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Paradoxical Cholinergic and Purinergic Vascular Reactivity of Rabbit Thoracic Aorta Cold-stored in University of Wisconsin Solution

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Abstract

Endothelial dysfunction has been reported in donor blood vessels destined for organ transplantation following cold-storage preservation with University of Wisconsin solution (UW). This was investigated in the present work. Segments of rabbit thoracic aorta were mounted on isometric fine-wire myographs at 37°C and gassed with 95% O_2/5% CO_2. Concentration-dependent vasodilatations to acetylcholine and adenosine-5′-triphosphate (ATP) were obtained in freshly-harvested rabbit aortic rings, with and without the endothelium, and after 8 days of cold-storage, at 4°C, in either UW, Krebs-Bülbring buffer (KBB) or saline. The action of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (100 μM) was evaluated upon the concentration-response curves to determine whether nitric oxide (NO) exerted any modulatory actions.

Endothelium-dependent, NO-mediated responses to acetylcholine were unaltered after eight days of storage in UW, reduced after storage in KBB and absent after removal of the vascular endothelium, saline storage or after testing in the presence of L-NAME, suggesting improved NO-mediated endothelial function with the use of UW. Structural preservation was also confirmed using scanning electron microscopy. In contrast, endothelium-dependent responses to ATP were unchanged after eight days of storage in KBB but were reduced after storage in UW and saline, suggesting purinergic (ATP) endothelial dysfunction after storage in UW. L-NAME markedly reduced vasodilatation to ATP in freshly harvested rings and after eight days of storage in KBB. This reduction was statistically significant ($P < 0{\cdot}05$, Student's two tailed, unpaired t-test) at $-\log$ (M) ATP concentrations of 5·5, 5·0, 4·5, 4·0 and 3·5. NO-dependent vasodilatation to ATP was not attenuated by L-NAME in UW-stored rings. Eight days' UW-storage of rabbit thoracic aortic rings appeared to have differential and paradoxical effects upon NO-dependent vasodilatation to acetylcholine and ATP.

Morphological observations using electron microscopy suggested that UW preserved the vascular endothelium and this was verified by retained vascular reactivity of endothelium-dependent vasodilatations to acetylcholine. UW-storage however, significantly reduced endothelium-dependent relaxation to ATP thereby suggesting that P_{2Y}-purinoceptors, which are located on the vascular endothelium, may be more susceptible to biodegradation than cholinergic receptors and may be responsible for endothelial dysfunction following transplantation.

The use of University of Wisconsin solution (UW) as a storage medium for donor organs has been established not only in the field of liver transplantation (Todo et al 1989) but also in heart (Wicomb et al 1993), lung (Hopkinson et al 1994) and small bowel (Schweizer et al 1994) transplantation. The justification for its use largely rests upon biochemical measurements of liver function enzymes and histological observations following transplantation after storage in UW.

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Segments of human iliac arteries harvested simultaneously with donor livers are often used as conduits to connect the donor liver's hepatic arterial supply to the recipient's systemic circulation during liver transplantation. Hepatic artery thrombosis occurs in 9–18% of transplants (Yanaga et al 1990) and often up to the tenth post operative day (Marujo et al 1991) suggesting that this may be due to poor vessel quality or deficiencies in storage and collection techniques. It has been proposed that impaired endothelial function of donor-organ vasculature may be induced by storage in specialised cold-preservation solutions and thus successful organ transplantation might be compromised (Clemens et al 1993). It has been established that optimum preservation should be achievable using UW rather than extra-cellular-type solutions such as Krebs Ringer, Eurocollins and Crystalloid Hyperkalaemic Cardioplegic Solution during liver transplantation (Todo et al 1989). However, hepatic sinusoidal endothelium may be particularly vulnerable to degradation during cold-storage and may result in liver-specific hypothermic injury which may be less important during cold-storage of other organs. It has been suggested that heart, lung, and kidney preservation in UW has few advantages compared with other extracellular solutions (Clavien et al 1992) and that UW-storage may specifically favour improved preservation of the hepatic vascular endothelium but this hypothesis remains to be proven.

