S-Adenosylmethionine Decreases Lipopolysaccharide-Induced Phosphodiesterase 4B2 and Attenuates Tumor Necrosis Factor Expression via cAMP/Protein Kinase A Pathway

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ABSTRACT

S-Adenosylmethionine (SAM) treatment has anti-inflammatory, cytoprotective effects against endotoxin-induced organ injury. An important component of the anti-inflammatory action of SAM involves down-regulation of the lipopolysaccharide (LPS)-induced transcriptional induction of tumor necrosis factor-α (TNF) expression by monocytes/macrophages. We examined the effect of SAM on expression and activity of LPS-induced up-regulation of phosphodiesterase 4 (PDE4), which regulates cellular cAMP levels and TNF expression. LPS treatment of RAW 264.7, a mouse macrophage cell line, led to the induction of Pde4b2 mRNA expression with no effect on Pde4a or Pde4d. SAM pretreatment led to a significant decrease in LPS-induced Pde4b2 expression. Moreover, these results demonstrate for the first time that inhibition of LPS-induced PDE4B2 up-regulation and increased cAMP-dependent PKA activation are significant mechanisms contributing to the anti-TNF effect of SAM. Moreover, these data also suggest that SAM may be used as an effective PDE4B inhibitor in the treatment of chronic inflammatory disorders in which TNF expression plays a significant pathogenic role.

Introduction

Lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, functions as the prototypical endotoxin causing the systemic inflammatory response syndrome, endotoxic shock, disseminated intravascular coagulation, and multiple organ failure. LPS-induced tissue injury and lethality are caused mainly by tumor necrosis factor-α (TNF), which is produced primarily by cells of the monocyte/macrophage lineage. Increased production of inflammatory cytokines such as TNF has been shown to play a dominant pathogenic role in multiple inflammatory disorders including arthritis, heart disease, and liver injury (Young et al., 2002; Tilg et al., 2006; Meshkani and Adeli, 2009; Gonzalez-Gay et al., 2010; Schaible et al., 2010). Hence, trimethylation on the Pde4b2 intronic promoter region. The SAM-mediated decrease in LPS-inducible Pde4b2 up-regulation resulted in an increase in cellular cAMP levels and activation of cAMP-dependent protein kinase A (PKA), which plays an inhibitory role in LPS-induced TNF production. In addition, SAM did not affect LPS-inducible inhibitor of nuclear factor-κB degradation or nuclear factor-κB (NF-κB)-p65 translocation into the nucleus but rather inhibited NF-κB transcriptional activity. These results demonstrate for the first time that inhibition of LPS-induced PDE4B2 up-regulation and increased cAMP-dependent PKA activation are significant mechanisms contributing to the anti-TNF effect of SAM. Moreover, these data also suggest that SAM may be used as an effective PDE4B inhibitor in the treatment of chronic inflammatory disorders in which TNF expression plays a significant pathogenic role.
appropriate regulation of LPS-inducible TNF production may play an important role in the development of clinical interventions aimed to reduce the many pathological conditions linked to TNF and is a target of various immunopharmacological strategies.

Work done by our group and others has shown that S-adenosylmethionine (SAM) supplementation can significantly attenuate LPS-induced TNF expression in monocytes/macrophages and Kupffer cells (Watson et al., 1999; Veal et al., 2004; Song et al., 2005), and this constitutes a major component of the ability of SAM to attenuate inflammation-related organ injury (Lieber et al., 1990; Barve et al., 2006). SAM is found in all living cells, where it functions as a methyl group donor in more than 100 different reactions catalyzed by methyltransferase enzymes (Dryden, 1999). As a principal methyl donor, SAM supports trans-methylation reactions and the synthesis and modification of several key cellular components including proteins, lipids, RNA, and DNA (Mato et al., 1997). Moreover, SAM controls essential metabolic pathways by regulating several important enzymatic reactions including those involved in polyamine biosynthesis and trans-sulfuration leading to GSH synthesis. Although the mechanisms of action of SAM have not been fully elucidated, our earlier work indicates that the ability of SAM to decrease TNF production in LPS-stimulated monocytes/macrophages probably involves increased cellular cAMP (Song et al., 2005). Increasing the cellular cAMP level with different types of cAMP enhancers has invariably resulted in the suppression of TNF production either in vitro or in vivo in LPS-stimulated monocytes/macrophages of both human and murine origin (Zidek, 1999; Jin and Conti, 2002; Jin et al., 2005; Ouagued et al., 2005; Gobejishvili et al., 2008).

