Biochemical Pharmacology, Vol. 57, pp. 1311–1319, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00032-5

Effect of Hydrazine upon Vitamin B_{12}-Dependent Methionine Synthase Activity and the Sulphur Amino Acid Pathway in Isolated Rat Hepatocytes

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ABSTRACT. The effect of the industrial chemical, hydrazine (4–12 mM), on methionine synthase (EC 2.1.1.13) activity and levels of the sulphur amino acids homocysteine, cysteine, and taurine as well as GSH were investigated *in vitro* in isolated rat hepatocyte suspensions and monolayers in order to explain some of the adverse *in vivo* effects of hydrazine. None of the concentrations of hydrazine were overtly cytotoxic in hepatocyte suspensions (measured as lactate dehydrogenase [LDH] leakage) after 3 hr. However, after 24 hr in culture cells treated with 12 mM, hydrazine showed a significant increase in LDH leakage. Methionine synthase activity was reduced by hydrazine (8 and 12 mM) in suspensions (by 45 and 55%, after 3 hr) and monolayers (12 mM; 65–80% after 24 hr). This was not due to nitric oxide production and the inhibitor of nitric oxide synthase, N^{ω}-nitro-L-arginine, failed to protect against the hydrazine-induced loss of ATP and GSH and the reduction in urea synthesis at 24 hr. Homocysteine export was increased by 6 mM hydrazine, and total taurine content of treated cells was increased by 12 mM hydrazine. Thus, hydrazine was found to have several important and possibly deleterious effects on some parts of the sulphur amino acid pathway. BIOCHEM PHARMACOL **57**;11: 1311–1319, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. hydrazine; methionine synthase; taurine; nitric oxide synthase; homocysteine; sulphur amino acids

Hydrazine is a highly reactive molecule used as a rocket propellant, an antioxidant in water systems, and to produce plastic blowing agents. It is also a minor metabolite of two important drugs, isoniazid and hydralazine [1, 2]. Experimentally, in rats, hydrazine causes depletion of ATP and GSH and the accumulation of triglycerides in the liver [3] as well as neurological effects [4]. It is also a putative carcinogen [5]. The mechanisms of hydrazine toxicity are not fully understood, although the accumulation of triglycerides is in part due to the increased mobilisation of fats from adipose tissue coupled with a reduction in transport of triglycerides out of the liver [6, 7]. Hydrazine has been reported to methylate DNA [8] and interfere in the urea cycle, with the result that citrulline levels are raised in the livers of experimental animals [9, 10]. However, these toxic effects have not precluded the use of hydrazine as a possible therapeutic agent in the treatment of terminally ill cancer patients and the cachexia associated with the disease [11].

The average human diet provides only 50% of the daily methionine requirement [12]. Methionine synthase is a vitamin B_{12} (cobalamin)-dependent enzyme responsible for synthesis of methionine from the by-product of methionine metabolism, homocysteine. It is also part of the folate pathway, as it forms tetrahydrofolate from methyltetrahydrofolate [13]. Methionine is required for the production of *S*-adenosylmethionine, polyamines, and proteins [14]. Dysfunctional methionine synthase, or deficiency of cobalamin or folate, has been associated with macrocytic anaemia [15], peripheral neuropathy [16], atherosclerosis [17], neural tube defects [18], and tumour formation [19]. Previous *in vitro* studies using highly purified enzyme have shown that methionine synthase can be inhibited by nitrous oxide [20], nitric oxide [21], and acetaldehyde [22], and is stimulated by polyamines [23]. The inhibition of methionine synthase by sodium nitroprusside has also been demonstrated in

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Received 8 July 1998; accepted 23 November 1998.

