Transpl Int (2005) 17: 699–706
DOI 10.1007/s00147-004-0788-2

ORIGINAL ARTICLE

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Loss of endothelium-dependent relaxation in abdominal aorta preserved in a co-storage system

Received: 22 August 2003
Revised: 2 August 2004
Accepted: 10 September 2004
Published online: 17 November 2004

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Abstract The potentially detrimental influence of parenchymal cells on endothelial function during preservation in UW solution was examined by co-storage of rat abdominal aortic rings with isolated liver cells. Cold storage of rings in UW solution alone for up to 96 h had no effect on the response to acetylcholine, though constriction was progressively lost. Co-storage of rings with liver cells resulted in no loss of sodium nitroprusside response, but the relaxation response to acetylcholine was reduced. The loss of acetylcholine response could not be attributed to Kupffer cells, the lowering of pH, oxygen depletion, or the loss of constriction. A similar loss of endothelial function was observed in rings stored in pieces of liver, kidney or heart. We conclude that parenchymal cells exude factors during preservation by cold storage which reversibly inhibit vascular NO production. These factors could significantly impair whole organ function on reperfusion.

Keywords Preservation · UW solution · Endothelial function · Hepatocytes · Liver · Cold storage

Abbreviations Ach: acetylcholine · SNP: sodium nitroprusside · UW solution: University of Wisconsin solution · NO: nitric oxide · KHB: Krebs-Henseleit bicarbonate buffer · KHH: Krebs-Henseleit HEPES buffer · LC: liver cells · HC: hepatocytes · KC: Kupffer cells

Introduction

Injury induced by hypothermic preservation in various organs can occur both to the parenchyma and to the vasculature, and vascular damage may be especially critical to subsequent organ function on reperfusion [1, 2]. Endothelial cells may be particularly susceptible to injury on reperfusion following hypothermic preservation [1]. These cells are susceptible to activation, initiating the up-regulation of adhesion molecules and cytokines, resulting in increased leukocyte adherence and subsequent infiltration [3]. Leukocyte infiltration can significantly increase organ injury on reperfusion [4, 5, 6]. The ability of endothelial cells to generate NO can also be compromized during organ preservation [7, 8]. NO itself can inhibit adhesion molecule expression [4], and can also inhibit pathways implicated in the development of graft vasculopathy [9, 10]. Preservation of the ability of the endothelium to generate NO may therefore be of considerable importance for both the short-term and long-term outcome of organ preservation. Understanding the factors which determine the preservation of endothelial function therefore has great clinical importance. Endothelial function under conditions of organ preservation have not, however, been extensively investigated.

Isolated rat aortic segments are a convenient in vitro system to study how preservation conditions affect the

vascular system. By co-storage with isolated liver cells it is possible to emulate to some degree the conditions experienced by vessels in the whole organ, and to dissect out the role played by parenchymal cells in endothelial changes observed following preservation. We have found that parenchymal cells appear to contribute a factor during hypothermic storage which causes impairment of endothelial function, not attributable to hypoxia or pH reduction.

Materials and methods

The principles of laboratory animal care (NIH publication No. 86–23, revised 1985) were followed.

Aortic ring preparation

A segment of the abdominal aorta between the renal artery and iliac branches was isolated from male Sprague-Dawley rats (300–325 g). After removal of the connective tissue, the segment was cut into approximately 8 rings (1–2 mm long). Following the storage period the rings were mounted in an organ perfusion bath, one end anchored and the other end connected to a force-displacement transducer (Grass Instruments FT-03C) for measurement of isometric contraction. The vessels were bathed in KHB (containing 118 mM NaCl, 4.6 mM KCl, 24 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 11 mM glucose), and also 1.2 mM $CaCl_2$ at a pH of 7.4 at 37°C with aeration (95% O_2:5% CO_2). A baseline tension of 1 g (10 mN) was established, and the vessels allowed to stabilize at this tension (about 30 min). Vessel constriction in response to 3 μM U46619, a thromboxane A_2 analogue which acts on smooth muscle cells, was measured. This was followed by measuring the relaxation response to sodium nitroprusside (1 nM–100 μM SNP, an endothelial cell-independent NO donor) or acetylcholine (1 nM–100 μM Ach, an endothelial cell-dependent generator of NO). For each storage condition 4–8 rat aorta preparations were used.

