S-adenosylmethionine (AdoMet) modulates endotoxin stimulated interleukin-10 production in monocytes

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S-adenosylmethionine (AdoMet) modulates endotoxin stimulated interleukin-10 production in monocytes. Am J Physiol Gastrointest Liver Physiol 284: G949–G955, 2003; 10.1152/ajpgi.00426.2002.—IL-10 is produced by a large variety of cells including monocytes, macrophages, B and T lymphocytes, as well as normal killer cells and is an important suppressor for both immunoproliferative and inflammatory responses. IL-10 exerts anti-inflammatory effects in the liver, and decreased monocyte synthesis of IL-10 is well documented in alcoholic cirrhosis. Intracellular deficiency of S-adenosylmethionine (AdoMet) is a hallmark of toxin-induced liver injury. Although the administration of exogenous AdoMet attenuates this injury, the mechanisms of its actions are not fully established. This study was performed to investigate the effect of exogenous AdoMet on IL-10 production in LPS-stimulated RAW 264.7 cells, a murine macrophage cell line. Our results demonstrated that exogenous AdoMet administration enhanced both protein production and gene expression of IL-10 in RAW 264.7 cells. Ethionine, an inhibitor for methionine adenosyltransferases, inhibited LPS-stimulated IL-10 both at the protein and mRNA levels. Exogenous AdoMet increased the intracellular cAMP concentration as early as 3 h and continued for 24 h after AdoMet treatment; however, the inhibitors for both adenylyl cyclase and PKA did not significantly affect IL-10 production. On the basis of these results, we conclude that AdoMet administration may exert its anti-inflammatory and hepatoprotective effects, at least in part, by enhancing LPS-stimulated IL-10 production.

inflammation; cytokines; liver injury

S-ADENOSYLMETHIONINE (SAM, SAm, or AdoMet) is produced from methionine and ATP by methionine adenosyltransferase (MAT). AdoMet is a key intermediate in the hepatic transsulfuration pathway and serves as a precursor for GSH as well as a methyl donor in most transmethylation reactions (12, 22). Clinical studies have reported that administration of stable salts of AdoMet has beneficial effects on many hepatic disorders ranging from cholestasis to alcoholic liver disease (36). Animal experiments have demonstrated that AdoMet has protective effects against a variety of hepatotoxic agents, including carbon tetrachloride, ethanol, and acetaminophen (4, 7, 20, 35). These hepatotoxins induce hepatic deficiencies of AdoMet and GSH. Although the mechanisms of its action are not fully established, one potential beneficial effect of AdoMet is attenuation of proinflammatory cytokine production by endotoxin (LPS)-stimulated monocytes/macrophages (6). TNF-α is a pleiotropic inflammatory cytokine and an important mediator of toxin-induced liver injury (2, 9, 17). It has recently been shown (38) that AdoMet supplementation to RAW 264.7, a murine macrophage cell line, decreases the amount of TNF-α released in the conditioned medium and the steady-state mRNA concentrations following LPS stimulation.

IL-10, initially named cytokine synthesis inhibitory factor, is an important suppressor of both immunoproliferative and inflammatory responses (30, 33). Exogenous IL-10 downregulates monocyte/macrophage effector functions including production of certain proinflammatory cytokines such as TNF-α and IL-1β (27). Inadequate monocyte production of IL-10 has been postulated to play a role in increased TNF production and subsequent liver injury in alcoholic liver disease (ALD) (18). In addition, IL-10 may also exert anti-inflammatory effects in the liver through inhibition of collagen gene transcription and increased collagenase expression by hepatic stellate cells (37). The effect(s) of AdoMet concentration (including exogenous administration of AdoMet) on IL-10 production in monocytes have not been examined. In the present study, we evaluated the effects of AdoMet both on IL-10 protein synthesis and gene expression in LPS-stimulated RAW cells.

