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Effect of cold storage in University of Wisconsin solution on the responses of porcine hepatic arteries to 5-hydroxytryptamine and bradykinin *in vitro*

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1 Responses to 5-hydroxytryptamine (5-HT), bradykinin and sodium nitroprusside (SNP) were examined in hepatic arteries of the pig 1 h after dissection (fresh) and following 24 h storage in either Ca^{2+}-free Krebs solution or the cryopreservative University of Wisconsin (UW) solution.

2 In fresh arteries contracted to approximately 40% of the maximum response to potassium with U46619, a thromboxane A_2-mimetic, concentration-response curves to 5-HT ($10^{-10}-10^{-5}$ M) were biphasic, with relaxation at low concentrations ($<10^{-8}$ M) and contraction at high concentrations. Bradykinin ($10^{-10}-10^{-7}$ M) produced concentration-dependent relaxation of precontracted fresh arteries with no apparent constrictor response.

3 Following 24 h storage in Ca^{2+}-free Krebs solution, relaxation responses to 5-HT and the sensitivity of the arteries to bradykinin were significantly reduced. Storage in UW solution did not affect relaxation responses to either 5-HT or bradykinin. Relaxation responses to SNP ($10^{-8}-10^{-3}$ M) were unaffected by storage in either solution.

4 Treatment of fresh arteries with N^G-nitro-L-arginine (L-NOARG, 10^{-4} M) significantly attenuated the relaxation response to 5-HT and displaced the bradykinin concentration-response curve four fold to the right with no affect on its maximum relaxation.

5 From these results it is concluded that endothelial cell function is better preserved during cold storage in UW solution than in Ca^{2+}-free Krebs solution.

Keywords: Porcine hepatic arteries; 5-hydroxytryptamine (5-HT); bradykinin; University of Wisconsin (UW) solution; cold storage

Introduction

The vascular endothelium plays an important role in the regulation of vascular tone through the release of various vasoactive factors such as nitric oxide (NO) and prostacylcin (PGI_2). The diffusion barrier properties of the endothelium probably also modulate vasoconstrictor responsiveness (Lew & Duling, 1992). Thus, any damage to the endothelium has the potential to cause major changes in vascular reactivity.

Cold ischaemic storage and reperfusion of donor organs often results in tissue damage characterized by loss of endothelial cell integrity on histological examination. Ischaemic injury of donor livers is a well recognised phenomenon and recent experimental evidence has implicated endothelial cell damage in the sinusoids as an important abnormality following ischaemia/reperfusion of the liver (McKeown *et al.*, 1988; Caldwell-Kenkell *et al.*, 1989). The cryopreservative University of Wisconsin (UW) solution is currently the solution of choice for the cold preservation of donor livers (Bismuth *et al.*, 1993). UW solution not only increases the functional organ storage time of donor livers, but also appears to reduce the incidence of primary liver malfunction, hepatic arterial thrombosis and graft failure following transplantation (D'Alessandro *et al.*, 1990). UW solution is calcium-free and contains a number of agents including adenosine, an inhibitor of platelet aggregation and free radical production; allopurinol, which inhibits free radical production; and glutathione, a free radical scavenger, which may help to preserve organ integrity during cold storage.

The superiority of UW solution as a cryopreservative may be due to the protection of endothelial cells (McKeown *et al.*, 1988; Caldwell-Kenkel *et al.*, 1989; Holloway *et al.*, 1990; Takei *et al.*, 1991; Eberl *et al.*, 1993). The majority of studies examining the effects of cold storage have been carried out in whole organ models; however, to date no pharmacological studies examining the direct effects of cold storage on hepatic arterial reactivity *in vitro* have been reported. Since the endothelial cell appears to be sensitive to preservation damage, the effect of cold storage on responses to endothelium-dependent vasodilator agents provides a simple method for assessing possible endothelial cell injury. Therefore the aim of this study was to examine the responses of isolated hepatic arteries of the pig to the endothelium-dependent vasodilator agents 5-hydroxytryptamine (5-HT) and bradykinin before and after 24 h cold storage to determine whether cold storage-induced damage to the endothelium is reduced by UW solution.

