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Evidence that *S*-adenosyl-L-methionine diastereoisomers may reduce ischaemia-reperfusion injury by interacting with purinoceptors in isolated rat liver

[1,3]J. Bruce Dunne, [2]Barry Alexander, [1,4]Roger Williams & [1,5]J. Michael Tredger

[1]Institute of Liver Studies and [2]Liver Sciences Unit, Academic Department of Surgery, King's College Hospital and School of Medicine and Dentistry, London SE5 9PJ.

1 Mechanisms underlying the haemodynamic activity of diastereoisomers of *S*-adenosyl-L-methionine (SAM) were investigated using inhibitors of purinoceptors and nitric oxide (NO) synthase in perfused rat livers damaged by sequential 24 h cold and 20 min rewarming ischaemia + reperfusion.

2 Stored livers were flushed with 10 ml saline alone (control) or with added (*R,S*) or (*S,S*) SAM (100 μM) and reperfused in the absence (control) or presence of 10 μM 8-phenyltheophylline (8-PT) or 100 μM L-*N*-monomethylarginine (L-NMMA).

3 Both SAM diastereoisomers rapidly increased blood flow and bile production *versus* controls ($P<0.001$) but the (*R,S*) isomer induced greater increases in blood flow and the (*S,S*) isomer greater increases in bile production: 625 *versus* 596 *versus* 518 ml blood flow and 100 *versus* 119 *versus* 56 mg bile production per g liver over 3 h in (*R,S*), (*S,S*) and control, respectively.

4 8-PT prevented the enhancement of blood flow by (*S,S*) SAM (529 *versus* 596 ml g^{-1} liver over 3 h for (*S,S*) SAM alone, $P<0.001$), but was without effect in control livers. 8-PT also reduced SAM-enhanced bile production: 51 *versus* 119 mg g^{-1} liver over 3 h, $P<0.001$. L-NMMA reduced blood flow and bile production similarly in the absence or presence of (*S,S*) SAM.

5 Thus, SAM may improve liver perfusion after ischaemia-reperfusion injury *via* stimulation of P_1 (A_2) purinoceptors at which SAM shows activity. The choleretic activity of (*S,S*) SAM is disproportionately greater than enhanced blood flow and may occur independently of a NO-dependent component of bile production.

Keywords: Ischaemia-reperfusion injury; isolated perfused rat liver; liver transplantation; nitric oxide; 8-phenyltheophylline; L-*N*-monomethylarginine; purinoceptors

Introduction

S-adenosylmethionine (SAM) is the endogenous methyl group donor and a central component of the methylation cycle which regulates key steps of transmethylation, transulphuration and polyamine synthesis (Finkelstein, 1994). The administration of exogenous SAM has been shown to ameliorate both clinical and experimental hepatic dysfunction, with efficacy demonstrated in acute and chronic cholestasis (Catalino *et al*., 1992; Manzillo *et al*., 1992; Bray *et al*., 1993) and in drug-induced hepatotoxicity (Bray *et al*., 1992; Corrales *et al*., 1992; Jover *et al*., 1992; Mesa *et al*., 1996). Cold ischaemia/reperfusion injuries which characterize the liver transplantation procedure are also reduced by administration of SAM (Dunne *et al*., 1994).

Liver grafts are damaged by periods of cold ischaemia (during storage), rewarming ischaemia (during implantation) and reperfusion injury at revascularization (Clavien *et al*., 1992). Hypothermic ischaemic injuries to the liver are limited using University of Wisconsin (UW) solution for graft storage (Belzer & Southard, 1988) but currently there are no routine measures effective against reperfusion injuries, which accentuate and augment ischaemic damage. Two promising approaches involve the displacement of UW from the graft just prior to reperfusion with a formulated flush solution, Carolina rinse (Currin *et al*., 1990) or portal vein blood (Emre *et al*., 1994). Improved early graft perfusion results in both cases. Carolina rinse has improved the outcome of liver transplantation experimentally (Currin *et al*., 1990) and clinically (Bachmann *et al*., 1997) and one of the most important components of Carolina rinse is thought to be adenosine (Bachmann *et al*., 1991).

We recently reported that SAM improved haemodynamic and metabolic indicators of liver function in preserved livers when administered at various stages of liver retrieval, storage and reperfusion (Dunne *et al*., 1997). When used in a simple flush solution, SAM rapidly and markedly improved tissue perfusion (Dunne *et al*., 1997). Together with earlier results showing a greater efficacy with SAM than equimolar amounts of *N*-acetylcysteine (Dunne *et al*., 1994), these observations suggest that benefits achieved simply through actions as a glutathione precursor are an unlikely mechanism. In this report we have examined alternative possibilities that SAM achieves haemodynamic benefit by actions on purinoceptors or through the stimulation of the nitric oxide (NO)-mediated vasodilatation.

Two diastereoisomers of SAM (*S,S* and *R,S*) arise during its pharmaceutical synthesis. These comprise 70 and 30%, respectively, of the synthetic butanedisulphonate salt whereas endogenous SAM consists of >95% of the (*S,S*) diastereoisomer *in vivo* (Anon, 1993; Hoffman, 1986). The aims of the

Present addresses: [3]Department of Surgery, School of Medicine, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112-2699, U.S.A; [4]Institute of Hepatology, University College London Medical School, 69–75 Chenies Mews, London WC1E 6HX; [5]Author for correspondence at: The Institute of Liver Studies, King's College School of Medicine and Dentistry, Bessemer Road, London SE5 9PJ.

present study were, firstly, to assess the relative biological activities of the (*R,S*) and (*S,S*) isomers of SAM used in flush solutions before reperfusion and secondly, to define a possible role for SAM in purinoceptor activation or NO release using 8-phenyltheophylline (8-PT) (Dalziel & Westfall, 1994) or L-*N*-monomethylarginine (L-NMMA) as a P_1-purinoceptor antagonist and NO synthase (NOS) inhibitor, respectively (Rees *et al*., 1989).

Methods

Animals

Male Lewis rats (300–400 g) were obtained from Charles River, England and fed CRM pelleted diet (SDS, Witham, U.K.) and tap water *ad libitum*. All procedures were performed under licence according to the Animals (Scientific Procedures) Act, 1986.

