San Diego, CA., U.S.A.).

Analysis of the radioligand binding assay Analysis of the saturation curve for specific binding to determine the B_{max} and K_D of rat aortic α_1-adrenoceptors was done using the Prism® program (GraphPad Software Inc., San Diego, CA, U.S.A.) assuming one binding site.

Comparative statistical tests The R_{max}'s and EC_{50}'s of the individual contractile concentration response curves were pooled for each group and differences in the pooled parameters between groups were statistically analysed using a repeated analysis of variance (ANOVA) with a Dunnett's post-test. The resultant EC_{50}'s from the vasorelaxant response curves were pooled and the groups compared using ANOVA with a Bonferroni post-test or a Student's *t*-test for two populations, where appropriate. The binding characteristics of rat aortic α_1-adrenoceptors were compared using a Student's *t*-test for two populations. All the results are presented as mean ± standard error of the mean. $P<0.05$ (two-tailed) was considered statistically significant.

Results

The effect of bile acids upon the in vitro *contractile response to NE and KCl*

In control arterial rings, NE (Figure 1A) or KCl (data not shown) caused concentration dependent increases in tension. Preincubation with increasing concentrations of the hydrophobic bile acid, DCA caused a concentration dependent decrease in the R_{max} and an increase in the EC_{50} of the contractile response curves to these two agonists with the greatest attenuation of the contractile response occurring at the 10^{-4} M concentration. When the aortic ring was incubated with the hydrophilic bile acid, CA, no attenuation of the contractile response to NE (Figure 1B) or KCl (data not shown) was observed, even at the highest concentration, 10^{-4} M. Tables 1 and 2 summarize the effects of the highest concentration, 10^{-4} M of the different bile acids on the R_{max} and EC_{50} of the contractile responses of rat abdominal aortic rings to NE and KCl. When comparing the attenuating effect of all the bile acids on the contractile responses to either agonist, the lipophilic bile acids, DCA and CDCA, were more effective in attenuating the contractile responses than the hydrophilic bile acids such as CA or TCA.

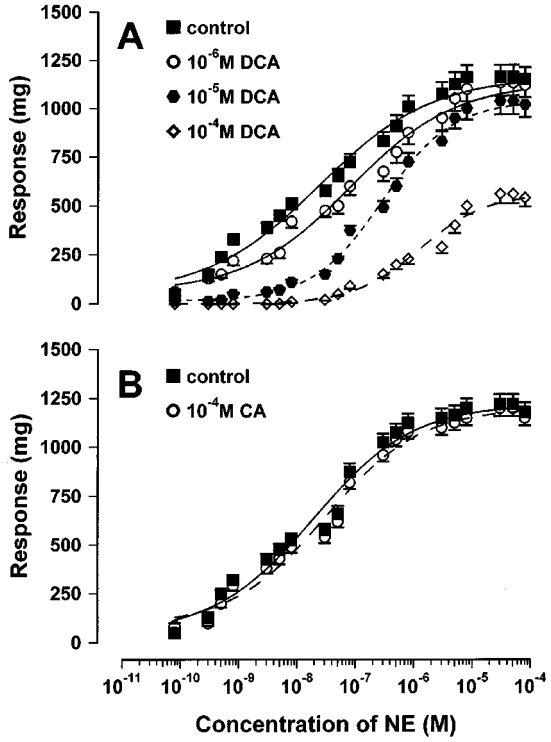

Figure 1 The effect of increasing concentrations of DCA (A) and CA (B) on the *in vitro* contractile response of rat aortic rings to NE. $n=5-7$ for each series of experiments.

Table 1 The effect of 10^{-4} M bile acids on the contractile response to NE of rat aortic rings

Bile acid	*Control* R_{max} (mg)	*Test* R_{max} (mg)	P	*Control* EC_{50} (nM)	*Test* EC_{50} (nM)	P
CA	1220±52	1200±42	n.s	13.7±1.3	18.7±3.3	n.s
DCA	1160±53	560±48	<0.0001	20.6±3.9	1718±75	<0.0001
CDCA	1163±63	600±54	<0.001	25.3±2.2	1500±66	<0.0001
UDCA	1180±58	1030±72	n.s	16.9±5.8	344±13	<0.001
TCA	1190±74	1190±80	n.s	19.1±2.8	20.8±4.1	n.s
TDCA	1113±72	1138±72	n.s	38.3±5.4	521±15	<0.0001
TCDCA	1313±74	1312±75	n.s	28.0±10.0	582±15	<0.0001
TUDCA	1255±109	1425±75	n.s	17.9±10.0	86.4±10.3	<0.002
GDCA	1150±22	1110±53	n.s	8.6±5.0	212±4.1	<0.0001