Methods

Experimental set-up

Pig livers were obtained from an abattoir (Large White pigs of either sex). Immediately after removal from the pig, the liver was placed in calcium-free Krebs solution (Ca^{2+}-free Krebs), stored on ice and transported to the laboratory within 30 min. A small section of the right lobe was placed in cool Ca^{2+}-free Krebs solution, and under a stereo microscope a branch of the hepatic artery was dissected free from the tissue. Segments of hepatic artery were either used immediately or placed in Ca^{2+}-free Krebs solution (storage control) or University of Wisconsin (UW) solution and stored on ice for a period of 24 h. Calcium-free Krebs solution was used as the control storage solution because UW solution does not contain calcium.

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Ring segments approximately 2 mm in length and 710 μm in diameter (s.d. 167 μm) were mounted in a Mulvany-Halpern style myograph for the measurement of isometric tension. Stainless steel wires (40 μm) were carefully passed through the lumen of the arteries and fastened to mounting supports attached to a force transducer and to a micrometer for the adjustment of vessel diameter. The myograph chambers were filled with Ca^{2+}-free Krebs solution (37°C) and bubbled with 95% O_2 and 5% CO_2. The arteries were then left to equilibrate for a period of 30 min. The length-tension relationship was determined as described by Mulvany & Halpern (1977) and the ring segments set at a passive tension calculated to produce 90% of the internal diameter that the artery would have if it were distended by a transmural pressure of 100 mmHg. The bathing solution was replaced with normal Krebs solution (i.e. containing 2.5 mM $CaCl_2$) and the arteries left to equilibrate for a minimum of 30 min. Following this period, arteries were contracted 2–3 times with a potassium depolarizing solution, (KPSS, 120 mM K^+ substituted for Na^+), to assess tissue viability and provide a reference contracture for subsequent data analysis.

Protocols

All experiments were performed in the presence of indomethacin (10^{-5} M) to inhibit the production of prostaglandins. Fresh and stored arteries were contracted with U46619, a thromboxane A_2-mimetic, to between 30% and 50% of their respective maximum contractions to KPSS. Upon reaching a steady level of contracture, single cumulative concentration-response curves (0.5 $\log_{10}$ increments) to either 5-hydroxytryptamine (5-HT) (10^{-10}–10^{-5} M), bradykinin (10^{-10}–10^{-7} M) or sodium nitroprusside (SNP) (10^{-8}–10^{-3} M) were generated. To test whether the relaxation responses to 5-HT and bradykinin were dependent on the release of endothelium-derived nitric oxide, fresh vessels were incubated for 30 min with the L-arginine analogue, N^G-nitro-L-arginine (L-NOARG, 10^{-4} M) prior to the construction of the concentration-response curves.

Solutions and drugs

The composition of the normal Krebs solution was as follows (mM): NaCl 119, KCl 4.69, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 5.5 and EDTA 0.026. Ca^{2+}-free Krebs solution was normal Krebs solution without $CaCl_2$. The composition of UW solution (DuPont Pharmaceuticals, Wilmington, DE, U.S.A.) is (g l^{-1}): Pentafraction 50, lactobionic acid (as lactone) 35.8, KH_2PO_4 3.4, $MgSO_4$ 1.23, raffinose 17.8, adenosine 1.34, allopurinol 0.14, glutathione 0.92, KOH to give a potassium concentration of 140 mEq l^{-1} and adjusted to pH 7.4 with NaOH.

The drugs used were U46619 (9,11-dideoxy-11α,9α epoxymethano-prostaglandin $F_{2\alpha}$), indomethacin, N^G-nitro-L-arginine, 5-hydroxytryptamine creatinine sulphate complex, (Sigma, St Louis, U.S.A.), bradykinin (Auspep, Parkville, Australia) and sodium nitroprusside (David Bull Laboratories, Mulgrave, Australia). Drugs were dissolved and diluted in water, with the following exceptions; U46619 (10^{-3} M in ethanol), N^G-nitro-L-arginine (10^{-2} M in 0.1 M $NaHCO_3$) and indomethacin (10^{-2} M in 0.1 M Na_2CO_3).

