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Endothelium-dependent Relaxation in Response to Ethanol in the Porcine Isolated Pulmonary Artery

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Abstract

Many drugs cannot be dissolved in distilled water and so other solvents such as ethanol, dimethylsulphoxide and methanol are used. Because very little is known about the direct effects of these three solvents on the cardiovascular system, we have examined their effects on isolated pulmonary and coronary arteries from the pig.

Increasing concentrations of ethanol, dimethylsulphoxide and methanol induced relaxation in porcine pulmonary (at 1·2% v/v, 59·9 ± 9·0% (n = 9), 55·9 ± 9·0% (n = 6) and 12·3 ± 6·4% (n = 8), respectively, of U46619-induced tone) and coronary arteries (at 1·2% v/v, 69·9 ± 7·1% (n = 10), 78·9 ± 6·1% (n = 7) and 12·9 ± 8·2% (n = 6) respectively, of U46619-induced tone). In the pulmonary arteries the relaxation in response to ethanol was found to be endothelium-dependent whereas the responses to dimethylsulphoxide and methanol were unaffected by removal of the endothelium. In the coronary arteries the relaxation to all three solvents was independent of the presence of the endothelium. Comparison of the sensitivity of the tissues to the solvents showed that ethanol and dimethylsulphoxide produced comparative responses in both the pulmonary and coronary arteries, whereas methanol was much less potent. The endothelium-dependent response to ethanol in the porcine pulmonary artery (maximum response, E_{max}, 67·1 ± 9·3% of U46619-induced tone, n = 7) was attenuated by the cyclooxygenase inhibitor, flurbiprofen (E_{max} 31·9 ± 12·0%, n = 7), the nitric oxide synthase inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester; E_{max} 23·5 ± 10·2%, n = 7)) and the combination of both inhibitors (E_{max} 18·3 ± 7·8%, n = 7). The residual relaxatory response to ethanol was abolished, and converted into a contractile response, both by removal of the endothelium (at 1·7% v/v ethanol 27·3 ± 11·5% of U46619-induced tone, n = 7) and by the addition of a low concentration of KCl (49·9 ± 10·3%, n = 6), suggesting the release of a non-prostanoid, non-nitric oxide factor from the endothelium. This response, however, was not attenuated by the cannabinoid receptor-antagonist SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl; 52·5 ± 4·3% relaxation, n = 8), suggesting that the factor released in this preparation by ethanol is not a cannabinoid.

The results of this study indicate that many solvents commonly used in pharmacological experiments have pronounced vasoactive properties. Methanol might be the vehicle of choice, because it was the least active solvent, whereas high concentrations of ethanol might influence vascular function at both the level of the smooth muscle and the endothelium, with the action on the endothelium involving the release of endothelium-derived relaxing factors.

While examining the effects of the calcium ionophore A23187 on the isolated pulmonary artery from the pig we noticed in control experiments that the vehicle, ethanol, also affected vasoconstrictor tone.

There have been relatively few studies of the effects of ethanol on isolated blood vessels. In the majority of these ethanol has been shown to cause concentration-dependent contraction (Kettunen et al

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1983) which in some cases has been shown to be endothelium-independent (Zhang et al 1993). However, in rat mesenteric arterioles in-vivo, ethanol caused relaxation via a non-prostanoid mechanism (Altura et al 1979). There have been no studies of the possible contribution of nitric oxide or endothelium-derived hyperpolarizing factors on vasorelaxatory responses to ethanol.

We have investigated the responses to ethanol in two preparations, the porcine isolated pulmonary artery (chosen because of the high sensitivity of this preparation to ethanol observed in our preliminary experiments) and, for comparison, the porcine isolated coronary artery (which is a commonly used vascular preparation; Cocks & Angus 1983). In addition to studying the effects of ethanol on these tissues, we have also examined the effects of two other solvents commonly used to dissolve pharmacological agents, dimethylsulphoxide (DMSO) and methanol.

Some of these results were presented at the British Pharmacological Society Meeting in Bristol in 1997 (Lawrence et al 1997).