Hypothermic storage conditions

Of the rings 4 were stored in a plastic centrifuge tube (1.5 ml) in 1 ml UW solution at 4°C. Some rings were stored with 2% (v/v) oxyrase (Mansfield, OH) or in UW solution equilibrated with argon to remove dissolved oxygen. Oxyrase is a cell-compatible enzyme mixture which has been shown to be effective at removing residual oxygen from cell suspensions [5]. Others were stored in UW solution at different pH values (7.4, 6.8, 6.5, 6.2, 6.0). In co-storage experiments, 4 rings were stored with 0.5 ml of a liver cell suspension (15 mg protein/ml, which typically represents 1×10^6 cells/ml) in UW solution and allowed to settle. After settling, the cells covered the rings completely.

Liver cell isolation

Liver cells (composed primarily of hepatocytes) were isolated by in situ perfusion digestion of the liver with collagenase D by a method previously described [11]. The liver cells were suspended in KHH which contained 118 mM NaCl, 4.7 mM KCl, 10 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 11.1 mM glucose, N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES 20 mM) and also 1.3 mM $CaCl_2$ at a pH of 7.4. Before use, all liver cell preparations underwent a preincubation period of 20 min at 37°C while continuously shaken and gassed with 95% O_2:5% CO_2. This preincubation period was used to allow the cells to repair isolation-induced damage, regenerate ATP, and reestablish a normal intracellular concentration of electrolytes. Cell viability was then assessed by the release of lactate dehydrogenase (LDH) and averaged less than 10% of total cellular LDH (>90% viability). After preparation, the final concentration of cell protein was determined by the Biuret method and adjusted as needed.

Cell fractionation

Resident liver macrophages (Kupffer cells) were isolated from the liver cell preparation, by a panning technique. Briefly, 10 ml of liver cell suspension (10 mg protein/ml, equivalent to 5.6×10^6 cells/ml [12]) in RPMI 1640 media with 10% fetal calf serum was plated onto polystyrene cell culture dishes (150×25 mm, Corning Corp., Corning, NY). The plates were first pretreated with RPMI 1640 media containing 10% fetal calf serum for 1 h at 37°C. The cell suspensions were incubated at 37°C for 3 h under 5% CO_2. The media was then poured off and collected into a clean tube and the plates were gently rinsed twice with 10 ml warm RPMI media with the washes added to the original media. These washes contained non-adherent hepatocytes. The adherent cells were removed from the plate by incubating the plates in 10 ml of calcium-free KHB at 4°C for 15 min. The cells were then removed by gentle scraping with a rubber policeman and saved. These adherent cells are termed Kupffer cells; they stain positive for non-specific esterase and phagocytose yeast cells labeled with Congo Red dye [13]. They are fully functional, as they respond vigorously to LPS by synthesizing IL-1$_\beta$, TNF$_\alpha$, PGE_2, nitric oxide, and IL-6 [13, 14, 15, 16]. In a sham control, the same procedure was followed but using silanized glass

Petri dishes instead of the polystyrene culture dishes, so that no adhesion occurred.

Statistics

The data are presented as the mean plus standard error of the mean. Differences between means of groups were evaluated by the unpaired Student's t-test with $p < 0.05$ taken as a statistically significant difference.

Results

When aortic rings were stored in UW solution at 4°C for up to 96 h, rewarmed in Krebs-Henseleit medium, and constricted with 3 μM U46619, the degree of constriction observed declined progressively (Fig. 1A, inset). However, there was no significant change in their relaxation response to Ach, when expressed as a percentage of constriction (Fig. 1A). By contrast, when fresh rings were constricted to a sub-maximal level by exposure to a low (0.1 μM) level of U46619 (Fig. 1A, inset, "L-U4"), their Ach response was enhanced, when expressed as a percentage of constriction (Fig. 1A, "L-U4"). This raises the possibility that the apparently unchanged Ach response with cold storage (Fig. 1A) could be masking an actual loss of relaxation response, since the relaxation response is expressed as a percentage of the constriction.

