

5'-Methylthioadenosine Modulates the Inflammatory Response to Endotoxin in Mice and in Rat Hepatocytes

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5'-methylthioadenosine (MTA) is a nucleoside generated from S-adenosylmethionine (AdoMet) during polyamine synthesis. Recent evidence indicates that AdoMet modulates *in vivo* the production of inflammatory mediators. We have evaluated the anti-inflammatory properties of MTA in bacterial lipopolysaccharide (LPS) challenged mice, murine macrophage RAW 264.7 cells, and isolated rat hepatocytes treated with pro-inflammatory cytokines. MTA administration completely prevented LPS-induced lethality. The life-sparing effect of MTA was accompanied by the suppression of circulating tumor necrosis factor- α (TNF- α), inducible NO synthase (iNOS) expression, and by the stimulation of IL-10 synthesis. These responses to MTA were also observed in LPS-treated RAW 264.7 cells. MTA prevented the transcriptional activation of iNOS by pro-inflammatory cytokines in isolated hepatocytes, and the induction of cyclooxygenase 2 (COX2) in RAW 264.7 cells. MTA inhibited the activation of p38 mitogen-activated protein kinase (MAPK), c-jun phosphorylation, inhibitor kappa B alpha (I κ B α) degradation, and nuclear factor κ B (NF κ B) activation, all of which are signaling pathways related to the generation of inflammatory mediators. These effects were independent of the metabolic conversion of MTA into AdoMet and the potential interaction of MTA with the cAMP signaling pathway, central to the anti-inflammatory actions of its structural analog adenosine. In conclusion, these observations demonstrate novel immunomodulatory properties for MTA that may be of value in the management of inflammatory diseases. (HEPATOLOGY 2004;39:1088–1098.)

Inflammatory reactions occur in response to a variety of pathogenic insults, including microbial products, tissue injury, and tumor cells. The primary purpose of this physiological reaction is to eliminate the pathogenic

insult and restore tissue integrity. However, when the magnitude or the duration of this response exceeds certain limits, consequences are always deleterious to the host. Therapeutic strategies aimed at the modulation of inflammation are thus of primary interest.

Abbreviations: LPS, bacterial lipopolysaccharide; TNF- α , tumor necrosis factor alpha; AdoMet, S-adenosylmethionine; iNOS, inducible NO synthase; IL-10, interleukin 10; MTA, 5'-methylthioadenosine; EMSA, electrophoretic mobility shift assay; MTAP, 5'-methylthioadenosine phosphorylase; PKA, protein kinase A; COX2, cyclooxygenase 2; I κ B α , inhibitor kappa B alpha; NF κ B, nuclear factor kappa B; MAPK, mitogen activated protein kinase.

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One of the most vigorous inflammatory responses is that triggered by bacterial products, such as lipopolysaccharide (LPS).¹ In mammals, LPS elicits a potent reaction involving the generation of numerous inflammatory mediators of diverse cellular origin such as cytokines, arachidonic acid metabolites, reactive oxygen species, and nitric oxide (NO).^{2,3} Among these, tumor necrosis factor- α (TNF- α) is a pro-inflammatory cytokine recognized as a central mediator of endotoxemia and other inflammatory processes, being also a key determinant in the induction of NO synthesis in response to LPS.^{4,5} Systemic exposure to LPS is frequently found in patients with liver cirrhosis, correlating with the degree of liver failure.³ The liver plays a central role in clearing LPS from the circulation and also responds to LPS, producing inflammatory cytokines and mediators.^{3,6} In this regard, multiple experimental evidence (animal models of liver damage including choline deficiency, CCl₄, and ethanol administration) and clinical observations indicate that LPS, mainly derived from the gastrointestinal tract, has an etiological role in liver injury.^{3,6,7}