METHODS

Materials. RAW 264.7 murine monocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA). AdoMet, as its 1,4-butanedisulphonate salt, was provided by Drs. R. O’Brian (Knoll Pharmaceuticals, Piscataway, NJ) and G. Stramentionoli (Knoll Farmaceutici, Milan, Italy). LPS (Escherichia coli O111:B4) was purchased from Difco Laboratories (Detroit, MI). Before use, LPS was dissolved in sterile, pyrogen-free water, sonicated, and diluted with sterilized HBSS. Penicillin, streptomycin, DMEM, tryp-

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sin, fetal bovine serum, and TRIzol reagent were purchased from Invitrogen (Grand Island, NY); 24- and 96-well plates were from Corning (Corning, NY); and murine IL-10, TNF-α, and cAMP ELISA kits were from Biosource International (Camarillo, CA). Purified hamster anti-mouse/rat TNF antibody, RiboQuant in vitro transcription kit, RiboQuant ribonuclease protection assay (RPA) kit, and mCK-2 RiboQuant mouse cytokine multi-probe template kit were from PharMingen (San Diego, CA). SQ 22536 and H-89 were from Calbiochem (San Diego, CA). All other reagents were of the highest purity available and, unless indicated otherwise, were obtained from Sigma (St. Louis, MO).

Cell culture. RAW 264.7 cells were cultured in DMEM containing 10% (vol/vol) fetal bovine serum, 2 mM glutamine, 5 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified O₂/CO₂ (19:1) atmosphere.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cell viability was assessed by examining cell number with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After treatments, cells were washed twice with PBS; then cell culture medium was removed and samples were suspended in 1 mg/ml MTT and incubated at 37°C for 2 h. After a 2-h incubation, 100 μl lysis buffer containing 20% SDS and 50% N,N-dimethylformamide were added and incubated at 37°C overnight. The optical density values were read at 570 nm

HPLC assay for intracellular AdoMet. The intracellular concentrations of AdoMet and S-adenosylhomocysteine (SAH) were measured as described in the method of Merali et al. (25). Cell pellets were mixed with 0.25 ml of 4% metaphosphoric acid (MPA) and centrifuged at 10,000 g for 2 min. The supernatants were collected for HPLC analysis. The HPLC system was equipped with a Waters 501 pump, a manual injector, and a 5-μm Hypersil C18 reverse-phase column (250 × 4.6 mm). The mobile phase consisted of 40 mM ammonium phosphate, 8 mM heptane sulfonic acid (ion-pairing reagent), and 6% acetonitrile (pH 5.0) and it was run isocratically at a constant rate of 1.0 ml/min. AdoMet and SAH were detected using a Waters 740 detector at 254 nm. The effects of exogenous AdoMet treatment on the release of IL-10 by LPS-stimulated RAW cell. The effect of exogenous AdoMet on IL-10 production by macrophages was determined by pretreating RAW cells with AdoMet for 2 h and then stimulating with LPS. After a number of preliminary studies in which the pretreatment period with AdoMet was varied from a few minutes to overnight, we designed this protocol to optimize the changes in LPS-stimulated IL-10 release by RAW cells without affecting their viability. Figure 2 summarizes the dose response of AdoMet-enhanced IL-10 release into the conditioned medium. Cells treated with 100 μM AdoMet demonstrated a significant increase in IL-10 production following stimulation by LPS. When the cells were pretreated with 1 mM AdoMet for 2 h, IL-10 production was increased to ~200% of that of control cells by 16 h after LPS stimulation. IL-10 production could not be detected in either untreated cells or those treated with 1 mM AdoMet without LPS (data not shown). The viability of these cells, measured by

ELISA assay for IL-10, TNF-α, and cAMP. IL-10 and TNF-α in conditioned medium and cAMP were quantified using ELISA kits in accordance with the manufacturer’s instructions. The detection limitation for IL-10 and TNF-α is 0.39 pmol/ml whereas samples for IL-10 assay were run undiluted, samples for TNF-α and cAMP were fivefold diluted. All assyas were run in triplicate.

RPA of IL-10 mRNA. Total RNA was isolated with TRIzol reagent (GIBCO-BRL) in accordance with the manufacturer’s instructions. IL-10 mRNA was assayed by RPA using RiboQuant multiprobe RNase protection system according to the manufacturer’s manual. Briefly, [32P]UTP-labeled RNA probe was prepared by in vitro transcription using multiprobe template set using mCK-2 as a template. Five to ten picograms of total RNA samples were hybridized overnight with purified RNA probe, after which free probe and other single-stranded RNA were digested with RNase A and T1 mix. The remaining RNase-protected probes were purified, resolved on 5% denaturing polyacrylamide gels, and quantitated by autoradiography.