The discovery that NO-dependent relaxation of the rabbit aorta to acetylcholine is entirely dependent upon endothelial integrity (Furchgott & Zawadzki 1980) provided an additional mechanism by which preservation damage to the hepatic vasculature could be assessed. It was previously established that purines are important vasoactive substances responsible for the regulation of hepatic vascular tone (Alexander et al 1992; Alexander 1996, 1998). Moreover, the observation that ATP could also elicit endothelium-dependent vasodilatation via the release of NO (Mathie et al 1991; Browse et al 1994) provided an additional parameter by which endothelial damage could be evaluated. Thus it was now possible to determine whether endothelial and smooth muscle function were altered by prolonged UW-storage using pharmacological measurements of vascular reactivity.

This hypothesis has been partially substantiated by a report that endothelial dysfunction was observed in UW-stored donor coronary vessels, where 5-HT-induced, endothelium-dependent, flow increases were decreased after storage for 4 h in UW compared with 4-h extracellular-type preservation with both Krebs' Ringer and Crystalloid Hyperkalaemic Cardioplegic Solution (Cartier et al 1993). Preservation of NO-mediated vascular reactivity remains a widely discussed and desirable objective for the retention of optimum conditions relating to the control of vascular tone (Sternbergh et al 1993; Anggard 1994). This is particularly important when removal of a thrombosed artery necessitates re-anastomosis using donor vessels previously stored in UW at 4°C for up to 8 days following the initial operation. Previous studies attempting to elucidate the underlying causes of this have been complicated by the heterogeneity of samples and variability of different drug regimens used. The aim of the present study, using a homogeneous population of male New Zealand White rabbits (2·2–2·9 kg) to limit the heterogeneity of data, was to determine whether endothelium-dependent, NO-mediated, responses to acetylcholine and adenosine-5′-triphosphate (ATP) differed after UW or extracellular-type (KBB) storage after 8 days at 4°C.

Materials and Methods

Operative procedures

Male New Zealand White rabbits, 2·2–2·9 kg, were killed by a lethal injection of sodium pentobarbitone through a cannulated marginal ear vein (Schedule I killing). Following thoraco-laparotomy, the thoracic aorta was gently dissected out avoiding vessel traction and 5-mm rings were cut and placed under 2-g tension upon fine-wire myographs, calibrated for isometric measurements in Krebs–Bülbring (KBB) buffer at 37°C and oxygenated with 95% O_2/5% CO_2. Samples of aortic rings from other rabbits were collected under comparable conditions and then stored at 4°C in either UW, KBB or saline (control) for 8 days. These were then mounted and tested upon fine-wire myographs in the manner described above.

Solutions and testing conditions

UW solution was kindly provided by Du Pont Pharmaceuticals Ltd, UK. KBB of the following composition (mM): NaCl 133, KCl 4·7, NaH_2PO_4 1·35, $NaHCO_3$ 20·0, $MgSO_4$ 0·61, glucose 7·8 and $CaCl_2$ 2·52, was chosen to reflect extracellular-type preservation and was freshly-prepared in the laboratory for KBB storage or fine-wire myograph testing. Acetylcholine chloride, adenosine-5′-triphosphate (ATP) , noradrenaline and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased

from Sigma Chemical Co. Ltd, Poole, Dorset. Acetylcholine, ATP and L-NAME were dissolved in fresh distilled water to provide 0·1 M solutions from which further dilutions were made. Noradrenaline was dissolved in 0·1 mM ascorbic acid to reduce the possibility of oxidation. The myographs were connected to a Grass FT03 isometric pressure transducer which converted vasodilatations or contractions of vascular smooth muscle to an electrical signal which was amplified and recorded on a Grass 79F Polygraph (Grass Instrument Co., Quincy, MA).

Experimental protocol

All vessels tested were mounted under 2 g tension in KBB at 37°C and flushed every 15 min with fresh KBB. After an equilibration period of 1 h, all vessels were preconstricted with a dose of noradrenaline which produced a submaximal degree of preconstriction, calculated from a concentration–response curve to noradrenaline, constructed at the start of every experiment. Endothelium-dependent vasodilatations to acetylcholine and to ATP in rabbit aortic rings were studied under normoxic and normothermic conditions in organ baths, on fresh (D0) samples and after 8 days of cold-storage (4°C) in either UW, KBB or saline. This storage time was chosen because this is the maximum time that vessels, which are used as conduits for liver transplants, may be used for re-anastomosis of thrombosed arteries following the initial transplantation. In some freshly-collected rings the endothelium was removed before testing. In a second group of rings, responses to acetylcholine and ATP were tested with and without the presence of the NO synthase inhibitor, L-NAME (100 μM). Cumulative concentration–response curves recording dose-related relaxation in isometric ring tension to increasing doses of acetylcholine, ATP and ATP were expressed as a percentage dilatation from the submaximal level of preconstriction produced by noradrenaline.

