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Protective Effect of S-Adenosylmethionine against Galactosamine-Induced Injury of Rat Hepatocytes in Primary Culture

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Summary

The protective effect of S-adenosylmethionine (SAMe) on D-galactosamine (GalN)-induced damage to rat hepatocytes was tested in primary cultures. SAMe at concentrations of 50 and 1000 mg/l significantly reduced lactate dehydrogenase release from cells injured by 40 mM GalN after 24 h of incubation. There were no significant changes in urea production after 24 h among tested groups, including control hepatocytes. Exposure of hepatocytes to GalN leads to 3.5-fold decrease in urea synthesis after 48 h in comparison with control cell cultures. Addition of the highest dose of SAMe (1000 mg/l) into the culture media attenuated this decrease by 180 %. None of the tested doses of SAMe (5, 25, 50 and 1000 mg/l) affected considerably the reduced activity of mitochondrial dehydrogenases. The content of reduced and oxidized glutathione in GalN-exposed cells was diminished to 1.5 % and 16 %, respectively, of the control values after 24 h. Using only the highest concentration SAMe increased significantly these contents. SAMe had no effect on dramatically decreased albumin synthesis. These findings indicate beneficial effect of SAMe, especially of the highest concentration, on GalN-induced toxicity to rat hepatocytes in primary culture. This action of SAMe seems to be associated with reduction of plasma membrane damage and increased synthesis of glutathione.

Key words

S-adenosylmethionine • Galactosamine • Hepatocytes *in vitro*

Introduction

D-galactosamine (GalN) is a highly selective hepatotoxin frequently used in animal experiments. *In vivo* GalN causes diffuse liver damage resembling viral hepatitis (Keppler *et al.* 1970). This toxin brings about the inhibition of RNA and protein synthesis *via* depletion of uridine nucleotides and accumulation of UDP-hexoseamines in hepatocytes (Keppler *et al.* 1970). In the rat, activation of Kupffer cells by endotoxin participates strongly on GalN toxicity (Kasravi *et al.* 1995, Stachlewitz *et al.* 1999). GalN itself increases sensitivity

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of the liver to TNF-α, which contributes to the injury (Leist *et al.* 1997). Although oxidative stress is probably not the primary cause of hepatocyte damage induced by GalN, increased production of reactive oxygen species has been reported *in vivo* (Yoshikawa *et al.* 1982) and *in vitro* (Quintero *et al.* 2002). It seems that GalN-induced depletion of hepatocyte glutathione is caused by inhibition of glutathione synthesis due to decreased activity of the enzymes of glutathione synthesis or increased activity of the enzymes involved in glutathione breakdown (McMillan and Jollow 1992).

S-adenosylmethionine (SAMe) participates in many biological reactions. SAMe is the most important methyl donor, a fundamental intermediate of the transsulphuration pathway, and takes part in polyamine biosynthesis (Friedel *et al.* 1989). SAMe is, through its transmethylation reactions, involved in synthesis and metabolism of many important compounds, such as nucleic acids, phospholipids, methyl-accepting proteins, hormones and neurotransmitters. In intracellular transsulphuration pathway, SAMe represents an important precursor of cysteine, taurine and glutathione (GSH), the major intracellular antioxidant. Endogenous SAMe is formed from methionine and ATP by the action of methionine adenosyltransferase. GalN inhibits this enzyme and causes depletion of GSH (Ozturk *et al.* 1986). Administration of SAMe was documented to increase GSH levels in carbon tetrachloride-treated rats (Corrales *et al.* 1992) and in hepatocytes cultured with GalN (Wu *et al.* 1996). Recently, SAMe was reported to regulate some liver functions (Mato *et al.* 2002).

The aim of our study was to evaluate potential protective effect of SAMe on GalN-induced hepatocyte injury in primary cultures. We particularly focused on estimation of functional parameters of hepatocytes in an effort to reveal possible mechanisms of SAMe action.