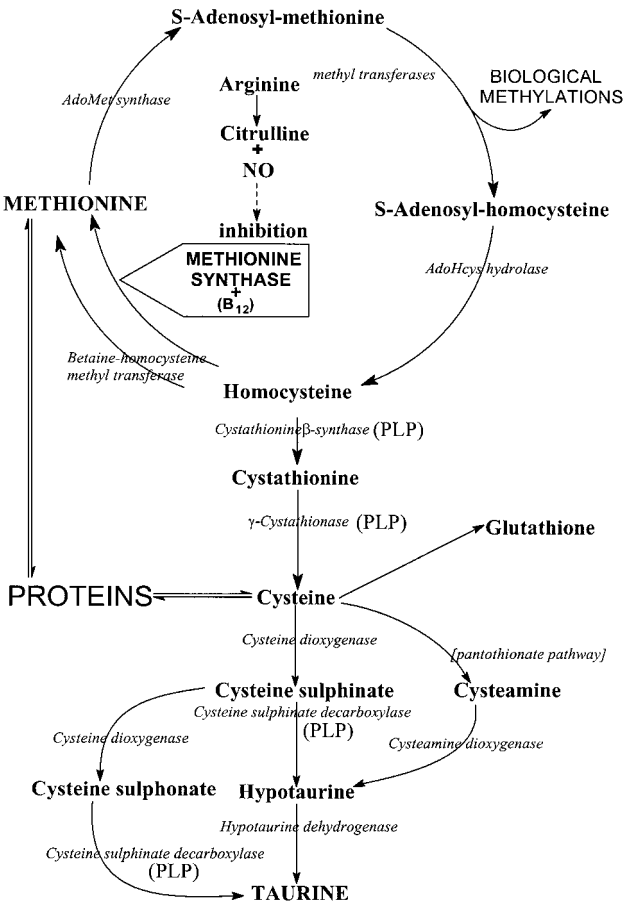

FIG. 1. Metabolic pathway to show the main sulphur amino acids resulting from methionine metabolism. An inhibition of methionine synthase (for example, by NO) would be expected to give rise to elevated homocysteine levels, possibly leading to an increase in cysteine and taurine levels [27, 28]. PLP: pyridoxal phosphate-dependent enzyme.

isolated hepatocytes [24], probably through the generation of NO* [25].

Inhibition of methionine synthase would be expected to result in an increase in homocysteine, an important risk factor for atherosclerosis [26]. Homocysteine may also be metabolised by the transulphuration pathway. Cystathionine β-synthase (requiring vitamin B_6 [pyridoxine] as a cofactor) converts homocysteine to cystathionine, which is in turn converted to cysteine (Fig. 1) [27, 28]. The end products of the transsulphuration pathway are taurine and sulphate [29].

Hydrazine has been shown to alter levels of sulphur compounds in rats given acute doses of the compound [30]. Thus, hydrazine will raise urinary levels of taurine in rats, and this despite the fact that hydrazine is known to inhibit pyridoxal phosphate (vitamin B_6)-requiring enzymes, which are present along the transsulphuration pathway leading to taurine synthesis (Fig. 1). The increase in taurine could result from raised cysteine levels due to reduced protein synthesis, increased protein degradation, reduced GSH synthesis, or an increase in homocysteine levels.

The inhibition of methionine synthase (for example by NO), would be expected to result in the accumulation of homocysteine, which may then be metabolised via intermediates in the transsulfuration pathway, to taurine. NO is produced by the enzyme NOS from the amino acid L-arginine. The products of this oxidation are NO and L-citrulline. NO is further oxidised to the inactive by-products NO_2^- and nitrate. Thus, an increase in L-citrulline or NO_2^- + nitrate can be used as a measure of increased NO synthesis, which may also indicate an increase in the inducible form of the enzyme NOS [31, 32]. Citrulline levels are raised in rats treated with hydrazine [10].

The following studies were carried out in isolated hepatocyte suspensions and in monolayers to see whether hydrazine: will increase homocysteine, cysteine and taurine levels *in vitro*; inhibits methionine synthase activity at concentrations which are not overtly cytotoxic; can be shown to generate NO; and whether any of the toxic effects of hydrazine or effects on methionine synthase activity can be blocked by using N^{ω}-nitro-L-arginine (NNA), an inhibitor of nitric oxide synthase (including the inducible form of NOS) [33].

MATERIALS AND METHODS

Chemicals

[^{14}C]5-Methyltetrahydrofolate (55 mCi/mmol) was obtained from Amersham and the AG1-X8 resin (chloride form, mesh size 200–400) from BioRad. 2,3-Diaminonaphthalene was obtained from Molecular Probes. The following cytokines were used: recombinant mouse tumour necrosis factor-alpha and recombinant mouse interleukin-1-β were purchased from Genzyme Co. Hydrazine hydrate, NNA, dithiothreitol, Williams' medium E with Glutamax®, insulin, recombinant mouse γ-interferon, lipopolysaccharide derived from *Escherichia coli* (strain 026.B6) and all other chemicals were obtained from Sigma. Ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate was obtained from Fluka (Sigma-Aldrich). All solvents were purchased from Rathbone.

Animals

Male Han Wistar outbred rats (180–220 g) (Glaxo Wellcome Research and Development) were used throughout.