Administration of S-adenosylmethionine (AdoMet; also abbreviated as SAME and SAM) in different experi-

mental models of liver injury has been shown to attenuate tissue damage and to improve survival in patients.^{8–10} An inflammatory component is a common denominator to many of the hepatotoxic conditions in which AdoMet alleviates liver injury,^{11–16} and there is increasing evidence indicating that the modulation of such an inflammatory response could be behind the beneficial effects of AdoMet. In this regard, it has been recently reported that AdoMet inhibits LPS-induced TNF- α production and inducible NO synthase (iNOS) expression *in vivo* and stimulates interleukin-10 (IL-10) production in macrophages.^{16–18} AdoMet displays a remarkable array of biological effects and is emerging as an important regulator of many cellular responses, including gene expression, cell proliferation, differentiation, and apoptosis; however, the molecular mechanisms behind these effects are not completely known.¹⁹ AdoMet, synthesized from L-methionine and ATP by the action of methionine adenosyltransferases (MAT), is the main methyl-group donor in methylation reactions, serves as a glutathione precursor in the liver, and participates in the synthesis of polyamines.^{19–21} During the synthesis of polyamines, decarboxylated AdoMet is converted into 5'-methylthioadenosine (MTA);²¹ MTA can also be formed from the spontaneous splitting of AdoMet under physiological conditions.²² It has been observed that MTA retains many of the effects of AdoMet, such as its antiproliferative and antineoplastic actions, regulation of apoptosis, and the prevention of CCl₄-induced damage in rat liver.^{23–26} Consequently, it is conceivable that some of the effects of AdoMet may be attributed to its conversion, either spontaneous or metabolic, into MTA. In the present work, we have addressed the immunomodulatory potential of MTA using a murine endotoxic shock model, as well as in RAW 264.7 macrophages and isolated hepatocytes. Our data demonstrate a potent anti-inflammatory profile for MTA.

Materials and Methods

Animal Experiments. Male C57BL6 mice (19–21 g of weight) were from Harlan (Barcelona, Spain). Studies were approved by the University of Navarra Committee on Animal Care and satisfied National Institutes of Health guidelines for humane treatment of animals. Mice were injected with endotoxin (*Salmonella typhimurium* LPS, Sigma, St. Louis, MO) dissolved in sterile, pyrogen-free saline. Mice were given 15 mg/kg LPS intraperitoneally (ip). Blood was collected and sera obtained at different times after LPS administration. Survival was monitored up to 96 hours after injections. Where indi-

cated, a single dose of 96 μ mol/kg ip MTA (Knoll Farmaceutici, Liscate, Italy) dissolved in dimethylsulfoxide (50 μ l/mouse) or vehicle was administered 30 minutes before LPS injection. This dose of MTA has been previously used in models of liver damage in rats.²⁶ No modifications in any of the parameters studied were observed in controls. Survival experiments were performed three times independently, all groups included 20 animals. Liver and lung tissues were removed at the indicated time points after treatments, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

Cell Culture. Murine macrophage-like RAW 264.7 cells and human A549 lung carcinoma cells were from the American Type Culture Collection (ATCC). Mouse Kupffer cells were isolated as described previously.²⁷ All cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine and antibiotics (Life Technologies). Rat hepatocytes were isolated from male Wistar rats (200 g of weight) by collagenase perfusion and cultured in Minimum Essential Medium (Life Technologies) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine and antibiotics as described previously.¹⁸ Cell viability was measured by Trypan blue exclusion. No significant differences were observed between controls and any of the treatments.

Treatments were performed for the indicated periods of time with 500 μ mol/L MTA, unless otherwise stated, (30 minutes before LPS, 1 μ g/mL, or cytokines) or with a mixture of pro-inflammatory cytokines (CM): human recombinant interleukin 1 β and TNF- α (100 UI/mL each) (Genzyme, Boston, MA).

Cytokine Measurements. TNF- α and IL-10 concentrations in serum and conditioned media were measured using enzyme-linked immunosorbent assay (ELISA) assay kits from BD Biosciences (San Diego, CA) and Biosource International (Camarillo, CA), respectively.

RNA Extraction and Northern Blot Analysis. Total RNA extraction and Northern blot analysis were carried out as reported previously.²⁴ The iNOS probe has been described,¹⁸ and the mouse TNF- α cDNA probe was generated by RT-PCR using the primers 5'-GAGTGA-CAAGCCTGTAGCCC-3' and 5'-CCCTTCTCCAGC-TGGAAGAC-3'. Blots were hybridized with an 18S rRNA probe as loading control.