Curve fit and statistical analysis

Responses have been expressed as a percentage of the maximum contraction induced to KPSS to minimize variation between tissues.

Concentration-response curves were computer-fitted using an iterative fit of the following equation:

$$\text{Response} = a + \frac{b}{1 + e^{-d(c+x)}}$$

where x = log concentration of drug; a = lower plateau of the curve; b = curve range; $c = -\log_{10}$ of the molar concentration required to give a 50% maximum response (pEC_{50}); d = slope parameter of the mid-point of the curve and e is the base of the natural logarithm. Biphasic concentration-response curves were computer-fitted using the same equation by fitting the relaxation and contraction phases individually.

Comparisons between the fitted pEC_{50} values and the absolute maximum responses (relaxation and/or contraction) of the concentration-response curves in both fresh, L-NOARG-treated and stored arteries were made by one-way analysis of variance (one-way ANOVA). If the ANOVA indicated that a difference existed a Dunnett's multiple comparisons test was used to determine the source of variation relative to the fresh vessels. All responses have been expressed as the mean ± s.e.mean. In all cases n represents the number of animals as only a single tissue from each animal was subjected to each protocol.

Results

Responses to 5-HT in fresh arteries

In arteries contracted with U46619, concentration-response curves to 5-HT were biphasic, with relaxations at low concentrations and contraction at high concentrations (Figure 1). The relaxation responses were rapid and transient and the maximum relaxation response was 79 ± 8% (n = 7) of the initial contraction and the pEC_{50} 9.11 ± 0.18. The maximum contractile response to 5-HT was 82 ± 6% of the maximum force to KPSS and the pEC_{50} 6.85 ± 0.14. The maximum relaxation response to 5-HT was reduced following treatment with L-NOARG to 17 ± 9%, (P < 0.01, n = 6) (Figure 3). L-NOARG had no affect on the maximum contractile response (84 ± 4%, P > 0.05) or the sensitivity to 5-HT (pEC_{50} 6.97 ± 0.10, P > 0.05).

Responses to bradykinin in fresh arteries

Bradykinin produced concentration-dependent relaxation of porcine hepatic arteries (pEC_{50} 9.29 ± 0.14, n = 8), (Figure 2). Unlike responses to 5-HT, bradykinin concentration-response curves were monophasic with no contractile response observed. Treatment with L-NOARG displaced the bradykinin concentration-response curve 4 fold to the right (pEC_{50} 8.69 ± 0.17, n = 6), however, this shift in the curve failed to reach statistical significance. L-NOARG had no effect on the maximum relaxation response to bradykinin (Figure 3).

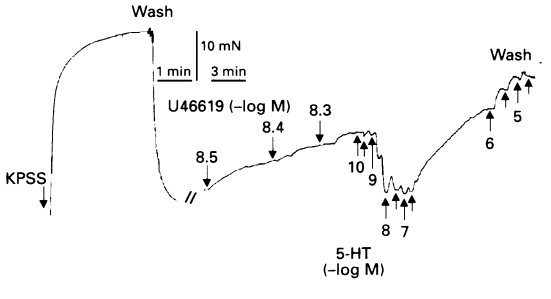

Figure 1 Chart recording of cumulative concentration-response curves to 5-hydroxytryptamine (5-HT) in the porcine hepatic artery. The artery was contracted with the thromboxane A_2-mimetic, U46619, to an initial force between 30% and 50% of the response to KPSS. Arteries were treated with indomethacin (10^{-5} M) prior to the construction of the curve. Concentrations of 5-HT were added to the bath in 0.5 log increments.