Materials and Methods

Drugs and solutions

Potassium chloride, absolute ethanol, DMSO and methanol were AnalaR reagents from BDH. U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$ methyl acetate), flurbiprofen, N^G-nitro-L-arginine methyl ester (L-NAME), acetylcholine chloride, substance P were from Sigma and SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl was obtained from Dr E. A. Boyd, Nottingham University. Stock solutions of U46619 (10mM), flurbiprofen (1mM) and SR141716A (10mM) were prepared in absolute ethanol and then, where necessary, diluted with distilled water. All other drugs were dissolved in distilled water.

The composition of the Krebs-Henseleit solution was (mM): NaCl 119; KCl 4·7; $MgSO_4.7H_2O$ 1·17; $NaHCO_3$ 24; $CaCl_2$ 1·25; KH_2PO_4 1·17; glucose 5·5.

Tissue samples

Lungs and hearts were removed within 30min from pigs killed in the abattoir and transported back to the laboratory in ice-cold, modified Krebs-Henseleit solution. The main pulmonary artery and coronary artery were located, dissected out and placed in Krebs-Henseleit solution containing 2% ficoll which had previously been oxygenated with 95% O_2–5% CO_2 and stored overnight at 4°C. The ficoll solution was used to prevent osmotic swelling of the cells (Lot et al 1993).

The next day the vessels (approximately 2–4mm diameter) were cleaned of excess connective tissue and cut into 5–6-mm segments. The endothelium of some segments was removed by gently rubbing the lumen of the vessel with a roughened metal rod. Two stainless-steel wires (0·2mm thick) were then placed in the artery; one was linked to a glass support and the other was connected by cotton to a Grass force-displacement transducer (Model FT03) connected to a Grass polygraph. The segments were then placed in an isolated organ bath containing Krebs-Henseleit solution (5mL) maintained at 37°C and oxygenated with 95% O_2–5% CO_2.

Experimental protocol

Initial resting tensions of 4 and 6g were applied to each segment of porcine pulmonary and coronary arteries, respectively, 30min after equilibration. The resting tension levelled off to approximately 1·5 and 2g, respectively, after a further 30min. The preparations were then stimulated with 60mM KCl until reproducible responses were obtained.

Rubbed and unrubbed tissues were exposed to the thromboxane mimetic, U46619 (1 to 30nM), to produce an amount of constrictor tone equivalent to approximately 80% of the response to 60mM KCl. Removal of the endothelium was confirmed by addition of 100nM acetylcholine to each pulmonary artery and 1μM substance P to all coronary artery segments. After equilibration, tissues were washed and left until a steady baseline was achieved (30–40min) before reconstricting to a similar level with U46619. When a steady response was obtained, increasing concentrations of ethanol, DMSO and methanol were added.

To establish which endothelium-derived relaxant factor(s) contributed to the endothelium-dependent responses to ethanol, the cyclooxygenase inhibitor, flurbiprofen (1μM) and the nitric oxide synthase inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester, 100μM) were added to tissues. Preparations were incubated with either U46619 or L-NAME before any addition of flurbiprofen, after which segments were left to equilibrate for at least 40min before being constricted to approximately 80% of the response to 60mM KCl, by use of U46619. Again, when a steady response was established increasing concentrations of ethanol were added.

To investigate the possibility that the L-NAME-resistant, flurbiprofen-resistant component of responses to ethanol involves an endothelium-derived hyperpolarizing factor, segments were exposed to Krebs-Henseleit solution containing 30mM KCl, produced by equimolar exchange of

K^+ for Na^+ ions. Tissues preconstricted by U46619 or 30mM KCl Krebs-Henseleit solution were then incubated with 1 μM flurbiprofen and 100 μM L-NAME to produce similar levels of tone, before addition of 1·7% v/v ethanol. Finally, to test the possibility that the L-NAME-resistant, flurbiprofen-insensitive relaxant was mediated by a cannabinoid, preparations incubated with 1 μM flurbiprofen and 100 μM L-NAME were exposed to the cannabinoid receptor-antagonist SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl; 10 μM) for 40min. Tone was again adjusted using U46619, and increasing concentrations of ethanol added.