Data are presented as mean ± s.e.mean. The example size for each experiment varied between 5 and 7 for individual series. *P* represents the significance of the difference from control (bile acid bath concentration = 0).

Table 2 The effect of 10^{-4} M bile acids on the contractile response to KCI of rat aortic rings

Bile acid	*Control* R_{max} (mg)	*Test* R_{max} (mg)	P	*Control* EC_{50} (mM)	*Test* EC_{50} (mM)	P
CA	650±18	600±18	<0.05	30.2±0.5	31.6±1.3	n.s
DCA	650±0	300±2	<0.0001	32.5±1.2	76.5±4	<0.0007
CDCA	640±51	330±13	<0.0001	34.7±1.2	63.1±1.4	<0.0001
UDCA	630±12	590±10	<0.01	33.4±1.4	44.2±1.6	<0.005
TCA	638±43	613±43	n.s	29.2±0.8	30.7±1.2	n.s

Data are presented as mean ± s.e.mean. The sample size for each experiment varied between 5 and 7 for each series. *P* represents the significance of the difference from control (bile acid concentration = 0).

Vasoactivity and hydrophobicity and/or lipophilicity

When the ability of the various bile acids at the 10^{-4} M concentration to blunt the contractile responses to NE, as measured by the degree of change in EC_{50} was correlated with the individual lipophilic capacity factors of Roda *et al*. (1990), the correlation was highly significant ($P<0.004$) (Figure 2). When the degree of change in EC_{50} was correlated with the individual bile acid hydrophobic indices of Heuman (1989), there was an almost significant correlation ($P<0.085$) between hydrophobicity and the degree of attenuation (data not shown). When KCl was used as the contractile agonist, the changes in EC_{50} significantly correlated with the lipophilic capacity factor ($P<0.02$) (Figure 3) and the hydrophobicity index ($P<0.0006$).

When R_{max} was correlated with the individual bile acid lipophilic capacity factor significant correlations were observed between lipophilicity and the degree of change in R_{max} (NE $P<0.015$; KCl $P<0.008$) (Figure 2). When R_{max} was correlated with the individual hydrophobic indices, significance was not found with NE ($P<0.15$) but was found with KCl ($P<0.02$). Irrespective of which agonist or index was used, the order of potency of vasoactivity of the bile acids was the same with DCA being the most potent and TCA being the least potent.

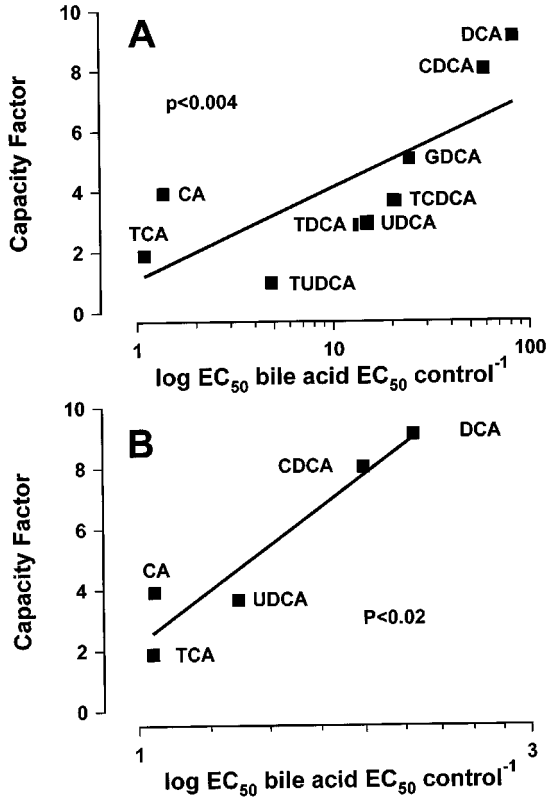

Figure 2 The relationship between the lipophilic capacity factor of Roda *et al*. (1990) of the nine bile acids and their ability to attenuate the contractile responses of the rat aortic rings to NE (A) and KCl (B).