The effect of parenchymal cells on arterial function was investigated by co-storage of arterial rings with preparations of isolated liver cells, which contain a number of different parenchymal cell types. The response of rings to SNP was unaffected by co-storage with liver cells (data not shown). On the other hand, when the rings were co-stored with liver cells (15 mg/ml) the endothelial response to Ach was significantly reduced (Fig. 1B). However, co-storage with liver cells tended to prevent some of the loss of constriction response to U46619 seen following the 48 h incubation. Since the % relaxation induced by Ach (in fresh rings) can depend on the degree of U46619-induced constriction (Fig. 1A), the reduced Ach response after co-storage with liver cells could result from reducing the cold storage-induced loss of U46619-induced constriction. To investigate this possibility, the data was sorted into two groups. In group 1, the mean constriction of rings incubated without liver cells (Fig. 1B, inset, C1) was chosen to be equal to the mean constriction of other rings incubated with liver cells (Fig. 1B, inset, HC1). Remaining data was put into group 2 (C2 and HC2). The mean constriction of C2 was less than that of C1, while the mean constriction of HC2 was more than that of HC1, reflecting the fact that for the entire dataset the co-storage with liver cells reduced the loss of constriction induced by cold storage in UW solution. However, the Ach response of HC1 was depressed compared with that of C1, even though their constriction was matched (Fig. 1B). Moreover, the Ach response of the C1 and C2 groups was identical, and the Ach response of the HC1 and HC2 groups was identical, such that the loss of Ach response seen in group 2 was identical to that seen in group1 (Fig. 1B). This shows that the loss of endothelial response to Ach seen with co-storage with liver cells cannot be attributed to protection from the loss of constriction response, and indeed is independent of the maximal degree of constriction achievable with U46619.

To test whether the loss of endothelial response to Ach was the result of a loss of response of the smooth muscle to nitric oxide, the relaxation response of rings to

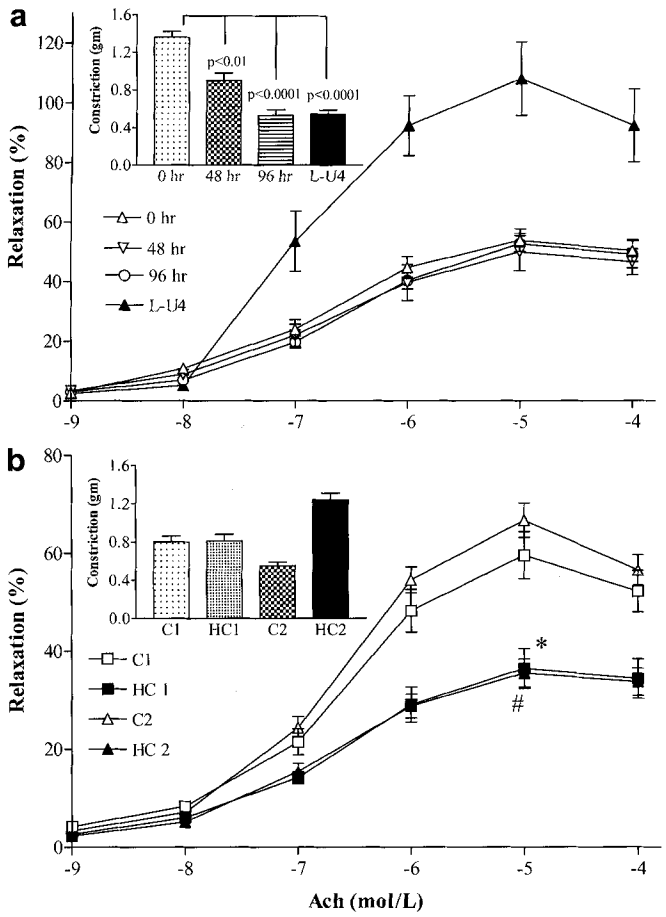

Fig. 1A, B The Ach response is reduced by co-storage of rings with liver cells. **A** Ach dose response curve on rat aortic rings stored from 0 to 96 h without liver cells. Points are mean ± SEM in 5–8 experiments. L-U4 is data from fresh rings constricted with only a low level (0.1 μM) of U46619. **B** Ach dose response curves on control rings and rings stored in 15 mg/ml liver cells for 48 h; *C* control, incubated without liver cells; *HC* incubated with liver cells. The numbers *1* & *2* signify two data groups divided according to constriction, as described in the text. $*p < 0.001$ and $\#p < 0.0001$ relative to control

