S-Adenosylmethionine attenuates the lipopolysaccharide-induced expression of the gene for tumour necrosis factor α

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Intracellular deficiency of *S*-adenosylmethionine (AdoMet) and elevated serum concentrations of tumour necrosis factor α (TNF) are hallmarks of toxin-induced liver injury. In these models, the administration of either exogenous AdoMet or antibody/soluble receptor for TNF attenuates the injury. We have demonstrated previously that the administration of exogenous AdoMet to AdoMet-deficient rats attenuated lipopolysaccharide (LPS)-induced liver injury and serum TNF concentrations. Here we report that AdoMet lowered the amount of TNF secreted by LPS-stimulated murine macrophage cells (RAW 264.7) in a dose-dependent manner. The inhibition of TNF release was correlated with changes in the steady-state TNF mRNA concentrations. Changes in TNF mRNA were not due to its altered

stability and might have been due to an attenuation of the transcription rate of the TNF gene. The inhibition of TNF release in RAW cells was not mediated by GSH because treatment with AdoMet did not increase intracellular GSH. In addition, *N*-acetylcysteine, whereas it did increase GSH concentration, had no effect on LPS-stimulated TNF release in these cells. Exogenous AdoMet also attenuated LPS-induced serum TNF levels in normal rats sensitized with lead. Thus AdoMet administration might exert its hepatoprotective effects at least in part by its inhibitory effect on expression of the gene for TNF.

Key words: *N*-acetylcysteine, cytokines, glutathione, hepatotoxins, liver injury.

INTRODUCTION

S-Adenosylmethionine (AdoMet) is a key intermediate in the hepatic trans-sulphuration pathway and serves as a precursor for GSH as well as the methyl donor in most transmethylation reactions [1,2]. Supplementation with exogenous AdoMet protects the liver from a wide variety of toxins, including carbon tetrachloride, ethanol and acetaminophen [3–6], although the mechanisms of its action are not fully established. These hepatotoxins induce hepatic deficiencies of AdoMet and GSH; it has been proposed that the beneficial effects of AdoMet might be mediated by the restoration of one or both of these important biological metabolites [7]. Tumour necrosis factor α (TNF), a pleiotropic inflammatory cytokine, is an important mediator in the development of liver injury caused by these toxins; serum TNF levels are elevated [8–10] and TNF-neutralizing antibodies or soluble TNF receptors attenuate the injury [11–13]. However, the effect of the administration of exogenous AdoMet on TNF production in these models of liver injury has not been examined.

Recently we reported that lipopolysaccharide (LPS)-induced liver injury in choline- and AdoMet-deficient rats was mediated by TNF [14]. Furthermore, we showed that the administration of exogenous AdoMet to these animals attenuated serum TNF and liver injury [15]. The attenuation of serum TNF in AdoMet-treated rats might have been the result of either a decreased rate of formation of TNF, or an increased rate of clearance of TNF from the systemic circulation. This study was not designed to discriminate between these two potential mechanisms. Here we examine the role of AdoMet in modulating LPS-induced TNF release in a murine macrophage cell line, RAW 264.7. We demonstrate that AdoMet supplementation decreases the amount of TNF released in the conditioned medium and the steady-state TNF mRNA concentrations elicited by LPS. We also dem-

onstrate that exogenous AdoMet attenuates LPS-induced serum TNF levels in normal, AdoMet-sufficient rats.

MATERIALS AND METHODS

Materials

AdoMet, as its 1,4-butanedisulphonate salt, as well as the sodium salt of its anion, were provided by Dr. Robert O'Brian (Knoll Pharmaceuticals Co., Piscataway, NJ, U.S.A.) and Dr. G. Stramentinoli (Knoll Farmaceutici, Milan, Italy). All other reagents were of the highest purity available and, unless indicated otherwise, were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell culture

RAW 264.7 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, U.S.A.) containing 4 mM *L*-glutamine, 1.0 mM sodium pyruvate, 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, U.S.A.), penicillin and streptomycin at 37 °C in a humidified air/CO₂ (19:1) atmosphere.

