S-adenosylmethionine (SAMe) modulates interleukin-10 and interleukin-6, but not TNF, production via the adenosine (A2) receptor

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Abstract

S-adenosylmethionine (SAMe) is the first product in methionine metabolism and serves as a precursor for glutathione (GSH) as well as a methyl donor in most transmethylation reactions. The administration of exogenous SAMe has beneficial effects in many types of liver diseases. One mechanism for the hepatoprotective action is its ability to regulate the immune system by modulating cytokine production from LPS stimulated monocytes. In the present study, we investigated possible mechanism(s) by which exogenous SAMe supplementation modulated production of TNF, IL-10 and IL-6 in LPS stimulated RAW 264.7 cells, a murine monocyte cell line. Our results demonstrated that exogenous SAMe supplementation inhibited TNF production but enhanced both IL-10 and IL-6 production. SAMe increased intracellular GSH level, however, N-acetyl cysteine (NAC), the GSH pro-drug, decreased the production of all three cytokines. Importantly, SAMe increased intracellular adenosine levels, and exogenous adenosine supplementation had effects similar to SAMe on TNF, IL-10 and IL-6 production. 3-Deaza-adenosine (DZA), a specific inhibitor of S-adenosylhomocysteine (SAH) hydrolase, blocked the elevation of IL-10 and IL-6 production induced by SAMe, which was rescued by the addition of exogenous adenosine. Furthermore, the enhancement of LPS-stimulated IL-10 and IL-6 production by both SAMe and adenosine was inhibited by ZM241385, a specific antagonist of the adenosine (A\textsubscript{2}) receptor. Our results suggest that increased adenosine levels with subsequent binding to the A\textsubscript{2} receptor account, at least in part, for SAMe modulation of IL-10 and IL-6, but not TNF production, from LPS stimulated monocytes.

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1. Introduction

The primary purpose of inflammatory reactions is to eliminate a pathogenic insult and restore tissue integrity, but abnormal magnitude or duration of this process is deleterious to the host. Lipopolysaccharide (LPS), a product of Gram-negative bacteria, is one of the strongest triggers of inflammatory responses. In mammals, LPS elicits a potent reaction involving the generation of numerous inflammatory mediators, such as cytokines, from diverse cellular origins. The liver is the central organ for clearing LPS from the circulation and also responds to LPS stimulated cytokine production. Multiple experimental studies and clinical observations indicate that LPS, mainly from gastrointestinal tract, plays an etiologic role in liver injury [1–3].

S-adenosylmethionine (SAMe) is the first product in the methionine metabolism pathway resulting from methionine adenosyltransferase (MAT)-catalyzed conversion. Abnormal SAMe content has been linked to the development of experimental and human liver diseases, and the adminis-
tration of stable salts of SAMe has beneficial effects on many hepatic disorders ranging from cholestasis to alcoholic liver disease [4–7]. Although the mechanisms of hepatoprotection by SAMe are not fully established, modulation of the inflammatory response by regulating LPS stimulated monocyte cytokine production is one likely mechanism. The modulation of LPS stimulated cytokine production in monocytes by exogenous SAMe was first reported by our group [8] with the observation that SAMe supplementation to RAW 264.7 cells, a murine monocyte cell line, decreased tumor necrosis factor α (TNF) production and steady-state mRNA concentrations following LPS stimulation. Furthermore, our recent studies showed that exogenous SAMe supplementation to RAW 264.7 cells also increased LPS-stimulated protein production and gene expression of IL-10 and IL-6, two critical anti-inflammatory and hepatoprotective cytokines [9,10].