Drugs

S-Adenosyl-L-methionine (SAM) was supplied as the 1,4 butanedisulphonate salt at >99% purity by BioResearch SpA (BASF Group, Milan, Italy) who also supplied the same salts of the (*S,S*) and (*R,S*) diastereoisomers which were stored at −70°C before use. 8-Phenyltheophylline (8-PT) and L-*N*-monomethylarginine (L-NMMA) were purchased from Sigma Chemical Co Ltd., Poole, Dorset, U.K.). All drugs were prepared immediately before their addition to perfusate.

Hepatectomy and perfusion

Rats were anaesthetized with an intraperitoneal injection of 60 mg kg^{-1} pentobarbitone sodium (Sagatal, RMB Animal Health, Dagenham, U.K.). The hepatic portal vein, bile duct and suprahepatic inferior vena cava were cannulated and the infrahepatic inferior vena cava ligated as previously described and the liver was removed (Dunne *et al*., 1994).

The perfusion circuit was located in a heated cabinet at 37°C and consisted of a Watson-Marlow peristaltic pump, a blood transfusion filter (Pall, SQ40S, Portsmouth, U.K.), a thin layer oxygenator over which was passed water-saturated 95% O_2/5% CO_2 and a constant head of 14 cm H_2O. The recirculating perfusate (120 ml) comprised bicarbonate buffered Ringer's (147 mM NaCl, 4 mM KCl and 2 mM $CaCl_2$), 2% bovine serum albumin (Sigma, Poole, U.K.), 20 U ml^{-1} heparin and 10 mM D-glucose. To ensure adequate oxygen delivery to the liver, washed erythrocytes from patients with haemochromatosis were suspended in the perfusate to a final mean haematocrit (15%) shown to provide adequate oxygenation at reduced viscosity (Alexander *et al*., 1994). Perfusate pH was maintained at 7.4 by the addition of $NaHCO_3$ as required.

Experimental protocol

Livers were flushed with and stored for 24 h at 0–4°C in UW containing 16 mg l^{-1} dexamethasone and 40 U l^{-1} insulin as recommended (ViaSpan, Du Pont Pharmaceuticals, Letchworth Garden City, U.K.). Subsequently, livers were rewarmed for 20 min by immersion in oxygenated bicarbonate buffered saline at 37°C. These periods were selected because they simulate prolonged storage intervals and anastomosis times known to impair outcome in rat liver transplantation (Sumimoto *et al*., 1990) although shorter cold ischaemia and longer rewarming ischaemia times are typical in man (Piratvisuth *et al*., 1995). After the sequential periods of cold and rewarming ischaemia, each liver was flushed with oxygenated bicarbonate-buffered saline at 37°C (10 ml saline flush) to remove UW and was perfused for 3 h.

The control group ($n=7$) comprised livers exposed to sequential cold and warm ischaemia and a saline flush and reperfused with standard perfusate (above). The six treatment groups comprised two treated only with the (*R,S*) or (*S,S*) diastereoisomers of SAM in the saline flush ($n=7$ for both) and two treated only with 8-PT or L-NMMA in the perfusate ($n=6$ for both). In the final two groups, livers were flushed with the endogenous (*S,S*) diastereoisomer of SAM, and 8-PT or L-NMMA was added to the perfusate ($n=7$ for both). SAM was used in the flush solution at a concentration of 100 μM. Bolus additions of 8-PT and L-NMMA were made to the perfusate just before reperfusion to achieve final concentrations of 10 and 100 μM, respectively. At 10 μM concentrations, 8-PT acts specifically on P_1 (A_2) receptors in the hepatic circulation (Ralevic *et al*., 1991). Animals were randomized between the treatment groups.

Samples and analysis

Samples of perfusate were taken at 0, 15, 30, 60, 120 and 180 min from the portal vein supply and from the suprahepatic IVC outflow. Portal samples (1 ml) were collected into EDTA for measurements of plasma markers of liver damage: aspartate aminotransferase (AST) (AST 10, Sigma, Poole, U.K.) and alanine aminotransferase (ALT) (kit from Boehringer Mannheim U.K., Lewes, East Sussex, used on a Cobas Mira analyzer, Roche Diagnostics, Welwyn Garden City, Hertfordshire, U.K.) for hepatocyte damage and purine nucleoside phosphorylase (PNP) (Rao *et al*., 1990) for endothelial cell damage. Venous outflow samples (1 ml) were collected into sodium fluoride for measurements of glucose and lactate (YSI 2300 Stat Plus, Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.). Both portal and venous outflow samples were analysed for blood gases (AVL 995-Hb, AVL Medical Instruments, Stone, U.K.). Bile was collected into pre-weighed tubes at the times shown above for the determination of bile flow in mg h^{-1} g^{-1} liver weight.

Calculations and statistics

Oxygen consumption was calculated according to Martin (1983). Oxygen delivery was determined from the product of blood flow and perfusate oxygen content, and oxygen extraction ratio (OER) by dividing oxygen consumption by oxygen delivery. Non-parametric statistical analyses were used throughout, with initial (15 min) data and values over the 3 h of perfusion (calculated as areas under the concentration *versus* time curve using the trapezoidal rule) compared using the Mann Whitney *U*-test. A value of $P<0.05$ was taken as being statistically significant.

Results

Blood flow and oxygen delivery

After sequential cold and warm ischaemia, liver blood flow at reperfusion was reduced to values around 3 ml min^{-1} g^{-1} liver as described in earlier studies (Dunne *et al*., 1994; Dunne *et al*.,

1997). Flushing livers with saline containing either the (*R,S*) or (*S,S*) diastereoisomers of SAM increased blood flow as early as 15 min after reperfusion, but this achieved significance only for the (*R,S*) isomer (3.44 ± 0.06 *versus* 2.93 ± 0.09 ml min^{-1} g^{-1} in control preparations using saline alone; $P < 0.001$) (Figure 1). Improved blood flow was maintained over the complete perfusion interval (3 h) with both the (*R,S*) and (*S,S*) isomers, (+21 and +15%, respectively, *versus* the saline flush: 625 and 596 *versus* 518 ml g^{-1} liver; $P < 0.001$ in both cases). The (*R,S*) isomer was significantly more effective overall ($P = 0.02$ *versus* the (*S,S*) isomer) (Figure 1). Oxygen delivery was influenced in an analogous manner by the two SAM diastereoisomers, with the (*R,S*) isomer more active than the (*S,S*) (mean increases over 3 h, +21% and +14%, respectively) (data not shown).