Animal studies

Animal studies were approved by the Animals Studies Subcommittee of the Lexington Veterans Affairs Medical Center. Male Sprague-Dawley rats, 100–120 g, were obtained from Harlan Sprague Dawley (Indianapolis, IN, U.S.A.). Rats were administered regular laboratory chow for 3 days, then injected intravenously with saline (vehicle control) or 100 μ g/kg body weight LPS (*Escherichia coli* 0111: B4 endotoxin; Difco Laboratories, Detroit, MI, U.S.A.) and 15 mg/kg body weight lead acetate [16] with or without AdoMet (170 μ mol/kg body weight).

Abbreviations used: AdoMet, *S*-adenosylmethionine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; TNF, tumour necrosis factor α .

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After 90 min, blood was drawn for TNF analyses; 24 h after treatment with LPS, rats were anaesthetized and blood was drawn for the measurement of liver transaminases. Animals were then killed; livers were removed and stored in liquid nitrogen for biochemical analyses.

Biochemical analyses

Intracellular and tissue AdoMet concentrations were assayed by reverse-phase HPLC with deproteinized extracts of cells or tissue [17]. Cellular GSH concentrations were assayed by HPLC analysis of dansylated protein-free cell extracts [18]. TNF in serum or conditioned medium was quantified by ELISA (Genzyme, Cambridge, MA, U.S.A.) in accordance with the manufacturer's instructions. Serum aspartate transaminase and alanine transaminase were assayed in the Clinical Laboratory of the University of Kentucky Medical Center. Viability was assessed by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the medium and measuring the amount of MTT converted to formazan in 1 h at 37 °C.

Northern blot analysis of TNF mRNA

Total RNA was isolated with Trizol Reagent (Gibco BRL) in accordance with the manufacturer's instructions. RNA was electrophoresed on a 1% (w/v) denaturing agarose gel and transferred to Genescreen nylon membrane (Dupont, Boston, MA, U.S.A.) with NorthernMax Rapid Transfer buffer (Ambion, Austin, TX, U.S.A.). Membranes were probed at 42 °C for 18 h with a 1.1 kb fragment of TNF cDNA (provided by Dr. Bruce Beutler [19]) labelled with the Prime It II random primer labelling kit (Stratagene, La Jolla, CA, U.S.A.). After autoradiography, membranes were stripped by immersion in boiling 0.1% SDS and reprobed with a murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific cDNA probe (Ambion, Austin, TX, U.S.A.).

Statistical analyses

Mean responses were analysed by one-way analysis of variance followed by a two-tailed Student's *t* test with an overall type I error rate of 0.05. Differences were considered significant if *P* < 0.05.

RESULTS

Treatment with AdoMet lowers the release of TNF by LPS-stimulated RAW cells

To determine the effect of exogenous AdoMet on TNF production by macrophages, RAW cells were pretreated with increasing concentrations of AdoMet for 2 h and then stimulated with LPS. This protocol was designed to optimize the changes in LPS-stimulated TNF release by RAW cells without affecting their viability and was finalized after a number of preliminary studies in which the duration of preincubation with AdoMet was varied from a few minutes to 18 hours. Results are summarized in Figure 1. AdoMet inhibited TNF release into the conditioned medium in a dose-dependent manner; a 50% decline was observed in cells pretreated with 1 mM AdoMet. Treatment with the AdoMet anion, 1,4-butanedisulphonate, did not alter the TNF concentrations (results not shown). The viability of these cells, as measured by the conversion of MTT to formazan, was unaffected by concentrations of AdoMet up to 1 mM (more than 90% viability). Treatment of cells with higher concentrations of AdoMet (e.g. 3 mM and higher), however, significantly lowered cellular viability to at most 70%.