Immunoblot Analysis. Tissues and cells were homogenized and Western blot analyses were performed as described previously.^{18,24} Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Equal loading was demonstrated with an anti-actin polyclonal antibody (Calbiochem, Darmstadt, Germany).

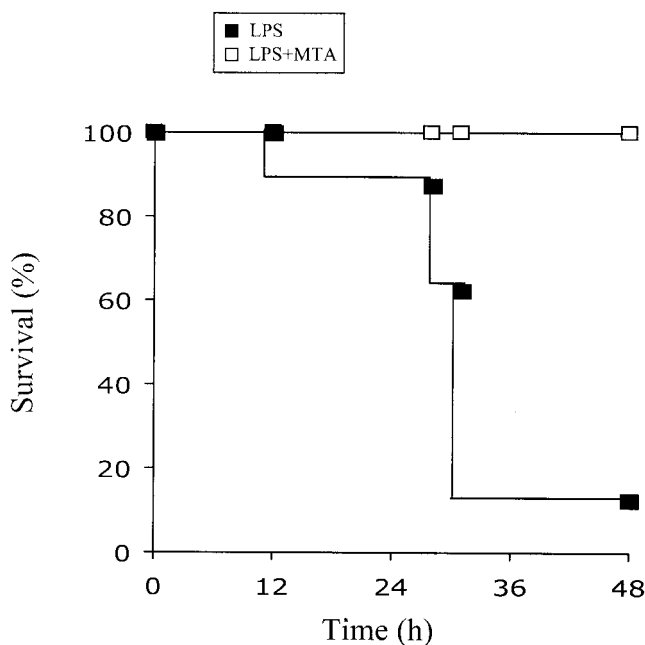


Fig. 1. MTA treatment prevents LPS-induced death in mice. Mice were injected with a single dose of MTA or vehicle followed 30 minutes later by a lethal dose of LPS.

Preparation of Nuclear Protein Extracts and Electrophoretic Mobility Shift Assay (EMSA). Nuclear protein extraction from hepatocytes and binding reactions were as described previously.¹⁸ DNA-protein complexes were resolved by electrophoresis on 4% polyacrylamide gels. The oligonucleotides used have been described.¹⁸

Transient Transfection of Rat Hepatocytes in Culture. Transfections were performed with 6.5 μ g of the reporter plasmid pGL3 NOS-8.3 (kindly provided by Dr. Joel Moss, NIH, Bethesda, MD), which harbors 8.3 kb of human iNOS promoter,²⁸ using the Tfx50 reagent (Promega, Madison, WI). After 36 hours, medium was changed and cells were pretreated with MTA. CM treatment was carried out for 24 hours, then cells were harvested and luciferase activity measured. Transfection efficiency was determined by cotransfection with the plasmid pRL-TK (1 μ g) (Promega). Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Determination of Nitrite Levels. NO production was measured as the accumulation of nitrite in culture medium after 24 hours of the onset of treatments as described previously.¹⁸

Statistics. Data are the means \pm SEM of at least three independent experiments performed in triplicate. Representative blots are shown. Statistical significance was estimated with Student's *t* test. A *P* value of less than .05 was considered significant.

Results

Effect of MTA on LPS-Induced Lethal Shock in Mice. Injection of LPS resulted in a 48-h survival rate of only 10%. In contrast, pretreatment with a single dose of MTA granted complete protection (100% survival rate at 48 hours, and no animal died thereafter for a total follow-up time of 96 h; Fig. 1).

Large quantities of NO, derived from the high capacity iNOS, are generated during Endotoxemia.²⁹ We determined the levels of iNOS protein in the lungs and liver of mice treated with LPS. iNOS expression was potently induced in both organs (Fig. 2), while pretreatment with a single dose of MTA blunted this effect.

We measured the levels of circulating TNF- α 90 minutes after LPS administration in mice that had been pretreated or not with MTA. Figure 3A shows that MTA completely prevented the elevation of TNF- α in serum. We also observed an increase in circulating IL-10 levels after LPS administration (Fig. 3B). Pretreatment with MTA resulted in a significant augmentation of LPS-induced IL-10 (Fig. 3B).