The addition to the perfusate of the purinoreceptor blocker 8-PT did not significantly affect liver blood flow after sequential cold and warm ischaemia (Figure 2A). However, 8-PT abolished the improvement in mean blood flow by the (*S,S*) isomer by 30 min (2.91 *versus* 3.39 *versus* 3.03 ml min^{-1} g^{-1} liver for (*S,S*) SAM + 8-PT, (*S,S*) SAM and control, respectively) and this was sustained for the remainder of the 3 h of perfusion (11% reduction in flow over 3 h *versus* (*S,S*) isomer alone, $P < 0.001$: 529 *versus* 596 *versus* 518 ml g^{-1} liver) (Figure 2A). Perfusate L-NMMA, the inhibitor of NOS, caused sustained reductions in liver blood flow in control preparations (of 36 ml g^{-1} liver over the 3 h perfusion, $P = 0.013$) (Figure 2B) and quantitatively similar reductions in the improvements in blood flow induced by the (*S,S*) isomer (35 ml g^{-1} liver $P > 0.05$) (Figure 2B). 8-PT also abolished the increase in oxygen delivery induced by (*S,S*) SAM but had no effect in saline-flushed livers, while L-NMMA was of equivalent efficacy in livers treated with saline or the (*S,S*) isomer (data not shown).

Bile production

Both the (*R,S*) and (*S,S*) isomers rapidly increased bile production, with flow at 15 min rising from 11.0 ± 2.9 mg h^{-1} g^{-1} liver in controls to 15.3 ± 2.1 and 20.5 ± 1.3 mg h^{-1} g^{-1} liver, respectively: $P > 0.05$ and 0.009) (Figure 3). Peak production of bile was at 60 min in every case and the increases in bile flow resulting from SAM treatment were sustained over the complete perfusion (Figure 3), with the (*S,S*) more active than the (*R,S*) diastereoisomer ($P = 0.03$ overall). Total bile production over 3 h rose from 56 (saline) to 119 and 100 mg g^{-1} liver with the (*S,S*) and (*R,S*) isomers, respectively ($P < 0.001$ in both cases).

The addition of 8-PT to the perfusate reduced total bile production in livers flushed with saline by 36% (from 56 to 36 mg g^{-1} liver over 3 h, $P < 0.02$), and by 57% in livers flushed with (*S,S*) SAM (from 119 to 51 mg g^{-1} liver, $P < 0.001$) (Figure 4A). 8-PT also appeared to act more rapidly in SAM-treated livers because reductions in bile flow were evident at 15 min (from 20.5 ± 1.3 to 12.9 ± 3.3 mg h^{-1} g^{-1}, $P = 0.06$) while comparable decreases were not achieved until 60 min in control livers flushed with saline (Figure 4A). Peak bile production was at 60 min in every case. L-NMMA reduced total bile production over 3 h by similar amounts (40 and 49 mg g^{-1} liver) in saline- and (*S,S*) SAM-treated livers, respectively (Figure 4B). In the former, this represented a 71% fall *versus* saline flush ($P < 0.001$) and production fell to

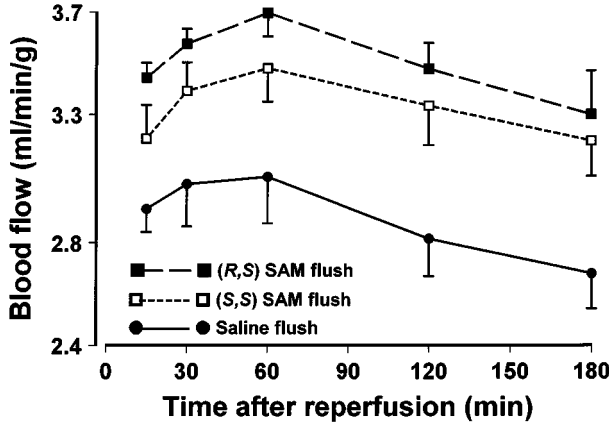

Figure 1 Influence of flushing livers with (*S,S*) or (*R,S*) isomers of SAM on hepatic blood flow. All livers were exposed to 24 h cold and 20 min rewarming ischaemia in UW preservation solution. Livers were reperfused after displacing UW with 10 ml saline alone (Saline flush) or containing 100 μM (*S,S*) SAM ((*S,S*) SAM flush) or 100 μM (*R,S*) SAM ((*R,S*) SAM flush). All data show means ± s.e.m. of seven determinations. Significant Mann Whitney *U*-test results: 15 min values – Saline *versus* (*R,S*) SAM, $P < 0.001$; area under the curve values (total over 3 h) – Saline *versus* (*S,S*) SAM and Saline *versus* (*R,S*) SAM $P < 0.001$; (*S,S*) SAM *versus* (*R,S*) SAM, $P = 0.02$.

