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Effect of S-Adenosylmethionine on Hepatic Injury from Sequential Cold and Warm Ischemia

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We investigated whether S-adenosylmethionine (SAM) treatment improved ischemic injury using perfused rat liver after sequential periods of 24 h cold and 20 min re-warming ischemia. SAM (100 μmol/L) was added to University of Wisconsin (UW) solution and Ringers lactate solution. After cold and sequential warm ischemia, releases of lactate dehydrogenase (LDH) and purine nucleoside phosphorylase (PNP) markedly increased during reperfusion. The increase in PNP was significantly reduced by SAM treatment. While the concentration of reduced glutathione (GSH) in ischemic livers significantly decreased, the concentration of glutathione disulfide (GSSG) increased. This decrease in GSH and increase in GSSG were suppressed by SAM treatment. Lipid peroxidation was elevated in cold and warm ischemic and reperfused livers, but this elevation was also prevented by SAM treatment. Hepatic ATP levels were decreased in the ischemic and reperfused livers to 42% of the control levels. However, treatment with SAM resulted in significantly higher ATP levels and preserved the concentration of AMP in ischemic livers. Our findings suggest that SAM prevents oxidative stress and lipid peroxidation and helps preserve hepatic energy metabolism.

Key words: S-Adenosylmethionine, UW solution, Total purine bases, Lipid peroxidation, Oxidative stress, Ischemia

INTRODUCTION

Ischemic injury to the liver is an unavoidable process during liver transplantation. Cold ischemia occurs during storage of the organ and warm ischemia while the anastomoses are made in the recipient, prior to flushing and reperfusion. Endothelial cell damage (Caldwell-Kenkel *et al.*, 1991) is an important feature of cold ischemia, while hepatocyte injury predominates during warm ischemia (Ikeda *et al.*, 1992).

Because of their high metabolic rate, hepatic cells are vulnerable to the deleterious influence of anoxia, however, the cause of cell death is not yet clear. A number of studies have suggested that ischemic cell death is a consequence of irreversible mitochondrial injury (Clemens *et al.*, 1983). Characteristic features of ischemic liver injury include a depletion of hepatic ATP and ADP (Harvey *et al.*, 1988), the rate of recovery from which is inversely correlated to the ischemic time (Kamiike *et al.*, 1988), impairment of mitochondrial respiratory function (Kim *et al.*, 1992), acidosis from glycolysis (Woods and Krebs, 1971), and the formation of reactive oxygen intermediates (Harvey *et al.*, 1988). However, the mechanisms of this association have not been fully clarified. The incidence of primary graft nonfunction (Furukawa *et al.*, 1991) and graft rejection (Howard *et al.*, 1990) have been related to the extent of these injuries, and cold ischemic times over twelve hours have been associated with an increased risk of graft loss (Adam *et al.*, 1992).

S-Adenosylmethionine (SAM) is an endogenous methyl group donor (Friedel *et al.*, 1989), and a reduced glutathione (GSH) and ATP precursor (Montero *et al.*, 1990) that reduces cholestasis. Recently Dunne *et al.* (1994) have shown that SAM produces sustained improvements in blood flow, oxygen delivery, bile production, and glucose homeostasis in a sequential cold and warm ischemia model that simulated the transplant procedure.

The aim of the present study was to examine whether treatment with SAM could decrease sequential cold and warm ischemic injury in rat liver. In particular, we investigated the effects of SAM on the mitochondrial energy metabolism.

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MATERIALS AND METHODS

Hepatectomy and perfusion

Male Sprague-Dawley rats, 250-300 g, were fasted for 18 h before the experiment, but allowed to drink tap water *ad libitum*. They were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) and systemically heparinized (400 IU/kg) via the penile vein. A midline incision was made to the abdomen, and the portal vein was cannulated with PE-190 and flushed with Krebs-Henseleit bicarbonate buffer (KHBB) containing (in mmol/L) NaCl, 118; KCl, 4.6; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose 5 (pH 7.4, 37°C). The flow rate was 3 ml/min/g liver and the perfusate was saturated with a 95% O_2-5% CO_2 gas mixture. After flushing had begun, the inferior vena cava was ligated above the right renal vein and cut distally. During flushing, the liver was dissected free from the rat and moved to the perfusion apparatus. The liver was flushed with 30 ml of Ringer's lactate solution (4°C) and stored in University of Wisconsin solution (UW, Du Pont Pharmaceuticals, DE, U.S.A.) for 24 h at 4°C. Livers were then immersed in oxygenated Ringers lactate solution at 37°C for 20 min. After sequential cold and warm ischemia, UW was flushed from liver with 10 ml of Ringer's lactate solution at 37°C and the liver was reperfused with KHBB for 60 min. Livers were perfused at a rate of ~4 ml/min/g liver essentially as previously described (Lee and Clemens, 1992). Samples of perfusate were taken after 0, 10, 20, 30, 40, 50 and 60 min of reperfusion. After 0 min and 60 min of reperfusion the median lobe of the liver was removed and then frozen in liquid nitrogen for purine nucleotide assay. The left lobe of the liver was removed and used for lipid peroxidation and glutathione assays.