MTA Modulates the Response of RAW 264.7 Macrophages to LPS. We next wanted to know if these systemic responses to MTA administration could be accounted for by the direct action of MTA on LPS target cells. RAW 264.7 cells were pretreated with increasing concentrations of MTA, treated with LPS for 6 hours, and TNF- α levels were determined in conditioned media. Figure 4A shows how MTA dose-dependently inhibited TNF- α production. To gain insight into the mechanism of action of MTA, TNF- α mRNA levels were measured. Figure 4B shows how MTA reduced the steady-state levels of TNF- α mRNA in RAW 264.7 cells at all times tested.

We examined the effect of MTA on LPS-induced IL-10 production by RAW 264.7 cells. Figure 4C shows how MTA treatment resulted in a dose-dependent potentiation of LPS-induced IL-10 levels.

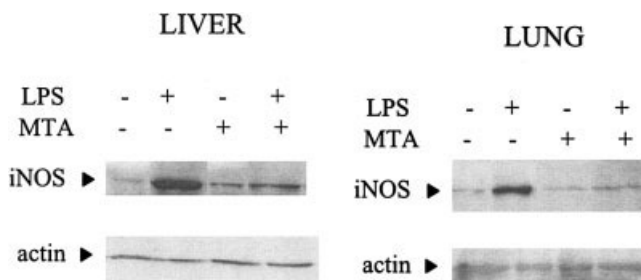


Fig. 2. MTA prevents the induction of iNOS gene expression in the liver and lungs of LPS-treated mice. Mice ($n = 6$ per group) were injected a single dose of MTA or vehicle, followed 30 minutes later by a dose of LPS. Six hours later, mice were killed. iNOS protein levels were determined in liver and lung by Western blotting.

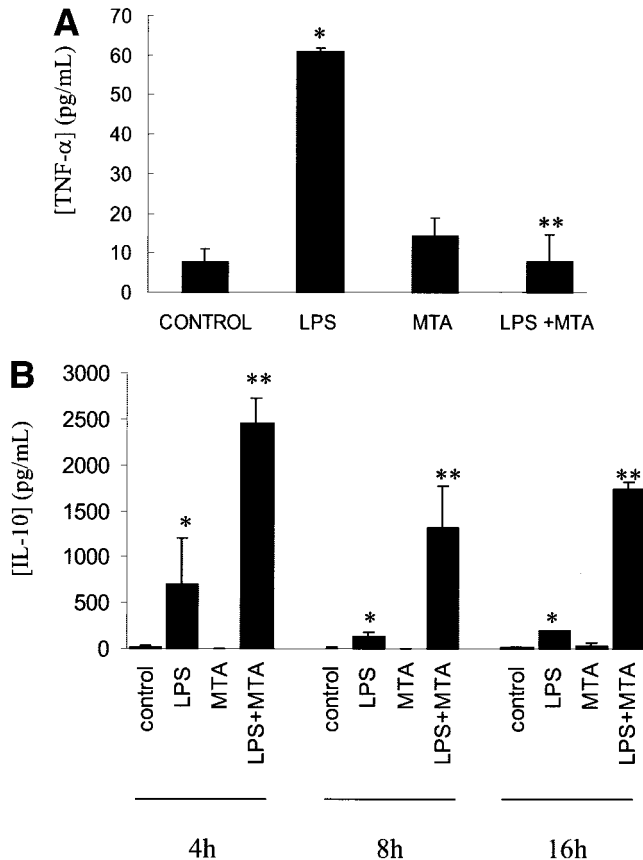


Fig. 3. MTA inhibits TNF- α and stimulates IL-10 production in LPS-treated mice. Mice ($n = 6$ per group) were treated with a single dose of MTA or vehicle 30 minutes before a dose of LPS. (A) Circulating TNF- α levels 90 minutes after LPS injection. * $P < .01$ compared to controls; ** $P < .01$ compared to LPS. (B) Circulating IL-10 levels at the indicated time points after LPS administration. * $P < .05$ compared to controls. ** $P < .05$ compared to LPS.