Data analysis

Unless otherwise stated, the effects of the solvents have been expressed in terms of the percentage of U46619-induced tone before addition of the solvent, and are shown as the mean ± s.e.m. of n observations. In some experiments, the maximum response (E_{max}) has been given; it is expressed as a percentage of U46619-induced tone. Differences between means were examined by use of the unpaired Student's *t*-test for single data comparisons; two-way analysis of variance was used for a comparison of concentration–response curves. $P < 0.05$ was considered to be indicative of statistical significance.

Results

Comparison of rubbed and unrubbed segments

There was no significant difference between the contractile responses to 60mM KCl of unrubbed and rubbed segments of porcine pulmonary artery (unrubbed 5·0 ± 0·4g; rubbed 4·3 ± 0·4g, n = 23) or of porcine coronary artery (unrubbed 6·7 ± 0·5g; rubbed 6·2 ± 0·4g, n = 23). The level of U46619-induced tone for unrubbed and rubbed segments as a percentage of the response to 60mM KCl in the pulmonary artery (unrubbed 55·4 ± 3·8%; rubbed 59·3 ± 4·7%, n = 23) and the coronary artery (unrubbed 70·3 ± 5·4%; rubbed 80·8 ± 6·8%, n = 23) were also not significantly different.

In unrubbed segments of the pulmonary artery, 100nM acetylcholine elicited 54·6 ± 6·5% (n = 14) relaxation of U46619-induced tone, but did not alter vascular tone in rubbed segments. In unrubbed segments of the coronary artery, 10nM substance P elicited 77·2 ± 5·1% (n = 22) relaxation of U46619-induced tone which again was abolished after rubbing of the endothelium. It was therefore concluded that rubbing the lumen of these vessels had adequately removed the endothelial layer. Ethanol (0·01–1·18% v/v) induced concentration-dependent relaxation of U46619-induced tone in unrubbed segments of the pulmonary and coronary arteries. The relaxation responses of the pulmonary and coronary arteries to 1·18% v/v ethanol were not significantly different, reaching 59·9 ± 9·0% (n = 9) and 69·9 ± 7·1% (n = 10), respectively, of U46619-induced tone (Figure 1). In rubbed segments of the coronary artery (Figure 1B), ethanol reduced U46619-induced tone to a similar extent, with a maximum response of 73·5 ± 8·2% (n = 10) whereas in rubbed segments of the pulmonary artery (Figure 1A) ethanol elicited a contraction (maximum response 32·4 ± 11·1%, n = 9) of the U46619-induced tone. A characteristic feature of endothelium-dependent relaxation to ethanol was that it was markedly slower than to acetylcholine (Figure 2).

Both DMSO (1·2% v/v) and methanol (1·2% v/v) elicited relaxation of U46619-induced tone in the pulmonary and coronary arteries (Table 1), with the response to DMSO being significantly greater than that to methanol. Endothelial denudation did not significantly alter the response to either DMSO or methanol. The addition of DMSO (up to 1·45% v/v) was shown to have no damaging effect on cellular mechanisms in the pulmonary artery—there was no alteration in the ability of these tissues to respond subsequently to 60mM KCl (unrubbed: 4·1 ± 0·6g before DMSO, 4·6 ± 0·5g after DMSO; rubbed: 2·9 ± 0·9g before DMSO, 2·9 ± 0·7g after DMSO).

Effect of flurbiprofen and L-NAME on responses to ethanol in the porcine pulmonary artery

We subsequently examined the endothelium-dependent response of the porcine pulmonary artery to ethanol in greater detail to establish which endothelium-derived relaxing factors were

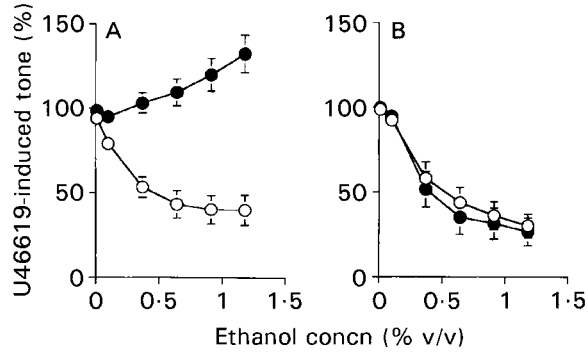

Figure 1. The effect of increasing concentrations of ethanol on unrubbed (○) and rubbed (●) segments of porcine isolated pulmonary artery (A) and the isolated coronary artery (B), preconstricted by administraton of the thromboxane mimetic, U46619. Responses are the means ± s.e.m. of nine or ten observations, expressed as a percentage of U46619-induced tone.