TNF, IL-10 and IL-6 play etiological roles in various types of liver disease. TNF, a pleiotropic inflammatory cytokine, is an important mediator in the development of liver diseases including alcoholic liver disease (ALD), nonalcoholic steatohepatitis (NASH), and the liver injury caused by a variety of toxins [11–14]. Interleukin-10 (IL-10), initially named cytokine synthesis inhibitory factor, is an important suppressor of both immunoproliferative and inflammatory responses. Exogenous IL-10 down-regulates monocyte/macrophage effector functions including the production of certain proinflammatory cytokines such as TNF [15,16]. 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) [17]. In addition, IL-10 may exert antifibrotic effects in the liver through the inhibition of collagen gene transcription and increased collagen expression by hepatic stellate cells [18]. Interleukin-6 (IL-6) is a multifunctional cytokine that has been classified as both a pro- and anti-inflammatory cytokine. However, current evidence suggests that IL-6 has primarily anti-apoptotic and anti-inflammatory effects. IL-6 has been shown to be protective in animal models of fulminant hepatic failure through mechanisms involving hepatocyte apoptosis [19]. Pretreatment with IL-6 protected both normal rats and IL-6-deficient mice from warm ischemia/reperfusion injury [20]. IL-6 induced hepatoprotection of steatotic liver isografts by preventing sinusoidal endothelial cell necroapoptosis and consequent amelioration of hepatic microcirculation disturbance, and protecting against hepatocyte death [21]. In addition to being an anti-apoptotic factor, recent reports have documented that IL-6 is a key factor in liver regeneration. IL-6 knockout mice also exhibit impaired liver regeneration which is reversed by IL-6 administration.

Although our previous studies have demonstrated that exogenous SAMe modulated protein production and gene expression of some critical cytokines involved in the pathogenesis of liver diseases, the mechanism(s) of this action are still unknown. Based on our previous results, the present study was carried out to investigate the possible mechanism(s) involved in the modulation by exogenous SAMe on the production of TNF, IL-10 and IL-6 in RAW cells stimulated by LPS.

2. Materials and methods

2.1. Cell culture

RAW 264.7 murine monocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7 cells were cultured in DMEM containing 10% (v/v) 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.

2.2. Compound

SAMe, as its 1,4-butanedisulfonate salt, was provided by Dr. Robert O’Brian (Knoll Pharmaceuticals Co., Piscataway, NJ, U.S.A.) and Dr. G. Stramentionoli (Knoll Farmaceutici, Milan, Italy). Lipopolysaccharide (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 Hanks’ balanced salt solution (HBSS). Penicillin, streptomycin, Dulbecco’s modified Eagle’s medium (DMEM), trypsin, and fetal bovine serum were purchased from Invitrogen Corporation (Grand Island, NY); 24-well and 96-well plates were from Corning Inc. (Corning, NY); and murine TNF-α, IL-10, IL-6 and cAMP ELISA kits were from Biosource International (Camarillo, CA). All other reagents were of the highest purity available and, unless indicated otherwise, and were obtained from Sigma (St. Louis, MO, U.S.A.).

2.3. MTT assay

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

2.4. HPLC assay for intracellular SAMe and adenosine

The intracellular concentrations of S-adenosylmethionine (SAMe) and adenosine were assayed by reverse-phase HPLC with deprotenized extracts of cells by a modified method of Merali et al. [22]. 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, 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 were run isocratically at a constant rate of 1.0 ml/min. SAMe and S-adenosylhomocysteine (SAH) were detected using a Waters 740 detector at 254 nm. Standard solutions of SAMe and SAH were prepared in 4% MPA. An internal standard, S-adenosylethionine (SAE), was added to all samples and standard solutions to a concentration of 100 nmol/ml. Protein concentrations were measured by protein assay kit from BioRad in accordance with the manufacturer’s instructions.

2.5. HPLC assay for intracellular glutathione (GSH)

Reduced glutathione (GSH), oxidized glutathione (GSSG) and cysteine in whole blood, cytosol and mitochondria were quantified by HPLC with electrochemical detection according to the method of Richie and Lang [23], with slight modifications. In brief, 20-μl samples were injected onto a reverse-phase C18 column (Val-U-Pak HP, fully endcapped ODS, 5 μm, 250×4.6 mm; ChromTech Inc.). The mobile phase, which consisted of a solution of 0.1 M monochloroacetic acid and 2 mM heptanesulfonic acid at pH 2.8 (98%) and acetonitrile (2%), was delivered at a flow rate of 1 mL/min. The compounds were detected in the eluant with a Bioanalytical Systems dual LC4B amperometric detector, using two Au/Hg electrodes in series with potentials of −1.2 V and +0.15 V for the upstream and downstream electrodes, respectively. Standard curves for the analytes were plotted as peak area versus concentration of the analyte.