sodium nitroprusside (SNP) was tested, following cold storage with or without liver cells. SNP (1 μM) resulted in 113.1 ± 4.4% relaxation in control stored rings, and caused 111.5 ± 4.2% relaxation after storage with liver cells (NS, $n = 8$). No significant effect of storage with liver cells on SNP-induced relaxation was detected at any SNP concentration between 10^{-9} and 10^{-5} M.

When the time of cold storage was varied, we found that the reduced response to Ach was evident after 24 h, but not after 4 h (Fig. 2A). The effect of liver cells depended on the liver cell concentration: a significant effect was evident at 5 mg/ml but not at 1 mg/ml, and the effect was near maximal at 15 mg/ml (Fig. 2B). In subsequent studies we used 15 mg/ml and 48 h storage as our standard condition to observe the liver cell effect.

We examined four hypotheses concerning the mechanism by which the liver cells exerted their effect.

First, we considered the possibility that the liver cells induced anoxia, which could result in endothelial injury on reoxygenation. We tested this by storing rings after gassing the storage solution with 95% N_2/5% CO_2 in the presence of oxyrase (without liver cells) to remove residual oxygen. Rings stored under these conditions had a normal Ach response (Fig. 3A), suggesting that the liver cell effect could not be explained by anaerobiosis induced by the liver cells.

Second, we considered the possibility that acidosis associated with ischemia could account for the liver cell effect. We tested this by storing rings in UW solution adjusted to a range of pH values, to determine how pH affected the Ach response. Rings stored at pH 6.8 had a normal response, but a progressive decline in the Ach response was seen as pH dropped from 6.8 to 6.0 (Fig. 3B). We also measured the pH decline which occurred when rings were incubated with liver cells (15 mg/ml), as measured by a small protein-tolerant pH electrode placed in the vicinity of the aortic ring. A decline of medium pH to 6.8 during 48 h cold storage was ob-

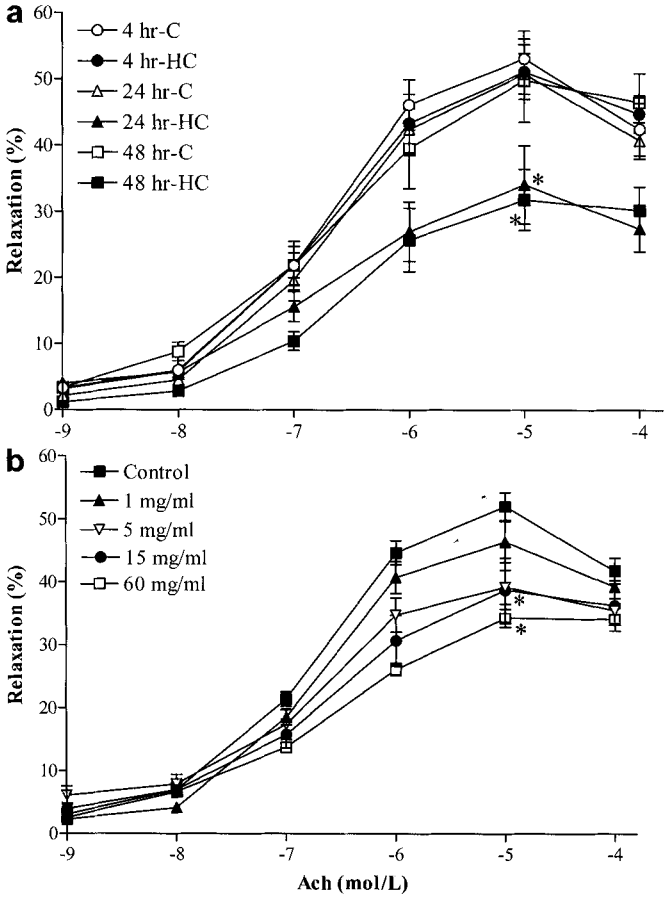

Fig. 2A, B Time and cell concentration dependence of the co-storage effect. **A** Ach dose response curve on rings stored 4, 24 and 48 h with or without 15 mg/ml liver cells (*HC*). **B** Ach dose response curve on rings stored 48 h in increasing concentrations of *HC* as shown; *C*, control (incubated without *HC*); *HC* incubated with HC. $*p < 0.05$ relative to control