Experimental protocol

Control livers: - The livers were removed from the animals as described above, flushed with oxygenated Ringer's lactate solution at 37°C, connected to the perfusion apparatus and perfused for 60 min. *Sequential cold and warm ischemic livers*: - The livers were treated as for cold ischemia, immersed for 20 min and reperfused for 60 min. *Sequential cold and warm ischemic livers treated with SAM*: - The livers were treated as for sequential cold and warm ischemia, by pretreatment with 100 μmol/L SAM (Sigma Chemical CO., U.S.A.) in UW solution and 100 μmol/L SAM in oxygenated Ringer's lactate solution.

Analysis of ATP and its catabolites

The median lobe of the liver was freeze-dried using liquid nitrogen, and the tissue then minced to a powder and extracted with 1.5 M perchloric acid. After centrifugation, neutralization, and final centrifugation, the extracts were kept in an ice bath until analysis. Hepatic concentrations of ATP, ADP, AMP, adenosine, inosine, hypoxanthine and xanthine in the extracts were measured using a Gilson HPLC unit. The system consisted of, two high performance pumps (model 306), a buffer mixer (model 811C), and a motor valve (Rheodyne 77251), for loading and injecting the samples. Adenine nucleotides and their catabolites were separated on Superspher® RP-18 (Merck, Darmstadt, F.R.G.), equipped with an UV detector (model 118). The column was then eluted at a flow rate of 0.7 ml/min with HPLC buffer I [2% H_3PO_4 (v/v) adjusted pH 4.0 with 25% NaOH] into which a linear gradient of buffer II (equal volumes of acetonitrile and methanol) was incorporated, to achieve 15% of the elutant in 15 min (Maessen *et al.*, 1988). During the next 5 min the mobile phase was linearly restored to 100% buffer I. ATP and its metabolite concentrations were calculated from the computer-integrated areas of the peaks in the chromatogram in relation to areas obtained for standard solutions. Based on the ATP, ADP and AMP concentrations energy charge (EC) was calculated by : - EC =(ATP+0.5 ADP)/(ATP+ADP+AMP).

Analytical procedures

Lactate dehydrogenase (LDH) activity, a marker of hepatocyte damage, was determined spectrophotometrically using Sigma kit # 228-50 (Sigma Chemical Co.). Purine nucleoside phosphorylase (PNP) activity, a marker of endothelial cells, was measured according to the method previously described by Hoffee *et al.* (1978). Total free glutathione and reduced glutathione (GSH) were determined in the acid extract using a previously published method (Brehe and Bruch, 1978). Glutathione disulfide (GSSG) was estimated by deducting GSH from the total glutathione concentration. The extent of lipid peroxidation in the liver was determined by measuring malondialdehyde (MDA), an end product of lipid peroxidation. The MDA contents in homogenates prepared as described by Masugi and Nakamura (1976) were measured using thiobarbituric acid. Protein was estimated using the Bradford (1976) dye binding method. Bovine serum gamma globulin was used as a standard.

Statistical analysis

Significant changes in various measured parameters were determined using the one-way ANOVA method. Differences between experimental groups were considered significant at $p<0.05$, and the appropriate Student-Newman-Keuls test was used for multiple comparisons. All results are presented as means ± S.E.M.

RESULTS

LDH and PNP

Release of LDH was negligible (approximately 20 ± 4 U/

L) over the whole test period in the control. After cold and sequential warm ischemia, LDH release in the ischemic livers was not different to that of the pre-ischemic livers. During reperfusion, however, LDH release from the perfused livers significantly increased to 1250 ± 160 U/L at 30 min. Treatment with SAM did not affect this increase (Fig. 1). In control livers, the level of PNP in the perfusate remained constant at approximately 50 ± 3 U/L throughout the experiment. Release of PNP after cold and warm ischemia was significantly higher than that of the control and there was an additional increase during reperfusion. The increase in PNP release was suppressed by SAM treatment (Fig. 2).