Figure 1 Effect of AdoMet on LPS-stimulated TNF release

RAW cells were plated at a density of 5×10^5 cells/ml and incubated overnight. AdoMet was added to the culture medium at various concentrations; after 2 h of incubation, LPS was added to the culture medium to a final concentration of 100 ng/ml. Cells were incubated in the continued presence of AdoMet and LPS for a further 6 h. Conditioned media were collected and TNF was quantified by ELISA as described in the Materials and methods section. Results are means $\pm$ S.D. for a representative experiment performed in triplicate and are expressed as percentages of the control value (no AdoMet). Points marked with an asterisk are significantly different (*P* < 0.05) from those of control cells.

Figure 2 Time course of TNF release by LPS-stimulated RAW cells in the presence and absence of AdoMet

Cells were plated as described in the legend to Figure 1, then pretreated with 1 mM AdoMet dissolved in medium (◇), or an equal volume of medium alone (□), for 2 h; they were then stimulated with 100 ng/ml LPS. Conditioned media were collected at the indicated times and analysed for TNF concentration by ELISA. Results are means $\pm$ S.D. for three separate experiments performed in duplicate. The mean response was compared by a repeated-measures analyses of variance. Since there was a significant treatment-versus-time interaction, we compared treated and untreated means at each time point using *t*-tests. Points marked with an asterisk are significantly different (*P* < 0.05) from control cells.

The time courses of LPS-stimulated TNF release in the presence and the absence of 1 mM AdoMet are shown in Figure 2. In the control cells (no AdoMet), TNF levels in the conditioned medium peaked 8 h after LPS stimulation. In AdoMet-treated cells, TNF release peaked earlier than in control cells (4 h after LPS stimulation) and the peak TNF levels were only one-quarter of those in control cells.

Figure 3 Changes in intracellular AdoMet concentration in AdoMet-treated cells

Cells were plated as described in the legend to Figure 1. As described in the legend to Figure 2, 1 mM AdoMet was added 2 h before treatment with LPS (■, ●), whereas cells that were not stimulated with LPS (□, ○) received an equal volume of medium. Cells were harvested at the indicated time points (after the addition of LPS or medium) and AdoMet was quantified as described in the text. Results are means $\pm$ S.D. for four separate determinations. Symbols: □, control; ■, control + LPS; ○, AdoMet; ●, AdoMet + LPS.

Table 1 Changes in intracellular GSH and TNF released into the conditioned medium after treatment with NAC or AdoMet

RAW cells were treated for 2 h with medium, 1 mM AdoMet or 5 mM NAC. The acid-soluble fraction was obtained as described in the Materials and methods section; dansylated extracts were subjected to HPLC analysis. TNF in the conditioned medium was measured by ELISA after 6 h stimulation with 100 ng/ml LPS. Results are means $\pm$ S.D. for four separate determinations. Values marked with an asterisk are significantly different from those of control cells ($P < 0.01$).

Treatment	[GSH] (nmol/mg of protein)	[TNF] (ng/ml)
Control	8.82 $\pm$ 0.21	75.8 $\pm$ 9.4
AdoMet	10.82 $\pm$ 0.05	29.0 $\pm$ 1.3*
NAC	15.73 $\pm$ 1.89*	78.8 $\pm$ 6.9

Intracellular AdoMet concentration is elevated in AdoMet-treated cells

Intracellular concentrations of AdoMet in treated and untreated RAW cells are shown in Figure 3. The basal concentration of AdoMet in RAW cells was 240 pmol/mg of protein and remained unchanged during the culture conditions used in these experiments. Treatment with 1 mM exogenous AdoMet resulted in a 5-fold elevation of intracellular AdoMet concentrations within 2 h of exposure; these levels remained elevated for at least 6 h. Interestingly, there was a slight but significant increase ($P < 0.05$) in endogenous AdoMet levels 6 h after stimulation with LPS alone.