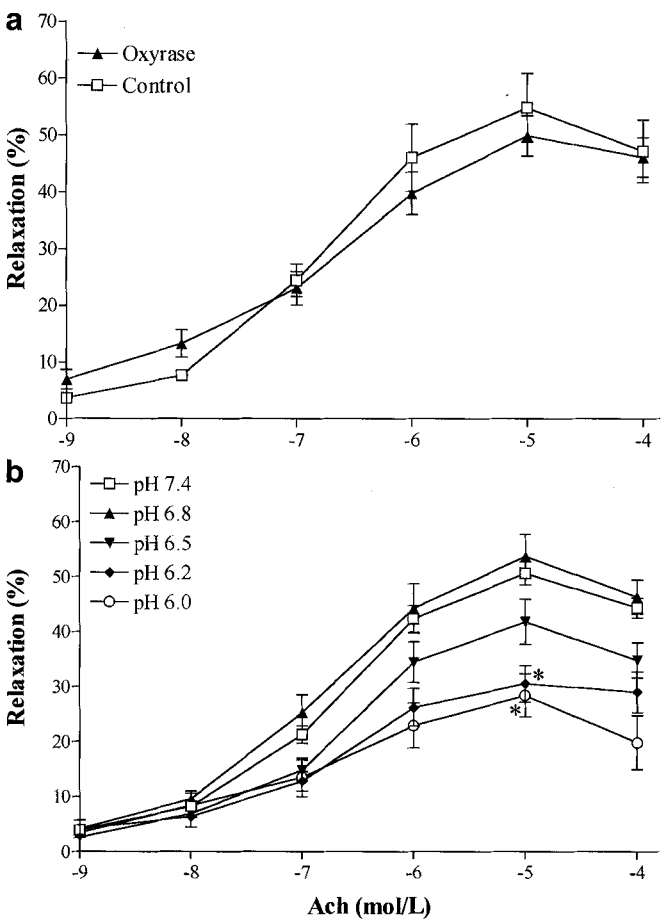

Fig. 3A, B The co-storage effect does not result from hypoxia or reduced pH. Ach dose response curves were measured on aortic rings **A** stored in UW ± oxyrase for 48 h (see Methods) or **B** measured on rings stored at decreasing pH values for 48 h as shown. $*p < 0.05$ relative to control

served. Since rings stored at this pH had a normal Ach response, this suggests that the liver cell effect cannot be explained by the mild acidosis induced by co-storage with liver cells.

Third, we considered the possibility that the loss of Ach response came from damage to the rings by residual enzymes or other factors carried over from the liver cell isolation process. To test this we compared the Ach response of rings stored with liver cells with that of rings stored with liver cells which had been washed extensively (3 extra washes with Krebs-Henseleit medium after isolation). Liver cells still impaired the Ach response equally well, even after extensive washing (Fig. 4A). This suggests that the liver cell effect cannot be explained by carryover of soluble components in the liver cell preparation which are toxic to the endothelial cells of the rings.