Glutathione and lipid peroxidation

The hepatic concentrations of GSH and GSSG in the control were 39.2 ± 3.6 nmol/mg protein and 3.5 ± 0.1 nmol/mg protein, respectively. After cold and sequential warm ischemia, no changes were observed in GSH levels in ischemic livers compared with pre-ischemic livers. After reperfusion, however, the concentration of GSH significantly decreased to 10.1 ± 0.9 nmol/mg protein. This decrease was prevented by SAM treatment. In contrast to what was observed for GSH, the concentration of GSSG during ischemia increased and then increased further after reperfusion. This increase was suppressed by SAM treatment. In the control, the level of MDA in the liver remained constant at approximately 0.65 nmol MDA formed/mg protein. In ischemic livers, on the other hand, the level of MDA started to increase after cold and warm ischemia and markedly increased to 2.4 times pre-ischemic values after reperfusion. SAM treatment prevented elevations in hepatic MDA (Table I).

Energy metabolism

Changes in adenine nucleotides and purine catabolites, which reflect energy metabolism in the hepatic tissue, during ischemia and after reperfusion, are shown in Table II. The value of cellular ATP in control livers was 6.42 ±

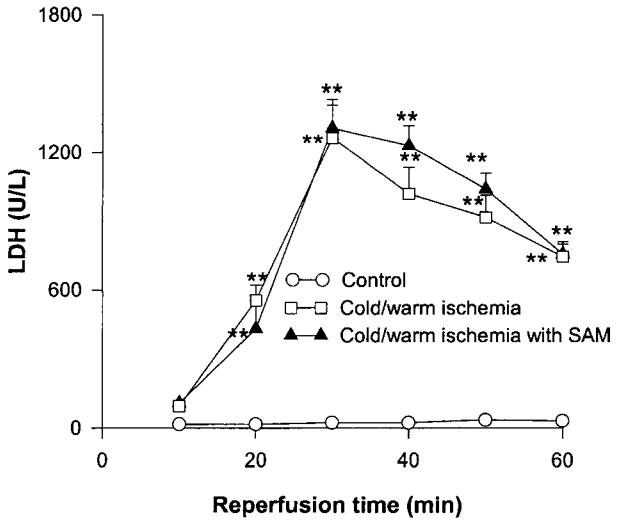

Fig. 1. Effect of SAM on the release of LDH in perfusate during reperfusion after cold and warm ischemia. Values are mean ± S.E.M. for 8 to 9 rats per group. **=Significantly different (p<0.01) from control.

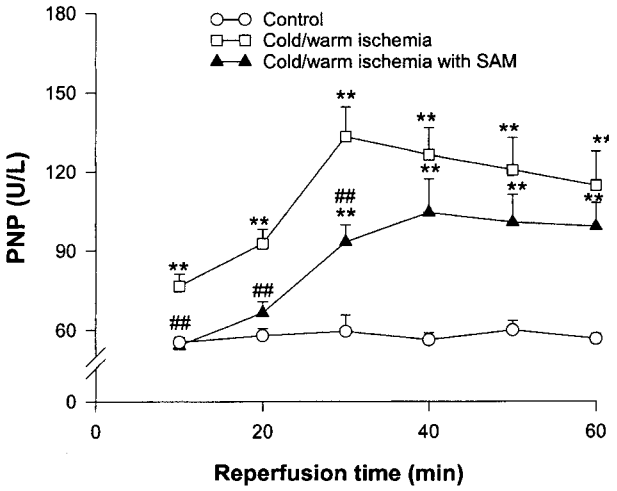

Fig. 2. Effect of SAM on the release of PNP in perfusate during reperfusion after cold and warm ischemia. Values are mean ± S.E.M. for 8 to 9 rats per group. **=Significantly different (p<0.01) from control. ##=Significantly different (p<0.01) from cold and warm ischemia.

Table I. Effect of SAM on hepatic concentrations of GSH, GSSG and lipid peroxidation after cold and warm ischemia and reperfusion

	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	MDA (nmol/mg protein)
Control	39.2 ± 3.6	3.5 ± 0.1	0.65 ± 0.04
Cold/warm IS	30.6 ± 2.7	4.7 ± 0.3**	0.79 ± 0.04*
Cold/warm IS+RP	10.1 ± 0.9** ++	9.7 ± 0.3** ++	1.56 ± 0.09** ++
Cold/warm IS+RP+SAM	16.2 ± 0.8** ++ ##	6.2 ± 0.4** + ##	1.11 ± 0.10** + ##

Values are means ± S.E.M for 5 to 8 rats per group.
*, **=Significantly different (p<0.05, p<0.01) from control.
+, ++=Significantly different (p<0.05, p<0.01) from cold/warm IS.
##=Significantly different (p<0.01) from cold/warm IS+RP.
IS, ischemia; RP, reperfusion.