Methods

Chemicals

Medium William´s E (without phenol red), fetal bovine serum, penicillin, streptomycin and glutamine were purchased from PAN BIOTECH GmbH (Germany); type I collagen, trypan blue, kit for lactate dehydrogenase, Urea Nitrogen kit, D-galactosamine, metaphosphoric acid, reduced glutathione, N-ethyl-maleimide, o-phthaldialdehyde, sodium phosphate (dibasic, anhydrous), sodium hydroxide, ethylene-diaminetetraacetic acid from Sigma-Aldrich (USA). Collagenase cruda was obtained from SEVAC (Czech Republic). Insulin (Actrapid, Hoechst, Germany), glucagon (Novo Nordisk, Denmark), prednisolon (Solu-Decortin, Merck, USA), MitoCapture Apoptosis Detection Kit (BioCat GmbH, Germany), Rat Albumin ELISA Quantification Kit (Bethyl Lab. Inc., USA) and Cell Proliferation Reagent WST-1 (Roche, Switzerland) were from the supplier mentioned in brackets.

Animals

Male albino Wistar rats (Biotest, Czech Republic) were housed at 23±1 °C, 55±10 % relative humidity, air exchange 12-14 times/h, and 12-hour light-dark cycle periods (6:00 h to 18:00 h). The animals had free access to standard laboratory rat chow (DOS 2B, Velaz, Czech Republic) and tap water. All animals received care according to the guidelines set by the institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

Hepatocyte isolation, culture and treatment

Hepatocytes were isolated from rats mentioned above with a body mass of 220-240 g by collagenase perfusion (Berry *et al.* 1991). The viability of freshly isolated hepatocytes was more than 90 % as confirmed by trypan blue exclusion. Isolated hepatocytes were suspended in William's E medium supplemented with fetal bovine serum (10 %), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (10 mg/ml), insulin (0.08 IU/ml), prednisolon (0.5 μg/ml), glucagon (0.008 μg/ml) and plated in collagen-coated Petri dishes (60 mm) at a density of $2x10^6$ cells/Petri dish. Hepatocytes were allowed to attach in a gassed atmosphere (5 % CO_2) at 37 °C for 2 h.

After the establishment of monolayers, the medium was removed and replaced with fresh medium containing 40 mM GalN and S-adenosylmethionine (SAMe) at concentrations of 5, 25, 50 or 1000 mg/l (these concentrations correspond to 12.5, 62.7, 125.5 μM or 2.5 mM). Then the hepatocytes were incubated for 24 h. After this period the medium was collected for biochemical assays and fresh medium with SAMe (without GalN) was added for further 24 h.

Biochemical assays

Hepatocyte toxicity was determined by lactate dehydrogenase activity (LDH) in the culture medium using a commercial kit from Sigma-Aldrich. Urea production, an important functional marker of

hepatocytes, was evaluated by a kit provided by Sigma-Aldrich.

Antioxidative conditions of hepatocytes were determined by the measurement of glutathione content. Reduced and oxidized form of glutathione were analyzed by reverse-phase high-performance liquid chromatography (Shimadzu, Japan) using slightly modified method of Hissin and Hilf (1976). The reverse-phase column Discovery C18, 15 cm x 4 mm, 5 μm (Supelco, USA) served for separation. Fluorometric detection (excitation wavelength 350 nm, emission wavelength 420 nm) followed a postcolumn derivatization reaction with o-phthalaldehyde. The CSW32 program (DataApex, Czech Republic) was used for collecting and processing of the chromatographic data.

The toxic effect of GalN was evaluated by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Primary culture of hepatocytes at a density of 3×10^4 cells/well (collagen-coated) was incubated with 40 mM GalN and SAMe at concentrations of 50 resp. 1000 mg/l for 24 h. Then all medium was removed, reagent WST-1 was added (1:20 final dilution) and the cells were incubated in a gassed atmosphere (5 % CO_2) at 37 °C for 120 min. The absorbance of the samples was measured using a microtiter plate reader Multiscan (USA) at dual wavelength mode of 450 and 690 nm respectively.