Isolation and Handling of Primary Rat Hepatocytes

Hepatocyte suspensions were prepared from rats using the two-stage collagenase perfusion, performed initially under anaesthesia (Hypnorm:Hypnovel:water 1:1:2) by the method described by Moldeus *et al.* [34] as previously described [35]. Cells were used in suspension if viability was greater than 90% (trypan blue exclusion) (1.5×10^6

* *Abbreviations:* LDH, lactate dehydrogenase; NNA, N^{ω}-nitro-L-arginine; NO, nitric oxide; NO_2^-, nitrite; TCA, trichloroacetic acid; and NOS, nitric oxide synthase.

cells/mL, 20 mL under CO_2 5%/O_2 95%, 37°) in Krebs–Henseleit buffer, in constantly rotating round-bottomed flasks. Cells were preincubated for 45 min to allow time for recovery from the isolation procedure, then treated with hydrazine hydrate (neutralised with HCl 1M) in a volume of 100 μl, to give a final concentration of 0, 4, 8, or 12 mM hydrazine. Samples of the suspensions (0.5 mL) were taken before the addition of hydrazine and after 1, 2, and 3 hr. Cells were rapidly sedimented (13,000 *g*, 1 sec) and the supernatant removed for LDH measurement. The sedimented cells were treated with 0.5 mL TCA (6.5%) and frozen (−80°) for the measurement of ATP and GSH. Cells in suspension (0.5 mL) were also sampled into TCA (10%) and frozen for subsequent measurement of urea and citrulline. After 3 hr, all the remaining cells were pelleted and assayed for methionine synthase activity (see below).

Hepatocyte monolayers were prepared from cells in the same way as suspensions, except that the cells were given two final washes and resuspended in sterile Williams' medium E with Glutamax® (0.75×10^6 cells/mL), insulin (10 μM), and 10% foetal bovine serum [36]. Aliquots of the cell suspension (20 mL) were placed into 75 cm^2 plastic culture flasks (GIBCO) and incubated for 3 hr (CO_2/air 5%/95%, 37°). After this time, cells were washed in PBS and the medium replaced with similar Williams' medium E with Glutamax®, but without serum. After 18 hr, the medium was replaced again and the cells were treated with neutralised hydrazine hydrate (100 μL), to give a final concentration of 6 or 12 mM, with or without NNA (0.5 mM). Positive control flasks were also prepared to ensure that it was possible to induce NOS and produce NO in lipopolysaccharide-(10 μg/mL) and cytokine-treated cells (recombinant mouse tumor necrosis factor-alpha, recombinant mouse interleukin-1-β, and recombinant gamma interferon, all 100 U/mL) [37]. Medium was removed after 8 or 24 hr and analysed for LDH leakage, homocysteine, cysteine, taurine, urea, and NO (as NO_2^-) as described below. Cells were also harvested after 8 and 24 hr by trypsinisation (the action of the trypsin being inhibited by media-containing serum), with the final cell suspension volume 5 mL. Cells from 0.5 mL aliquots were sedimented (400 *g*, 3 min) and treated with TCA (6.5%, 0.5 mL) and immediately frozen (−80°) for subsequent measurement of ATP, GSH, homocysteine, cysteine, and taurine as described below. The remaining cells (4.5 mL) were sedimented and stored at −80° for analysis of methionine synthase activity. Additional media and cell samples were taken from the flasks treated with cytokines after 4 hr.

Incubation of Purified Methionine Synthase with Hydrazine

To assess the direct action of hydrazine on the activity of methionine synthase, hydrazine (12 mM) was incubated with the highly purified enzyme [24] and enzyme activity assessed as described below.

ATP Measurement

ATP was determined in hepatocytes using luciferase according to the method of Stanley and Williams [38] as previously described [39]. Briefly, acid extracts were prepared from the 0.5 mL aliquots of cells (TCA, 6.5%, 0.5 mL, 13,500 *g*, 4°, 5 min), aliquots of acid supernatant (10 μL) were treated with Luciferase–Firefly lantern extract (100 μL) in buffer (2 mL), and the bioluminescence produced was measured with a Thorn EMI photon detection system.

Reduced Glutathione Measurement

GSH was determined as the total non-protein sulphydryls in the acid supernatant cell extract by the method of Hissin and Hilf [40] as previously described [35]. This method was used as greater than 95% of liver total non-protein sulphydryls are present as GSH [41].

LDH Leakage

LDH leakage [42] and uptake of trypan blue were used as markers of cytotoxicity *in vitro*, and were determined as previously described [35]. LDH activity was expressed as a percentage of the total LDH present in cells after sonication of an aliquot of cell suspension or as units of activity in the culture medium of hepatocyte monolayers.

Urea Measurement

Urea and citrulline were measured in 0.5 mL aliquots of cell culture media treated with TCA (10%), using a Sigma diagnostic kit (procedure No. 535), a method which is based on that of Cocker [7, 43].