Inhibition of TNF release by AdoMet is not mediated by GSH

In some cells, AdoMet is a precursor for the important intracellular thiol GSH. Because reduced thiols have been reported to modulate TNF release by primary macrophages [20–23], it was necessary to determine whether the effects of AdoMet supplementation were mediated by GSH. As shown in Table 1,

Figure 4 Northern blot analysis of TNF mRNA in the presence and absence of AdoMet

After 2 h of pretreatment with 1 mM AdoMet or medium alone, RAW cells were stimulated with 100 ng/ml LPS in the continued presence of AdoMet. Cells were collected at the indicated times after exposure to LPS, then RNA was isolated for Northern blot analyses as described in the Materials and methods section. Autoradiographs of TNF (upper panels) and GAPDH (lower panels). This is a representative example of three separate experiments.

treatment of RAW cells with 1 mM AdoMet did not significantly affect intracellular GSH although it inhibited TNF release by 40%. LPS had no effect on GSH under these conditions. To provide further evidence that GSH was not modulating TNF release, cells were incubated with another pro-GSH molecule, *N*-acetylcysteine (NAC) for 2 h and then with LPS. Treatment with NAC doubled intracellular GSH levels but did not affect the amount of TNF released by these cells. The viability of RAW cells was not affected by treatment with up to 5 mM NAC (viability at least 90%).

Exogenous AdoMet modulates LPS-induced TNF gene expression

Northern blot analyses were performed to determine whether the effect of AdoMet on TNF expression could be attributed to changes in the amount of TNF mRNA. The results are shown in Figure 4. As reported by others [24], unstimulated RAW cells had detectable levels of TNF mRNA; treatment with AdoMet did not affect these levels. On exposure to LPS, TNF mRNA levels peaked 2–4 h after stimulation. Peak mRNA levels were slightly lower in AdoMet-treated cells and returned to baseline levels within 12 h. In control cells, however, there was a prolonged elevation of the TNF mRNA after stimulation with LPS, which did not return to baseline even after 24 h.

The steady-state mRNA level is primarily a function of the rate of its degradation and the rate of its formation. The rate of degradation is reflected in the stability of the mRNA. To determine the stability of TNF mRNA, both AdoMet-treated and control cells were incubated with LPS for 2 h and then incubated with actinomycin D (10 μ g/ml) to block further transcription. TNF mRNA was assayed at various time points, as shown in Figure 5. The rate of change in TNF message was unaffected by treatment with AdoMet; changes observed in the Northern blots were therefore probably due to changes in the rate of formation of TNF mRNA.

Treatment with AdoMet attenuates serum TNF levels in LPS/lead acetate-stimulated rats

To determine whether the observation that AdoMet inhibits TNF expression in RAW cells could be reproduced *in vivo*, rats were administered LPS plus lead acetate with or without co-

Figure 5 Effect of AdoMet treatment on the stability of the TNF mRNA

RAW cells were pretreated with 1 mM AdoMet or medium alone for 2 h, then LPS was added to a final concentration of 100 ng/ml; after 2 h, actinomycin D (ActD) was added to a final concentration of 10 μ g/ml and cells were collected at regular intervals for Northern blot analysis as described in the legend to Figure 4. Upper panel: autoradiograph of control and AdoMet-treated cells. Lower panel: results from densitometric analysis. Results are the ratios of the density of the TNF band to the density of the GAPDH band, and are expressed as percentages of the value at 0 min for each of the two treatments (□, control; ○, AdoMet). This is a representative example of three separate experiments.

administration of AdoMet ($n = 7$). TNF was undetectable in the serum of rats injected with saline. LPS-induced serum TNF concentrations (90 min post-LPS/lead acetate administration) in AdoMet-treated animals were significantly lower than those in the animals not receiving AdoMet (52.3 ± 7.09 compared with 69.0 ± 4.33 ng/ml; $P < 0.0005$). Hepatic AdoMet levels were the same in animals administered LPS plus lead acetate with or without exogenous AdoMet (96.9 ± 10.7 and 94.3 ± 14.4 nmol/mg protein respectively). No liver injury, as measured by serum transaminase levels, was detected in rats administered LPS, with or without AdoMet supplementation, or in rats injected with saline.