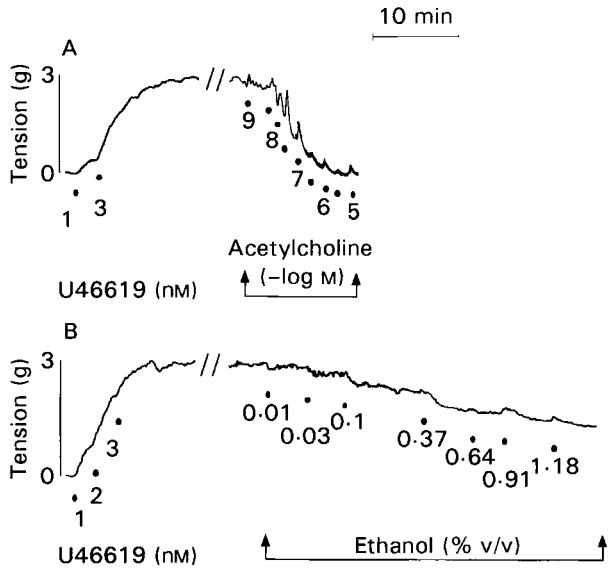

Figure 2. Representative traces of the effects of increasing concentrations of acetylcholine (A) and ethanol (B) on unrubbed segments of the porcine isolated pulmonary artery, preconstricted by administraton of the thromboxane mimetic, U46619. The time-course of the response to ethanol is much longer than that of the response to acetylcholine.

involved. This was achieved by the use of two inhibitors—the cyclooxygenase inhibitor, flurbiprofen (1 μM) and the nitric oxide synthase inhibitor, L-NAME (100 μM). Flurbiprofen alone, L-NAME alone and flurbiprofen plus L-NAME significantly reduced the response to ethanol by a similar amount (E_{max}: control 67·1 ± 9·3%, flurbiprofen 31·9 ± 12·0% (n = 7); control 78·8 ± 4·8%, L-NAME 23·5 ± 10·2%, (n = 7); control 70·8 ± 11·8%, flurbiprofen plus L-NAME 18·3 ± 7·8%, (n = 7)). There was no significant difference between the responses to ethanol in the presence of each inhibitor alone, or in the presence of both inhibitors (Figure 3); a small relaxatory response to ethanol remained in each instance.

The effect of KCl, SR141716A and removal of the endothelium on ethanol-induced relaxation in the presence of flurbiprofen and L-NAME

As shown in Table 2, 1·7% v/v ethanol elicited relaxation of the porcine pulmonary artery in the

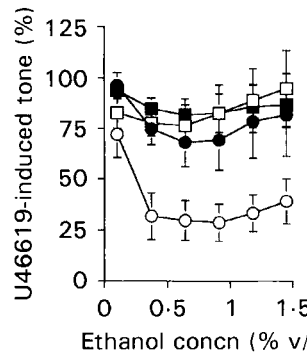

Figure 3. The effect of ethanol on U46619-induced tone of unrubbed segments of the porcine isolated pulmonary artery in the absence (○) or presence of 1 μM flurbiprofen (●), 100 μM L-NAME (□) or a combination of both (■). Responses are the means ± s.e.m. of seven observations, expressed as a percentage of U46619-induced tone.