Statistical analyses. All data are expressed as means ± SD. Statistical analysis was performed using one-way ANOVA and further analyzed by Newman-Keuls test for statistical difference. Differences between treatments were considered to be statistically significant at P < 0.05.

RESULTS

Effects of AdoMet treatment on intracellular AdoMet concentration. The effects of exogenous AdoMet treatment of RAW cells on intracellular concentrations of AdoMet are shown in Fig. 1. The basal concentration of AdoMet in RAW cells was 912.36 ± 253.6 pmol/mg protein and remained unchanged during the culture conditions used in these experiments. Treatment with 1 mM exogenous AdoMet resulted in a threefold elevation of intracellular AdoMet concentrations within 2 h of exposure. These levels remained elevated over 8 h.

Effects of AdoMet treatment on the release of IL-10 by LPS-stimulated RAW cell. The effect of exogenous AdoMet on IL-10 production by macrophages was determined by pretreating RAW cells with AdoMet for 2 h and then stimulating with LPS. After a number of preliminary studies in which the pretreatment period with AdoMet was varied from a few minutes to overnight, we designed this protocol to optimize the changes in LPS-stimulated IL-10 release by RAW cells without affecting their viability. Figure 2 summarizes the dose response of AdoMet-enhanced IL-10 release into the conditioned medium. Cells treated with 100 μM AdoMet demonstrated a significant increase in IL-10 production following stimulation by LPS. When the cells were pretreated with 1 mM AdoMet for 2 h, IL-10 production was increased to ~200% of that of control cells by 16 h after LPS stimulation. IL-10 production could not be detected in either untreated cells or those treated with 1 mM AdoMet without LPS (data not shown). The viability of these cells, measured by

![Fig. 1. Changes in intracellular S-adenosylmethionine (AdoMet) concentration in AdoMet-treated cells. Raw cells were plated at a density of 5 × 10⁵ cells/ml and incubated overnight; then cells were treated with or without (UT) the presence of 1 mM AdoMet. Cells were harvested at the indicated time points, and intracellular AdoMet concentrations were quantified by HPLC as described in METHODS. Values represent means ± SD from 3 separate experiments. *P < 0.05 compared with control cells.](http://ajpgi.physiology.org/DownloadedFrom/10220336byJune26,2017)
the conversion of MTT to formazan, was unaffected by concentrations of AdoMet up to 1 mM (90% viability). Treatment of cells with higher concentrations of AdoMet (e.g., 3 mM), however, significantly lowered cellular viability to 70% (data not shown).

Time course changes of LPS-stimulated IL-10 release in the absence or presence of 1 mM AdoMet. In the control cells (no AdoMet), IL-10 levels demonstrated a time-dependent increase during the 24-h culture period (from 34.99 ± 9.9 pg/ml in 2 h to 635.65 ± 48.45 pg/ml in 24 h) after LPS stimulation (Fig. 3). Compared with control cells, AdoMet-treated cells produced a higher level of IL-10 by 8 h after LPS stimulation. By 24 h after LPS stimulation, IL-10 production by AdoMet-treated cells reached almost twice that in control cells (635.65 ± 48.45 pg/ml in control cells vs. 1,113.53 ± 85.49 pg/ml in AdoMet-treated cells).

Effects of AdoMet and anti-IL-10 antibody on LPS-stimulated TNF production. As expected, exogenous AdoMet (2 mM) inhibited TNF production by >50% (23 ± 0.8 pg/ml in LPS treatment vs. 11.9 ± 1.3 pg/ml in AdoMet + LPS treatment) at 18 h after LPS stimulation. Pretreatment with anti-IL-10 antibody (10 μg/ml) modestly but significantly attenuated the TNF inhibitory effects of AdoMet (15.4 ± 0.6 pg/ml).