2.6. ELISA assay for TNF, IL-10, IL-6 and intracellular cyclic AMP (cAMP)

TNF, IL-10 and IL-6 in conditioned medium were quantified using ELISA kits in accordance with the manufacturer’s instructions. The detection limitation for TNF, IL-10, IL-6 and cAMP are 13.5, 4.0, 4.0, and 0.39 pg/ml, respectively. Whereas the samples for the IL-6 and IL-10 assay were run undiluted, samples for TNF and cAMP were diluted 5-fold. All assays were run in triplicate.

2.7. Statistical analyses

All data are expressed as mean±S.D. Statistical analysis was performed using one-way ANOVA and further analyzed by Newman–Keul’s test for statistical difference. Differences between treatments were considered to be statistically significant at P<0.05.

3. Results

3.1. Effects of SAMe treatment on intracellular SAMe concentration

The effects of exogenous SAMe supplementation to RAW cells on intracellular concentrations of SAMe are shown in Table 1. Treatment with 1 mM exogenous SAMe resulted in an 8-fold elevation of intracellular SAMe concentrations within 2 h. These levels remained elevated over 8 h.

3.2. Effects of SAMe treatment on the release of TNF, IL-10 and IL-6 from LPS stimulated RAW cell

The effect of exogenous SAMe on TNF, IL-10 and IL-6 production by LPS stimulated RAW cells was determined by pre-treating cells with SAMe for 2 h and then stimulating with 100 ng/ml LPS. Fig. 1 summarizes the dose-responses of TNF, IL-10 and IL-6 production. Whereas SAMe pretreatment decreased TNF production in a dose-response fashion following LPS stimulation (A), both IL-10 (B) and IL-6 (C) production was increased by SAMe in a dose-dependent manner. Compared to untreated cells, cells treated with up to 1 mM SAMe without LPS did not show significant changes in TNF, IL-10 and IL-6 production (data not shown). The viability of these cells, measured by MTT assay, was unaffected by concentrations of SAMe up to 1 mM (more than 90% viability).

3.3. Is GSH involved in the modulation of SAMe on production of TNF, IL-10 and IL-6?

The effect of exogenous SAMe on intracellular GSH concentrations in RAW cells was determined by HPLC as described in Materials and methods and the results are

Table 1
Effect of exogenous SAMe supplementation on intracellular SAMe level (nmol/mg protein)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td>0.216±0.04a</td>
<td>0.205±0.09b</td>
<td>0.229±0.06b</td>
<td>0.283±0.1b</td>
<td>0.238±0.08b</td>
</tr>
<tr>
<td>SAMe</td>
<td>0.209±0.09a</td>
<td>1.686±0.129b</td>
<td>2.22±0.313b</td>
<td>2.015±0.451b</td>
<td>0.591±0.05b</td>
</tr>
</tbody>
</table>

SAMe at 1 mM was added and left in the medium. Samples were collected at different time points for HPLC assay.

a Data represent mean±S.D.

b Statistically significant from corresponding UT (P<0.01).
summarized in Fig. 2A. The basal concentration of GSH in RAW cells was approximately \(7.5 \pm 0.8\) nmol/mg protein and remained unchanged during the culture conditions used in these experiments. Treatment with 1 mM exogenous SAMe resulted in a minor, but significant elevation of intracellular GSH concentrations within 8 h of exposure and remained elevated over 24 h. To investigate whether the modulation of SAMe on TNF, IL-10 and IL-6 by SAMe resulted from its effects on intracellular GSH level, the effects of exogenous N-acetylcysteine (NAC) on the production of these cytokines were evaluated by pre-treating RAW cells with different concentrations of NAC for 2 h and then stimulating with LPS. Fig. 2B shows the results of
TNF, IL-10 and IL-6 levels 16 h after LPS stimulation. Whereas NAC lowered LPS-stimulated TNF and IL-6 production in a dose-dependent manner, both doses of NAC decreased IL-10 production mildly but significantly in comparison to cells treated with LPS alone, but there was no difference between two NAC doses.

3.4. Effects of SAMe supplementation on intracellular adenosine concentration

The effect of SAMe on intracellular adenosine concentrations was determined by HPLC as described in Materials and methods and the results are summarized in Fig. 3. The basal concentration of adenosine in RAW cells was below the detectable limitation of our HPLC system. Treatment with 1 mM exogenous SAMe resulted in a significant elevation of intracellular adenosine concentration within 2 h of SAMe addition which peaked at 8 h.