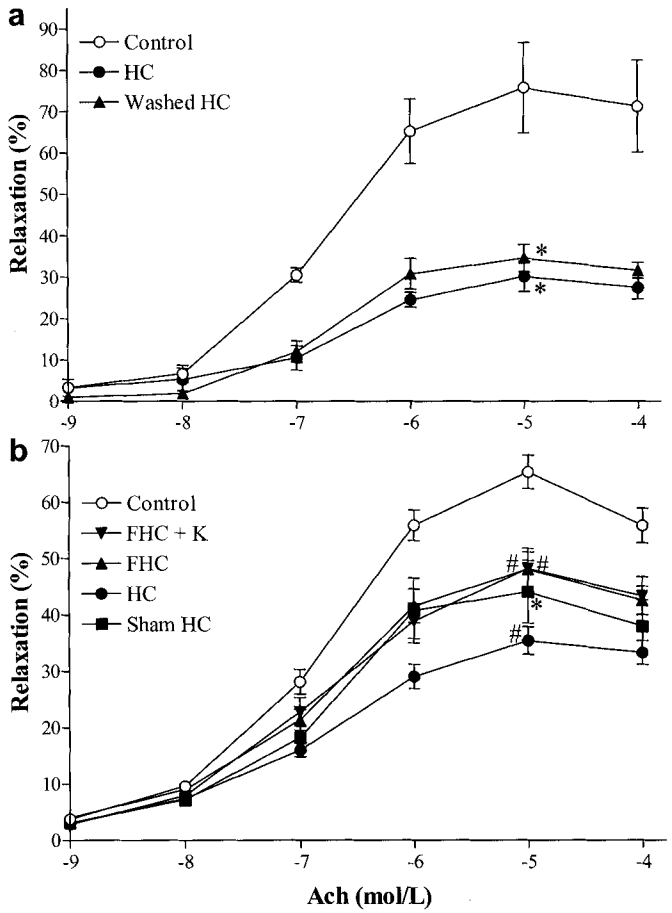

Fig. 4A, B The co-storage effect does not result from soluble contaminants or from Kupffer cells. **A** Ach dose response curves ($n=3$) in rings stored in HC washed 3 times. *$p<0.05$ relative to control in unpaired t-test. **B** Ach dose response curve on controls ($n=6$), rings stored in HC ($n=16$), in fractionated HC with Kupffer cells removed (FHC) ($n=6$), in fractionated HC with Kupffer cells removed then recombined with the Kupffer cell fraction (FHC + K) ($n=3$), and in sham HC where the fractionation procedure was followed but modified so that no selective fractionation occurred (see Methods) ($n=3$). *$p<0.001$ and #$p<0.0001$ relative to control

Since Kupffer cells have been implicated in injury to the liver on reperfusion following ischemia [17], the loss of Ach response could possibly be attributed to these cells. To test this we separated the cell types in the liver cell preparation by selective adhesion to culture plates (see Methods), resulting in fractions either enriched in Kupffer cells or hepatocytes free of Kupffer cells. These fractions were then used for co-storage with rings, alone or in combination. We found that fractionated hepatocytes free of Kupffer cells (FHC) did not impair the endothelial response to Ach quite as much as unfractionated liver cells (HC, Fig. 4B). However, when rings were incubated with a mixture of the fraction without Kupffer cells and the fraction enriched in Kupffer cells (FHC + K), the Ach response was no different from that of rings incubated with hepatocytes free of Kupfer cells (Fig. 4B). This suggests that the Kupffer cells did not contribute to the loss of Ach response. Moreover, when the liver cell preparation was subjected to a sham fractionation procedure (using silanized glass plates such that no cell fractionation actually occurred), rings incubated with these cells showed the same loss of Ach response as hepatocytes free of Kupffer cells (sham HC, Fig. 4B). This reinforces the conclusion that the Kupffer cells did not contribute to the loss of Ach response, and further suggests that the small degree of amelioration of the liver cell effect observed with fractionated liver cells was related to additional changes to the cells induced by the fractionation procedure.

Next we investigated the reversibility of the liver cell effect on Ach response. If the effect were mediated by a soluble component generated by the liver cells during cold storage which transferred to the rings, then the component could wash out on re-equilibration of the rings with warm Krebs-Henseleit medium after storage. This clearly does not happen quickly, since the vessels are routinely incubated in warm Krebs-Henseleit medium for 30 min before testing. If this time was extended to 90 min without measuring the Ach response at 30 min, a slight amelioration of the loss of Ach response was observed, but most of the loss of Ach response was still present (% relaxation: control tested at 30 min, 63.9 ± 3.1, with HC tested at 30 min, 31.9 ± 4.2; control tested at 90 min, 71.1 ± 3.4, with HC tested at 90 min, 43.1 ± 2.6; $p<0.001$ for HC effect at both times). Surprisingly, however, if the Ach response was tested at 30 min then washed, and then retested at 90 min, the loss of Ach response was completely reversed (% relaxation: with HC tested at 90 min following a test at 30 min, 67.7 ± 5.0; p NS). Since the test involves contraction with U46619 followed by relaxation with increasing doses of Ach, we investigated which of these components of the test was responsible for the reversal. When rings co-incubated with liver cells were exposed to