We next examined if the induction of iNOS by LPS could be modulated by MTA in RAW 264.7 and Kupffer cells. Figure 5A shows that MTA attenuated LPS-mediated iNOS protein induction. This effect resulted in reduced production of NO, as demonstrated by nitrite levels in RAW 264.7 culture medium (1.8 ± 0.4 nmol/ 10^6 cells in controls vs. 66.8 ± 3.3 in LPS and 42.9 ± 2.3 in MTA plus LPS; $P < .05$).

Cyclooxygenase 2 (COX2) expression can be induced by LPS and pro-inflammatory cytokines in macrophages.^{30,31} We measured COX2 protein levels in RAW 264.7 cells treated with LPS or CM. Figure 5B shows that MTA attenuated the induction of COX2 by LPS and completely abolished the effect of CM.

iNOS and TNF- α induction by LPS are dependent on the NF- κ B pathway, which involves the degradation of inhibitor- κ B (I κ B).^{29,32} In RAW 264.7 cells, we observed that LPS dramatically reduced I κ B α contents between 15 and 30 minutes after its addition, and this response was attenuated by MTA (Fig. 6A).

Activation of the p38 mitogen-activated protein kinase (MAPK) cascade is an important step in the production of pro-inflammatory cytokines and the induction of COX2 by LPS in macrophages.^{31,33} MTA attenuated the time-dependent activation of this kinase in RAW 264.7 cells, while the total cellular levels of p38 MAPK were not affected (Fig. 6B). LPS activates the transient phosphorylation of c-jun in macrophages, which is required for the transcriptional activation of COX2.³¹ Accordingly, we observed that LPS treatment of RAW 264.7 cells elicited the phosphorylation of c-jun (Fig. 6C). This effect was completely prevented by MTA (Fig. 6C).

MTA Modulates the Response of Hepatocytes to CM. We evaluated whether MTA could modulate the response of hepatocytes to pro-inflammatory cytokines. Figure 7A shows that MTA dose-dependently reduced iNOS mRNA levels induced after 3 hours of CM stimulation. The same response was observed in isolated mouse hepatocytes (data not shown). This resulted in decreased NO production, as demonstrated by nitrite levels in culture medium (7.2 ± 1.6 nmol/ 10^6 cells vs. 83.3 ± 7.2 in CM and 43.3 ± 7.8 in CM plus MTA; $P < .05$).

Induction of iNOS expression in hepatocytes results from enhanced iNOS gene transcription.^{29,32} We tested if MTA could modulate CM-mediated transactivation of iNOS promoter by CM. For this purpose, hepatocytes were transfected with a human iNOS promoter-luciferase reporter construct. MTA treatment reduced the levels of luciferase activity induced by CM (Fig. 7B). Empty vector activity was not affected by any stimuli (not shown). Given the central role played by NF- κ B in cytokine-mediated induction of iNOS expression,^{29,32} we studied the effect of MTA on NF- κ B binding activity in hepatocytes by EMSA. Figure 7C shows that CM-mediated stimulation of NF- κ B binding was inhibited by MTA. Complex specificity was demonstrated by competition assays with excess unlabeled NF- κ B consensus or mutated oligonucleotides (not shown). Furthermore, CM-elicited degradation of I κ B α protein was attenuated by MTA (Fig. 7D).

MTA Effects Are Not Mediated Through Its Conversion Into AdoMet. We have previously reported that AdoMet inhibits CM-mediated induction of iNOS expression in rat hepatocytes.¹⁸ MTA can be converted to methionine through the methionine salvage pathway so that MTA addition to cells may raise the AdoMet pool.^{21,22} We observed that MTA inhibition of LPS-induced TNF- α production by RAW 264.7 cells was preserved in the presence of cycloleucine, an inhibitor of MAT activity³⁴ (Fig. 8A). Cycloleucine significantly reduced cellular AdoMet contents without affecting viability (not shown). This suggests AdoMet is not the