Intracellular levels of cAMP as well as cGMP are precisely regulated by the coordinated control of its rate of synthesis via adenylyl cyclase activity and its rate of degradation via a large family of phosphodiesterases (PDEs). There are 11 different members of the mammalian class I PDE super family, (PDE1–PDE11), which through the complexity formed by multiple genes within the families and a large number of PDE splicing variants serve to fine-tune cyclic nucleotide signals and contribute to specificity in the functional responses of cells to a variety of extracellular stimuli (Conti and Beavo, 2007; Houslay, 2010). Distinct kinetic and functional properties are attributed to PDE families with some specific hydrolyzing cAMP (PDE4, 7, and 8), some both cAMP and GMP (PDE1, 2, 3, 10, and 11), and others only cGMP (PDE5, 6, and 9) (Conti and Beavo, 2007; Houslay, 2010). Of the cAMP-specific PDEs, the PDE4 family is widely expressed and is the current therapeutic target of selective inhibitors for the treatment of inflammatory diseases, such as asthma and chronic obstructive pulmonary disease, as well as depression and cognitive deficits (Spina, 2008; Houslay, 2010). PDE4 represents a large complex family, with four genes (PDE4A/B/C/D) encoding more than 20 distinct PDE4 isoforms as a consequence of distinct promoter usage and mRNA splicing; of note, these isoforms have similar catalytic activities but distinct cellular functions. The distinct cellular functions exist because of differences in specific intracellular targeting and signaling complex formation with various binding partners, which generate the temporal and compartmentalized dynamics of cAMP levels (Conti and Beavo, 2007; Houslay, 2010).

In monocytes/macrophages, LPS-mediated activation of TLR-4, a transmembrane LPS receptor, leads to downstream signaling, which plays a critical role in the production of TNF (Beutler and Rietschel, 2003). Of importance, in monocytes/macrophages that predominantly express isoforms of PDE4 A, B, and D, it has been established that PDE4B is involved in LPS-induced signaling mediated by TLR-4 and is essential for LPS-induced TNF expression (Jin and Conti, 2002; Jin et al., 2005). Several studies including ours have shown that LPS-inducible TNF production by monocytes is markedly decreased when PDE activity is blocked by PDE4-specific inhibitors (Kwak et al., 2005; Ouagued et al., 2005; Gobejishvili et al., 2008). Peritoneal and lung macrophages, as well as peripheral leukocytes from Pde4b knockout mice, showed significantly attenuated production of TNF in response to LPS, whereas the responses of Pde4d(--/-) mice were similar to those of wild-type mice (Jin and Conti, 2002). Moreover, LPS stimulation of mouse peritoneal macrophages resulted in up-regulation of only Pde4b, and Pde4b(--/-) mice were protected from LPS-induced shock (Jin et al., 2005). Hence, in the present work we examined the effects of SAM on LPS-induced PDE4B expression and activity as a potential mechanism underpinning the anti-TNF activity of SAM. The results obtained demonstrate for the first time that SAM can significantly suppress LPS-induced up-regulation of PDE4B expression/activity, increase cellular cAMP levels, and decrease LPS-induced TNF expression. Furthermore, in conjunction with increasing cellular cAMP levels, SAM increases PKA activity and decreases NF-κB transcriptional activity, both of which play a significant role in down-regulating LPS-induced TNF expression.

Materials and Methods

Materials. The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA). LPS (Escherichia coli 0111:B4) was purchased from Difco (Detroit, MI). Before use, LPS was dissolved in sterile, pyrogen-free water, sonicated, and diluted with sterilized phosphate-buffered saline. Stabilized SAM salt (S-(5′-adenosyl)-L-methionine p-toluensulfonate salt), phospho-CREB (Ser133), CREB antibodies, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin, streptomycin, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA); the murine TNF-α ELISA kit was from BioSource International (Camarillo, CA). N-[2-p-Bromocinnamylaminoethyl]-5-isoquinolinesulfonamide (H-89) and p65 antibody were from Enzo Life Sciences (Farmingdale, NY), and β-actin antibody was from Cell Signaling Technology (Danvers, MA). IgG and phospho-PKA IIa reg (Ser96) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies specific for mouse PDE4B2 was a generous gift from Dr. Marco Conti (University of California, San Francisco, CA).

Isolation of Human CD14+ Monocytes from Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells from healthy volunteers were isolated by Ficoll-Paque PREMIUM (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient centrifugation. CD14+ monocytes were further purified by positive selection using magnetic cell sorting CD14+ microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) as described by the manufacturer. Purity of CD14+ cells was determined using a FACSCanto II flow cytometry system (BD, Franklin Lakes, NJ).
Cell Culture and Treatments. RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium and CD14+ monocytes were cultured in RPMI 1640 medium, both containing 10% (v/v) fetal bovine serum, 10 U/ml penicillin, and 10 μg/ml streptomycin at 37°C in a humidified 5% CO2 atmosphere. For RNA extraction and PDE assay, cells were plated at 2 × 106 per well in 2 ml of medium in six-well plates and treated for 2 or 4 h after LPS stimulation, respectively. For Western blot analysis and chromatin immunoprecipitation assay, cells were plated in 100-mm dishes at a density of 8 × 106 per dish in 10 ml of medium. For TNF ELISA, 24-well plates were used, in which cells were plated at 0.6 × 106 per well in 1 ml of medium and treated for 8 h with LPS. In all experiments, cells were pretreated with SAM for 16 h, and LPS was used at a concentration of 100 ng/ml.