Table II. Effect of SAM on adenine nucleotide metabolism in liver tissue after cold and warm ischemia and reperfusion

	Control	IS	IS/RP	IS/RP+SAM
ATP	6.42 ± 0.46	1.08 ± 0.03**	2.71 ± 0.09** ++	4.62 ± 0.18** ++ ##
ADP	2.37 ± 0.31	0.82 ± 0.06**	0.90 ± 0.06**	0.88 ± 0.04**
AMP	3.69 ± 0.36	4.14 ± 0.33	2.00 ± 0.12** ++	3.44 ± 0.19##
Adenosine	1.53 ± 0.09	0.79 ± 0.11**	1.20 ± 0.16	0.62 ± 0.07** ##
Inosine	1.73 ± 0.37	2.17 ± 0.25	1.37 ± 0.12** +	0.82 ± 0.05* ++ ##
Hypoxanthine	0.71 ± 0.10	3.20 ± 0.21**	0.88 ± 0.08++	0.87 ± 0.07++
Xanthine	0.80 ± 0.11	0.73 ± 0.04	0.61 ± 0.02+	0.66 ± 0.04

Values are means ± S.E.M for 5 to 7 rats per group.
Units are μmol/g liver.
*, **=Significantly different ($p<0.05$, $p<0.01$) from control.
+, ++=Significantly different ($p<0.05$, $p<0.01$) from ischemic group.
##=Significantly different ($p<0.01$) from cold/warm IS+RP.
IS, ischemia; RP, reperfusion.

0.46 μmol/g liver weight. The level of ATP in the liver decreased rapidly after cold and warm ischemia, while the level of ADP decreased gradually. Following reperfusion in ischemic livers, hepatic ATP levels were restored but were found to be approximately 42% of the control levels. ADP levels remained at 38% of the control levels and the AMP levels remained lower than the values observed in control livers. The corresponding hepatic ATP levels of livers pretreated with SAM after reperfusion were found to be 72% of control levels. Hepatic AMP levels were restored to control levels. The hepatic energy charge was significantly reduced by cold and warm ischemia but restored to the level of the control liver after reperfusion (Fig. 3). Poor recovery of the energy charge was only observed in the SAM-treated group. During the cold and warm ischemic period, the level of hypoxanthine markedly increased to 4.5 times the control values. However, after reperfusion, concentrations of hypoxanthine were decreased. In the SAM-treated group, the total concentrations of purine catabolites were lower than those in the control livers. In particular, the levels of adenosine and inosine significantly decreased in the SAM-treated livers.

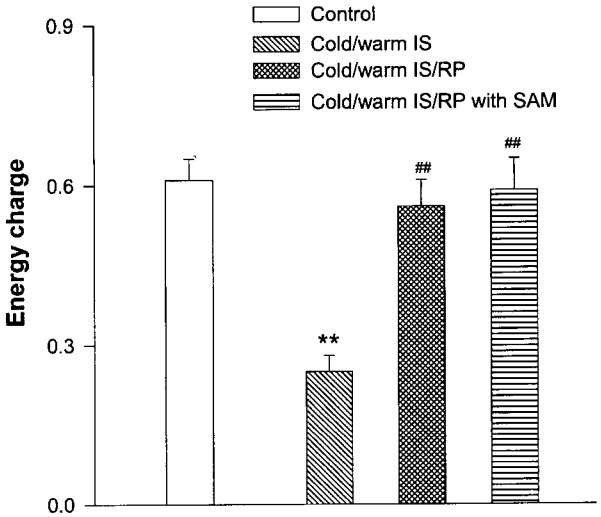

Fig. 3. Effect of SAM on energy charge in liver tissue after cold and warm ischemia and reperfusion. Values are mean ± S.E.M. for 5 to 7 rats per group. **=Significantly different ($p<0.01$) from control. ##=Significantly different ($p<0.01$) from cold and warm ischemia. IS, ischemia; RP, reperfusion.

DISCUSSION

We have demonstrated that the exposure of cold-preserved livers to a subsequent period of warm ischemia and reperfusion results in a marked deterioration of liver function. SAM limited hemodynamic and metabolic features of this ischemic damage to the donor livers, when included in the preservation solution, and added to Ringers lactate solution.