To evaluate functional capacity of cultured hepatocytes the amount of albumin secreted into the culture medium during incubation period was measured using the commercial ELISA kit from Bethyl Lab. Inc., USA.

Light microscopy

The morphological changes of hepatocytes in culture were examined using inverted microscope with phase contrast Olympus CK 40 (Japan). Microphotographs were obtained by digital camera Olympus Camedia C 4040.

Detection of mitochondrial membrane potential

Mitochondrial membrane potential was determined using hepatocyte uptake of MitoCapture. In intact cells, MitoCapture accumulates and aggregates in the mitochondria, giving them a bright red fluorescence (Em_{max} 590 nm). In cells with the altered mitochondrial membrane potential, MitoCapture cannot aggregate in the mitochondria and thus it remains in the cytoplasm in its monomer form, fluorescing green (Em_{max} 530 nm). Hepatocytes were incubated with MitoCapture according to the protocol of the apoptosis detection kit, then the cells were washed twice with fresh media and MMP was visualized by epifluorescence microscope Nicon Eclipse E-400 (Japan) equipped with the digital color matrix camera COOL1300 (VDS, Germany). Photographs were taken by Image Analysis System LIM LUCIA DI (Laboratory Imaging Ltd., Czech Republic) and analyzed. Results are expressed as percentage of cells containing mitochondria with low membrane potential.

Statistical analysis

Due to normal variation among different primary culture preparations, the results of a typical experiment were presented. Experiments were repeated three times using different hepatocyte preparations. Similar trends were observed in each experiment. All values are expressed as means ± SD. The statistical significance was analyzed using one-way ANOVA test, Tukey-Kramer's post-hoc test was used for multiple comparisons between groups (GraphPad InStat 3.06 for Windows, GraphPad Software, USA). Statistical significance was set at $p<0.05$.

Results

GalN at the concentration of 40 mmol/l caused severe injury to rat hepatocytes in primary culture after 24 h of incubation. As is shown in Figures 1 and 2, GalN induced dramatic increase in LDH activity in the culture medium after 24 and 48 h and rapid fall in activities of mitochondrial dehydrogenases after 24 h. Urea genesis was not affected by GalN during the first 24 h, but more than a 3.5-fold decrease in comparison with controls was observed at 48 h (Fig. 3). In contrast, albumin production was reduced to 11 % of control cell in hepatocytes treated with GalN at 24 h (Fig. 4) and almost blocked at 48 h (1.1 %). Figures 5 and 6 show a rapid decrease in the reduced and oxidized (GSSG) form of glutathione in GalN-injured hepatocytes after 24 h. There was more than a 10-fold decrease in GSH/GSSG ratio in affected cells compared to controls (calculated from the data shown in Figs 5 and 6). Mitochondrial membrane potential, measured using fluorescent probe MitoCapture, was fully abolished by GalN in comparison with the controls, where only 10 % of cells contained lowly polarized mitochondria (Fig. 7).

Significant reduction of LDH release caused by SAMe was observed in hepatocyte cultures incubated

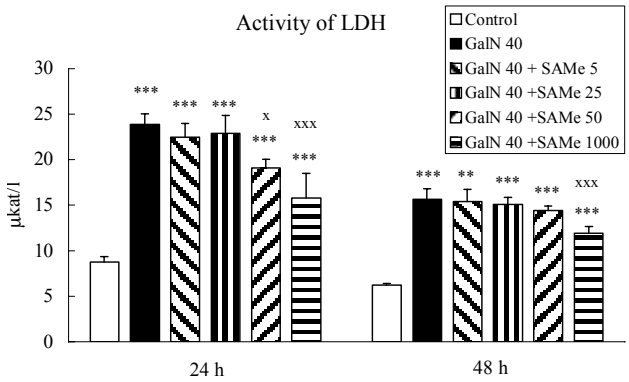

Fig. 1. Effect of SAMe (5, 25, 50 and 1000 mg/l) on release of LDH from hepatocytes injured by 40 mM GalN after 24 and 48 h. Data are means ± S.D. (n=6); *** p<0.001 compared with controls; [x] p<0.05, [xxx] p<0.001 compared with 40 mM GalN (in appropriate time periods).