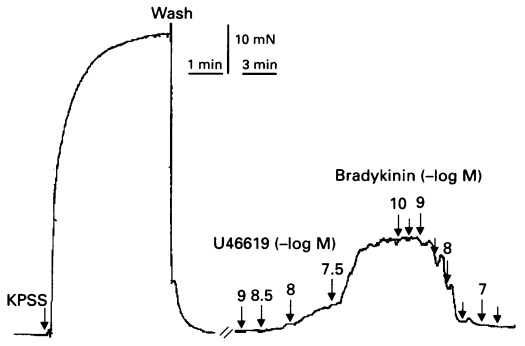

Figure 2 Chart recording of cumulative concentration-response curves to bradykinin (BK) in the porcine hepatic artery. The artery was contracted with the thromboxane A_2-mimetic, U46619, to an initial force between 30 and 50% of the response to KPSS. Arteries were treated with indomethacin (10^{-5} M) prior to the construction of the curve. Concentrations of BK were added to the bath in 0.5 log increments.

Effect of cold storage in Ca^{2+}-free Krebs and UW solution

The maximum relaxation response to 5-HT was significantly reduced following 24 h storage in Ca^{2+}-free Krebs solution but not by storage in UW solution ($32 \pm 7\%$, $P<0.01$, $n=6$ and $57 \pm 10\%$ $P>0.05$, $n=5$) (Figure 4). The contraction phase of the concentration-response curve to 5-HT was attenuated in both Ca^{2+}-free Krebs-stored ($67.1 \pm 2.3\%$, $P>0.05$) and UW-stored arteries ($53.8 \pm 5.8\%$, $P<0.01$).

The bradykinin concentration-response curve was shifted 5 fold to the right following storage in Ca^{2+}-free Krebs solution ($pEC_{50}=8.60 \pm 0.23$, $P<0.05$, $n=7$). The maximum relaxation response to bradykinin was also reduced; however, the reduction failed to reach statistical significance (Figure 4). There was no significant change in the sensitivity of the arteries or maximum responses to bradykinin following storage in UW solution ($pEC_{50}=8.84 \pm 0.17$, $n=6$) (Figure 4). Storage in Ca^{2+}-free Krebs solution or UW solution had no effect on the sensitivity or maximum relaxation mediated by SNP (Figure 4).

To obtain comparable starting conditions, the initial level of active force was set to similar values in all treatment groups by titrating the precontraction level to 30% – 50% of the maximum response to KPSS. However, when expressed as active pressure, to correct for differences in vessel size, the response to KPSS was larger in the UW-stored arteries than in the Krebs-stored arteries, which in turn were larger than the fresh arteries (108 ± 6 kPa, 81 ± 5 kPa, and 65 ± 3 kPa respectively). This increase in the size of the response to KPSS with storage means

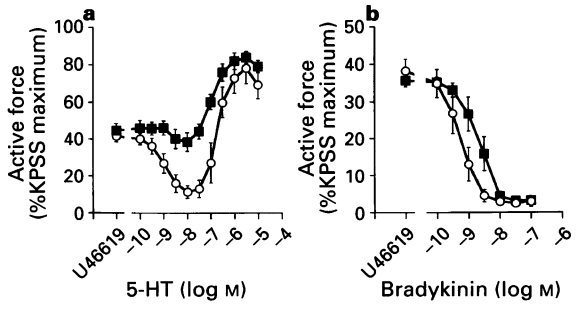

Figure 3 Cumulative concentration-response curves to (a) 5-hydroxytryptamine (5-HT) and (b) bradykinin in the absence (○) and presence of N^G-nitro-L-arginine (L-NOARG, 10^{-4} M) (■) in porcine hepatic arteries contracted by the thromboxane A_2-mimetic, U46619. Each point represents the arithmetic mean with the s.e.mean.