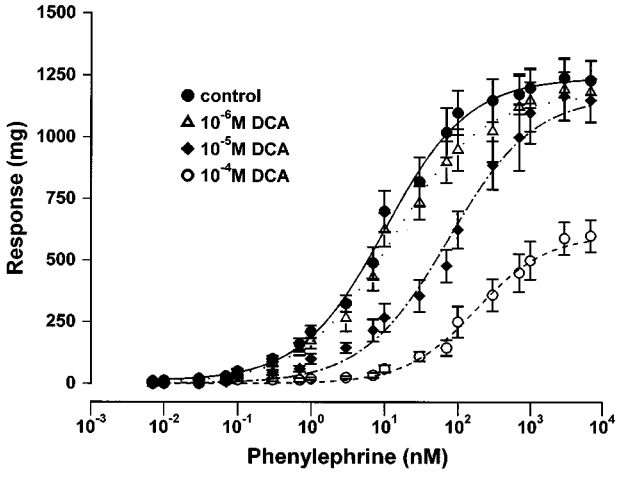

Figure 3 The effect of increasing concentrations of DCA on the *in vitro* contractile response of rat aortic rings to the selective α_1-adrenoceptor agonist, phenylephrine. $n=5-7$ for each series of experiments.

Bile-acid induced vasorelaxation in precontracted arterial rings

In aortic rings precontracted with either 30 nM phenylephrine or 30 mM KCl, the addition of increasing concentrations of DCA and CDCA caused a concentration dependent relaxation whereas the addition of CA, TCA and UDCA had no effect (data not shown).

Effect of bile acids on the contractile response of the rat aorta to α_1-adrenoceptor agonists

Phenylephrine (Figure 3) and methoxamine (data not shown) caused a concentration dependent increase in tension. Preincubation with increasing concentrations of DCA caused concentration dependent attenuation of the response curve. The greatest attenuation of the contractile response occurred at the 10^{-4} M DCA concentration (Figure 3). When methoxamine was used as the contractile agonist, DCA attenuated the concentration response curve (data not shown).

Effects of bile acids on the binding characteristics of rat aortic α_1-adrenoceptors

In vascular smooth muscle membranes, the K_D of α_1-adrenoceptors was 5.9 ± 0.7 pM ($n=4$) with a B_{max} of 81.6 ± 12.4 fmole mg protein^{-1}. Incubation of vascular smooth muscle membranes with 10^{-4} M DCA significantly increased the K_D of the α_1-adrenoceptors (14.1 ± 2.1 pM; $n=4$; $P<0.001$) without affecting their B_{max} (84.4 ± 14.3 fmole mg protein^{-1}). When vascular smooth muscle membranes were incubated with 10^{-4} M TUDCA, the K_D and B_{max} of α_1-adrenoceptors were not significantly different from control (K_D (control vs TUDCA): 6.9 ± 0.5 pM vs 6.5 ± 1.1 pM; B_{max}: 77.1 ± 2.6 fmole mg protein^{-1} vs 74.6 ± 3.5 fmole mg protein^{-1}).

Table 3 The effect of denuding the endothelium, and pretreatment with L-NAME and indomethacin on the vasorelaxant response to DCA of rat aortic rings

Arterial ring preparation	*EC_{50} (μM)*	P
Control (endothelium-intact)	9.8 ± 0.7	
Endothelium-denuded	13.0 ± 0.7	n.s.
Control	13.4 ± 0.7	
Pretreated with 10^{-4} M L-NAME	11.8 ± 0.7	n.s.
Control	11.4 ± 0.7	
Pretreated with 10^{-5} M indomethacin	10.6 ± 0.7	n.s.

The values shown represent the $EC_{50}\pm$standard error of the mean of the relaxant response curves. $n=5-7$ experiments. n.s. = not significant.

Bile acid binding site experiment

No binding was observed when vascular smooth muscle membranes were incubated with all concentrations of radiolabelled ^{14}C-DCA.