3.5. Is adenosine involved in the modulation of SAMe on production of TNF, IL-10 and IL-6?

To investigate the possibility that the modulation of SAMe on TNF, IL-10 and IL-6 resulted from its effects on intracellular adenosine level, exogenous adenosine at 1 mM was added to the media for 2 h before LPS stimulation and its effects on the cytokine production were determined and results were summarized in Fig. 4. Whereas exogenous adenosine lowered LPS-stimulated TNF production, both IL-10 and IL-6 production was significantly increased in comparison to cells treated with LPS alone, which mimicked SAMe’s effects.

DZA is a specific inhibitor of SAH hydrolase which catalyzes the hydrolysis of SAH to homocysteine and adenosine, therefore the addition of DZA to the medium can inhibit the production of adenosine from SAMe. The effect of DZA on SAMe-modulated production of TNF, IL-10 and IL-6 from LPS-stimulated RAW cells were shown in Fig. 5. The presence of DZA in the media further down-regulated SAMe-modulated TNF decrease and addition of adenosine could not rescue the decrease induced by DZA (Fig. 5A). In contrast to TNF production, the elevation of both IL-10 and IL-6 production by SAMe was blocked by DZA and the addition of adenosine partially rescued SAMe function inhibited by DZA (Fig. 5B and C).

3.6. The effect of SAMe supplementation on intracellular cAMP level

The effect of exogenous SAMe on cytosolic cAMP concentrations in RAW cells was determined by ELISA, and the results are summarized in Fig. 6. Cells treated with 1 mM SAMe showed an increase in intracellular cAMP concentrations after 3 h and intracellular levels of cAMP in SAMe-treated cells remained significantly elevated for at least 12 h.

3.7. Antagonist of adenosine receptor A2 abolished modulation of SAMe and adenosine on LPS-stimulated IL-10 and IL-6 production, but had no effect on TNF

To determine whether or not the adenosine (A2) receptor (AR), a subtype of ARs mainly expressed on monocytes/macrophages, was involved in the enhancement of SAMe on LPS-stimulated TNF, IL-10 and IL-6 production, ZM241385, a selective antagonist of the A2 receptor, was added to the media at a dose of 30 μM before LPS stimulation and results are shown in Fig. 7. ZM241385 itself had no effect on LPS-stimulated TNF, IL-
10 and IL-6 production from monocytes. While ZM241385 did not alter the decrease of LPS stimulated TNF production by SAMe, it inhibited the elevation of both IL-10 and IL-6 production resulting from SAMe pretreatment. To verify that this inhibition by ZM241385 was due to its antagonism on A2 receptor, RAW cells were pretreated with adenosine (1 mM) for 2 h, and then stimulated with LPS in the absence or presence of adenosine. Our results showed that ZM241385 not only completely inhibited the enhancement of IL-10 and IL-6 production by adenosine pretreatment, it also inhibited the decrease of LPS-stimulated TNF production resulting from adenosine treatment.

4. Discussion

SAMe, synthesized from methionine and ATP in a reaction catalyzed by methionine adenosyltransferase (Fig. 8), is a key intermediate in the hepatic transsulfuration pathway and serves as a precursor for glutathione (GSH) as well as a methyl donor in most transmethylation reactions. Methyl groups from SAMe are added to methyl acceptors and SAMe is converted to S-adenosylhomocysteine (SAH) which is converted to homocysteine and adenosine in a reaction catalyzed by SAH hydrolase. In the liver, homocysteine can be metabolized to cysteine, a rate-limiting amino acid for glutathione synthesis, by the transsulfuration pathway, or used for the re-synthesis of methionine [24]. The administration of SAMe in different experimental models of liver injury has been shown to attenuate tissue damage and to improve survival. Since inflammatory reactions are implicated in most of liver diseases alleviated by SAMe, increasing evidence has emerged that the beneficial effects of SAMe could result from its capacity to modulate inflammatory reactions [4–7]. Although our previous studies have shown that SAMe modulated TNF, IL-10 and IL-6 production from LPS stimulated monocytes/
macrophages, the mechanism(s) behind these effects are not completely known. Considering that some of the metabolites in the SAMe metabolism pathway may influence cytokine metabolism, it is conceivable that some of the effects of SAMe may be attributed to its conversion to one or more of its metabolites. For example, a very recent study has reported that 5'-methylthioadenosine (MTA), a metabolite in the methionine/SAMe metabolism pathway, recapitulated SAMe’s effect on TNF production in both RAW cells and Kupffer cells [25]. In the present study we investigated the possible involvement of other metabolites of SAMe, particularly glutathione and adenosine, in the modulation of SAMe on TNF, IL-10 and IL-6 production from LPS stimulated RAW cells.