Electronmicroscopy

For transmission electron microscopy, specimen rings were removed from either UW- or KBB-storage after 8 days. These were minced and placed in fresh gluteraldehyde cacodylate solution for 1 h, followed by cacodylate buffer. They were then postfixed in 1% osmium tetroxide and embedded in epon. One-micron sections were then prepared and stained with toluidine blue. Sections with surface endothelial cells were identified, thin-sectioned, stained with uranyl acetate and examined with a Corinth AE1 electron microscope at 2500–15 000 magnification.

Statistical analysis

Results from concentration–response curves were expressed as the mean ± the standard error of the mean (s.e.m.). The data were checked for a normal distribution and a Student's two-tailed, unpaired *t*-test was applied to test for differences between responses, $P < 0{\cdot}05$ being taken as indicative of a significant difference. Where multivariate analysis was required, Newman-Keuls Analysis of Variance, followed by Student's two-tailed unpaired *t*-test with Bonferonni adjustment, where differences were significant, was applied.

Results

In the rabbit thoracic aorta noradrenaline produced comparable concentration–dependent increases in vascular tone which was measured as a gradual increase in isometric tension upon the pressure transducers. This enabled cumulative concentration–response curves to be constructed from which the maximum degree of preconstriction could be calculated at the start of every experiment. No significant differences were found between any of the groups tested and consequently all vessels were preconstricted with a −log (M) concentration of 2×10^{-6} noradrenaline which consistently produced an average of 71·3 ± 2·4% submaximal constriction (Gryf-Lowczowski et al 1997).

Cholinergic responses

Acetylcholine produced concentration- and endothelium-dependent vasodilatation in freshly-collected rings and rings which were stored in UW for 8 days (Figure 1). Removal of endothelium abolished vasodilatation to acetylcholine. After 8 days of cold-storage, percentage relaxation was significantly greater with UW- than KBB-storage at −log (M) acetylcholine concentrations of 7·0 ($P < 0{\cdot}05$), 6·5 ($P < 0{\cdot}05$) and 6·0 ($P < 0{\cdot}01$). Responses after UW-storage for 8 days were not significantly different from those of freshly collected rings (Figure 1). Vasodilatation to acetylcholine was, again, completely abolished after 8 days of saline, KBB- or UW-storage in the presence of L-NAME and therefore produced an identical curve to that produced by de-endothelialization (Figure 1).

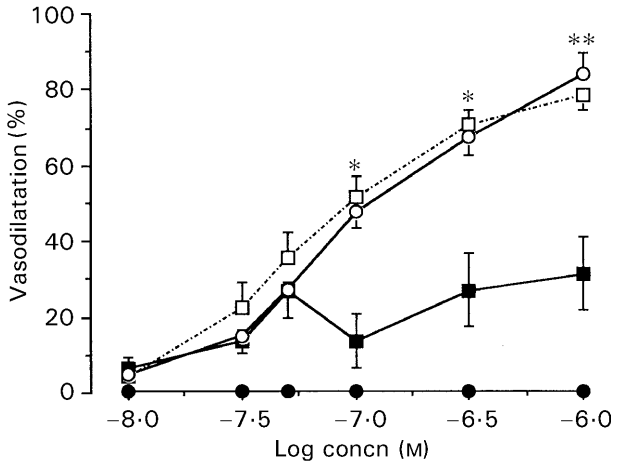

Figure 1. Percentage vasodilatation to acetylcholine of preconstricted sections of rabbit aorta on freshly collected tissue (–□–) and after removal of endothelium (–●–) at day 0, on harvesting, and also following 8 days of storage in UW (–○–) or KBB (–■–). Vasodilatation to acetylcholine was significantly greater in UW- than in KBB-stored arteries after 8 days of storage and was not significantly different to that seen in freshly collected arteries. Vasodilatation was totally abolished after de-endothelialization, saline-storage or in the presence of L-NAME. *$P < 0.05$. ** $P < 0.01$, UW vs KBB.