The role of the endothelium in bile acid-induced vasorelaxation

Addition of increasing concentrations of DCA to pre-contracted aortic rings whose endothelium was intact caused vasorelaxation in a concentration-dependent manner (Figure 4A). A near identical vasorelaxant response to DCA was observed when the aortic ring was denuded of its endothelium (Figure 4A). In fact, the EC_{50}'s of the vasorelaxant response curves were not significantly different from each other (Table 3). The vasorelaxant responses to DCA in pre-contracted aortic rings pretreated with 10^{-4} M L-NAME were not significantly different from those seen when DCA was added to pre-contracted aortic rings not treated with L-NAME (Figure 4B; Table 3). Pretreatment with indomethacin did not modify the vasorelaxant response to DCA (Figure 4C; Table 3).

Effect of DCA on the extent of lipid peroxidation in rat aortic membranes

The hydrophobic bile acid, DCA, increased the extent of lipid peroxidation in rat vascular smooth muscle membranes in a dose-dependent manner with significance ($P<0.05$) observed at the 10^{-4} M concentration (Figure 5).

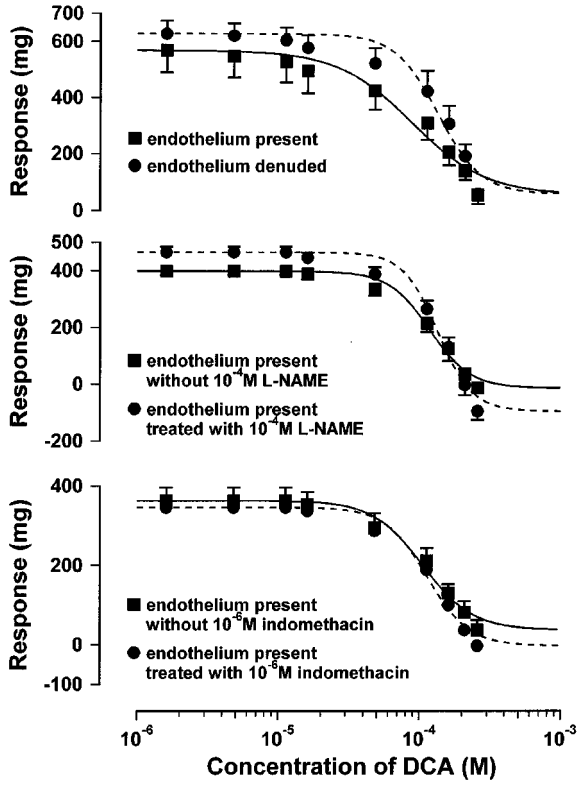

Figure 4 The effect of denuding the endothelium (top), and treatment with L-NAME (middle) and indomethacin (bottom) on the vasorelaxant response to DCA in rat aortic rings precontracted with phenylephrine. $n=5-7$ for each series of experiments.

Effect of DCA on membrane fluidity assessed by fluorescence anisotropy

Although a tendency for a concentration-dependent reduction in the anistropy parameter was observed, increasing doses of DCA over the concentration range, $5\times10^{-5}-10^{-4}$ M, did not significantly alter membrane fluidity when using the DPH probe (Figure 6A). As expected, A_2C markedly increased membrane fluidity (data not shown). When the 2-AS or 6-AS probes were used, DCA significantly ($P<0.0006$; $P<0.004$, respectively) increased fluorescence whereas no difference in steady state anisotropy was seen with the 9-As, 12-As and 16-AP probes (Figure 6B).

Effect of DCA on membrane fluidity assessed by determining the cholesterol : phospholipid molar ratio

Incubating vascular smooth muscle membranes with 10^{-4} M DCA did not significantly alter either their cholesterol (224.4 ± 5.8 μg mg protein^{-1} [control] vs 226.3 ± 7.2 μg mg protein^{-1} [test]) or phospholipid (315.5 ± 18.4 μg mg protein^{-1} [control] vs 315.6 ± 5.9 μg mg protein^{-1} [test]) contents.