RNA Isolation and Real-Time PCR Analysis. Total RNA was isolated from RAW 264.7 cells using TRIzol reagent (Invitrogen) and treated with DNase I to remove any contaminating genomic DNA (RQ1 RNase-Free DNase; Promega, Madison, WI). For real-time PCR, the first-strand cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD). The reverse transcription was performed using 10 ng of total RNA. The reverse transcription conditions were 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR was performed in triplicate with an ABI Prism 7500 sequence detection system and PerfeCTa SYBR Green II (Quanta Biosciences). Mouse 18S rRNA and TNF-specific primers were purchased from Superarray (S2) and the untreated sample (S1) calculated as fold change

<table>
<thead>
<tr>
<th>Primer</th>
<th>Mouse Pde4a pan-specific</th>
<th>Mouse Pde4b pan-specific</th>
<th>Mouse Pde4d pan-specific</th>
<th>Mouse Pde4b1, 3, 4 limited isoform-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5'-GCAGCTCTTCTTGGAGAAGTC-3'</td>
<td>5'-GCCACCTTCGAAAGTTAGA-3'</td>
<td>5'-GAGTCTCCAGGCTTCCAGCTCA-3'</td>
<td>5'-GCCACCTTCGAAAGTTAGA-3'</td>
</tr>
<tr>
<td>R1</td>
<td>5'-GTTATCCCTCCACCATGCTCA-3'</td>
<td>5'-GCGTCCACACCTCTTGGAG-3'</td>
<td>5'-GAAGTCTCCAGGCTTCCAGCTCA-3'</td>
<td>5'-GAGTCTCCAGGCTTCCAGCTCA-3'</td>
</tr>
<tr>
<td>F2</td>
<td>5'-GCAGCTCTTCTTGGAGAAGTC-3'</td>
<td>5'-GCCACCTTCGAAAGTTAGA-3'</td>
<td>5'-GAGTCTCCAGGCTTCCAGCTCA-3'</td>
<td>5'-GCCACCTTCGAAAGTTAGA-3'</td>
</tr>
<tr>
<td>R2</td>
<td>5'-GTTATCCCTCCACCATGCTCA-3'</td>
<td>5'-GCGTCCACACCTCTTGGAG-3'</td>
<td>5'-GAAGTCTCCAGGCTTCCAGCTCA-3'</td>
<td>5'-GAGTCTCCAGGCTTCCAGCTCA-3'</td>
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ΔCt[normalized ChIP] = C[ChIP] – C[Input] – log2(input dilution factor)

The data are presented as fold difference between the treated sample (S2) and the untreated sample (S1) calculated as fold change = 2^ΔΔCt[S2–S1]. The ΔΔCt = ΔCt[S2–S1] = ΔCt[S2: normalized ChIP] – ΔCt[S1: normalized ChIP].

Cytoplasmic and Nuclear Protein Extraction. Cells were washed twice with phosphate-buffered saline and lysed by hypotonic shock in cytoplasmic extraction buffer (10 mM HEPES, pH 7.8, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, protease inhibitor cocktail, and phosphatase inhibitors). The cells were collected by scraping and the nuclei were separated from the cytosolic proteins by low-speed centrifugation at 1500g for 5 min at 4°C. The supernatant (cytoplasmic extract) was stored at −70°C. The nuclear pellet was resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.8, 520 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.2% Nonidet P-40, protease inhibitor cocktail, and phosphatase inhibitors) and incubated on ice for 60 min. The samples were centrifuged at 100,000g for 15 min at 4°C, and the supernatant containing nuclear proteins was stored in small aliquots at −70°C until further use. The protein concentration in extracts was measured using Bio-Rad Dye Reagent (Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer’s protocol.

Western Blot Analysis. Proteins (30–50 μg) were analyzed by SDS-polyacrylamide gel electrophoresis using a Bio-Rad Laborato-
ries electrophoresis system, followed by immunoblotting according to the manufacturer’s instructions. Immunoreactive bands were visualized using enhanced chemiluminescence light detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Detection of β-actin served as a loading control. Quantification was performed with Scion Image analysis software (Scion Corporation, Frederick, MD).