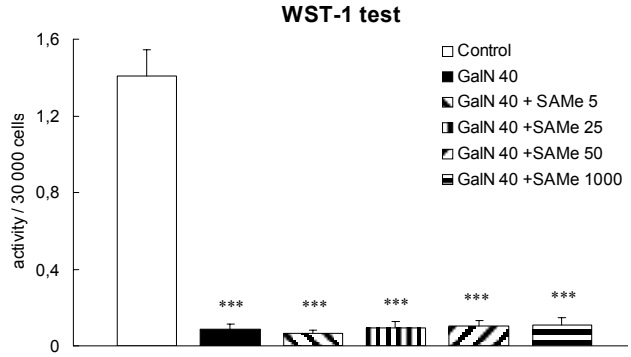

Fig. 2. Effect of SAMe (5, 25, 50 and 1000 mg/l) on activities of mitochondrial dehydrogenases of hepatocytes injured by 40 mM GalN after 24 h. Data are means ± S.D. (n=8). *** p<0.001 compared with controls.

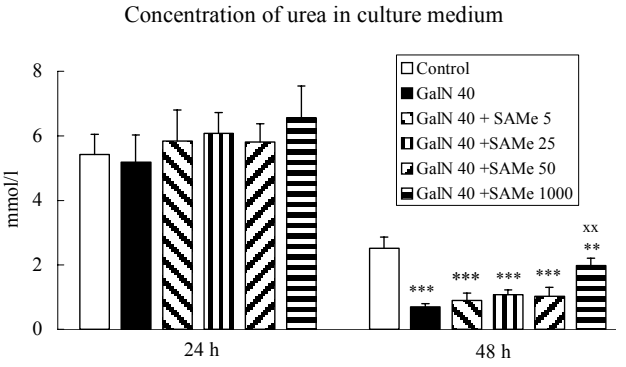

Fig. 3. Effect of SAMe (5, 25, 50 and 1000 mg/l) on urea production by GalN-injured hepatocytes after 24 and 48 h. Data are means ± S.D. (n=6). ** p<0.01, *** p<0.001 compared with controls after 48 h; [xx] p<0.01 compared with 40 mM GalN after 48 h.

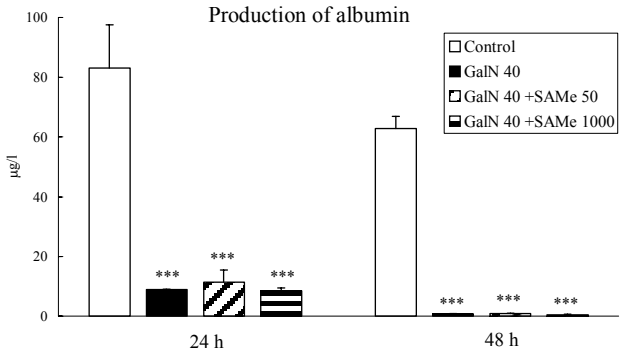

Fig. 4. Effect of SAMe (50 and 1000 mg/l) on albumin synthesis by GalN-injured hepatocytes after 24 and 48 h. Data are means ± S.D. (n=3). *** p<0.001 compared with controls (in appropriate time periods).