Discussion

Bile acids are amphiphilic steroids synthesized from free cholesterol in the hepatocyte and are essential for the solubilization of lipids in bile and the gastrointestinal tract, the induction and maintenance of bile flow, and the absorption of fat from the gastrointestinal tract (Heaton, 1972; Hofmann, 1977; Radominska *et al*., 1993). In addition to these well-known actions in the gastrointestinal tract, one of the least known effects of bile acids is their vasorelaxant action (Bomzon *et al*., 1984; Pak *et al*., 1994). In this study, we examined some aspects of the mechanism of vasorelaxant action of bile acids. Our data indicate that the vasorelaxant activity of an individual bile acid is related to its lipophilicity (Roda *et al*., 1990). Of all the bile acids tested, this study showed that the dihydroxy lipophilic bile acids, DCA and CDCA, possess more intrinsic vasoactivity than trihydroxy hydrophilic bile acids, such as CA and the amine conjugates. In fact, we established an order of potency for vasorelaxant activity of bile acids is DCA > CDCA > GDCA > TDCA > TCDCA with TCA, CA and TUDCA having little or no vasoactivity. This order could have been predicted since

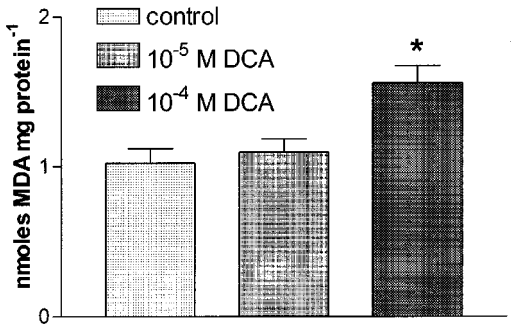

Figure 5 The effect of increasing concentrations of DCA on the extent of lipid peroxidation in rat vascular smooth muscle membranes. The data are presented as mean $\pm$ s.e.mean and represent the cumulated absolute nmoles amounts of MDA equivalents mg protein^{-1} from five experiments. *$P<0.05$ and represents the significance of the difference from control.

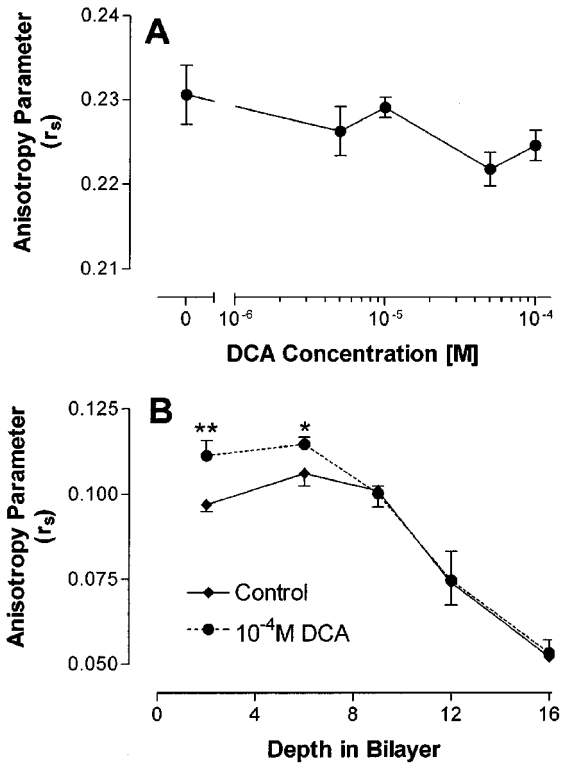

Figure 6 (A) The effects of increasing concentrations of DCA on the static fluidity of rat vascular smooth muscle membranes measured by the fluorescent probe, DPH. Data are presented as mean ± standard deviation from two different membrane preparations. (B) The effect of 10^{-4} M DCA on the steady state fluidity of rat vascular smooth muscle membranes measured by the n-AS and n-AP fluorescent probes. *$P<0.004$; **$P<0.0006$ and represents the significance of the difference from control at the equivalent point. Data are presented as mean ± s.d. from two different membrane preparations except for the 16-AP probe where only one measurement was made (see text for explanation).