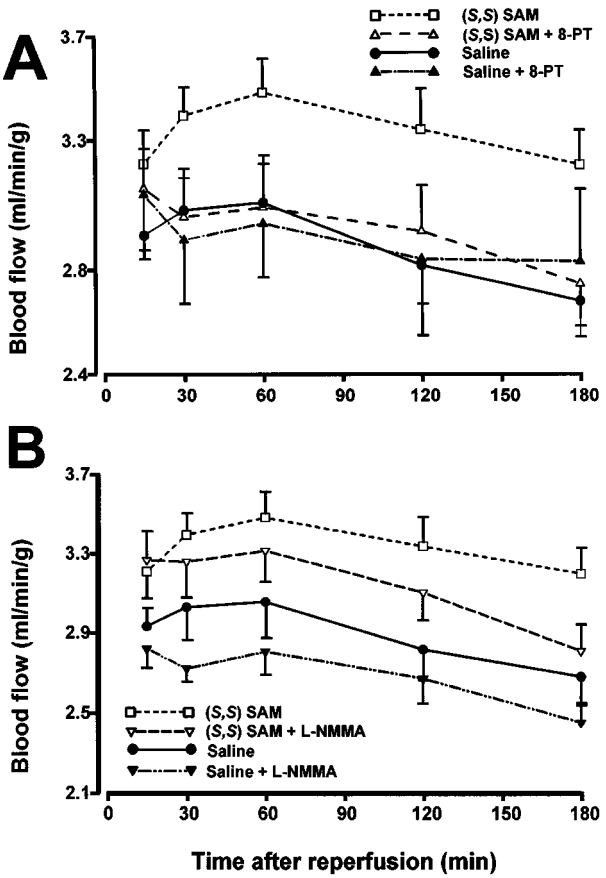

Figure 2 Influence of (A) purinergic receptor blockade and (B) inhibition of nitric oxide synthesis on baseline and (*S,S*) SAM-induced hepatic blood flow. Livers were flushed with saline (Saline) or (*S,S*) SAM ((*S,S*) SAM) before reperfusion in the presence of 8-phenyltheophylline (10 μM)(A) or L-*N*-monomethylarginine (100 μM L^{-1})(L-NMMA)(B). All data are means ± s.e.m. values of six or seven determinations. Significant Mann Whitney *U*-test results: (A) area under the curve values (total over 3 h) – Saline *versus* (*S,S*) SAM, $P < 0.001$; (*S,S*) SAM *versus* (*S,S*) SAM + 8-PT, $P < 0.001$; (B) 15 min values – Saline + L-NMMA *versus* (*S,S*) SAM + L-NMMA, $P = 0.03$; area under the curve values – Saline *versus* Saline + L-NMMA, $P = 0.013$; Saline *versus* (*S,S*) SAM, $P < 0.001$; Saline + L-NMMA *versus* (*S,S*) SAM + L-NMMA, $P < 0.001$.

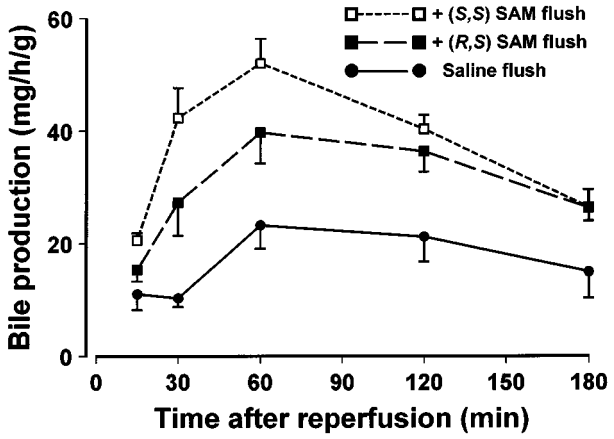

Figure 3 Influence of flushing livers with (*S,S*) or (*R,S*) isomers of SAM on bile production. The legend to Figure 1 applies. Significant Mann Whitney *U*-test results: 15 min values-Saline *versus* (*S,S*) SAM, $P<0.009$; area under the curve values (total over 3 h) Saline *versus* (*S,S*) SAM and Saline *versus* (*R,S*) SAM, $P<0.001$; (*S,S*) SAM *versus* (*R,S*) SAM, $P=0.03$.

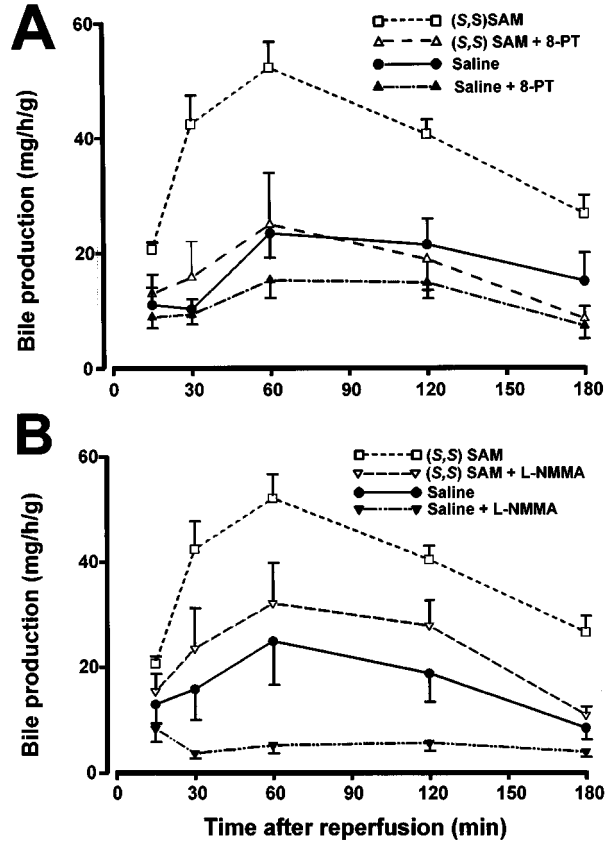

Figure 4 Influence of (A) purinergic receptor blockade and (B) inhibition of nitric oxide synthesis on baseline and (*S,S*) SAM-induced bile production. The legend to Figure 2 applies. Significant Mann Whitney *U*-test results: (A) 15 min values−Saline *versus* (*S,S*) SAM, $P=0.009$; area under the curve values (total over 3 h)−Saline *versus* (*S,S*) SAM, $P<0.001$; Saline *versus* Saline+8-PT, $P=0.02$; (*S,S*) SAM *versus* (*S,S*) SAM+8-PT, $P<0.001$; (B) 15 min values−Saline *versus* (*S,S*) SAM, $P=0.009$; area under the curve values−Saline *versus* Saline+L-NMMA, $P=0.001$; Saline *versus* (*S,S*) SAM, $P<0.001$; Saline+L-NMMA *versus* (*S,S*) SAM+L-NMMA, $P<0.001$; (*S,S*) SAM *versus* (*S,S*) SAM+L-NMMA, $P<0.001$.

only 16 mg g^{-1} liver over 3 h (Figure 4B). The typical peak of bile production at 60 min was abolished in saline-flushed livers treated with L-NMMA and peak flow was at 15 min. The more extensive production of bile noted after (*S,S*) SAM was reduced by 41% by L-NMMA over 3 h (from 119 to 70 mg g^{-1}, $P<0.001$). In both control and SAM-treated livers, reductions in bile flow were evident from 15 min (Figure 4B).