The data on PNP release suggest that the action of SAM may be related to maintaining the integrity of endothelial cells that are damaged during cold and warm ischemia and reperfusion. Although there is some controversy about the use of PNP as an indicator of endothelial injury (Brass and Mody, 1995), the enzyme is believed to originate primarily from damaged endothelial cells (Rao *et al.*, 1990), but it is also released from damaged hepatocytes (Brass and Mody, 1995). The greater metabolic activity in damaged hepatocytes or the shedding of cytosolic components in blebs, formed during ischemia and reperfusion (Lemasters and Thurman, 1991), may account for the increased perfusate level of LDH. Our results show that the concentrations of LDH in perfusate increased after 20 min of reperfusion and peaked at 30 min. No hepatoprotective effects versus cold and warm ischemia and reperfusion injury could be attributed to SAM.

Accumulating evidence indicates that oxgen-derived free radicals play a major role in producing the microvascular and parenchymal cell damage associated with reperfusion of ischemic tissues (Drugas *et al.*, 1991). Glutathione (GSH) plays an important role as a free radical scavenger. Previous studies have shown that GSH is depleted from cells exposed to warm ischemia and GSH-depleted cells are more sensitive

to a loss of viability upon re-oxygenation (Vreugdenhil *et al.*, 1990). In cold and warm ischemic livers, the concentration of hepatic GSH remained unchanged during ischemia, but significantly decreases after reperfusion. In contrast, the concentration of GSSG increases after ischemia and further increases after reperfusion. This decrease in GSH and increase in GSSG were significantly prevented by SAM treatment. SAM increases intracellular GSH concentrations (Ponsoda *et al.*, 1991), which are known to maintain the activity of its sulfhydryl groups (Corrales *et al.*, 1992). Donor treatment with SAM may also increase GSH by stimulating cystathionine β-synthetases (Finkelstein *et al.*, 1975). These two effects may counteract the consequences of increased tissue oxygenation after reperfusion, which is capable of impairing liver function by the excessive production of oxygen free radicals and the induction of oxidative stress.

Free radical attack on biological membranes, such as the plasma membrane, and the mitochondria and the endoplasmic reticulum, can lead to the oxidative destruction of membranes polyunsaturated fatty acids by lipid peroxidation. This may cause alterations in biomembrane-associated functions and structure, and may disrupt the function of the cell or of subcellular organelles. In ischemic livers, the level of MDA started to increase after cold and warm ischemia and markedly increased after reperfusion. Treatment with S-adenosylmethionine significantly prevented lipid peroxidation. The protective effect of SAM may be attributable to its scavenging of reactive oxygen species (Dunne *et al.*, 1997), and to its ability to sustain GSH production.

Changes in cellular levels of adenine nucleotides and their metabolites have been analyzed in ischemic tissues by many researchers. However, these studies have usually been confined to specific metabolites. In the present study, ATP levels and the total content of adenine nucleotides in ischemic livers were very low. However, a marked increase in the hypoxanthine level was observed at the end of the ischemic period. Purine metabolite accumulated in a rat liver during cold and warm ischemia decreased after reperfusion. Under physiologic conditions, AMP is rapidly converted to ATP within both the cytosolic and mitochondrial compartments. The ATP and AMP concentrations in the SAM-treated livers increased. The explanation for these findings may be that SAM is involved in the regeneration of ATP.

Energy charge (EC) is a parameter that represents the cellular energy status on the basis of adenine nucleotide concentrations (Atkinson, 1977). In the present study, EC was reduced after cold and warm ischemia but was restored to the level of control livers after reperfusion. There was no significant difference between the control livers and the SAM-treated livers. Thus there is a discrepancy between the time course of change in ATP and EC levels after ischemia and reperfusion. It is believed that this discrepancy is attributable to the decrease in total adenine nucleotides after cold and warm ischemia and reperfusion.

The total content of purine bases remained unchanged after ischemia, but decreased after the period of reperfusion. Recently, Kobayashi *et al.* (1991) showed that once the cellular membranes are damaged by free radical attack, they become leaky to the constituents of adenine nucleotides. The adenine nucleotides are converted to nucleosides in the interstitial spaces, and taken up by the endothelial cells (Rounds *et al.*, 1994), where they are catabolized to hypoxanthine, since the sinusoidal endothelial cells are abundant in xanthine oxidase (Brunder *et al.*, 1982). They may also be more vulnerable to oxygen radicals generated by the xanthine oxidase when the blood supply is restored. Treatment with SAM in this study slowed the degradation of ATP and restored the level of the purine bases.

We have demonstrated that treatment with SAM has the effect of reducing hepatocellular damage caused by cold and warm ischemia and reperfusion. Our findings suggest that SAM prevents oxidative stress and lipid peroxidation and preserves hepatic energy metabolism.

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