Methionine Synthase Activity

Cell pellets were stored at −80° until assay. Pellets were re-suspended in 750 μL of homogenising buffer (50 mM phosphate buffer containing 0.1 M NaCl and 50 mg/L phenylmethylsulphonylflouride, pH 7.2) and were homogenised by sonicating for 3 × 5 sec using a probe sonicator. The homogenate was centrifuged (50,000 *g*, 10 min) and the supernatant assayed immediately for methionine synthase activity, using a modified version of Weissbach *et al.* [44]. The reaction mixtures contained potassium phosphate buffer (50 mM, pH 7.2), 400 μM [DL]-homocysteine, 35 μM *S*-adenosylmethionine, 236 μM methyltetrahydrofolate (2658 dpm/nmol), 60 μM hydroxycobalamin, 25 mM dithiothreitol, and the enzyme source (total volume 300 μL). The incubations were carried out in light-protected stoppered serum vials under nitrogen. Samples were preincubated for 5 min (37°) prior to initiation of the reaction by the addition of homocysteine through a syringe. Incubations (37°) were performed for 60 min. The enzyme reaction was terminated by the addition of 400 μL ice-cold

water and solutions immediately passed through 0.5 × 5.0-cm columns of BioRad AG1-X8 resin. [^{14}C]-Methionine was eluted with 2 mL water, collected and quantitated by scintillation spectrometry, and enzyme activity was expressed as nmol/hr/mg protein.

HPLC Analysis of Total Homocysteine and Cysteine in Cell Culture Media

Levels of total (oxidised and reduced) homocysteine and cysteine were measured by fluorimetric detection after separation by HPLC, using a modified version of Fortin and Gernst [45]. Briefly, samples were reduced using 10% tri-*n*-butylphosphine in dimethylformamide for 30 min at 4° then derivatised with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate for 60 min at 60°. Samples were injected onto a reverse phase C18 column (LiChrospher® 100 RP-18, 5 μm, 125 × 4 mm I.D.) fitted with a guard column and eluted with an isocratic gradient of 0.1 M acetate buffer containing 2% methanol at a flow rate of 0.8 mL/min. *N*-Acetylcysteine was used as an internal standard. Cysteine was eluted after 2.5 min, homocysteine after 3.5 min, and *N*-acetylcysteine after 6.0 min.

HPLC Analysis of Taurine

Taurine was measured in media and cells by a modification of the method of Waterfield [46]. Media (1000 μL) or acid cell extract (250 μL) were passed though Dowex columns (0.5 mL Dowex-1-X4, Cl^- form on 1.5 mL Dowex-50W-X8, H^+ form) and the taurine eluted with UHQ water (4 mL) as previously described. Homoserine was added to the eluates as internal standard (20 μL, 100 μM), then frozen (liquid N_2) and freeze-dried overnight. Samples were then reconstituted in mobile-phase buffer (1 mL) and analysed by HPLC using a Beckman autosampler and quantitated using fluorimetric detection as previously described. Although taurine is concentrated by cells, a significant amount is transported/leaked into the media from damaged cells or by cells with an osmotic imbalance. This gives another measure of membrane integrity and/or osmotic status of the cells. For this reason, results are shown as the total amount of taurine present in the cells and media as well as the media concentration.

Determination of Nitric Oxide

NO was determined in the media of hepatocyte monolayers as NO_2^- after treatment with nitrate reductase by measuring both the chemiluminescent product of its reaction with ozone [47] and the fluorescent product following the reaction of NO_2^- with 2,3-diaminonaphthalene in 96-well plates using a CytoFluor 4000 plate reader [48].

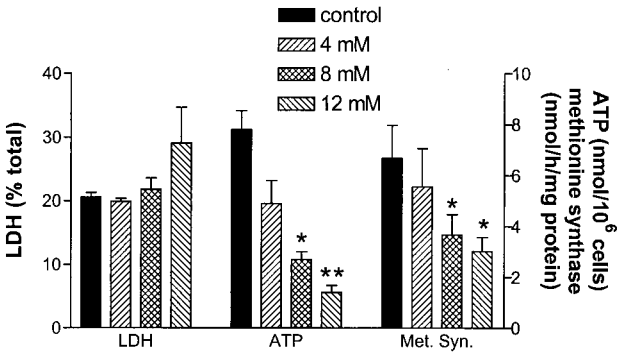

FIG. 2. Effect of hydrazine (0, 4, 8, or 12 mM) on LDH leakage (as % of total), intracellular ATP levels, and methionine synthase activity in 3 hr incubations of isolated hepatocyte suspensions. Values are means ± SEM of four different experiments. *$P < 0.05$, **$P < 0.01$ (control cells compared with treated cells using Dunnett's test). Met. Syn., methionine synthase.

Protein Assay

Protein concentration in cell cytosol was determined using the BioRad protein assay based on the method of Bradford [49], using BSA as the standard.