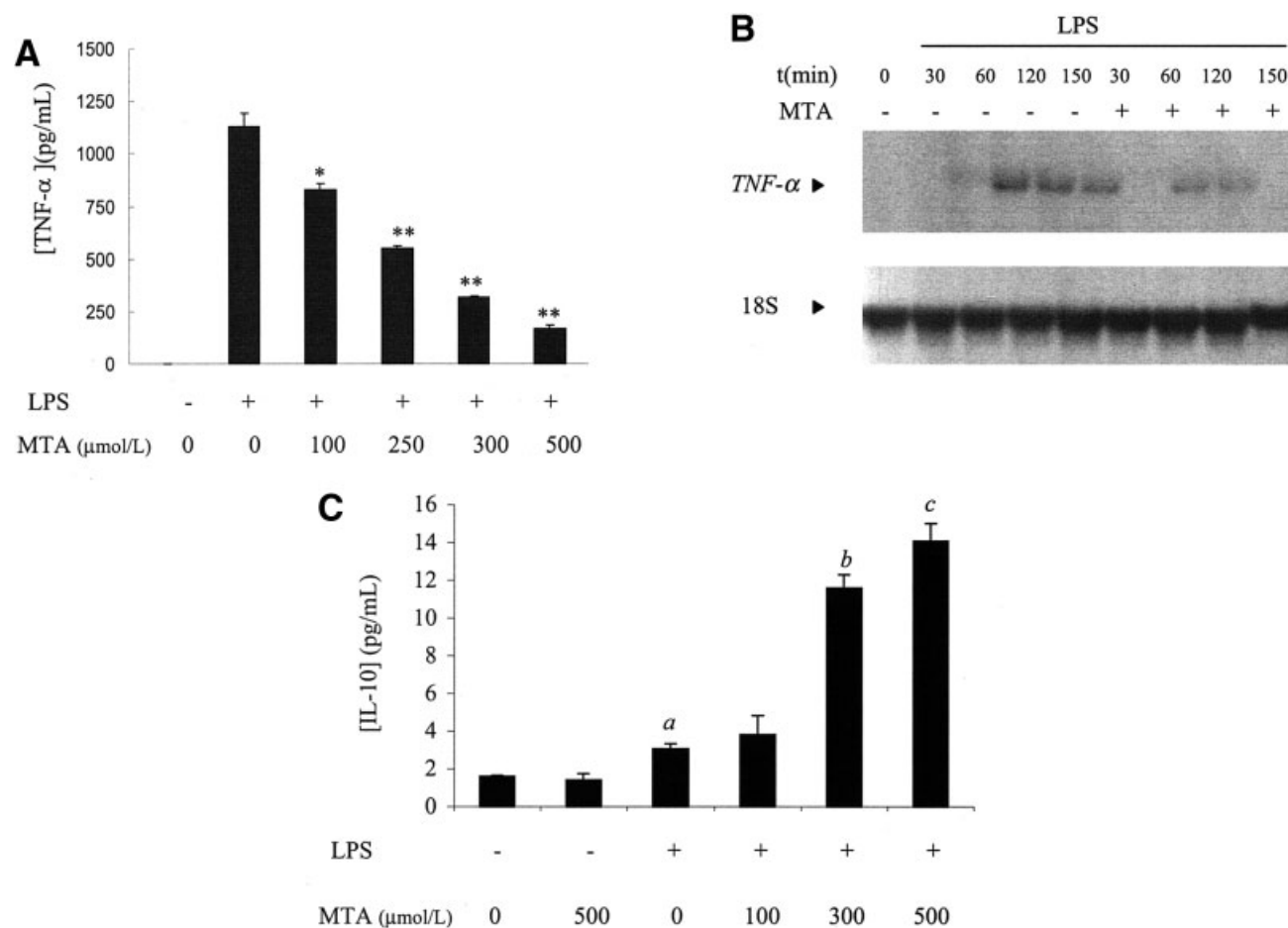


Fig. 4. MTA inhibits LPS-induced TNF- α production and potentiates the effect of LPS on IL-10 production in RAW 264.7 cells. Cells (10^5 /well) were pretreated with MTA and then with LPS. (A) TNF- α concentrations in conditioned media after 6 hours of LPS treatment in the presence of MTA. * $P < .05$, ** $P < .01$ compared to LPS. (B) Cells were pretreated or not with MTA and then with LPS. TNF- α mRNA levels were measured by Northern blotting. (C) IL-10 concentrations in conditioned media from cells treated with MTA and LPS for 18 hours. $a P < .05$ compared to control, $b P < .01$ compared to LPS alone, $c P < .01$ compared to LPS alone, and $P < .05$ compared to LPS plus MTA at 300 $\mu\text{mol/L}$.