lipophilicity is, in part, related to the number of hydroxyl groups on the steroid nucleus. DCA has one less hydroxyl group than CA making it more lipophilic. It is also known that amide conjugation also increases hydrophilicity (Heuman, 1989; Roda *et al*., 1990) and this would be reflected by reduced vasorelaxant activity. We did observe this effect with several bile acids. For example, the responses to DCA are more pronounced than those observed when TDCA or GDCA was used. In the order of potency, the vasoactive status of bile acids such as DCA and TCA is unequivocal. On the other hand, the exact vasoactive status of UDCA is uncertain since at the 10^{-4} M concentration, this bile acid attenuated the contractile response to NE and KCl, but did not relax precontracted aortic rings. One cannot also overlook the result of the competitive radioligand experiments with ^{3}H-prazosin with hydrophilic TUDCA where we reported no effect on α_1-adrenoceptor affinity or density, despite this acid modestly attenuating contractility. Furthermore, statistical significance was always observed when vasoactivity was correlated with lipophilicity and not always observed when correlated with hydrophobicity. Moreover, the correlation was of greater significance when KCl was used as the contractile agonist when compared to NE. Overall, our observations on the vasoactivity of bile acids are consistent with observations of the actions of bile acids in other biological systems where other investigators have shown lipophilic bile acids are biologically more active than hydrophilic bile acids (Hofmann & Roda, 1984). However, it seems that some unexplained subtle quantitative differences apparently exist in this relationship when applied to bile acids and vasorelaxation. Hence, we propose that this relationship may only be used as a general guideline to predict vasorelaxant action especially when considering bile acids whose hydrophobic–hydrophilic balance is less obvious.

In vascular smooth muscle, the mechanisms of vasorelaxation are diverse and are uniformly targeted towards reducing the availability of free intracellular calcium. In the present study, we focussed on three possible mechanisms of bile acid-induced vasorelaxation, namely bile acids interacting with a bile acid binding site present in the vascular smooth muscle membrane; bile acids antagonizing a prototypal contractile receptor, the α_1-adrenoceptor; and bile acids stimulating the release of endothelium-derived relaxant factors. The results of our experiments demonstrate that the vasorelaxant activity of lipophilic bile acids is not dependent upon stimulation of a surface membrane bile acid binding site or the endothelium. Instead, our results suggest that bile acid-induced vasorelaxation is mediated by antagonizing α_1-adrenoceptors, a phenomenon that itself is also linked to the lipophilicity of an individual bile acid. DCA attenuated the contractile response to the selective α_1-adrenoceptor agonists, phenylephrine and methoxamine. DCA at the same concentration also lowered the affinity of aortic α_1-adrenoceptors without a change in density. Therefore, we concluded that the basis of vasorelaxant activity of hydrophobic bile acids could be linked to their ability to antagonize vascular α_1-adrenoceptors by lowering receptor affinity. This mechanism of vasorelaxation involving bile acid action on the affinity of receptors, such as vascular α_1-adrenoceptors, should not be considered as unitary because bile acids also attenuate contractile responses to other agonists, such as arginine vasopressin (Pak *et al*., 1994) and 5-hydroxytryptamine (Bomzon & Said, unpublished data).

In 1980, Hirata & Axelrod proposed that proper receptor function was, in part, dependent upon an optimal membrane environment. The membrane environment itself may be adversely affected by ROS whose prime targets are the polyunsaturated fatty acids in cell membranes (Cheeseman & Slater, 1993; Freeman & Crapo, 1982; Jourd'Heuil *et al*., 1995). The deleterious action of ROS on adrenoceptors has also been described (Kaneko *et al*., 1991; Persad *et al*., 1997; Schimke *et al*., 1995; Williams *et al*., 1995). At micromolar concentrations, lipophilic bile acids, such as DCA, can stimulate the generation of ROS and increase the extent of lipid peroxidation (Craven *et al*., 1987; DeRubertis & Craven, 1987; Sokol *et al*., 1993). Against this background, we considered this information as a potential explanation for reduced affinity of vascular α_1-adrenoceptors by DCA. Accordingly, we examined the effect of the lipophilic bile acid, DCA, on the fluidity and the extent of lipid peroxidation in rat vascular smooth muscle membranes. We found that DCA increased the extent of lipid peroxidation in these membranes. MDA is an end-product of peroxidative decomposition of polyeonic fatty acids in the lipid peroxidation process; and its presence in tissues is indicative of ROS activity and the extent of lipid peroxidation process and ROS activity accumulation in tissues is indicative of the extent of lipid peroxidation (Gutteridge, 1995; Halliwell & Chirico, 1998; Holley & Cheeseman, 1993). The stimulatory action of micromolar concentrations of lipophilic bile acids, such as DCA, on ROS generation and subsequent initiation of the lipid peroxidation process has been described in epithelial cells (Craven *et al*., 1987; DeRubertis & Craven, 1987). Micromolar concentrations of the same bile acid can also promote the generation of ROS to cause lipid peroxidation in hepatocytes (Sokol *et al*., 1993) and macrophages (Ljubuncic *et al*., 1996). To the best of