Statistics

Data from the cell suspensions were analysed using analysis of variance and Dunnett's test for comparison of several treatment groups with a single control. As different isolations of cells resulted in very different amounts of methionine synthase activity, the activity of the enzyme was compared before and after treatment using the same cell isolation as the control group using a paired *t* test. Significance was set at >95%.

RESULTS

Effect of Hydrazine upon Cells in Suspension after Three Hours

TOXICITY MARKERS. LDH leakage, a measure of membrane integrity, was not significantly raised with any of the hydrazine concentrations tested (Fig. 2). The ATP level, a sensitive marker of metabolic competence, was significantly reduced at concentrations of hydrazine which also affected methionine synthase activity (8 and 12 mM) (Fig. 2).

METHIONINE SYNTHASE ACTIVITY. The activity of vitamin B_{12}-dependent methionine synthase was measured in the cells after treatment with various concentrations of hydrazine for 3 hr. A significant reduction in enzyme activity was noted with concentrations of 8 and 12 mM (Fig. 2).

Effect of Hydrazine upon Cultured Hepatocytes at 8 and 24 Hours

TOXICITY MARKERS. There was no significant difference in LDH leakage between control and 6 mM hydrazine-

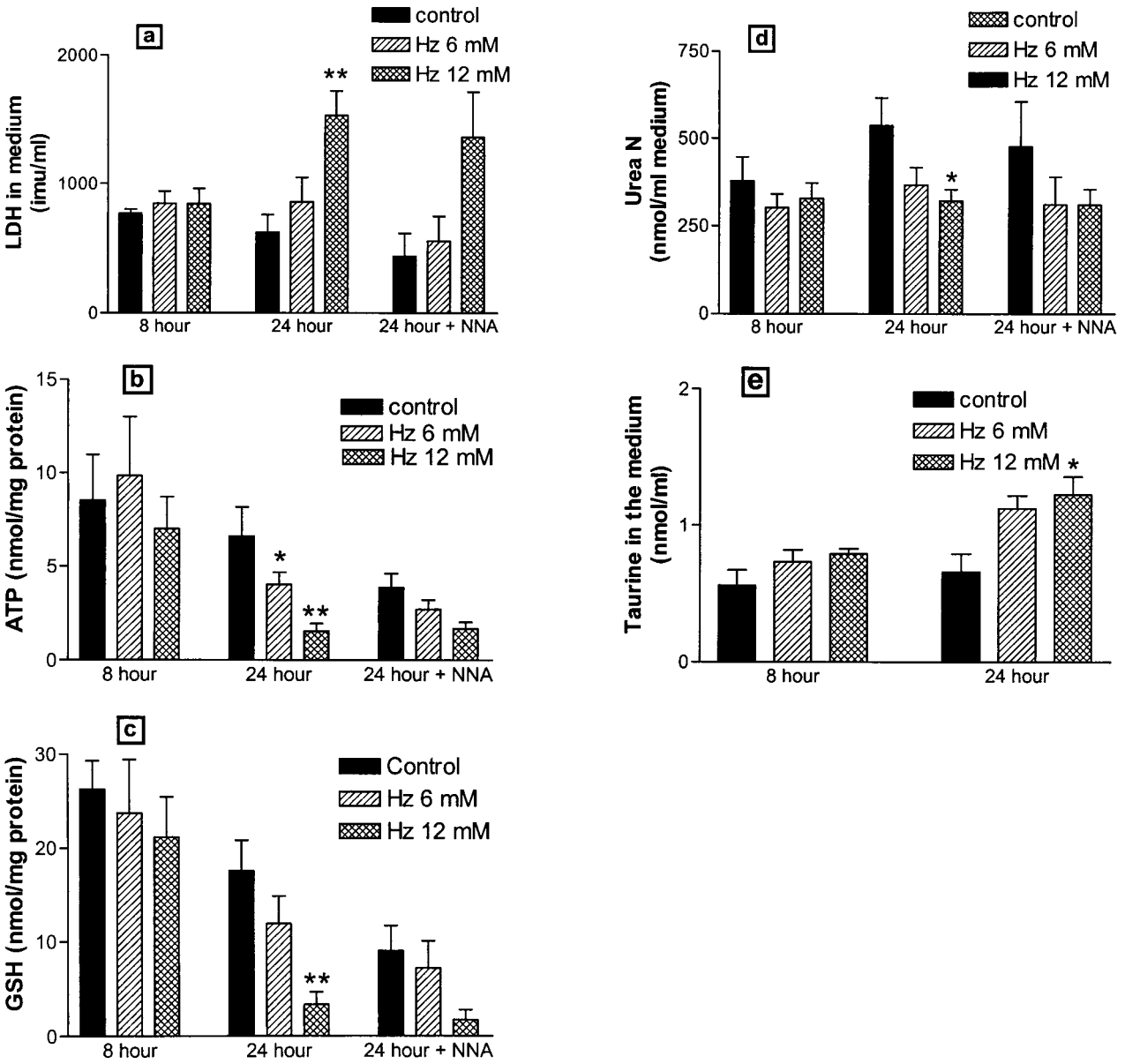

FIG. 3. Effect of hydrazine (Hz: 0, 6, or 12 mM) on (a) LDH leakage, (b) intracellular ATP, (c) intracellular GSH levels, (d) total urea levels, and (e) taurine concentration in the medium in 8- and 24- hr incubations of isolated hepatocyte monolayer cultures; (a)–(d) with and without the addition of 0.5 mM NNA. Values are means ± SEM of 4–7 different experiments. *P < 0.05, **P < 0.01 (control cells compared with treated cells using Dunnett's test).

treated cells at either time point. However, 12 mM hydrazine caused a significant increase in LDH leakage after 24 hr, showing that the membrane integrity of these cells had been compromised (Fig. 3a). After 8 hr ATP levels were very similar for all cells, but at 24 hr there was a significant reduction in levels for treated cells (Fig. 3b); similarly, GSH levels were decreased at 24 hr by 12 mM hydrazine (Fig. 3c). Urea levels were also measured in the media and found to be similar in all cells at 8 hr, but significantly reduced at 24 hr (Fig. 3d) by 12 mM hydrazine. The