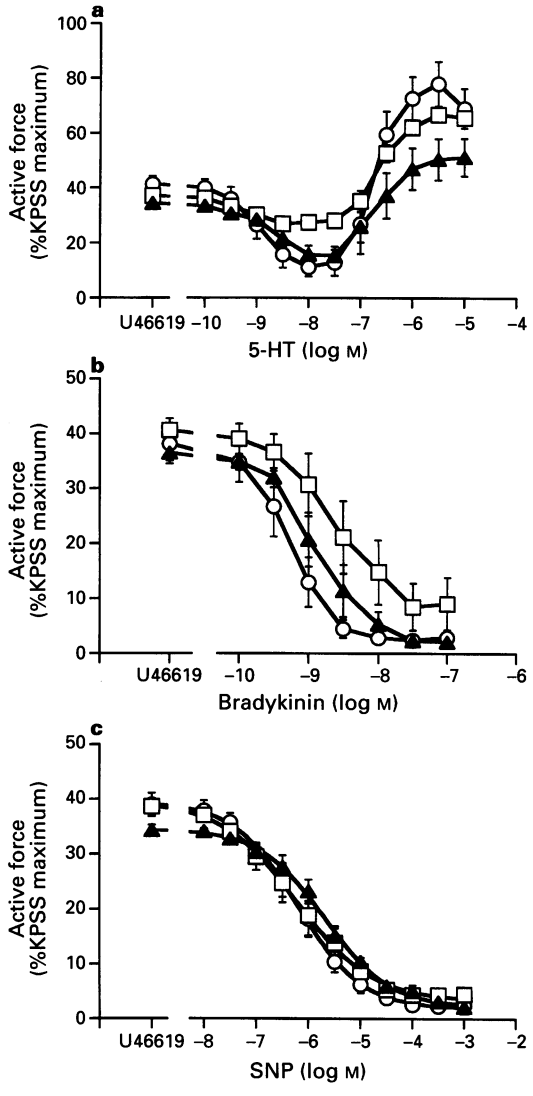

Figure 4 Cumulative concentration-response curves to (a) 5-hydroxytryptamine (5-HT), (b) bradykinin and (c) sodium nitroprusside (SNP) in fresh porcine hepatic arteries (○) and following cold storage in Ca^{2+}-free Krebs solution (□) and University of Wisconsin solution (UW) (▲). Arteries were contracted with the thromboxane A_2-mimetic, U46619. Each point represents the arithmetic mean with the s.e.mean.

the initial level of active force in the treatment groups was not the same. However, the larger absolute level of precontraction in UW-stored arteries would tend to decrease the fractional relaxation compared to Ca^{2+}-free Krebs-stored arteries. Thus, the maintained relaxation response to 5-HT and sensitivity to bradykinin cannot simply be the result of the different levels of precontraction.

Discussion

The main result of this study was that a prolonged period of cold storage in Ca^{2+}-free Krebs solution but not UW solution reduced the relaxation response to 5-HT and the sensitivity of the arteries to bradykinin. These results suggest that UW solution is able to preserve endothelial cell function during periods of cold storage.

Involvement of nitric oxide

In the porcine hepatic artery 5-HT has a dual action, with low concentrations eliciting relaxation and high concentrations

contraction. An endothelium-dependent component in 5-HT-induced relaxation *in vitro* was first recognised by Cocks & Angus (1983) in ring segments of dog and pig coronary artery and this property has been demonstrated in isolated arteries and veins from a variety of species including rabbit jugular vein (Martin *et al.*, 1992), and pig and rat vena cava (Sumner, 1991; Bodelsson *et al.*, 1993) and pig pulmonary artery (Glusa & Richter, 1993). L-Arginine analogues were able to inhibit relaxations in some of these tissues (rabbit jugular vein, pig vena cava and pig pulmonary artery) suggesting that the relaxation was mediated by NO; however, direct vascular smooth muscle relaxation has also been shown to contribute to the relaxation response to 5-HT (Mylechrane, 1990). The relaxation response to 5-HT in the porcine hepatic artery is probably mediated by the release of endothelium-derived NO rather than direct smooth muscle relaxation since treatment of the vessels with L-NOARG greatly attenuated the relaxation phase of the 5-HT concentration-response curve.

No attempt was made to identify the endothelial 5-HT receptors responsible for mediating relaxation in porcine hepatic artery. Current evidence suggests that two distinct receptor subtypes are involved in endothelium-dependent relaxation to 5-HT. In pig coronary artery (Schoeffter & Hoyer, 1990) and human and bovine cerebral arteries (Hamel & Boucher, 1991; Hamel *et al.*, 1993), endothelium-dependent relaxation has been claimed to be mediated by 5-HT_{1D} receptors. In contrast, 5-HT receptors mediating endothelium-dependent relaxations in rabbit and rat jugular vein (Martin *et al.*, 1987; Ellis *et al.*, 1995) and pig vena cava (Sumner *et al.*, 1991) share pharmacological features with the 5-HT_2 receptor class and are likely to be of the 5-HT_{2B} receptor subtype (Ellis *et al.*, 1995).