Oxygen consumption and extraction ratio (OER)

Oxygen consumption rose continuously from 30 min in livers flushed with saline, but peaked at 2 h after treatment with either SAM isomer (Figure 5A). Although neither isomer affected total oxygen consumption significantly over the 3 h perfusion, values were consistently higher during the initial reperfusion after treatment with (*S,S*) SAM. Over 3 h, consumption was 7% higher for the (*S,S*) and 16% lower for the (*R,S*) isomer: 387 *versus* 304 *versus* 361 μmol g^{-1} liver in controls ($P=0.005$ for (*R,S*) *versus* (*S,S*)) (Figure 5A). However, the increase in consumption with (*S,S*) SAM was matched by a 21% improvement in delivery and OER was unchanged overall (Figure 5B). In contrast, the decrease in consumption with the (*R,S*) isomer, coupled with a 14% increase in delivery, resulted in an overall fall in mean OER (from 0.237 to 0.164 over 3 h, $P<0.001$ *versus* saline or the (*S,S*) isomer (mean, 0.220), (Figure 5B).

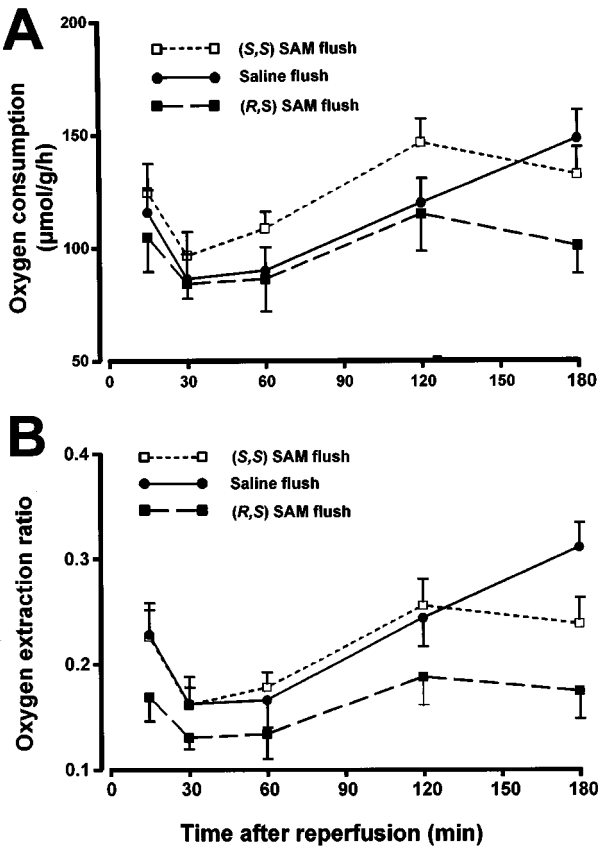

Figure 5 Influence of flushing livers with (*S,S*) or (*R,S*) isomers of SAM on (A) oxygen consumption and (B) oxygen extraction ratio. The legend to Figure 1 applies. Significant Mann Whitney *U*-test results: (A) area under the curve values (total over 3 h)−(*S,S*) SAM *versus* (*R,S*) SAM, $P=0.005$; (B) area under the curve values−Saline *versus* (*R,S*) SAM, $P<0.001$; (*S,S*) SAM *versus* (*R,S*) SAM, $P=0.001$.

There was no effect of 8-PT on oxygen consumption over the 3 h perfusion, but L-NMMA reduced consumption by 13% after flushing with the (*S,S*) isomer ($P=0.02$): mean, 339 *versus* 387 μmol g^{-1} liver. OER was unchanged by 8-PT but was decreased by L-NMMA after flushing with the (*S,S*) isomer ($P=0.04$ initially and overall).

Glucose and lactate release

Glucose was released into the perfusate at the start of reperfusion in all groups (Figure 6A). This initial release of glucose was greater after flushing with (*S,S*) SAM than the (*R,S*) isomer (0.77 ± 0.02 *versus* 0.64 ± 0.05 mmol g^{-1}, $P=0.02$; *cf* 0.73 ± 0.03 mmol g^{-1} after saline flush). Over the remaining interval, glucose concentrations in perfusate progressively decreased and converged for the two SAM isomers, and in both cases net uptake over 3 h was more rapid than in livers flushed with saline (Figure 6A). 8-PT decreased glucose re-uptake, with total glucose release after treatment with (*S,S*) SAM increased from 0.42 to 0.54 mmol over 3 h ($P=0.04$). There was no corresponding effect of L-NMMA.

Lactate was released from perfused livers for the first 60 min of reperfusion after which time net re-uptake occurred. (Figure 6B). There were no significant effects on net lactate release into the perfusate with either of the SAM isomers or 8-PT. L-NMMA did not affect perfusate lactate concentrations in untreated livers but increased lactate release by 49% (from 0.4 to 0.6 mmol, $P=0.03$) after flushing with (*S,S*) SAM (Figure 6B). This seemed likely to result from the impairment in oxygen consumption induced by L-NMMA after SAM treatment as described above.

Enzyme release

The extent of hepatocellular injury detected during the 3 h perfusion was not different between the treatment groups as judged from similar progressive rises in perfusate AST and ALT (data not shown). In such circumstances, disproportionate changes in PNP release can be considered to reflect damage to endothelial cells (Dunne *et al*., 1997). PNP release into the perfusate was not affected by either SAM isomer at 15 min, but this initial release was increased when either 8-PT or L-NMMA was used, especially in conjunction with (*S,S*) SAM (1.89 and 1.96 IU L^{-1} g^{-1} liver, respectively *versus* 1.05 for SAM alone, $P<0.001$ in both cases) (Figure 7). PNP concentrations were higher throughout the 3 h perfusion in the presence of either 8-PT or L-NMMA, and total release increased from 465 to 521 IU L^{-1} g^{-1} liver (+12%) and from 410 to 587 IU L^{-1} g^{-1} liver (+43%) with 8-PT ($P>0.05$ and 0.002 *versus* saline and SAM flush, respectively) (Figure 7A) and to 522 (+12%) and 604 IU L^{-1} g^{-1} liver (+27%) with L-NMMA ($P=0.05$ and <0.001, respectively) (Figure 7B).