with SAMe at doses of 50 (p<0.05) and 1000 (p<0.001) mg/l for the first 24 h and with the highest dose of SAMe (1000 mg/l) for 48 h (Fig. 1). GalN decreased activities of mitochondrial dehydrogenases which were not improved by SAMe at any concentration that we tested (Fig. 2). SAMe also did not affect functional capacity of hepatocytes in the first 24 h of incubation as evaluated by urea synthesis and albumin production (Figs 3 and 4). Concentration of urea in culture medium was significantly higher in hepatocytes treated with SAMe at the dose of 1000 mg/l after 48 h, urea production was elevated by 120 % in comparison with damaged hepatocytes without protection. In our experimental conditions, albumin production was not influenced by SAMe. Content of both GSH and GSSG was significantly increased only in cells treated with the highest dose of SAMe (Figs. 5 and 6). At any used concentration SAMe did not change intracellular GSH/GSSG ratio in comparison with GalN alone. Only the highest concentration of SAMe was able to increase significantly a number of cells (48 %, p<0.001) with polarized mitochondria (Fig. 7).

Morphological changes are shown on microphotographs in Figure 8. Incubation of hepatocytes with GalN (Fig. 8b) is associated with discontinuities of the plasma membrane, spherical shape, highly granular cytoplasm and marked reduction in brightness contrast between the nucleus and cytoplasm in comparison with intact cells (Fig. 8a). Treatment with SAMe (1000 mg/l) considerably reduced morphological changes induced by GalN (Fig. 8c).

Discussion

GalN is a well-known hepatotoxin causing injury to hepatocytes *in vivo* (Keppler *et al.* 1970, Ferenčíková *et al.* 2003) and *in vitro* (Tran-Thi *et al.* 1985, Kučera *et al.* 2006). SAMe, a naturally occurring substance in almost all mammalian cells, is necessary for

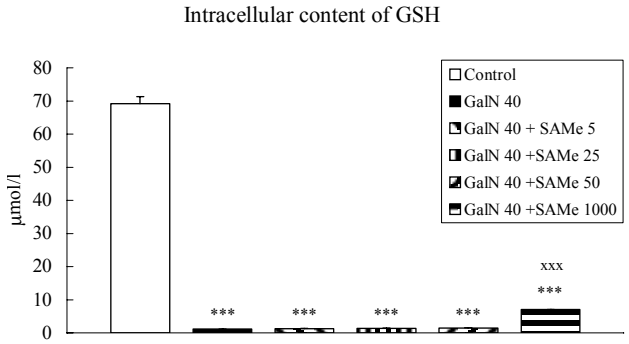

Fig. 5. Effect of SAMe (5, 25, 50 and 1000 mg/l) on intracellular content of GSH after 24 h. Data are means ± S.D. (n=3). *** p<0.001 compared with controls; ^{xxx} p<0.001 compared with 40 mM GalN.

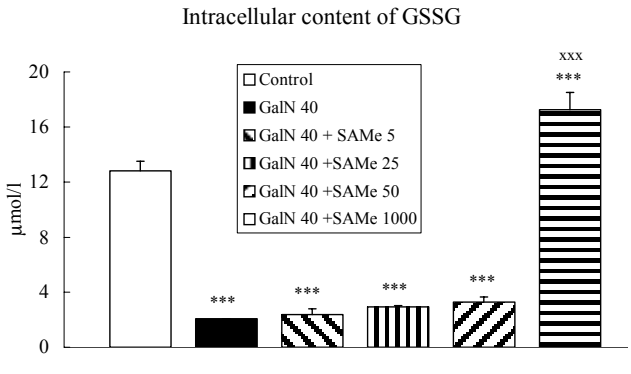

Fig. 6. Effect of SAMe (5, 25, 50 and 1000 mg/l) on intracellular content of GSSG after 24 h. Data are means ± S.D. (n=3). *** p<0.001 compared with controls; ^{xxx} p<0.001 compared with 40 mM GalN.