PDE4 Enzymatic Assay. PDE4-specific enzymatic activity was determined using a PDE4 assay kit (FabGennix International Inc., Frisco, TX) as described previously (Gobejishvili et al., 2008). In brief, cells were lysed after treatment using SoliBuffer (FanGennix International Inc.), and 25 μg of protein was used per assay. Assays were performed in duplicate; the final concentration of CAMP and rolipram was 2 and 10 μM, respectively. PDE4 activity was estimated from the difference between total and rolipram-resistant PDE activity. Linearity of the enzyme activity was assessed over the range from 500 pg to 5 ng of cAMP hydrolyzed/min/mg protein, achieved by adding 2 to 7 μg of partially purified PDE4A5. Rolipram at 100 μM inhibited 95 to 98% of the PDE4 activity of partially purified PDE4 enzymes.

Plasmids and Transfections. An NF-κB-luciferase reporter construct was obtained from Clontech (Mountain View, CA). RAW 264.7 cells were transfected with NF-κB-luciferase reporter plasmids using Lipofectamine LTX reagent (Invitrogen). Cells were seeded in 24-well plates for 24 h before transfection. For transfection, 0.3 μg of total DNA was used per well. Cells were cotransfected with a β-galactosidase expression vector as a control. After transfection, cells were incubated in a humidified atmosphere of 5% CO2 at 37°C for 24 h. After 24 h, the transfected cells were treated with SAM (0.25–1 mM) overnight (16 h) and stimulated with LPS (100 ng/ml) for 2 h. After treatment, total RNA was extracted and analyzed for TNF mRNA expression by real-time PCR. As expected, LPS strongly induced TNF (Fig. 1A). A linear mixed-effects model (see Materials and Methods) was fitted to the relative expression values, with fitted regression curves plotted in Fig. 1B, which showed that SAM pretreatment significantly attenuated LPS-induced TNF expression in a dose-dependent manner. The estimated slope for the effect of SAM pretreatment on relative expression of LPS-induced TNF expression was −64.71 (95% confidence interval −46.66 to −82.75), suggesting an ∼50% decrease in LPS-induced TNF mRNA per 0.77 mM SAM. In most of the subsequent experiments SAM was used at a concentration of 0.75 mM.

**Results**

SAM Pretreatment Decreases LPS-Induced TNF Expression in a Dose-Dependent Manner. The effect of SAM supplementation on TNF mRNA expression in RAW 264.7 cells was determined by pretreating cells with different doses of SAM (0.25–1 mM) for 16 h and further stimulating them with LPS (100 ng/ml) for 2 h. After treatment, total RNA was extracted and analyzed for TNF mRNA expression by real-time PCR. As expected, LPS strongly induced TNF (Fig. 1A). A linear mixed-effects model fitted to the relative expression values, with fitted regression curves plotted in Fig. 1B, which showed that SAM pretreatment significantly attenuated LPS-induced TNF expression in a dose-dependent manner. The estimated slope for the effect of SAM pretreatment on relative expression of LPS-induced TNF expression was −64.71 (95% confidence interval −46.66 to −82.75), suggesting an ∼50% decrease in LPS-induced TNF mRNA per 0.77 mM SAM. In most of the subsequent experiments SAM was used at a concentration of 0.75 mM.

![Fig. 1](https://example.com/figure1.png)  
**Fig. 1.** Effect of SAM pretreatment on LPS-induced TNF expression in RAW cells. A, TNF mRNA in UT and LPS-treated cells 2 h after LPS stimulation was quantified using real-time PCR. ***p < 0.001 compared with UT. B, cells were pretreated with increasing concentrations of SAM (0.25–1 mM) overnight (16 h) and stimulated with LPS for 2 h before RNA extraction. TNF mRNA was quantified using real-time PCR. Expression values are presented as percentages relative to LPS-induced expression (which was set to 100%) without SAM treatment. Each experiment is plotted with a different symbol, and the fitted regression line is shown.
**TABLE 2**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PDE4 Activity</th>
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<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pmol cAMP hydrolyzed/min/mg protein</td>
<td>% relative to UT</td>
<td>pmol cAMP hydrolyzed/min/mg protein</td>
<td>% relative to UT</td>
</tr>
<tr>
<td>UT</td>
<td>13.76 (100)</td>
<td>10.49 (100)</td>
<td>9.14 (100)</td>
<td></td>
</tr>
<tr>
<td>LPS (100 ng/ml)</td>
<td>24.11 (175.2)</td>
<td>16.13 (153.8)</td>
<td>17.88 (195.6)</td>
<td></td>
</tr>
<tr>
<td>SAM (0.75 mM)</td>
<td>9.38 (68.2)</td>
<td>8.81 (84)</td>
<td>5.31 (58.1)</td>
<td></td>
</tr>
<tr>
<td>SAM (0.75 mM) + LPS</td>
<td>17.92 (130.3)</td>
<td>12.87 (122.7)</td>
<td>10.02 (109.6)</td>
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</table>