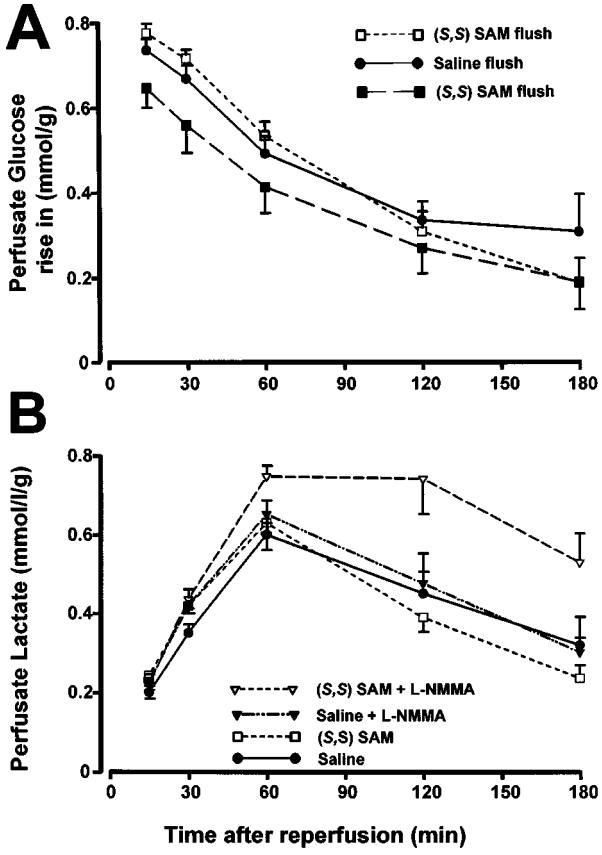

Figure 6 Influence of flushing livers with SAM diastereoisomers on (A) perfusate glucose and (B) nitric oxide synthesis inhibition of perfusate lactate. The legends to Figures 1 and 2 apply. Significant Mann Whitney *U*-test results: (A) 15 min values–(*S,S*) SAM *versus* (*R,S*) SAM, $P=0.02$; (B) area under the curve values (total over 3 h)–(*S,S*) SAM *versus* (*S,S*) SAM + L-NMMA, $P=0.03$; Saline + L-NMMA *versus* (*S,S*) SAM + L-NMMA, $P=0.02$.

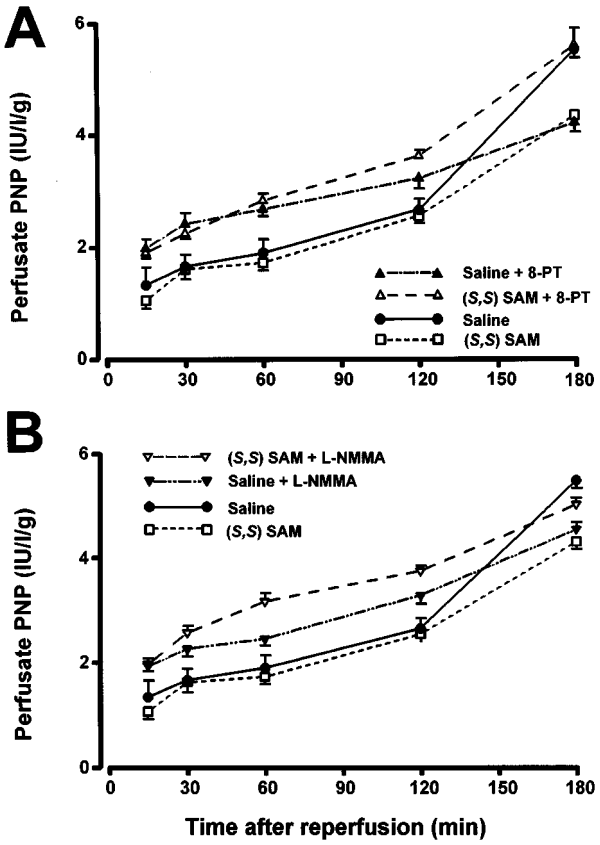

Figure 7 Influence of (A) purinergic receptor blockade and (B) inhibition of nitric oxide synthesis on baseline and (*S,S*) SAM-induced PNP release. The legend to Figure 2 applies. Significant Mann Whitney *U*-test results: (A) 15 min values–(*S,S*) SAM *versus* (*S,S*) SAM + 8-PT, $P<0.001$; area under the curve values (total over 3 h)–(*S,S*) SAM *versus* (*S,S*) SAM + 8-PT, $P=0.02$; (B) 15 min values–(*S,S*) SAM *versus* (*S,S*) SAM + L-NMMA, $P<0.001$; area under the curve values–(*S,S*) SAM *versus* (*S,S*) SAM + L-NMMA, $P<0.001$.

Discussion

S-Adenosylmethionine (100 μM) administered in a flush solution conferred significant protection against the haemodynamic and functional abnormalities induced in rat livers after sequential periods of cold and rewarming ischaemia. The flush, simulating that used to remove UW solution from transplanted human liver grafts just prior to reperfusion, remained within the liver for typical periods of 1–2 min yet provided benefit over the entire 3 h study period. Moreover, two diastereoisomers of SAM (the endogenous (*S,S*) and pharmaceutically-derived (*R,S*) products) showed distinct characteristics in improving blood flow and bile production, with equimolar amounts of the former more choleretic and of the latter more active haemodynamically.