appearance of taurine in the media was not significantly different from control at 8 hr, but at 24 hr cells treated with both 6 and 12 mM hydrazine showed increased media taurine concentrations, which were significant with 12 mM hydrazine (Fig. 3e). As taurine is a much smaller entity than LDH, it may mark the early stages of loss of membrane integrity or may reflect increased export of taurine due to osmotic stress being imposed on the cells. Thus, 12 mM hydrazine resulted in cell death (measured as loss of membrane integrity), and both 6 and 12 mM hydrazine resulted in some biochemical dysfunction.

Methionine Synthase Activity

Methionine synthase activity was not changed significantly in the cultured hepatocytes after 8 hr, but was inhibited by 12 mM hydrazine after 24 hr. Large variations in control activity were found between batches of hepatocytes. For

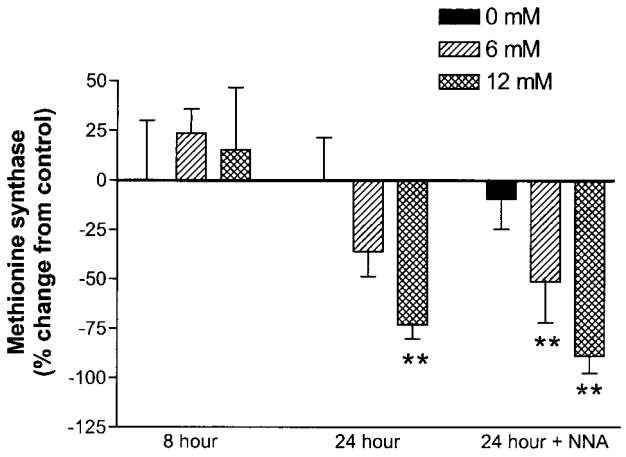

FIG. 4. Methionine synthase activity in hepatocyte monolayer cultures expressed as % of control values at 8 and 24 hr, after the addition of hydrazine (0, 6, or 12 mM) and NNA. Values are means ± SEM of 4–7 different experiments. **P < 0.01, paired *t*-test.

each individual batch of cells, the trend of hydrazine inhibition was the same (Fig. 4).

Hydrazine (12 mM) was not found to cause the same degree of inhibition of purified methionine synthase as that found in isolated hepatocytes (30% compared to 52% in suspensions and 65–80% in monolayers).

Homocysteine and Cysteine Concentration in the Cell Culture Media and Total (Cytosol and Media) Taurine Concentration

After 8 hr of hydrazine treatment, no statistical difference was found in homocysteine concentration between control and treated cells. After 24 hr, there was a significant increase in homocysteine concentrations in the media of cells treated with 6 mM hydrazine, but not 12 mM hydrazine (Fig. 5a).

Although cysteine was consumed by control cells (media concentration 497 μM), little or no cysteine was taken up from the media by the treated cells (Fig. 5b). Total taurine levels were slightly raised by hydrazine treatment, although the amount of taurine produced was small compared to the amount of homocysteine exported into the cell culture media (Fig. 5c). The concentrations of homocysteine and cysteine in the cell cytosol were measured, but were below the level of detection (results not shown).