Effect of Ethionine and cycloleucine on IL-10 production by LPS-stimulated RAW cells. Ethionine is a competitive inhibitor of methyltransferases and induces a functional AdoMet deficiency. To evaluate whether AdoMet deficiency may inhibit LPS-stimulated IL-10 production, 3 mM ethionine was added 2 h before LPS stimulation. More than 75% of IL-10 production elicited by LPS was inhibited by ethionine treatment. The viability of RAW cells was not affected by treatment with 3 mM ethionine (viability >90%). Cycloleucine also induces functional AdoMet deficiency (inhibits nonhepatic MAT activity). Cycloleucine at 20 and 40 mM significantly decreased LPS-stimulated IL-10 production by 24 and 47%, respectively. Adding AdoMet significantly reversed the cycloleucine inhibition (data not shown).

Effects of exogenous AdoMet and ethionine on LPS-induced IL-10 gene expression. RTPAs were performed to determine whether the effects of AdoMet and ethionine on IL-10 expression could be attributed to

Fig. 2. Effect of AdoMet on LPS-stimulated IL-10 release. Cells were plated as described in the legend to Fig. 1. AdoMet was added to the culture medium at various concentrations; after 2-h 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 16 h. Conditioned media were collected, and IL-10 was quantified by ELISA as described in METHODS. Values represent means ± SD from 3 separate experiments and are expressed as fold of control value (no AdoMet). *P < 0.05 compared with control cells.

Fig. 3. Time course of IL-10 release by LPS-stimulated RAW cells in the presence and absence of AdoMet. Cells were plated as described in the legend to Fig. 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 analyzed for IL-10 concentration by ELISA. Values represent means ± SD from 3 separate experiments. *P < 0.05 compared with control cells.

Fig. 4. Time course of IL-10 release by LPS-stimulated RAW cells in the presence and absence of ethionine. Cells were plated as described in the legend to Fig. 1, then pretreated with 3 mM ethionine 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 analyzed for IL-10 concentration by ELISA. Values represent means ± SD from 3 separate experiments. *P < 0.05 compared with control cells.
changes in IL-10 mRNA levels following exposure to LPS. IL-10 mRNA was detected 4 h after stimulation in both control (LPS alone) and AdoMet-treated cells, but AdoMet-treated cells expressed higher levels of IL-10 mRNA than control cells (Fig. 5). Whereas it was undetectable at 24 h in control cells, IL-10 mRNA expression in AdoMet-treated cells was prolonged to at least 24 h after stimulation. Ethionine-treated cells expressed lower amounts of IL-10 mRNA than either control or AdoMet-treated cells by 8 h after LPS-stimulation, and IL-10 mRNA returned to undetectable levels in 24 h after stimulation.

**Role of cAMP pathway in the elevation of IL-10 production.** The effect of exogenous AdoMet on cytosolic cAMP concentrations in macrophages was determined by ELISA, and the results are summarized in Fig. 6. Cells treated with 1 mM AdoMet showed an increase in intracellular cAMP concentrations from a basal value of 8.84 ± 1.35 to 14.08 ± 0.78 pg/mg protein at 3 h. Intracellular levels of cAMP in AdoMet-treated cells remained significantly elevated for 24 h. To investigate whether the enhancement of IL-10 by AdoMet treatment resulted from increased intracellular cAMP concentration, the inhibitors for adenylyl cyclase and PKA were added to the medium before AdoMet administration, and the results are shown in Fig. 7. Either 1 mM SQ 22536 (inhibitor of adenylyl cyclase) or 1 μM H-89 (inhibitor of PKA) was added to the medium 2 h before AdoMet administration, and LPS was added 2 h later. AdoMet treatment elevated LPS-stimulated IL-10 production significantly. SQ 22536 pretreatment did not change the enhancement of IL-10 by AdoMet administration, whereas H-89 lowered the enhancement slightly.