**SAM Pretreatment Attenuates PDE4 Activity.** Suppression of LPS-induced TNF expression in macrophages can be achieved by inhibition of PDE4, which is critical for LPS signaling and TNF expression (Jin and Conti, 2002; Jin et al., 2005). Hence, we examined whether the SAM effect on TNF was a result of decreased PDE4 activity. Cells were pretreated with 0.75 mM SAM for 16 h followed by stimulation with LPS (100 ng/ml) for 4 h. Cellular extracts were assayed for PDE4-specific activity. The time point for PDE4 activity was selected on the basis of previously established expression optima (Gobejishvili et al., 2008). As expected, LPS stimulation led to the induction of PDE4 activity. Of note, SAM pretreatment markedly attenuated both LPS-induced up-regulation and basal PDE4 activity (Table 2).

**Effect of SAM on LPS-Inducible PDE4 Isoforms and cAMP Levels in Murine Macrophages.** Previous studies in primary mouse macrophages have shown that PDE4B is the predominant LPS-inducible PDE (Jin and Conti, 2002; Jin et al., 2005). To assess whether the same PDE4 subfamily is induced in RAW 264.7 macrophages upon LPS stimulation, cells were treated with LPS for 2 h, total RNA was extracted, and Pde4a, Pde4b, and Pde4d mRNAs were quantified by real-time PCR. In agreement with earlier observations, we found that only Pde4b was induced by LPS (Fig. 2A). Subsequently we screened for the expression of isoforms Pde4b1 to Pde4b5 in response to LPS and found that Pde4b2 was the predominant LPS-induced Pde4b isoform (Fig. 2B). No expression was detected for Pde4b isoforms 1, 3, or 4 and LPS-inducible Pde4b5 mRNA was <1% of the Pde4b2 level.

We then examined the effect of SAM on LPS-induced expression of PDE4B2 mRNA and protein. PDE4B2 mRNA and protein were quantified in cells pretreated with SAM and stimulated with LPS. In agreement with its effects on the LPS-induced PDE4 activity, SAM decreased the up-regulation of both PDE4B2 mRNA and protein expression (Fig. 3, A and B) and resulted in a significant increase in LPS-induced intracellular cAMP levels (Fig. 3C). Significant differences in Pde4b2 mRNA were found between LPS-induced and untreated cells (p < 0.001) and between LPS-induced cells and cells pretreated with SAM at 0.25 mM (p = 0.03), 0.5 mM (p = 0.001), 0.75 mM (p < 0.001), and 1.0 mM (p < 0.001) (linear mixed model) (Supplemental Table 3). Figure 3A plots the fitted regression curves from the linear mixed-effects model, for expression values relative to LPS-induced expression. The estimated slope for the effect of SAM pretreatment on relative expression of LPS-induced Pde4b2 expression was −37.4 (95% confidence interval –22.24 to –52.62) (Supplemental Table 3).

**Effect of SAM on LPS-Induced Up-Regulation of TNF and PDE4B2 in Human CD14+ Monocytes.** The effect of SAM on LPS-induced TNF and PDE4B2 expression was also examined in primary human monocytes. CD14+ monocytes were purified from human peripheral blood mononuclear cells as described under Materials and Methods and treated with different concentrations of SAM and subsequent stimulation with LPS. SAM was able to significantly blunt LPS induction of TNF mRNA (Fig. 4A; Supplemental Table 4), PDE4B2 mRNA (Fig. 4B; Supplemental Table 4), and PDE4 activity (Table 3). The effect of SAM pretreatment on LPS-induced up-regulation of TNF and PDE4B2 mRNA expression was even more marked in CD14+ cells compared with that in RAW cells, with levels of 0.25 mM resulting in >50% reduction of mRNA (Fig. 4, A and B).

**Effect of SAM on the Methylation Status of Histone H3 Associated with the Pde4b2 Promoter Region.** Histone lysine methylation is an important posttranslational modification that specifies the transcriptional state of a gene (Kouzarides, 2002). In relation to the inhibition of gene transcription, a distinct link between histone H3 lysine 9 (H3K9) trimethylation (H3K9Me3) and transcriptional repression has been established (Kouzarides, 2002; Hublitz et al., 2009). Furthermore, relevant to the present work, it has been demonstrated that the promoter H3K9 trimethylation causing transcriptional repression occurs in a SAM-dependent manner (Wang et al., 2003). Hence, we examined the effect of SAM treatment on the H3K9 trimethylation of the mouse Pde4b2 intronic promoter region as a potential mechanism underlying its repressive effect on the Pde4b2 mRNA expression. The mouse Pde4b2 promoter location was defined by