U46619 but no Ach at 30 min, and then washed, the liver cell effect was reversed when tested at 90 min (% relaxation: with HC tested at 90 min following a challenge with U46619 at 30 min, 65.8 ± 7.5; p NS), while rings exposed to Ach but no U46619 at 30 min, and then washed, showed little reversal (% relaxation: with HC tested at 90 min following a challenge with Ach at 30 min, 38.5 ± 2.6; $p < 0.001$). This shows that the loss of Ach response induced by liver cells is not irreversible damage, and that early constriction of the rings with U46619 can restore the Ach response which, when tested later, is otherwise lost.

Finally, we investigated how the liver cell effect compared with the effect which might be expected in the intact whole organ. To do this we incubated aortic rings in slits cut into lobes of whole liver which had been flushed with UW solution as for preservation by cold storage. For the control in this experiment (liver control, Fig. 5A), rings were inserted into the liver and then retrieved immediately and stored in UW solution, to allow for any damage induced by manipulation of the rings. Storage of the rings in the liver resulted in a similar degree of loss of Ach response as incubation with liver cells (in liver, Fig. 5A). This suggests that the microenvironment of the cold stored liver is well emulated by the co-storage with the liver cell preparation. Furthermore, this effect was not limited to the liver. Storage of rings in slits cut into whole rat hearts or kidneys flushed with UW solution similarly impaired the Ach response (Fig. 5B).

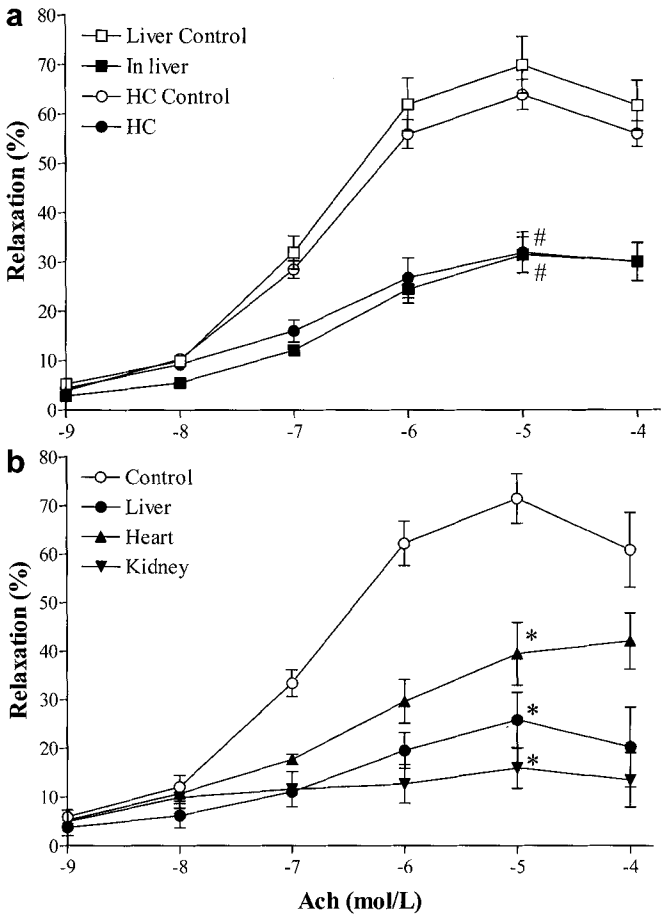

Fig. 5A, B The co-storage effect mimics the effect of intact tissue. **A** Ach dose response curve on rings stored for 48 h in 15 mg/ml isolated liver cells (*HC*) or inside lobes of liver pre-perfused with UW solution ($n = 14$–18). #$p < 0.0001$ relative to control. **B** Ach dose response curve on rings stored in liver, heart or kidney pre-perfused with UW solution, $n = 3$. * $p < 0.05$ relative to control

Discussion

While cold storage of arterial rings in UW solution for 48 h impaired the constriction response to U46619 (Fig. 1A), co-storage with liver cells largely prevented that loss (Fig 1B). However, co-storage with liver cells caused an impairment of endothelial function, as judged by the reduced Ach response (Fig. 1B) in the face of unaltered sensitivity to SNP. Ach acts on endothelial cells to produce NO, while SNP relaxes smooth muscle cells by endothelial cell-independent release of NO. These results suggest that during liver preservation with UW solution, parenchymal cells contribute to endothelial but not smooth muscle dysfunction.