Extensive evidence shows that intracellular redox status (GSH level) modulates cytokine production in LPS stimulated monocytes/macrophages [26,27]. In our study, we observed a mildly increased intracellular GSH level after SAMe supplementation. To determine the contribution of elevated intracellular GSH to SAMe-modulated TNF, IL-10 and IL-6 production, we examined the effects of exogenous N-acetylcysteine (NAC), a widely used GSH precursor, on LPS stimulated production of these three cytokines. It was clearly demonstrated that exogenous NAC decreased the production of all cytokines examined in current study, indicating that the enhancement of intracellular GSH levels by SAMe did not account for its modulation on IL-10 and IL-6 production, which were increased by SAMe. Although both SAMe and NAC decreased TNF production, it is unlikely that the enhancement of GSH by SAMe plays a major role in decreasing TNF production considering the concentrations of NAC and SAMe we used, the magnitude of GSH enhancement by SAMe, and the magnitude of TNF decrease by both SAMe and NAC.

Adenosine is another metabolite in the SAMe metabolism pathway which may be involved in SAMe modulation on TNF, IL-10 and IL-6 production because both in vivo and in vitro studies have demonstrated that adenosine exhibits potent anti-inflammatory properties. Adenosine inhibited LPS-induced TNF-α [28] production and enhanced IL-10 [29] secretion by human monocytes. Signaling of these effects is mainly attributed to the
activation of the A2 receptor; a subtype of adenosine receptors expressed on the cells of immune system, and elevated cAMP levels [30]. In the current study, we observed that exogenous SAMe increased intracellular adenosine and cAMP concentration and that exogenous adenosine treatment demonstrated a similar pattern of modulation on TNF, IL-10 and IL-6 production to SAMe.

Adenosine is a direct product of SAH hydrolysis through a reaction catalyzed by SAH hydrolase (Fig. 8). The inhibition of SAH hydrolase would block the production of adenosine by this pathway even in the presence of precursors, like SAMe. To verify the involvement of adenosine, we used DZA, a specific inhibitor of SAH hydrolase, to block adenosine production. Our data clearly demonstrated that the blockade of adenosine production prevented the elevation of both IL-10 and IL-6 induced by SAMe, which could be partially rescued by the addition of adenosine, indicating that increased intracellular adenosine level induced by SAMe is involved in its modulation on IL-10 and IL-6 production. Since the initial step of adenosine signaling is the activation of the adenosine A2 receptor, a subtype of adenosine receptors expressed on the cells of immune system, we used ZM241385, a selective antagonist of A2 receptor [31], which was added 30 min before LPS stimulation and our data showed that ZM241385 completely blocked the influence of adenosine on the production of the three cytokines from RAW cells stimulated with LPS, indicating that A2 receptor is the central receptor responsible for adenosine regulated cytokine production from monocytes/macrophages. Whereas ZM241385 also completely inhibited the enhancement of LPS-stimulated IL-10 and IL-6 production by SAMe, it had no effect on the decrease of TNF levels, suggesting that the modulation of IL-10 and IL-6, but not TNF production by SAMe treatment in monocytes was A2 receptor-mediated. Although the possibility exists that SAMe could bind and activate A2 receptor directly, ZM241385 was added to the media at 90 min after SAMe treatment in our in vitro model, and thus it seems unlikely that SAMe acts as a receptor agonist.

In conclusion, the present study demonstrated that SAMe modulated TNF, IL-10 and IL-6 production from LPS stimulated RAW cells by different mechanisms. We have shown that SAMe increased IL-10 and IL-6 production by activating the adenosine (A2) receptor which is involved, at least in part, in increased intracellular adenosine levels. The exact mechanism(s) of SAMe modulation on TNF metabolism in monocytes/macrophages remains to be further investigated.

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