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**Fig. 2.** LPS induces only the Pde4b2 isoform in RAW cells. Cells were stimulated with LPS for 2 h before RNA extraction. mRNA was quantified using real-time PCR and is represented as fold change over UT as described under Materials and Methods. A, induction of Pde4a, Pde4b, and Pde4d mRNA in response to LPS. B, induction of Pde4b2 mRNA in response to LPS. Data are presented as mean ± S.D. (n = 3). **+, p < 0.001 compared with UT.
sequence similarity to the rat Pde4b2 promoter (Monaco et al., 1994) and the 5' terminus of the mouse REFSSEQ mRNA NM_001177980.1. The effect of SAM treatment on H3K9 methylation at the Pde4b2 promoter was examined by ChIP analysis using an H3K9Me3-specific antibody and the promoter region primer pairs shown in Fig. 5A. Regions I and II interrogated for H3K9Me3 status are located within 700 bp upstream of the transcriptional start site (TSS), whereas regions III and IV are located within 400 bp downstream of the TSS in the 5' untranslated region. In comparison with the SAM untreated cells, a distinct increase in the trimethylation of H3K9 in all four regions of the Pde4b2 intronic promoter was observed in SAM-treated cells, under both basal and LPS-stimulated conditions (Fig. 5B). Of note, the greatest increases in the SAM-mediated H3K9Me3 levels were observed in region III, which overlaps the transcriptional start site. Commensurate with the LPS-inducible transcriptional activation of the Pde4b2 gene, in SAM untreated cells, there was a gradual decrease in H3K9Me3 levels at 30 and 60 min upon LPS stimulation. In comparison, in SAM-treated cells, the H3K9Me3 levels remained unchanged at 30 min.
and showed a decline only at 60 min after LPS stimulation. It should be noted that, because the basal H3K9Me3 levels were higher in SAM-treated cells, the net levels of H3K9Me3 after LPS stimulation remained significantly higher than those in SAM-untreated cells. These data strongly suggest that the increase in SAM-dependent histone H3K9 trimethylation associated with the Pde4b2 intronic promoter region leads to the transcriptional repression and consequent decrease in the LPS-inducible Pde4b2 mRNA expression.

**Role of PKA on SAM-Mediated Decrease in LPS-Induced TNF Expression.** Because PDE4 inhibition and the consequent increase in cAMP levels leads to activation of cAMP-dependent PKA, we examined the effect of SAM supplementation on PKA activation. Cells pretreated with SAM were stimulated with 100 ng/ml LPS, and cytoplasmic and nuclear lysates were obtained for Western blot analysis. SAM pretreatment did not alter the nuclear levels of LPS-induced p65, demonstrating that there was no effect on NF-κB activation (Fig. 7A, compare lanes 2 and 3). After degradation, IκBα returned to the basal level in 60 min in both SAM-treated and untreated cells (Fig. 7A, lanes 2, 3 and 6, 7). These data show that SAM pretreatment does not affect LPS-induced IκBα proteolysis. The effect of SAM on LPS-induced NF-κB activation, as indicated by the nuclear translocation of p65, was also examined. Nuclear proteins extracted from cells pretreated with 0.75 mM SAM followed by stimulation with LPS (100 ng/ml) for 30 min were examined by Western blot analysis. SAM pretreatment did not alter the nuclear levels of LPS-induced p65, demonstrating that there was no effect on NF-κB activation (Fig. 7B).

**SAM Down-Regulates LPS-Stimulated NF-κB Transcriptional Activity.** Because LPS-induced NF-κB activation was not altered, the effect of SAM on LPS-stimulated NF-κB transcriptional activity was examined. Cells were transiently transfected with a reporter construct carrying a luciferase gene under the control of an NF-κB promoter containing three tandem repeats of the κB sequence (κB-luc). LPS stimulation for 6 h increased NF-κB-dependent transcription approximately 4-fold. Of note, in contrast to its effect on NF-κB activation, SAM pretreatment significantly attenuated LPS-stimulated NF-κB activity (Fig. 8). These results are in agreement with our earlier findings (Gobejishvili et al., 2006, 2008), showing that increased cellular cAMP levels achieved by PDE4 inhibition have no effect on LPS-inducible NF-κB activation but can significantly decrease transcriptional stimulation by NF-κB.

**Discussion**

An important characteristic of SAM is its ability to reduce the production of the inflammatory cytokine TNF, which
plays a critical pathogenic role in multiple clinical disorders mediated by inflammation. Work from our research group and others has demonstrated that exogenous SAM supplementation can attenuate endotoxin-stimulated TNF production both in vitro and in vivo and can also attenuate inflammation-related organ injury in vivo (Lieber et al., 1990; Watson et al., 1999; Song et al., 2005; Barve et al., 2006). In particular, SAM supplementation has been shown to signif-