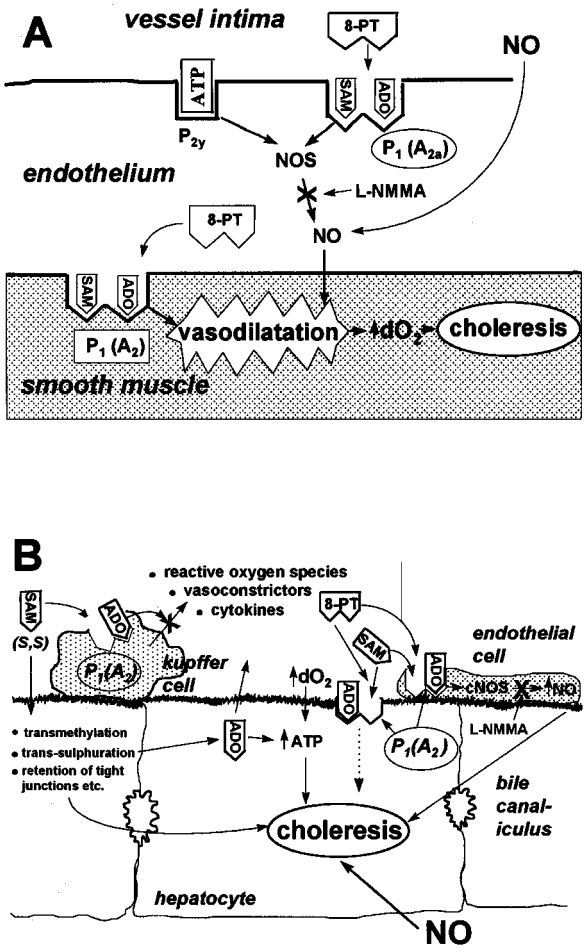

Figure 8 Putative actions of SAM on (A) hepatic blood flow and (B) bile production after ischaemia/reperfusion injury. The following summary applies but additional details may be found in the Discussion. (A) SAM, like adenosine (ADO), appears to act at P_1 (A_2) receptors on smooth muscle and endothelial cells (P_1 (A_{2a}) subtype), mediating effects on blood flow which were totally attenuated by the P_1 (A_2) receptor antagonist, 8-PT. In untreated livers there appeared to be minimal activation of such receptors with endogenous agonists because 8-PT was essentially without effect. L-NMMA, an inhibitor of NOS, partly attenuated liver blood flow, and by similar amounts in untreated and SAM-treated livers. This NO-dependent component of blood flow may originate *via* NOS-dependent P_1 (A_{2a}) or P_{2Y} purinoceptors, or exogenous NO production, and the specific interactions of SAM at these stages are unknown. (B) SAM appears to enhance bile production primarily through actions at P_1 (A_2) receptors since 8-PT almost totally attenuated its effects. The localization of the P_1 (A_2) receptors responsible was not investigated but may include sinusoidal endothelium or hepatocytes themselves. Further indirect actions *via* Kupffer cells, improved blood flow and oxygen delivery (dO_2) may contribute to its actions. Because bile production was enhanced more by (*S,S*) than by (*R,S*) SAM, this suggests additional contributions *via* SAM catabolism (including ADO production) from which (*R,S*) SAM is excluded. Residual bile production in untreated livers is primarily NO-dependent since it is almost totally inhibited by L-NMMA. In SAM-treated livers, L-NMMA inhibited bile production by quantitatively similar amounts, and actions involving the effects of SAM at P_1 (A_{2a}) receptors on endothelium (acting *via* constitutive NOS) cannot be excluded.

The observation that the improved tissue perfusion induced by the endogenous (*S,S*) SAM diastereoisomer was almost totally abolished by purinoreceptor blockade with 8-PT is highly suggestive of adenosine receptor involvement. The characteristics of purinoceptors in the rabbit hepatic vasculature have been defined using ranked order of agonist potencies (Mathie *et al*., 1991; Ralevic *et al*., 1991). Vasodilatation in the liver may be achieved either *via* stimulation of the A_2 sub-type of P_1 receptors on smooth muscle (Ralevic *et al*., 1991; Vials & Burnstock, 1993) or the P_{2Y} receptors on vascular endothelium (Browse *et al*., 1994), with a third possibility the stimulation of the A_{2a} subtype of A_2 receptors found on vascular endothelium. Only vasodilatation mediated at P_1 (A_2) receptors (including the A_{2a} subtype) is antagonized by 8-phenyltheophylline (Fredholm *et al*., 1994). In contrast, L-NMMA will inhibit effects mediated *via* both P_{2Y} and P_1 (A_{2a}) receptors since NOS is involved in the response (Vials & Burnstock, 1993; Browse *et al*., 1994). Because 8-PT specifically inhibited the improved tissue perfusion induced by SAM, whereas L-NMMA reduced blood flow by similar amounts in livers flushed with saline or SAM, we can conclude that SAM appears to increase portal venous blood flow *via* the A_2 sub-types of P_1 purinoceptors. The same data suggest that the action of SAM cannot be mediated exclusively at P_1 (A_{2a}) receptors (currently believed to be located on vascular endothelium but possibly also on sinusoidal epithelium as shown in Figure 8A). The vasodilatation of vascular smooth muscle induced by SAM may be achieved at resistance sites currently believed to be located post-sinusoidally in rat liver (Yang *et al*., 1995). However P_1 (A_2) receptors are also found on Kupffer cells and adenosine is known to inhibit their activation *in vitro* (Reinstein *et al*., 1994). Analogous actions by SAM would inhibit the release of cytokines and vasoconstictors from Kupffer cells activated by ischaemia/reperfusion injury (Lemasters & Thurman, 1991), so complementing the vasodilatation it induced directly. Neither Kupffer cell activity nor the localization of the receptors involved in the responses to SAM were investigated specifically in this study but their involvement in the putative mechanisms of action of SAM are shown in Figure 8B.

It is not clear whether the comparable reductions in blood flow induced by L-NMMA in the absence or presence of SAM were achieved *via* identical mechanisms, so multiple NO-dependent pathways for maintaining vasodilatation in untreated livers may be operative as shown in Figure 8. Our data are compatible with recent evidence for some dependence of hepatic microvascular blood flow on nitric oxide after hepatic warm ischaemia and reperfusion injury (Kobayashi *et al*., 1995; Koeppel *et al*., 1997), with possible sites of action the endothelial and/or stellate (Ito) cells (Moncada *et al*., 1991; Rockey *et al*., 1995). Injury to these cells, like that to the vascular endothelium and Kupffer cells, has been shown to increase progressively with the duration of ischaemia (Lemasters & Thurman, 1991; Ohno *et al*., 1994). In our study prolonged preservation and rewarming times were selected (Methods) to maximally compromise liver function and the potential benefits of SAM, but efficacy should remain over

shorter intervals through the same mechanisms of action. Although we did not evaluate the benefits of SAM after shorter episodes of ischaemia in this study, SAM reduced various indicators of liver injury in grafts preserved from 5.3 – 19.3 h in a clinical placebo controlled pilot study performed in liver graft recipients transplanted at our center, (Wong *et al*., 1998).