mediator of MTA's effect. Additional evidence for the AdoMet-independent effects of MTA was obtained in A549 cells. These cells lack MTA phosphorylase (MTAP), and thus are not able to utilize MTA as a precursor for AdoMet.³⁵ MTA significantly reduced iNOS protein levels induced by LPS plus TNF- α in A549 cells (Fig. 8B).

MTA Effects Are Independent of the cAMP-PKA Pathway. MTA interacts with adenosine purinergic receptors.³⁶ Elevation of intracellular cAMP levels, and the cAMP-PKA pathway, is central to the anti-inflammatory effects of adenosine.³⁷ As previously reported, the cAMP-inducing agent forskolin downregulated LPS-induced TNF- α production, while H89 (a PKA inhibitor) strongly potentiated this effect in RAW 264.7 cells^{38,39} (Fig. 9A). In the presence of MTA, the effect of forskolin on TNF- α was enhanced, while that of H89 was abolished (Fig. 9A). We also tested the effect of PKA inhibition on the potentiation by MTA of LPS-induced IL-10

production. In the presence of H89, the stimulatory effect of MTA on IL-10 production was preserved (Fig. 9B).

Discussion

We have evaluated the anti-inflammatory properties of the naturally occurring nucleoside MTA. In a model of acute systemic inflammation induced by LPS administration to mice, we observed that MTA completely protected from LPS-induced death. One of the hallmarks of LPS-induced systemic toxicity is the activation of iNOS gene expression and the massive NO production in diverse cellular backgrounds, a systemic response implicated in the lethality of sepsis.^{29,40} We observed that the induction of iNOS was significantly attenuated by MTA in the liver and lungs of LPS-treated mice. TNF- α is an early-released cytokine recognized as a central mediator of endotoxemia and systemic inflammation, which significantly contributes to the activation of iNOS gene expression.⁴ In this *in*