The loss of response to Ach could not be explained by either hypoxia (Fig. 3A) or acidosis (Fig. 3B) resulting from co-storage with liver cells. It was not the result of exposure to a soluble contaminant in the liver cell preparation, since extra washing of the preparation had no effect on the loss of Ach response (Fig. 4A). The ability of storage in intact liver to reproduce the liver cell effect further supports the view that this effect is not an artifact of cell isolation (Fig. 5A). We were also surprised to find that the effect of the liver cell preparation could not be attributed to the presence of Kupffer cells, since the effect remained after removal of the Kupffer cell fraction and was also unaffected by their restoration (Fig. 4B). Kupffer cells have been implicated in injury to the liver on reperfusion following cold ischemia in UW solution [18], and especially in injury-susceptible fatty livers [19] or livers from non-heart-beating donors [17]. Our results suggest rather that hepatocytes themselves can injure endothelial function, since hepatocytes predominate in the FHC fraction and this fraction shows the co-storage effect (Fig. 4B). The small reduction of the co-storage effect seen after the cell fractionation procedure appears to be related to aspects of the procedure other than the fractionation, since the small reduction was also evident in the sham control where cells went through the procedure but without separation into fractions (Fig. 4B).

The ability of constriction with U46619 to reverse the co-storage effect is consistent with the notion that a

regulatory pathway is altered by an agent produced by hepatocytes, as U46619 is a specific agonist for TP (thromboxane) receptors, and U46619 will induce a specific response. However, TP receptors are located in the smooth muscle cells, and it is unclear how U46619 action on the smooth muscle cells will restore endothelial cell function. The interaction between endothelial cells and the underlying smooth muscle is complex. In addition to production of the well known relaxant nitric oxide, endothelial cells can also influence smooth muscle via hyperpolarizing factors [20]. These appear to be epoxyeicosatrienoic acids (EETs) generated by endothelial cytochrome p450 epoxygenases, which activate Ca-dependent K channels in smooth muscle resulting in hyperpolarization [20]. Hyperpolarization can in some arteries feed back to endothelial cells via myoendothelial gap junctions [21]. Just how such feedback mechanisms could operate to promote endothelial function in preserved arteries, as found here, is however unknown.

Endothelial dysfunction is emerging as a commonly observed consequence of ischemia reperfusion injury which may have significant detrimental consequences for the organ. Tsao et al. [22] established that endothelial injury occurred very early in reperfusion, and that the injury was the result of superoxide production. Expression of the adhesion molecule P-selectin was subsequently found to be up-regulated maximally within 10–20 min of reperfusion [23]. Blocking P-selectin-inhibited leukocyte adherence and accumulation in a splanchnic ischemia/reperfusion model, resulting in reduced tissue injury [24]. Furthermore, NO was found to inhibit leukocyte adhesion [25], and an NO donor reduced P-selectin expression in splanchnic ischemia/reperfusion [26]. These studies led to the concept of an "endothelial trigger followed by neutrophil amplification" in ischemia-reperfusion injury [4], and established that events which occur early in reperfusion have a significant impact on subsequent tissue survival. Interaction between the damaged endothelium and blood components is then undoubtedly important for the subsequent development of injury seen in vivo. For example, complement activation can play an important role in promoting endothelial cell activation on reoxygenation after hypoxia, and such activation may be linked to low NO/cGMP [27]. We conclude therefore that since, as shown here, parenchymal cells depress endothelial nitric oxide production following cold storage in UW solution, then, even if the effect is only temporary, it could have a significant impact on organ recovery on blood reperfusion.

Acknowledgements Supported by grants from NIH (DK-18624 and DK-35143) to Dr. Southard.

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