Fig. 6. Inhibition of PKA reverses the SAM effect on LPS-induced TNF expression. A, cells were pretreated with SAM (0.75 mM) and stimulated with LPS for 30 min. Cytoplasmic lysates were analyzed for phospho-PKA (pPKA, S96) and (B) nuclear phospho-CREB (pCREB, S133) levels by Western blotting (a representative Western blot is shown). β-Actin and total CREB were used as loading controls. C, cells were pretreated with SAM (0.75 mM) in the presence and absence of PKA inhibitor H-89 (10 μM) and stimulated with LPS for 2 h before RNA extraction. TNF mRNA was quantified using real-time PCR and is represented as fold change over UT. Data are presented as mean ± S.D. (n = 3). *, p < 0.05 compared with LPS; #, p < 0.05 compared with S+LPS. D, cells were pretreated with SAM (0.75 mM) in the presence and absence of PKA inhibitor H-89 (10 μM) and stimulated with LPS for 8 h. Cell-free supernatants were assayed for TNF using an ELISA kit. Data are presented as mean ± S.D. (n = 3). *, p < 0.05 compared with LPS; #, p < 0.05 compared with S+LPS. S, SAM.

Fig. 7. SAM does not affect LPS-induced IκBα degradation and p65 translocation into nucleus. A, RAW cells were treated with SAM (0.75 mM) and stimulated with LPS for the indicated treatments and times. Cytoplasmic extracts were prepared, and Western blot analysis was performed by using anti-IκBα antibody. β-Actin was used as a loading control (a representative Western blot is shown). B, cells pretreated with SAM were stimulated with LPS for 30 min. Nuclear lysates were collected, and p65 levels were analyzed by Western blotting (a representative Western blot is shown); β-actin was used as a loading control.
Effect of SAM on LPS-Induced PDE4B and TNF Expression

Fig. 8. Down-regulation of LPS-induced NF-κB transcriptional activity by SAM (S). Cells were transfected with a luciferase reporter construct containing the NF-κB-responsive IκB promoter and a β-galactosidase control plasmid as described under Materials and Methods. The transfected cells pretreated with SAM (0.75 mM) were stimulated with LPS for 6 h. Cytoplasmic extracts were prepared and assayed for luciferase activity. A β-galactosidase enzyme assay was performed on the same lysates to normalize for transfection efficiency. Data are presented as mean ± S.D. (n = 3), **, p < 0.01 compared with UT; ***, p < 0.001 compared with LPS-stimulated.

significantly attenuate LPS-induced TNF expression in monocytes/macrophages and Kupffer cells (Veal et al., 2004). Our previous research showed that SAM leads to an increase in cellular cAMP levels in monocytes, which correlates with the decrease in LPS-inducible TNF expression (Song et al., 2005). In addition, we have shown that decreased cellular cAMP levels caused by increased PDE4B expression play a causal role in LPS-induced TNF expression in ethanol-activated priming of monocytes/macrophages (Gobejishvili et al., 2006, 2008). On the basis of these observations, it was important to examine the impact of SAM on the LPS-inducible PDE4 expression and activity and resultant cellular cAMP levels, as a potential mechanism for its anti-inflammatory effect.

As observed previously, SAM supplementation resulted in a dose-dependent decrease in LPS-induced TNF expression (Figs. 1B and 4A). Examination of the potential involvement of cellular cAMP levels and PDE4B expression as a mechanism of SAM anti-inflammatory function showed that SAM effectively decreased LPS-induced up-regulation of PDE4B expression; in particular, SAM decreased PDE4B mRNA and protein expression and increased cellular cAMP levels (Figs. 3 and 4B). SAM is the principal biological methyl donor and mediates the methylation of histones and DNA, which can have a tremendous affect on the epigenetic regulation of regional and global gene activities. Histone methylation plays a distinctive role in the regulation of both transcriptional activation and repression of genes (Kouzarides, 2002). In the context of transcriptional repression and site-specific methylation events, a causal link between promoter-associated H3K9Me3 and transcriptional repression has been established (Hublitz et al., 2009). Of note, this H3K9 trimethylation and transcriptional repression occurs via specific histone methyl transferases in a SAM-dependent manner (Wang et al., 2003). Hence, alterations in the transcriptionally repressive histone methylation modifications on the Pde4b2 intronic promoter region were examined as a potential mechanism underlying SAM-mediated suppression of Pde4b2 mRNA expression (Fig. 5). The data obtained in Fig. 5 strongly suggest that SAM treatment could be affecting H3K9-specific histone methyl transferases, causing the observed increase in the promoter-associated H3K9 trimethylation and leading to the suppression of LPS-induced up-regulation of PDE4B mRNA expression.

The observed anti-inflammatory mechanism of SAM is supported by findings that demonstrated the critical role of PDE4B2 in LPS-induced TNF expression in mouse macrophages (Jin et al., 2005). In addition, these observations are in agreement with studies demonstrating that decreasing PDE4 activity with specific inhibitors causes a significant down-regulation in LPS-induced TNF expression and an inflammatory response in vitro and in vivo (Kwak et al., 2005; Ouagued et al., 2005; Hertz et al., 2009). Taken together, these observations strongly suggest that inhibition of LPS-induced up-regulation of PDE4B2 plays a major role in the ability of SAM to attenuate LPS-induced TNF production and related organ injury.