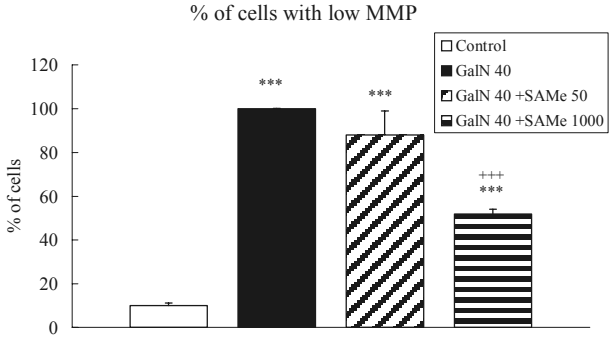

Fig. 7. Effect of SAMe (50 and 1000 mg/l) on percentage of hepatocytes containing mitochondria with low membrane potential after 24 h. Data are means ± S.D. *** p<0.001 compared with controls; $^{+++}$p<0.001 compared with 40 mM GalN.

transsulphuration and transmethylation reactions and polyamine synthesis (Friedel *et al.* 1989). Numerous clinical studies in man have shown that the administration of SAMe has a favorable effect on liver disorders (Friedel *et al.* 1989, Ponsoda *et al.* 1991, Martinez-Chantar *et al.* 2002). In experimental models, the administration of stable salts of SAMe has been reported to preserve hepatocytes against the action of several toxins such as tert-butylhydroperoxide (Lotková *et al.* 2005), carbon tetrachloride (Tsuji *et al.* 1990, Gasso *et al.* 1996), thioacetamide (Mesa *et al.* 1996) *in vivo* and *in vitro* and also galactosamine (Stramentinoli *et al.* 1978, Wu *et al.* 1996).

Although Wu *et al.* (1996) examined the effect of SAMe in high concentrations (mM) on hepatocytes damaged by GalN *in vitro*, lower doses as well as the functional capacity of hepatocytes were not studied. Therefore, we wanted to clarify functional properties of hepatocytes injured by GalN and to evaluate protective action of SAMe in both lower (µM) and higher (mM) concentrations on GalN-damaged rat hepatocytes in primary culture.

GalN prevents the synthesis of critical membrane proteins, which together with the accumulation of various metabolites of GalN are suspected to cause parenchymal cell oncosis in the liver (El-Mofty *et al.* 1975). *In vitro* damage to the plasma membrane can be examined by measurement of LDH release from injured cells into the culture medium. In our experiments, 40 mM GalN was confirmed to produce severe plasma membrane damage. The LDH release induced by GalN tended to decline with increasing SAMe concentration. Nevertheless, significant reduction was reached at concentrations of 50 and 1000 mg/l after 24 h and 1000 mg/l after 48 h of incubation (Fig. 1). Endogenous SAMe produced in hepatocytes is predominantly utilized in the transmethylation reaction (Mato *et al.* 2002). GalN inhibits *in vitro* incorporation of L-methionine into macromolecules (Ozturk *et al.* 1986). This inhibition is associated with a decrease of SAMe and an increase of methionine in the injured cells thus leading to the suppression of the methylation of important biomolecules such as phospholipids and nucleic acids. Exogenous SAMe would be expected to enhance intracellular content of SAMe and to recover disturbed transmethylation and transsulphuration reactions.

SAMe at the concentrations up to 200 µM does not enter hepatocytes significantly and therefore cannot participate directly on intracellular events (Bontemps and van den Berghe 1997). Moreover, SAMe most probably does not have specific transport system for its transfer across plasma membrane in mammalian cells in contrast to the mitochondrial membrane, where a specific, carrier-mediated system was found (Horne *et al.* 1997). SAMe added at micromolar concentrations to suspension of