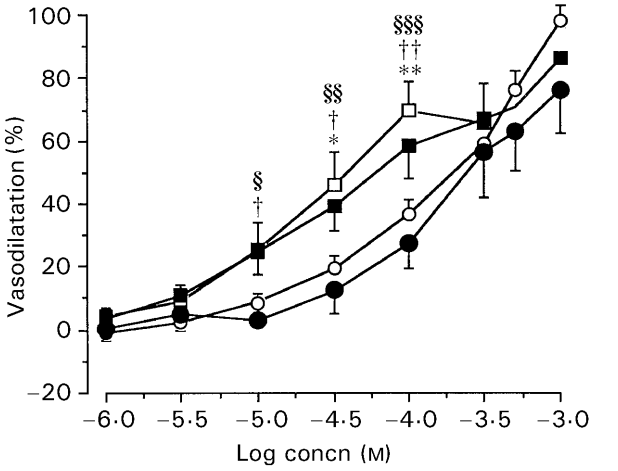

Figure 2. Concentration–response curves to ATP in freshly collected tissues (–□–) and after 8 days of storage of rabbit aorta in UW (○), KBB (■) or saline (●). Vasodilatation to ATP was significantly less in UW arteries vs freshly prepared arteries, * $P < 0.05$, ** $P < 0.01$; KBB- vs UW-stored arteries † $P < 0.05$, †† $P < 0.01$; and in freshly prepared vs saline-stored arteries § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$.

Effect of storage on ATP-induced vasodilatation

ATP produced dose-dependent vasodilatation in freshly-collected rings and after storage in UW, KBB and saline (Figure 2). No significant differences were found between any of the 3 types of storage. However vasodilatation to ATP was significantly less after 8 days of UW- and saline-storage compared with freshly-collected arteries at −log (M) ATP concentrations of 5·0 ($P < 0.05$), 4·5 ($P < 0.01$) and 4·0 ($P < 0.01$, Figure 2). This implied that UW-storage induced a deterioration in ATP-induced vasodilatation after 8 days of storage that was comparable with that measured after 8 days of storage in saline.

Effect of storage on nitric oxide-dependent vasodilatation to ATP

There were no significant differences between any of the groups of arteries studied after attenuation with L-NAME (Figure 3a). This therefore suggested that either all were attenuated with L-NAME to the same degree, that none were attenuated by L-NAME and that ATP induced vasodilatation by an NO-independent mechanism, or that a mixture of NO-dependent and -independent vasodilatation was being observed. L-NAME significantly reduced vasodilatation to ATP in

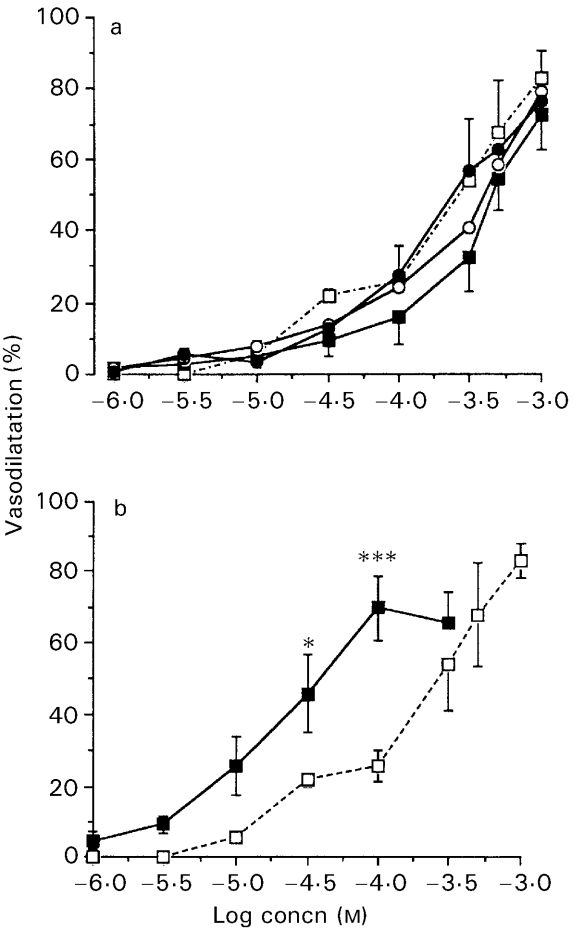

Figure 3. (a) Percentage vasodilatation to ATP after 8 days of cold-storage of rabbit aorta in KBB (■), UW (○) or saline (●). These were compared with responses measured in freshly-collected arteries (–□–) in the presence of L-NAME. There were no significant differences between the groups after attenuation with L-NAME. (b) Percentage vasodilatation to ATP in freshly-collected arteries before (■) and after (–□–) attenuation with L-NAME, * $P < 0.05$, *** $P < 0.001$.