An unresolved issue is the identity of the agonist involved: whether this is SAM itself or breakdown products such as adenosine or methylthioadenosine produced in similar proportions (Engstrom & Benevenga, 1987) during the decarboxylation and transmethylation of SAM respectively (Friedel *et al*., 1989). To our knowledge, there is no record that SAM can act directly on A_2 purinoreceptors, but this cannot be discounted for several reasons. First is its rapid action; second, the high (100 μmol L^{-1}) concentrations used in flush solutions in this study at which weak agonist activity could be expressed; third, the specific blockade achieved with 8-PT and finally, the fact that adenosine is already released in excess from ischaemic liver (Arnold & Cysyk, 1986). Chemical analysis of the (*S,S*) SAM showed that $<0.01\%$ was present as adenosine (Analytical report, BASF Pharma), equating to a concentration of <10 nmol L^{-1} at which adenosine can exhibit significant effects (Fredholm *et al*., 1994). Nonetheless, the half-life of adenosine in blood is said to be in the order of one second (Moser *et al*., 1989), so sustained benefit might not be expected unless continuous catabolism of SAM occurred.

This is also feasible given that at least 50% of the flush solution was subsequently displaced into the perfusate and SAM had a half-life exceeding 90 min in our perfusion system (J.B. Dunne, J.M. Tredger, H. Thom, P. Giulidori, C. Di Padova, unpublished observation). Methylthioadenosine (MTA), which was present as 0.13% of (*S,S*) SAM (Analytical report, BASF Pharma), has been reported to demonstrate weak agonist properties at P_1 purinoceptors and is antagonized by 8-PT (Munshi *et al*., 1988; Nishida *et al*., 1985). An additional consideration must be the dissimilar activities of the (*S,S*) and (*R,S*) diastereoisomers of SAM, with equimolar amounts of the latter demonstrating greater haemodynamic activity. (*R,S*) SAM did not contain greater amounts of adenosine or MTA (content, $<0.01\%$ and 0.09%, respectively), so contaminants seem unlikely to be responsible. Degradation of SAM diastereoisomers in aqueous conditions involves enzymic and chemical reactions, with the latter producing MTA, adenine and the alternative racemer (i.e. (*S,S*) SAM from (*R,S*) SAM and *vice versa*). However, existing evidence suggests that (*R,S*) SAM is sterically excluded from the enzymic reactions which lead to products including MTA and adenosine (De La Haba *et al*., 1959; Hoffman, 1986). Together, these observations suggest that SAM itself may be the active purinergic agonist in our studies, although the relative affinities of (*R,S*) and (*S,S*) SAM for purinoceptors are currently unknown.

Bile flow was significantly reduced by both 8-PT and L-NMMA in control livers flushed with saline. This suggests that, as with blood flow, there are both NO-dependent and purinergic pathways maintaining residual bile production in livers reperfused after ischaemic injury. In the normal rat liver, two NO-dependent pathways influencing bile production are described. The first appears to reflect exogenous NO production, probably mediating increases in oxidized glutathione and bile-acid-independent bile flow (Trauner *et al*., 1997). The second appears to require neuronal NO, with neuronal nitric oxide synthase being abundant in nerve fibres densely surrounding the interlobular bile ducts (Esteban *et al*., 1997). It is not clear whether either mechanism would contribute significantly to bile production in an ischaemic perfused rat liver lacking innervation and an intact endothelium. Consequently, an additional NO-dependent mechanism may maintain bile production, counteracting the cholestatic effects of NO produced intracellularly and impairing canalicular motility through 5′-cyclic GMP-mediated disruption of inositol-1,4,5-triphosphate receptor / calcium channels, and the calcium signals that cause bile canalicular contraction (Dufour *et al*., 1995).

The responses of bile production to 8-PT and L-NMMA differed in livers treated with (*S,S*) SAM, where the increase in bile flow was almost completely abolished with 8-PT but only partly by L-NMMA. The latter suggests, firstly, that SAM supplements that component of bile flow in ischaemic livers dependent on purinergic stimulation (directly or through improved hepatic blood flow) and secondly, that NO might act as a second messenger for part of this response. The possible sites at which SAM might interact with purinoceptors in liver and affect bile production are shown in Figure 8B. Their precise localization and involvement in bile production was beyond the remit of the present study and remains to be elucidated. It is believed that bile flow depends on the perfusion fraction of the liver but additional considerations must apply in our model of sequential cold and warm ischaemia for two reasons. The first is that a 7% reduction in blood flow in control livers treated with L-NMMA was associated with a corresponding 71% reduction in bile production and, the second that proportionality to blood flow was also not maintained using the two diastereoisomers of SAM, with the (*R,S*) isomer improving blood flow more than equimolar amounts of the (*S,S*) isomer, but the opposite true for bile production. Additional factors which may have facilitated bile production over the 3 h perfusion interval may include tissue oxygenation and its metabolic sequelae as well as actions of SAM affecting bile formation. Thus, oxygen extraction ratio values were higher for the (*S,S*) isomer than its (*R,S*) racemer over the 3 h perfusion period as a result of lower oxygen consumption and increased oxygen delivery and this may contribute to its greater choleretic activity *via* enhanced ATP production (Sumimoto *et al*., 1988). An additional consideration may be the participation of administered (*S,S*) SAM in transmethylation and trans-sulphuration reactions excluded to its (*R,S*) racemer by steric considerations (De La Haba *et al*., 1959; Hoffman, 1986) and of known benefit to bile production (Friedel *et al*., 1989). These might also include acute interactions such as those documented by Roman *et al*., (1996) where SAM was thought to maintain tight junction integrity in rat hepatocyte couplets by effects on the pericanalicular cytoskeleton. These possible mechanisms are summarized in Figure 8B.

In conclusion, NO production appears to play a greater part in maintaining liver blood flow and bile production in ischaemic livers than does vasodilatation mediated at A_2 purinoceptors. SAM improves blood flow in ischaemic livers when incorporated into a short pre-reperfusion flush and this appears to be regulated through actions at A_2 purinoceptors. It remains to be established whether this is mediated by the compound itself or *via* its metabolites. The haemodynamic benefits of SAM translate into improvements in bile production which are disproportionate to blood flow. Independent benefits of SAM on bile production, related particularly to the endogenous (*S,S*) diastereoisomer, appear important in choleresis.

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