Suppression of TNF production mediated by inhibition of PDE4B is cAMP-dependent and requires PKA (Jin et al., 2005). Work examining the role of PKA-anchoring proteins has further emphasized the critical role of PKA in the anti-inflammatory effects of cAMP and down-regulation of LPS-induced TNF expression (Wall et al., 2009). We show here that SAM-mediated inhibition of LPS-induced up-regulation of PDE4B led to an increase in cellular cAMP and activation of PKA (Figs. 3C and 6A and B). The role for PKA in the SAM-mediated decrease in LPS-inducible TNF expression was further supported by the observation that PKA inhibition reverses the SAM-inhibitory effect on TNF expression (Fig. 6, C and D). These data strongly suggest that cAMP-dependent PKA activation plays a significant role in attenuating LPS-induced TNF expression and the anti-inflammatory mechanism of SAM. Although increased cellular cAMP levels and downstream signaling decrease LPS-inducible TNF expression, it is significant to note that work done by Beavo’s group (Hertz et al., 2009) shows that during differentiation of monocytes/macrophages, in the absence of LPS, enhanced cellular cAMP levels caused by agonists can lead to a large increase in the expression of several proinflammatory chemokines.

Numerous studies have demonstrated the critical role of NF-κB in the LPS-inducible transcriptional induction of the TNF gene (Haas et al., 1990; Müller et al., 1993; Barnes and Karin, 1997). LPS mediates its effects on NF-κB nuclear translocation by the targeted phosphorylation and subsequent degradation of IκBα, which results in the release of p65 from the IκBα-p65 complex and its translocation into the nucleus, DNA binding, and transactivation of target genes such as TNF. Examination of the effect of SAM on LPS-induced IκBα proteolysis and NF-κB nuclear translocation showed that none of these events is affected by SAM (Fig. 7). Furthermore, in agreement with previous work (Veal et al., 2004), we showed that SAM inhibited NF-κB transcriptional activity, as demonstrated by the suppression of the LPS-inducible NF-κB promoter reporter activity (Fig. 8). Taken together, these data suggest that SAM does not affect LPS-inducible TLR-4-mediated signaling events involved in NF-κB activation and nuclear translocation but rather...
influences the nuclear events leading to NF-κB-dependent transcription. It is likely that the SAM-mediated increase in cellular cAMP caused by inhibition of LPS-induced up-regulation of PDE4B2 contributes to its anti-NF-κB effects. Our earlier work along with several other observations shows that cAMP represses NF-κB-mediated transcription and TNF mRNA expression without affecting NF-κB activation/DNA binding activity (Ollivier et al., 1996; Newman et al., 1998; Shames et al., 2001; Gobejishvili et al., 2006, 2008). Work examining the anti-TNF effects of cAMP showed that LPS-induced c-fos is stabilized by cAMP, leading to the accumulation of the c-Fos protein, which directly interacts with p65 and impedes its recruitment to the TNF promoter and decreases transcription (Koga et al., 2009). The possibility that LPS/cAMP-mediated effects on c-fos play a role in decreasing TNF expression in SAM-treated cells is currently being investigated.

The results of this study demonstrate for the first time that the anti-inflammatory mechanism of SAM can be attributed primarily to its ability to inhibit LPS-induced PDE4B2 activity and expression, increase cellular cAMP levels, and modulate PKA and NF-κB activity. Interest in inhibition of PDE4 activity as a potential therapeutic approach has been increasing in the last decade because of its significant role in regulating inflammatory responses (Spina, 2008). Indeed, PDE4 inhibitors have been demonstrated to possess both immunomodulatory and anti-inflammatory actions; however, despite reports of the efficacy of PDE4 inhibitors, their therapeutic application is hampered by the presence of significant dose-limiting side effects, including the gastrointestinal side effects of nausea, vomiting, and diarrhea and the development of vascular injury (Spina, 2008). In contrast, SAM has been used in several clinical studies and has a very favorable safety profile, comparable to that of placebo (Bottilogeri, 2002). Hence, the present findings showing that SAM effectively decreases LPS-induced PDE4 gene expression and activity and TNF production, along with its safe tolerability profile, strongly support its therapeutic potential in the treatment of inflammatory disorders.

Authorship Contributions

Participated in research design: Gobejishvili, McClain, and Barve. Conducted experiments: Gobejishvili, Avila, Barker, Ghare, and Kirpich.

Contributed new reagents or analytic tools: Barker and Henderson. Performed data analysis: Gobejishvili, Barker, and Brock.

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References


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