Influence of NNA on Hydrazine Toxicity in Cultured Hepatocytes

TOXICITY MARKERS. NNA did not protect the cells against hydrazine-induced toxicity. LDH leakage was of the same magnitude as samples not containing NNA (Fig. 3a), while ATP levels, GSH levels, and urea concentration were not significantly different in cells treated with hydrazine alone (Fig. 3, b and c). Indeed, the cells treated with NNA

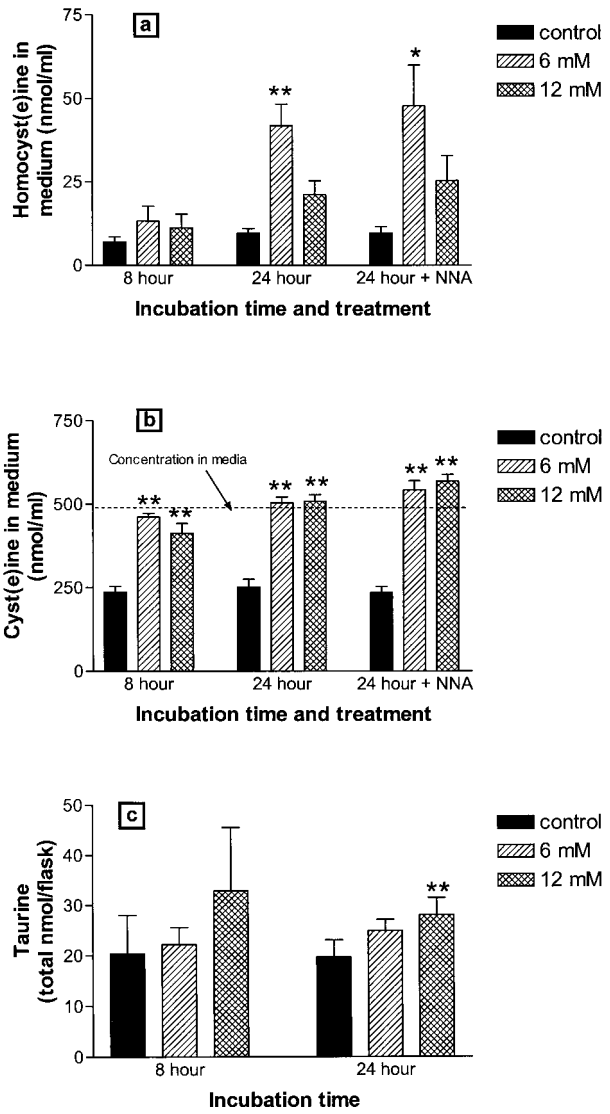

FIG. 5. Concentration of (a) homocysteine and (b) cysteine in the medium of hepatocyte monolayers 8 and 24 hr after the addition of hydrazine (0, 6, or 12 mM) and NNA (N^{ω}-nitro-L-arginine, 0.5 mM) to the cells. (c) Total taurine content of cells and media in flasks 8 and 24 hr after the addition of hydrazine. (0, 6, or 12 mM). Values are means ± SEM of 4–7 different experiments. *P < 0.05, **P < 0.01 (control cells compared with treated cells using Dunnett's test).

alone appeared to be slightly more compromised after 24 hr than cells not treated with NNA.

METHIONINE SYNTHASE ACTIVITY. Inhibition of enzyme activity by hydrazine was not reduced by the addition of NNA indeed, there was a highly significant reduction in enzyme activity in these cells (Fig. 4).

Homocysteine and Cysteine Concentrations in the Cell Culture Media

The addition of NNA to the cell culture flasks did not alter the effects of hydrazine on the medium concentration of

these metabolites (Fig. 5). Taurine was not measured in NNA-treated cells.

Nitrite Generation

Although the hepatocytes treated with cytokines generated NO (36–40 μM NO_2^- vs 0 μM NO_2^- in controls at 24 hr), no increase in NO_2^- levels was seen in the control cells or hydrazine-treated samples with either method used (data not shown).

DISCUSSION

The full toxicological profile of hydrazine is not fully understood, but inhibition of methionine synthase may explain some features. Thus, hydrazine causes fatty liver in several species, a phenomenon which has characteristics in common with alcohol toxicity. Inhibition of methionine synthase by alcohol has been proposed to be involved in fatty liver formation [50].

Initial experiments used isolated rat hepatocytes in suspension, incubated in three different concentrations of hydrazine previously shown not to be overtly cytotoxic but known to cause some biochemical changes [7]. Methionine synthase activity was found to be inhibited, after 3 hr, by hydrazine (8 and 12 mM), without overt toxicity measured as LDH leakage.