our knowledge, the data presented in this study is the first report of micromolar concentrations of DCA increasing the extent of lipid peroxidation in vascular smooth muscle membranes.

It is well known that bile acids can solubilize membranes by promoting cholesterol and phospholipid loss from membranes (Güldütuna *et al*., 1993). However, membrane solubilization occurs when membranes are exposed to millimolar concentrations of bile acids (Güldütuna *et al*., 1993) and would anticipate a change in membrane fluidity. Although membranes are not solubilized at micromolar concentrations of bile acids, their effect on the fluidity of vascular smooth muscle membranes at these concentrations is not known. In the present study in which vascular smooth muscle membranes were exposed to 10^{-4} M DCA, we were unable to detect any changes in membrane fluidity using DPH fluorescence anisotropy and the cholesterol : phospholipid molar ratio. This conclusion is in agreement with our previous observations in purified rat cardiac membranes (Gazawi *et al*., 2000) where we reported that micromolar concentrations of the bile acids. DCA, TDCA, CDCA and TCDCA, had no effect on membrane fluidity or cause loss of cholesterol or phospholipids.

ROS preferentially attack polyunsaturated fatty acids resulting in, amongst other phenomena, a reduction in membrane fluidity. Jourd'heuil and his colleagues (Jourd'Heuil *et al*., 1995) have shown that the outer membrane hemileaflet is susceptible to peroxidation. As micromolar concentrations of DCA increase the extent of lipid peroxidation, one would anticipate a preferential reduction in the fluidity of superficial layers of the membrane. DCA increased the fluorescence of the 2-AS fluorescent probe. Increased fluorescence, although quantitatively smaller, was also detected when 6-AS fluorescent probe was used. As the 2- or 6-AS fluorescent probes reflect the motional freedom of lipids in the superficial layers of the membrane, it seems that DCA oxidatively attacks the superficial layers of the membrane to preferentially reduce its fluidity. As we did not know the exact orientation of the membrane in these experiments, we are not able to state with any certainty whether the superficial layers of the membranes corresponds to the outer membrane hemileaflet.

Focusing on the results of our experiments in which we tried to determine whether bile acids interact with a bile acid binding site on the vascular smooth muscle membrane, our experiments involving ^{14}C-DCA failed to establish that vascular smooth muscle membranes contain a binding site for bile acids. In the cell membrane of hepatocytes and intestinal epithelia, a bile acid carrier or transport system has been described (Bouscarel *et al*., 1995; 1999) for transporting bile acids into these cells. These transport systems are an integral component of the enterohepatic circulation of bile acids. From a functional point of view, there appears to be little value in vascular smooth muscle membrane having its own binding site or carrier system for bile acids as these compounds are needed for the digestion and absorption of lipids in the intestinal tract. In fact, the portal circulation is the only vascular smooth muscle exposed to high concentrations of bile acids; and the vasorelaxation observed in this circulation in healthy individuals during digestion has not been attributed to elevated portal blood concentrations of hydrophobic bile acids.

In 1994, Lee and his colleagues (Pak *et al*., 1994) reported that bile acid-induced vasorelaxation was not dependent upon the presence of the endothelium. For their experiments, they assessed the vasorelaxant action of TDCA in endothelium-denuded isolated rat arterial rings and the perfused mesenteric circulation or the identical preparations pretreated with 10^{-4} M L-NAME. Using a more lipophilic bile acid, DCA, in an aortic ring preparation, denuded of its endothelium or pretreated with L-NAME, as well as indomethacin, we confirmed that bile acid-induced vasorelaxation is not dependent upon the endothelium.