**DISCUSSION**

IL-10 is a critical anti-inflammatory cytokine that inhibits the synthesis of proinflammatory cytokines in monocytes, macrophages, lymphocytes, and neutrophils (33). Exogenous administration of IL-10 has been shown to be hepatoprotective in multiple experimental models of liver injury such as LPS + galactosamine (28), *C. parvum* + LPS (32), and concanavalin A (Con A) (15). Exogenous IL-10 has also been used to decrease fibrosis in hepatitis C patients who are nonresponders to interferon therapy (29) and to control the inflammatory response during localized inflammation such as rheumatoid arthritis or systemic inflammation such as sepsis (8). With the use of IL-10 knockout mice, IL-10 has been shown to inhibit neutrophil infiltration and fibrosis in a carbon tetrachloride model of liver injury (21). Similarly, IL-10 knockout mice were shown to be more sensitive to acetaminophen hepatotoxicity, demonstrating the importance of endogenous anti-inflammatory pathways in attenuating toxin/xenobiotic-induced hepatotoxicity (3). Lastly, IL-10 knockout mice were shown to be more sensitive to alcohol-induced liver injury in a model of alcohol + LPS hepatotoxicity (14).

Patients with alcoholic cirrhosis have a well-characterized decrease in LPS-stimulated IL-10 production (18, 34). In vitro studies have suggested that decreased IL-10 production plays a major role in the dysregulated TNF metabolism observed in ALD (18). Patients with ALD have a significantly higher incidence of an IL-10 polymorphism (13). Mice chronically fed alcohol have decreased LPS-stimulated IL-10 production in conjunction with increased liver injury (14). Similarly, incubation of a macrophage cell line with alcohol on a chronic basis causes decreased LPS-stimulated IL-10 production (34). Thus there is substantial evidence implicating inadequate IL-10 levels in many types of experimental and clinical liver disease, and there is an emerging rationale for exogenous IL-10 therapy or...
used to study cytokine metabolism, it appears that AdoMet concentrations critically regulate LPS-stimulated IL-10 production. AdoMet also inhibits TNF production. With the use of anti-IL-10 antibody, we showed that a small, but significant, component of AdoMet’s anti-TNF activity may be mediated through enhanced IL-10 production.

Data on whether or not AdoMet can be taken up intact by cells are still controversial (10, 39). We observed an approximately threefold increase in intracellular AdoMet concentration after supplementation with 1 mM exogenous AdoMet, although we did not examine the mechanisms by which this increase occurred. Such an increase could have resulted from either a change in its endogenous metabolism or from cellular uptake of the exogenous AdoMet.

There are multiple potential mechanisms whereby exogenous AdoMet may increase LPS-stimulated IL-10, with one possibility being increased cAMP. The anti-inflammatory functions of cAMP-elevating agents have been demonstrated in several cell systems including inhibition of TNF synthesis and enhancement of IL-10 synthesis both in human and murine monocytes. Previous studies (1, 5, 26, 31) reported that dibutyryl cAMP, an exogenous cAMP, upregulated IL-10 production both in LPS-stimulated human monocytes and Con A-stimulated CD8+ T cell clones. Drugs that elevate intracellular cAMP (e.g., iloprost, pentoxifylline, prostaglandin E2) augmented LPS-induced IL-10 production both at the protein and mRNA levels in human monocytes (11, 16). We demonstrated for the first time that supplementation of exogenous AdoMet to RAW cells increased intracellular cAMP concentrations as early as 3 h after AdoMet treatment. To investigate whether enhancement of IL-10 production in RAW cells treated with AdoMet involved the cAMP pathway, inhibitors for both adenylyl cyclase and PKA were used. Inhibition of adenylyl cyclase had no effect on IL-10 production, whereas the inhibition of PKA lowered IL-10 production only slightly. Thus, although the cAMP levels increased, it seems unlikely that the cAMP pathway plays a major role in the enhancement of LPS-induced IL-10 production in RAW cells treated with AdoMet.

In conclusion, this research demonstrates that AdoMet concentrations critically regulate LPS-stimulated IL-10 production in monocytes and that AdoMet elevated protein synthesis and gene expression of IL-10 in LPS-stimulated RAW cells. Although the focus of our research and this discussion has been on liver disease, the implications of this work extend far beyond hepatic diseases to other processes ranging from sepsis to rheumatoid arthritis, where modulation of IL-10 appears to play an important role in disease activity/progression.

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