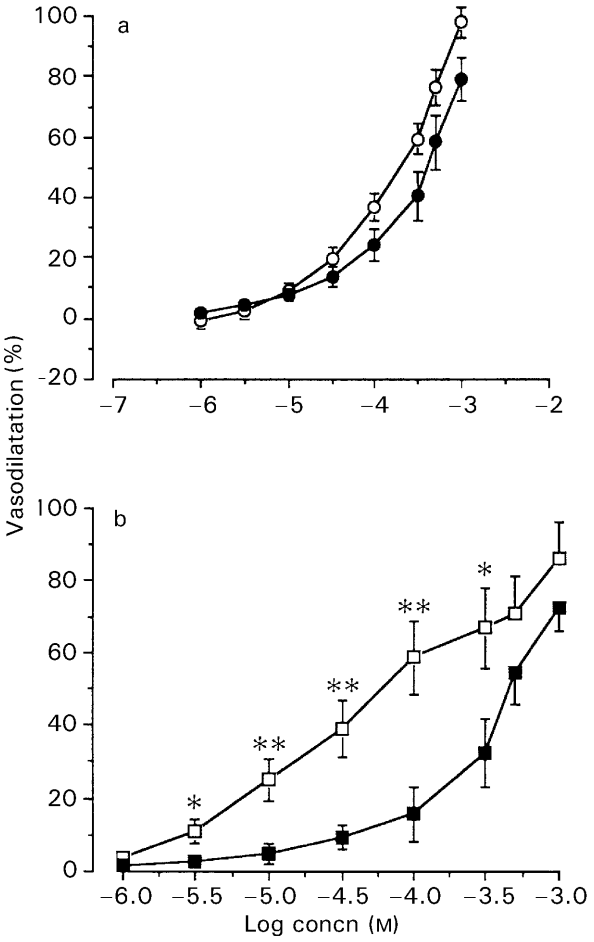

Figure 4. (a) Percentage vasodilatation to ATP before (□) and after (■) attenuation with L-NAME following storage of rabbit aorta in UW, * $P < 0.05$. (b) Percentage vasodilatation to ATP before (□) and after (■) attenuation with L-NAME following storage in KBB, * $P < 0.05$, ** $P < 0.01$.