presence of 100 μM L-NAME and 1 μM flurbiprofen (27·9 ± 7·7%, n = 7) whereas removal of the endothelium revealed a contractile response to ethanol (27·3 ± 11·5% of U46619-induced tone, n = 7). Thus, the residual L-NAME-resistant, flurbiprofen-insensitive response to ethanol is endothelium-dependent. Substitution of U46619 by 30 mM KCl Krebs-Henseleit solution (which elicited a similar amount of vasoconstriction-U46619 71·7 ± 9·5% of the response to 60 mM KCl; KCl 78·0 ± 13·0%, n = 6) also converted the relaxation to 1·7% v/v ethanol into a contraction (49·9 ± 10·3% of induced tone, n = 6). However, the endothelium-dependent relaxation to 1·7% v/v ethanol was significantly enhanced in the presence of 10 μM SR141716A (control 17·4 ± 11·7% relaxation, SR141716A 52·5 ± 4·3% relaxation, n = 8).

Discussion

Responses to solvents

Our results show that ethanol induced a concentration-dependent relaxation of isolated pulmonary and coronary arteries taken from the pig. Whereas the responses to ethanol were endothelium-dependent in the pulmonary arteries, those in

Table 1. Responses of unrubbed and rubbed segments of porcine isolated pulmonary and coronary arteries, preconstricted with the thromboxane mimetic U46619, to dimethylsulphoxide and methanol.

	Treatment	Pulmonary artery	n	Coronary artery	n
Dimethylsulphoxide	With endothelium	55·9 ± 9·0*	6	78·9 ± 6·1*	7
(1·2% v/v)	Without endothelium	60·4 ± 3·6*	6	78·6 ± 6·2*	7
Methanol	With endothelium	12·3 ± 6·4	8	12·9 ± 8·2	6
(1·2% v/v)	Without endothelium	30·9 ± 9·2	8	20·1 ± 4·0	6

Relaxation is expressed as a percentage of U46619-induced tone, and are shown as means ± s.e.m. of n observations. *$P < 0·05$, significantly greater than the corresponding response to methanol.

Table 2. The effect of 1·7% v/v ethanol on vascular tone in the presence 1 μM flurbiprofen and 100 μM L-NAME in unrubbed and rubbed segments of the porcine isolated pulmonary artery, in the absence and presence of 30 mM KCl Krebs-Henseleit solution and the absence and presence of 10 μM SR141716A.

Treatment	n	Control (unrubbed)	Treated
Rubbed	7	−27·9 ± 7·7%	+27·3 ± 11·5%*
Plus KCl	6	−32·2 ± 4·3%	+49·9 ± 10·3%*
Plus SR141716A	8	−17·4 ± 11·7%	−52·5 ± 4·3%*

Where necessary U46619 was used to induce tone equivalent to 80% of the response to 60 mM KCl. Responses have been expressed as a percentage of U46619-induced tone, where a negative value denotes a relaxation response and a positive value denotes a contractile response above the level of U46619-precontracted tone. Values are the means ± s.e.m. of n observations. *$P < 0·05$, significantly different from control.

the coronary arteries were endothelium-independent. Although two other solvents, DMSO and methanol, also induced relaxation in the pulmonary and coronary arteries, neither response was dependent on a functional endothelium.

The potencies of each solvent in each vessel were similar, but ethanol and DMSO were more potent than methanol. These findings suggest that if a non-aqueous solvent is required to dissolve a drug, the least vasoactive vehicle available is methanol.

The effects of DMSO and methanol on isolated blood vessels have not previously been documented. DMSO has, however, been shown to relax perfused cat and dog hearts (Shlafer & Karnow 1975), dilate pial vessels in-situ in compressed monkey brains (De La Torre et al 1973) and induce vasodilatation in-vivo in vessels from pedicle flaps of rats (Adamson et al 1966). Although the mechanisms of these responses have not been identified, it is clear that DMSO is not exerting a non-specific, irreversible effect in our preparations, because the response to 60 mM KCl was unchanged after exposure to DMSO (1·45% v/v).