In isolated hepatocyte monolayers, hydrazine (6 and 12 mM) had no effect on methionine synthase activity after 8 hr, although at 24 hr significant inhibition was seen with 12 mM hydrazine. Thus, hepatocyte suspensions were more susceptible to the toxic effects of hydrazine, although this could be due to the shorter recovery period of cells in suspension after isolation (45 min vs 24 hr). Monolayer cells are also provided with many nutrients, whilst suspended cells were incubated only in a balanced salt solution. The analysis of LDH leakage, ATP and GSH concentrations, and urea synthesis suggested that at 8 hr the cells were fully competent. However, after 24 hr, a dose-dependent increase in LDH activity and taurine concentration in the media (12 mM) and a decrease in ATP, GSH, and urea concentrations was seen, indicating toxic effects on the cells. The inhibition of methionine synthase could therefore be due to the general toxicity of hydrazine. As hydrazine caused less inhibition of purified methionine synthase than in hepatocytes, it is suggested that a metabolite of hydrazine or other metabolic factor may cause some of the inhibition of methionine synthase activity.

The export of homocysteine, one of the substrates of methionine synthase, was measured in the cell culture media at 8 and 24 hr. In control cells, there was only a small increase in homocysteine levels over the 24-hr time period, which was not significant. Hydrazine (6 mM), however, caused a significant increase in homocysteine concentrations after 24 hr which had probably accumulated over the 8–24 hr period. This was despite the fact that methionine synthase activity was not significantly inhibited, although it was reduced. It is possible that there had been some recovery of enzyme activity and/or that some of the increase in homocysteine was due to inhibition of cystathionine β-synthase. Cystathionine β-synthase is a pyridoxal phosphate-requiring enzyme, and hydrazine has been reported to inhibit pyridoxal phosphate-dependent enzymes [51]. Interestingly, homocysteine export was not greatly increased by 12 mM hydrazine, compared to that seen with the 6 mM. This suggests that the cells treated with the 12 mM hydrazine were so metabolically compromised that they were either no longer able to produce homocysteine or to export it. However, no accumulation of homocysteine was found in cells (data not shown). In order to clarify this anomaly, the dose–response relationship between hydrazine concentration and homocysteine export and the time scale for these changes is currently being investigated.

Cysteine was present in the medium (Williams' medium E) at a concentration of 497 μM. Control cells were able to utilise this cysteine during the course of the experiment, although all the uptake appeared to occur within the first 8 hr of the incubation, as the concentration of cysteine in the media was reduced to 250 μM, and remained at this level until the end of the experiment. It is probable that, after an initial uptake of cysteine into the cells, an equilibrium was established between free cysteine, protein cysteine, GSH cysteine, and that metabolised to taurine. In the presence of hydrazine, cysteine uptake apparently ceased, although any cysteine resulting from the catabolism of GSH and/or homocysteine could not be distinguished from that already present in the media. It may be that cysteine was still being utilised in the hydrazine-treated cells, but that the free cysteine concentrations were maintained at levels similar to medium concentrations, possibly by increased endogenous synthesis, for example from the excess homocysteine. Although not significantly increased, the levels of cysteine in the medium were slightly raised; indeed, the high concentration of cysteine already present in the medium made slight increases in concentration difficult to determine. The reduced GSH levels may also reflect the apparent lack of cysteine utilisation/uptake, or it may simply be a measure of general cytotoxicity.

Significantly more taurine in total was synthesised by cells treated with 12 mM hydrazine after 24 hr than control cells, although the amount was much less than and not proportional to the increase in homocysteine concentration in the medium. This could have been due to an inhibition of cystathionine β-synthase or the rapid export of homocysteine into the medium.

Thus, hydrazine had effects upon the sulphur amino acid pathway which could in part be explained by the inhibition of methionine synthase.

As NO is known to inhibit methionine synthase, NNA, an inhibitor of NOS was added to hydrazine-treated cells to investigate whether it would protect the cells from hydrazine toxicity. The results show that NNA had no protective effect and that there was also no increase in NO_2^- levels in the hydrazine-treated cells, despite the fact that

cytokines did result in the generation of NO, measured as NO_2^-. Thus, it appears to be very unlikely that the effects of hydrazine on methionine synthase *in vitro* are mediated through the generation of NO either directly or indirectly by the induction of NOS.

In summary, hydrazine inhibited vitamin B_{12}-dependent methionine synthase activity *in vitro*, which may have resulted in the increased synthesis of homocysteine which was subsequently exported into the media. These factors may account for some aspects of hydrazine toxicity *in vitro*. The increase in homocysteine could also account for the slight increase in taurine levels which were found and are consistent with the raised taurine levels of urinary taurine found in hydrazine-treated rats [7, 30].

The effect of hydrazine upon the enzymes of the sulphur amino acid pathway, in particular cystathionine β-synthase, are currently under investigation.

S. K. is a Maplethorpe Fellow.

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