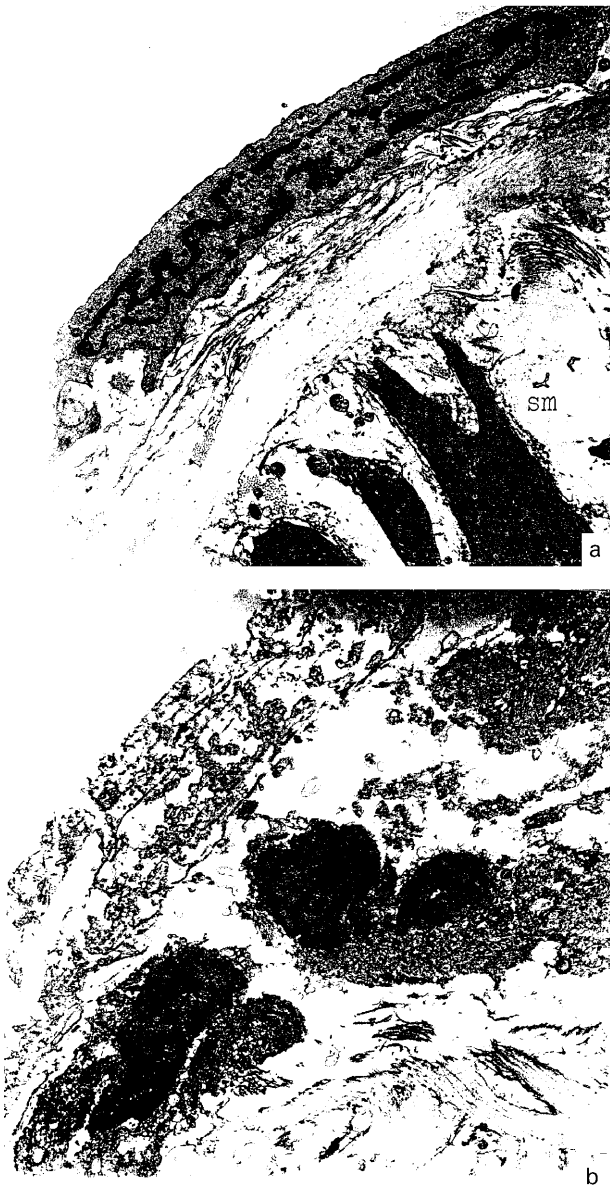

Figure 5. (a) Electron photomicrograph of the intima and subadjacent media of a KBB-stored section of rabbit aorta at × 15 000 magnification. The endothelial and smooth muscle morphology has been severely damaged. (b) Electron photomicrograph of the intima and subadjacent media of a UW-stored rabbit aorta at 15 000 magnification. Normal endothelial and smooth muscle morphology appears to have been preserved (e = endothelium, sm = smooth muscle).

freshly-collected arteries and this difference was statistically significant at −log (M) ATP concentrations of 4·5 and 4·0 ($P < 0.05$ and $P < 0.001$, respectively, Figure 3b). However, L-NAME only attenuated vasodilatation to ATP after 8 days of UW-storage at a −log (M) ATP concentration of 3·0 ($P < 0.05$, Figure 4a), thereby suggesting that ATP could only elicit NO-independent vasodilatation except at the highest doses used. In contrast, L-NAME markedly attenuated ATP-induced vasodilatation in KBB-stored arteries after 8 days of storage and this attenuation was statistically significant at −log (M) ATP concentrations of 5·5, 5·0, 4·5, 4·0 and 3·5 ($P < 0.05$, 0·01, 0·01, 0·01 and 0·05, respectively, Figure 4b).

Structural preservation after UW- or KBB-storage

Electron microscopy confirmed markedly improved endothelial preservation after storage in UW compared with storage in KBB. Mitochondria and endoplasmic reticulum appeared unaltered, even after 8 days of UW-preservation. KBB-storage resulted in severe endothelial degeneration (Figure 5).

Discussion

The present study demonstrated that endothelium-dependent responses to acetylcholine were

unaltered after 8 days of storage in UW but reduced after storage in KBB. These responses were totally abolished after de-endothelialization and saline-storage thus suggesting improved retention of endothelial function was achieved with the use of UW compared with use of KBB for storage. Complete abolition of acetylcholine-induced vasodilatation by 100 μM L-NAME after both KBB- and UW-storage suggested that this was entirely due to NO-mediated responses. These pharmacological measurements of vascular reactivity were confirmed by virtually unaltered electron-microscopic morphology of rabbit aortic endothelium after 8 days of UW-storage and reduced endothelial preservation after KBB-storage in both the present study and in an earlier investigation (Gryf-Lowczowski et al 1997).

However, although endothelium-dependent responses to acetylcholine were maintained after 8 days of UW-storage and significantly reduced after KBB-storage, vasodilatation to ATP was maintained after 8 days of KBB- and reduced after UW-storage. There were no significant differences in ATP-induced vasodilatation in UW-, and saline-stored rings. In fresh arteries, L-NAME only partially attenuated the vasodilatation to ATP at -4 and $-4{\cdot}5$ log (M) ATP suggesting that NO-dependent and -independent mechanisms were involved at this concentration. In addition, ATP appeared to induce further vasodilatation at higher concentrations that was also NO-independent since this was not attenuated by L-NAME. ATP-induced vasodilatation after 8 days of UW-storage was not attenuated by L-NAME and therefore suggested that this occurred via an NO-independent mechanism. In contrast, ATP-induced vasodilatation was almost totally attenuated by L-NAME in KBB-stored rings thus implicating an NO-dependent mechanism. Since there were no significant differences between KBB-stored and freshly-harvested rings to ATP-induced vasodilatation, in the presence of L-NAME, these factors therefore suggested that ATP-induced vasodilatation in UW-stored rings occurred predominantly via an NO-independent mechanism and that in KBB-stored rings it occurred via a predominantly NO-dependent, but endothelium-independent, mechanism. Since the vascular endothelium was preserved in UW-stored rings and virtually destroyed in KBB-stored rings, this suggested that the ATP-induced vasodilatation in these segments of rabbit thoracic aorta, tested under the present conditions, was due to an NO-dependent, but endothelium-independent, mechanism. Moreover, storage in UW preserved the NO-independent fraction of ATP-induced vasodilatation because this was not attenuated by L-NAME. Therefore KBB-storage retained NO-dependent, endothelium-independent, ATP-induced vasodilatation and UW-storage retained NO-independent, ATP-induced vasodilatation which was probably also endothelium-independent because there were no significant differences between UW-stored, KBB-stored and freshly harvested rings. Not all P_{2Y}-purinoceptors are located on the vascular endothelium. Studies in the isolated rabbit hepatic artery have shown that the smooth muscle compartment could be an alternative location of P_{2Y}-purinoceptors (Brizzolara & Burnstock 1991) or smooth muscle nucleoside P_3-purinoceptors (Chinellato et al 1992). ATP-induced vasodilatation in the human forearm has also been shown to be NO independent since this was not attenuated by L-NAME (Rongen et al 1994). In addition, the ATP-sensitive potassium (K_{ATP})-channel blocker, glibenclamide did not attenuate ATP-induced vasodilatation in other studies which used human mammary arteries (Vroom et al 1994). ATP may also have induced vasodilatation via mechanisms other than NO-dependent, P_{2Y}-vasodilatation. ATP may also be catabolized to adenosine and elicit vasodilatation via P_1 (A_2)-purinoceptors which are predominantly located on the vascular smooth muscle compartment (Browse et al 1994; Alexander 1998).