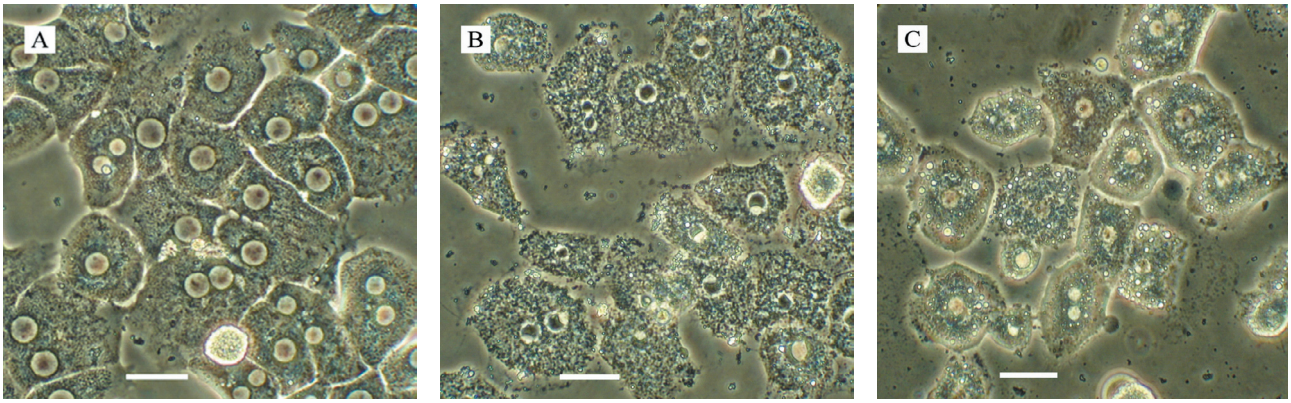

Fig. 8. Microphotographs of intact hepatocytes (Fig. 8a), cells damaged by 40 mM GalN (Fig. 8b) and treated with 40 mM GalN and SAMe (1000 mg/l) after 24 h (Fig. 8c) (phase contrast, magnification 400x for all microphotographs, bar 10 μm).

isolated rat hepatocytes is utilized mainly to methylate phospholipids located on the outer surface of the plasma membrane (Bontemps and van den Berghe 1997).

Cleavage of the tetrazolium salt (WST-1) by mitochondrial dehydrogenases proceeds only in viable cells, thus the dramatic decrease induced by GalN (Fig. 2) indicates massive reduction in cell viability. SAMe even in the supreme dose was not effective in abolishing this decrease.

GalN also influences metabolic functions of hepatocytes, including urea formation. In contrast to the situation *in vivo*, where high doses of GalN already decreased urea synthesis 24 h after GalN administration (Yokoyama *et al.* 2005), in our experimental *in vitro* conditions this hepatotoxin inhibited urea synthesis as late as during the second 24-hour period of incubation (Fig. 3). After 48 h, GalN decreased urea production by more than 3.5-fold in comparison with controls. Only the highest SAMe concentration (that could penetrate cell membrane) enhanced the production of urea. On the other hand, GalN induces early suppression of albumin production (Fig. 4), most probably by inhibition of messenger RNA due to reduced uridine pool in hepatocytes. SAMe did not increase albumin synthesis because it does not apparently influence the content of uridine in hepatocytes.

We have found that GalN causes a dramatic decrease in intracellular GSH after 24 h (Fig. 5) which corresponds well with earlier findings (McMillan and Jollow 1992, Wu *et al.* 1996). McMillan and Jollow (1992) reported that GalN-induced slow fall in hepatocyte GSH level is consistent with the inhibition of GSH synthesis. Sufficient amount of free cysteine synthesized from SAMe is required for the formation of GSH. As mentioned above, methionine adenosyltransferase (MAT) plays a crucial role in SAMe synthesis. In adult hepatocytes only MAT I/III, as a transcriptional product of *MAT1A* gene, participates in the synthesis of SAMe (Kotb *et al.* 1997). GalN was shown to inhibit this enzyme and cause GSH depletion (Ozturk *et al.* 1986). Administration of SAMe is able to abolish this effect *in vivo* (Stramentinoli *et al.* 1978) and *in vitro* (Wu *et al.* 1996). It has been observed that MAT I/III activity depends on the redox status of the cell (Pajares *et al.* 1992). The molecule of rat MAT I/III contains ten cysteine residues and thus is very sensitive to thiol group modification (Mato *et al.* 2002), as for instance under prooxidant conditions. Impairment in MAT activity compromises cellular synthesis of SAMe, and this situation may have an impact on essential metabolic pathways in the liver in which SAMe participates.