The activity of vascular α_1-adrenoceptors is an important determinant of vascular tone. In this report, we have described that the mechanism of bile acid-induced vasorelaxation is characteristic of lipophilic bile acids and could be mediated by antagonism of vascular α_1-adrenoceptors by reducing their affinity. However, the action of lipophilic bile acids on the physicochemical microenvironment of the membrane possibly mediated by ROS and the resultant impact on α_1-adrenoceptor function should also be considered. Based upon the data presented in this communication, it seems reasonable to conclude that DCA-induced reduction in fluidity and stimulation of lipid peroxidation in vascular smooth muscle membranes could account for ability of this bile acid to reduce the affinity of vascular α_1-adrenoceptors. Although the data presented in this study favour this concept, further studies are needed to establish a causal relationship between the deleterious effect of DCA on vascular α_1-adrenoceptors and its action on membrane fluidity and lipid peroxidation. It should also be remembered that lipophilic bile acids also affect other mechanisms known to be involved in vasorelaxation. These include inhibition of calcium entry through voltage-dependent calcium channels (Binah *et al*., 1987; Pak *et al*., 1994) and facilitating potassium efflux through potassium channels (Kotake *et al*., 1989). From such studies, the basis of bile acid-induced vasorelaxation is obviously diverse. It also should not be forgotten that bile acids inhibit mitochondrial function (Krähenbühl *et al*., 1994). Moreover, ROS themselves are vasorelaxants by virtue of their ability to open ATP-sensitive potassium channels (Wei *et al*., 1998) and to impair cellular signalling associated with calcium by interacting with various ion transport proteins underlying transmembrane signal transduction (Kourie, 1998). Instead, the ability of lipophilic bile acids, such as DCA, to reduce the affinity of α_1-adrenoceptors and to increase membrane rigidity and the extent of lipid peroxidation in vascular smooth muscle membranes should rather be perceived as merely one facet of a multifaceted mechanism whereby bile acids relax vascular smooth muscle.

Do these data have any clinical relevance? Cholestatic patients and animals exhibit attenuated responsiveness to sympathomimetic stimulation and this phenomenon has been proposed as one of the underlying mechanisms of reduced vascular resistance in these individuals (Bomzon *et al*., 1996). Bile acids accumulate in the plasma of cholestatic patients with the total fasting serum bile acids concentrations may reach levels as high as $500-800\ \mu M\ l^{-1}$, but most reports indicate concentration ranges of $100-250\ \mu M\ l^{-1}$ (Schaffner *et al*., 1971; Ostrow, 1993). Accordingly, bile acids have been implicated as mediators of the reduction in systemic vascular resistance in cholestasis (Bomzon *et al*., 1996). At concentrations equivalent to that seen in cholestatic individuals, we observed that lipophilic bile acids reduced the affinity of α_1-adrenoceptors that may be due to their pro-oxidant action. Therefore, our data lend further support to the idea that the plasma accumulation of bile acids contribute to the reduction in systemic vascular resistance in cholestasis.

To conclude, this study has established that bile acids are non-selective vasorelaxants. This vasorelaxant activity correlates with the lipophilicity of a bile acid with lipophilic bile acids possessing greater vasorelaxant activity than hydrophilic species. This study has also shown that the possible mechanism

of the vasorelaxant action of lipophilic bile acids might be due to an antagonist action on a prototypal contractile receptor system, the α_1-adrenoceptor, that expresses itself as a lowering of the affinity of the receptor without changing its density. Our data also indicates that lipophilic bile acids can modify the membrane microenvironment through augmentation of the lipid peroxidation process. By virtue of the change in the membrane microenvironment, the behavior of surface membrane contractile receptors may be impaired. In addition, we have confirmed that bile acid-induced vasorelaxation is not endothelium-dependent. At the same time, our data indicate that bile acid-induced vasorelaxation is not linked to an interaction with a bile acid binding site on the membranes of vascular smooth muscle cells. Overall, we believe that the process of bile acid-induced vasorelaxation is a multifaceted process encompassing numerous membrane processes that interact with each other to ultimately restrict transmembrane influx of calcium.

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