The present study demonstrated ATP activity to be, at least partially, NO-dependent since ATP-induced vasodilatation in KBB-stored and freshly-harvested rings was attenuated by L-NAME. Other studies have recently shown that ATP may elicit vasodilatation through NO release by simultaneous activation of P_{2Y}- and A_2-purinoceptors (Browse et al 1997). L-NAME did not totally abolish ATP-induced vasodilatation and the residual component may have been due to adenosine P_1 (A_2)-induced vasodilatation. In order to confirm this, a suitable P_1 (A_2)-receptor antagonist, such as 8-phenyltheophylline, could have been added to the organ baths in the presence of L-NAME during construction of concentration-response curves to ATP (Mathie & Alexander 1990). This was not attempted during the present study.

The fact that the ATP-induced vasodilatation was NO-dependent in KBB-stored rings, however, still did not preclude the possibility that adenosine may also have been acting via an NO-dependent mechanism, since NO-mediated adenosine vasodilatation was demonstrated in the isolated perfused guinea-pig heart (Vials & Burnstock 1993). The fact that ATP-induced vasodilatation was insensitive to L-NAME attenuation in UW-stored rings could possibly have been due to two reasons. Firstly, the vascular endothelium could have been damaged by UW-storage, although the

morphological data from the present study do not support this postulate. Electron microscopy of KBB-stored rings revealed marked endothelial destruction whereas endothelial morphology was preserved in UW-stored vessels. Therefore the retained ATP/NO-mediated purinergic activity in KBB- compared with UW-stored rings was unlikely to be related to structural preservation of the vascular endothelium. Secondly, P_{2Y}-purinoceptors, located on the vascular endothelium, were selectively damaged by UW-storage and ATP could therefore only elicit NO-dependent, endothelium-independent, vasodilatation, possibly via P_1 (A_2)-purinoceptors. Alternatively, and less likely, UW-storage may have caused swelling of the vascular endothelium which may have reduced diffusion of L-NAME into the smooth muscle compartment. The vessel rings were not weighed before and after storage in UW and therefore this hypothesis remains to be substantiated.

In summary, ATP-induced, NO-dependent, endothelium-independent vasodilatation appeared to be best preserved in KBB-stored vessels and acetylcholine-induced, endothelium- and NO-dependent vasodilatation best preserved in UW-stored vessels. Therefore UW-preservation is not universally compatible with the preservation of all vascular receptors which induce vasodilatation via the release of NO. Reduced purinergic ATP- and NO-mediated endothelial function was demonstrated after UW- compared with KBB-storage of rabbit aortae according to the pharmacological criteria of vascular reactivity used in the present study. Reductions in purinergic, NO-mediated, endothelial function after UW-storage of human hepatic arteries therefore, could potentially reduce efficient liver sinusoidal perfusion and compromise successful transplantation. Purinergic, NO-dependent dysfunction after UW-storage could therefore provide an explanation for previous reports of increased hepatic vascular resistance following transplantation (Ikeda et al 1990). Therefore although UW-storage of rabbit thoracic aortae may apparently favour improved retention of vascular morphology according to electron microscopic observations, vascular pharmacological tests suggest this to be detrimental to the retention of normal purinergic (ATP) NO-dependent vascular reactivity.

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