Only the highest dose of SAMe (1000 mg/l) was able to increase intracellular GSH compared to GalN alone (Fig. 5). GSSG was increased in the same manner (Fig. 6). Interestingly, GSH/GSSG depressed after the incubation with GalN was not affected by SAMe treatment. Although the highest concentration of SAMe raised GSH, there was no alteration in the redox status of hepatocytes.

Kupffer cells play an important role in the pathogenesis of GalN-induced hepatocyte injury (MacDonald *et al.* 1987, McMillan and Jollow 1995, Stachlewitz *et al.* 1999). Activated liver macrophages produce high levels of inflammatory cytokines including tumor necrosis factor α (TNF-α). Cytotoxic action of TNF-α is in particular mediated by increased production of reactive oxygen species (ROS) (Schultze-Osthoff *et al.* 1992). On the other hand, Osawa *et al.* (2001) revealed increased ROS generation as a key event in the sensitization of hepatocytes to TNF-α. The resynthesis of

GSH is inhibited in the presence of GalN and its intracellular level drops; consequently, excessive accumulation of ROS sensitizes hepatocytes to TNF-α. TNF-α also stimulates hepatocytes to produce nitric oxide (Abou-Elella *et al.* 2002) that play both deleterious and beneficial role in liver injury (Farghali *et al.* 1997). TNF-α can also initiate survival signaling (e.g. by nuclear factor-κB), which has antiapoptotic effects (Beg and Baltimore 1996).

Besides the protective effect of SAMe against GSH depletion, the beneficial effect of SAMe against GalN toxicity can be explained by findings of Arias-Diaz *et al.* (1996) that SAMe protects isolated hepatocytes against cytokine-incited toxicity. Furthermore, Arias-Diaz *et al.* (1995) also observed that exogenous SAMe suppressed the release of TNF-α by lipopolysaccharide-stimulated pulmonary macrophages *in vitro* may contribute to explanation of the SAMe effect on GalN toxicity.

Previous study (Miyahara *et al.* 1982) revealed mitochondrial damage in GalN-induced hepatic lesion. Our results (Fig. 7) documented the ability of GalN to abolish completely high mitochondrial membrane potential in hepatocytes after 24 h. Opening of a high-conductance mitochondrial permeability transition pores increases the permeability of inner mitochondrial membrane resulting in the collapse of the MMP, disruption of ionic homeostasis, release of cytochrome c and, subsequently, to cell death (Drahota *et al.* 2005, Lemasters *et al.* 1999). Enhanced production of ROS has been shown to be responsible for opening permeability transition pores (Lemasters *et al.* 1999). Increased generation of ROS by GalN and depletion of GSH leads to the loss of MMP. SAMe at the concentration of 1000 mg/l lowered the number of cells with low MMP. This effect can be achieved by enhanced synthesis of GSH.

The protective effect of SAMe against GalN toxicity *in vitro* could be accomplished by several mechanisms. SAMe may preserve membrane structure by increasing reduced methylation of phospholipids and preventing of lipid peroxidation (Song *et al.* 2003). SAMe, when entering into the cell, can also act as a precursor of GSH and serve for other reactions including methylation of important biological molecules and polyamine synthesis. In conclusion, our results clearly document significant protective effect of high concentration of SAMe compared to GalN-induced hepatocyte injury *in vitro*. Nevertheless, further experiments are required to elucidate other possible effects of SAMe, namely its role in protection of cytokine-induced toxicity.

Abbreviations

GalN – galactosamine; GSH – reduced form of glutathione; GSSG – oxidized form of glutathione; LDH – lactate dehydrogenase; MAT – methionine adenosyl-transferase; MMP – mitochondrial membrane potential; ROS – reactive oxygen species; SAMe – S-adenosyl-methionine; TNF-α – tumor necrosis factor α; UDP – uridine diphosphate.

Acknowledgements

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