The relaxation responses to ethanol apparent in the porcine pulmonary and coronary arteries are consistent with the findings of Altura et al (1979) on rat mesenteric arterioles in-situ, but contrast with those obtained from other vascular preparations, for example canine isolated pulmonary arteries and the basilar and middle cerebral arteries from piglets, sheep, dogs and baboons, for which it has been demonstrated that ethanol (0·05 to 3·50% v/v) induces contractions (Kettunen et al 1983) which, in some instances, are endothelium-independent (Zhang et al 1993). Contractile responses to ethanol might be Ca^{2+}-dependent in smooth muscle cells, because removal of extracellular Ca^{2+} attenuated contractile responses in the canine middle cerebral arteries (Zhang et al 1993) and Johnson et al (1996) demonstrated that ethanol elicited an increase in intracellular Ca^{2+} levels in vascular smooth muscle and endothelial cells maintained in culture. This is consistent with our results in the porcine pulmonary arteries, where, in the absence of the endothelial layer, a contractile response was noted which could be explained by an increase in intracellular Ca^{2+} levels in smooth muscle cells (Nelson et al 1990), whereas in the presence of the endothelium we saw a relaxant response, which could be because of an increase in intracellular Ca^{2+} levels in endothelial cells (Lückhoff & Busse 1990). However, as relaxatory responses to ethanol in the porcine coronary artery were endothelium-independent, it can be concluded that there are fundamental differences at the level of the endothelium and smooth muscle between the pulmonary and coronary arteries from the pig.

Although there are several possible triggers of this increase in intracellular Ca^{2+} levels, for example, fluidization of the membrane (Chin & Goldstein 1977), which might explain the slow time-course of our responses to ethanol, or the production of a hypoxic state (Doekel et al 1978), it remains unclear which mechanism is implicated in vascular responses to ethanol.

Endothelium-derived factors involved in responses to ethanol in the porcine pulmonary artery

Because the relaxation to ethanol in the porcine pulmonary artery is endothelium-dependent, we decided to investigate further the characteristics of this effect. It is well documented that the endothelium can release a variety of factors capable of relaxing vascular smooth muscle, the most prevalent being nitric oxide (Moncada et al 1987), dilator prostanoids (Smith et al 1994) and endothelium-derived hyperpolarizing factor (Randall et al 1996). Our work supports a role for both nitric oxide and dilator prostanoids in the response of the pulmonary arteries to ethanol because inhibitors of nitric oxide synthase and cyclooxygenase both attenuated the relaxations. This contrasts with responses in rat mesenteric arterioles in-vivo, where inhibition of prostaglandin synthetase failed to affect relaxatory responses to comparable concentrations of ethanol (Altura et al 1979).

Although our results implicate nitric oxide and dilator prostanoids in the response to ethanol, the inhibitors in combination failed to mimic the effect of removing the endothelium, i.e. flurbiprofen and L-NAME failed to reveal the constrictor effect of ethanol. This raised the possibility that a third endothelium-derived factor was released by ethanol. The residual non-prostanoid, non-nitric oxide

relaxation response was abolished, and converted into a contractile response, both by rubbing of the endothelium and by the presence of an increased extracellular concentration of KCl. On the basis of similar observations in the rat isolated perfused mesentery it has recently been suggested that this endothelium-derived non-prostanoid, non-nitric oxide factor is a cannabinoid (Randall et al 1996). However, the cannabinoid receptor antagonist, SR141716A (Rinaldi-Carmona et al 1994), failed to block the residual relaxation response to ethanol in the pulmonary artery, and even significantly enhanced it. It seems, therefore, that in the porcine pulmonary artery the non-prostanoid, non-nitric oxide factor released by ethanol is not a cannabinoid substance.

The overall conclusion from these results is that if a non-aqueous solvent has to be used to dissolve a potential vasoactive compound, methanol might be the vehicle of choice, because of its relative lack of vascular activity. However, if ethanol or DMSO are used, adequate controls must be present to take into account the effects of these solvents, and care should be taken in interpreting the results obtained after use of these solvents owing to the possibility of their altering cellular mechanisms. Our results, with those of other workers, have demonstrated substantial heterogeneity among the effects of these solvents, not only between species, but also between arteries isolated from different vascular beds. In addition, we have shown that the action of ethanol in the porcine isolated pulmonary artery is complex, involving the release of several endothelium-derived vasoactive factors and direct vasoconstriction.

Acknowledgements

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