DISCUSSION

In the present study, AdoMet attenuated LPS-induced TNF concentrations in both the tissue culture and animal systems. In the tissue culture model, supplementation of RAW cells with exogenous AdoMet inhibited TNF expression at the transcriptional level. This conclusion was supported by the observations that AdoMet supplementation lowered both the amount of TNF protein released and the induction of TNF mRNA in response to LPS. In addition, the change in TNF mRNA was not attributed to its lower stability. In the animal model, the

administration of AdoMet resulted in 25% lower LPS-induced serum TNF levels. Injection of LPS plus lead acetate to normal, AdoMet-sufficient rats did not cause liver injury and evoked serum TNF levels that were comparable to those observed in AdoMet-sufficient rats in our previous study [15]. In that study, the administration of AdoMet to AdoMet-deficient animals markedly attenuated LPS-induced serum TNF levels. The present study shows that AdoMet deficiency might not be a prerequisite for exogenous AdoMet to affect serum TNF concentrations.

In AdoMet-treated RAW cells, we noted a 5-fold increase in intracellular AdoMet, although we did not examine the mechanisms by which this increase might have occurred. Such an increase could have resulted from either changes in its endogenous metabolism or from cellular uptake of the exogenous AdoMet. With regard to the cellular uptake of AdoMet, it should be noted that there is a disagreement over the ability of cells to incorporate exogenous AdoMet into intracellular pools. For example, a number of studies, including our own [15], have reported that exogenous AdoMet might be taken up by liver or isolated hepatocytes [25–27]. However, others have reported that exogenous AdoMet is not taken up by hepatocytes and that only its methyl group is incorporated into the phospholipid moiety of cellular membranes [28–30]. It is possible that the uptake of exogenous AdoMet by a cell might be affected by its concentration. Most of the above studies used rather low concentrations of AdoMet (at most 50 μ M), whereas in our studies on RAW cells a concentration of 1 mM AdoMet was used. In fact, one of these reports [30] did note an increase in intracellular AdoMet when concentrations of over 200 μ M were used.

The present work demonstrated that the inhibition of TNF release in AdoMet-treated RAW cells was not mediated by changes in the intracellular GSH pool. This conclusion was based on the observation that treatment with AdoMet did not affect GSH concentrations in RAW cells. This conclusion was further supported by incubating RAW cells with another pro-GSH molecule, NAC, to increase intracellular GSH concentrations. Although this treatment did increase GSH levels, it did not affect the amount of TNF secreted after stimulation with LPS. This finding is in contrast with reports that NAC can inhibit TNF release by LPS-stimulated peritoneal macrophages [20], Kupffer cells [21], and alveolar macrophages [22,23]. However, each of these studies employed primary cell cultures, whereas RAW cells are a transformed cell line and therefore might have a different susceptibility to changes in redox status. In addition, the NAC concentrations used in the cited studies ranged from 10 to 50 mM, compared with the 5 mM concentration used for RAW cells. We found that concentrations of NAC higher than 5 mM were toxic to RAW cells, but 5 mM NAC was sufficient to increase GSH concentrations and thus test the hypothesis that GSH modulates LPS-induced TNF released by RAW cells. These results demonstrate that TNF production by RAW cells is modulated by AdoMet administration, and that this modulation does not involve the conversion of AdoMet to GSH.

This study strongly suggests that the hepatoprotective effect of AdoMet in toxin-induced liver injury is mediated, at least in part, by the suppression of TNF expression and raises the possibility that other pathological conditions involving TNF might also be attenuated by AdoMet. TNF has been implicated in the aetiology of sepsis, cerebral malaria, rheumatoid arthritis and inflammatory bowel disease [31–34] and AdoMet might prove to be a useful therapeutic intervention for these patients as well. At the very least, a more detailed understanding of mechanisms of the TNF-modulating effects of AdoMet will lead to the identification of novel therapeutic targets.

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