Bradykinin was a more effective relaxing agent than 5-HT in porcine hepatic arteries. The inability of L-NOARG to inhibit significantly the relaxation responses to bradykinin is in contrast to its significantly inhibitory effect on the maximum relaxation response to 5-HT. The ability of L-NOARG to inhibit the relaxation response to 5-HT but not bradykinin may be due to (1) the different receptor systems stimulating the release of different endothelium-derived relaxing factors (EDRFs) (Boulanger *et al.*, 1989; Hoeffner *et al.*, 1989), (2) differences in the efficiency of receptor occupancy-effect coupling (Martin *et al.*, 1992) and (3) a shift in the balance between smooth muscle 5-HT_{2A} receptors mediating contraction and endothelial cell 5-HT receptors mediating relaxation following removal of NO synthesis.

Effects of cold storage

Cold-storage of porcine hepatic arteries in Ca^{2+}-free Krebs solution reduced their maximum relaxation response to 5-HT and sensitivity to bradykinin. Since cold-storage in Ca^{2+}-free Krebs solution did not affect responses to SNP, but produced similar changes in arterial reactivity as treatment with L-NOARG these results may suggest endothelial cell dysfunction. It is possible that this dysfunction is specific to the pathways of NO synthesis or release but to settle this point would require further experiments.

Cold storage in UW solution maintained the relaxation responses to both 5-HT and bradykinin, an effect consistent with UW solution conserving endothelial cell function. These functional studies are in general agreement with the results of other studies which have found the endothelium of the hepatic microcirculation to be particularly sensitive to cold storage damage, and that storage in UW solution appears to protect the endothelium (McKeown *et al.*, 1988; Caldwell-Kenkel *et al.*, 1989, 1990; Holloway *et al.*, 1990). These studies evaluated endothelial cell integrity of the microcirculation in whole perfused liver preparations using morphological and cytochemical indices. Eberl *et al.* (1993) examined the effects of cold storage on bovine cultured aortic endothelial cells and found that UW solution protected cellular integrity better than the cryopreservatives, Brettschneider's HTK solution and Euro-Collins solution. Despite the different preparations and methods used to examine endothelial cell integrity, the results from each study point to endothelial cell dysfunction or damage as the major consequence of a prolonged period of cold storage. The results of this study show for the first time that changes in the reactivity of the hepatic artery occur as a direct consequence of such damage.

An important aspect to consider is the possible differences between storage of isolated arteries and whole livers. It may be that in whole livers separate events are taking place which may be harmful to the vascular endothelium, such as the release of cytotoxic mediators from non-endothelial cells. The reactivity of arteries taken from cold-stored livers was not examined in the present study so whether arterial reactivity differs between stored isolated arteries and whole livers remains to be established.

An unexpected finding of cold-storage, both in Ca^{2+}-free Krebs- and UW solution, was the reduced contractile responses to 5-HT. In contrast, responses to the K^+ depolarizing solution were enhanced following cold storage. This suggests that changes in arterial function are not simply limited to the endothelium, but may also involve complex changes in smooth muscle reactivity.

Conclusions

The results of this study suggest that in isolated hepatic arteries of the pig relaxation responses to 5-HT and bradykinin involve the release of NO. The small residual relaxation response to 5-HT following treatment of the arteries with L-NOARG and the poor ability of L-NOARG to inhibit the relaxation response to bradykinin suggests that other EDRFs distinct from NO and prostaglandins may be involved in the relaxation response to these agents. A prolonged period of cold storage in physiological saline solution had similar effects to L-NOARG treatment which may suggest possible dysfunction of the NO synthesis pathway within the endothelial cells. It is concluded that cold-storage induced changes in endothelial cell function are reduced by UW solution compared to Ca^{2+}-free Krebs solution.

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