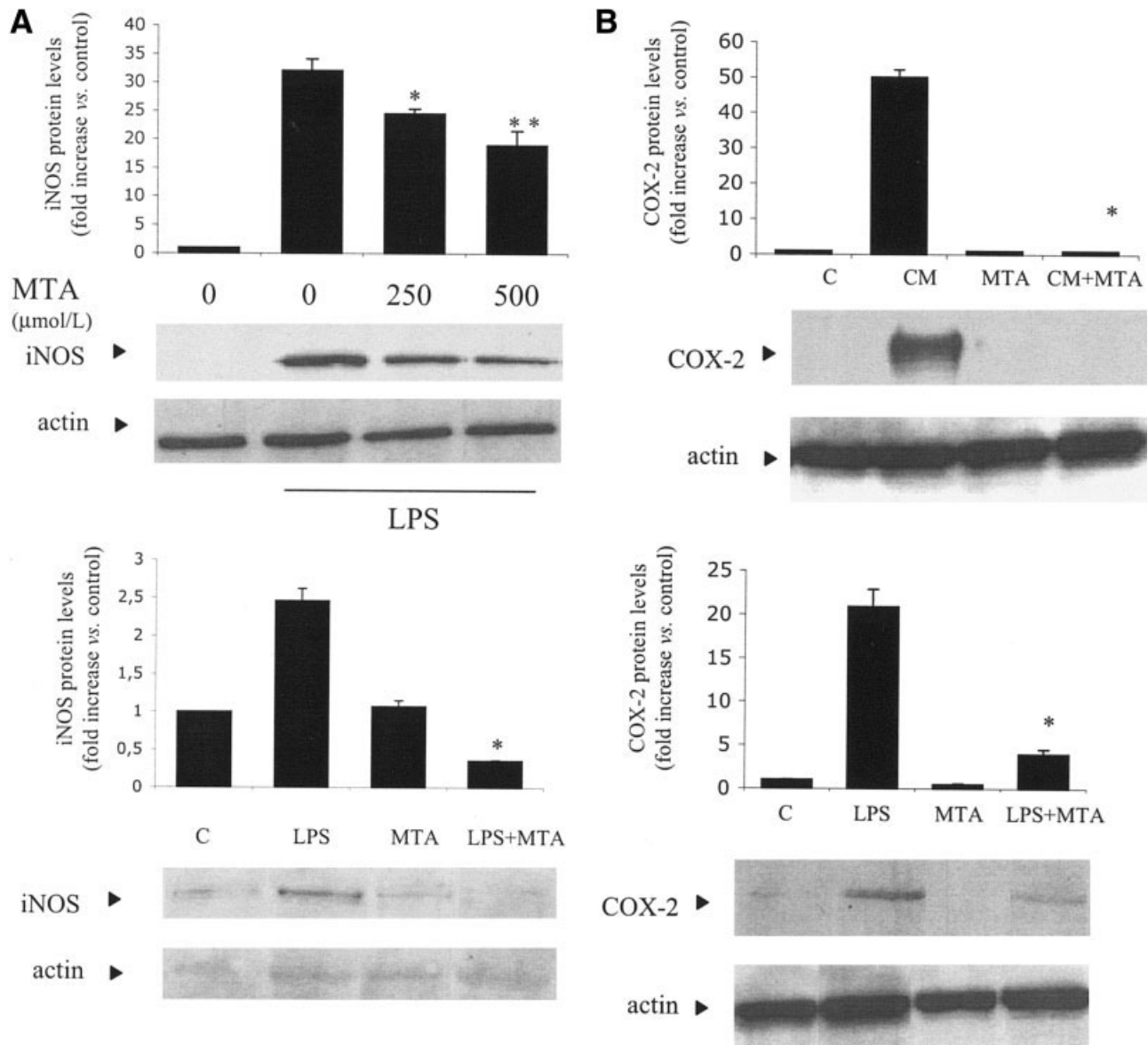


Fig. 5. MTA inhibits the effects of LPS and CM in RAW 264.7 and Kupffer cells. (A) Cells were pretreated with MTA and then LPS was added to cultures. iNOS protein levels were determined by Western blotting after 6 hours or 20 hours for Kupffer cells. RAW 264.7 cells upper panel, $*P < .05$ compared to LPS, $**P < .05$ compared to MTA 250 $\mu\text{mol/L}$. Kupffer cells lower panel, $*P < .05$ compared to LPS. (B) RAW 264.7 cells were pretreated or not with MTA (500 $\mu\text{mol/L}$) and then with CM (upper panel) or LPS (lower panel) for 18 hours. COX2 expression was determined by Western blotting. $*P < .01$ compared to CM or LPS alone.

in vivo model, we show that MTA markedly reduced circulating TNF- α levels. This action of MTA may be relevant to its life-sparing effect, because the production of TNF- α is thought to be central in the mortality of endotoxemia.^{4,41} Moreover, we also observed that the production of the anti-inflammatory cytokine IL-10 was potently stimulated by MTA treatment. IL-10 is an important component of the endogenous response of the organism to modulate the extent of inflammation, inhibiting the synthesis of cytokines such as TNF- α , and NO production by macrophages.^{19,42}

We have examined whether these *in vivo* responses could be mediated by the direct interaction of MTA with target inflammatory cells. Our results show that MTA inhibited LPS-induced TNF- α production and iNOS gene expression, while it potentiated the release of IL-10 in RAW 264.7 cells. Furthermore, we also observed that LPS and CM-induced COX2 expression, an enzyme that significantly contributes pro-inflammatory mediators such as prostaglandin E₂³⁰ was also down-regulated by MTA. These observations suggest that a similar response might occur *in vivo* in Kupffer

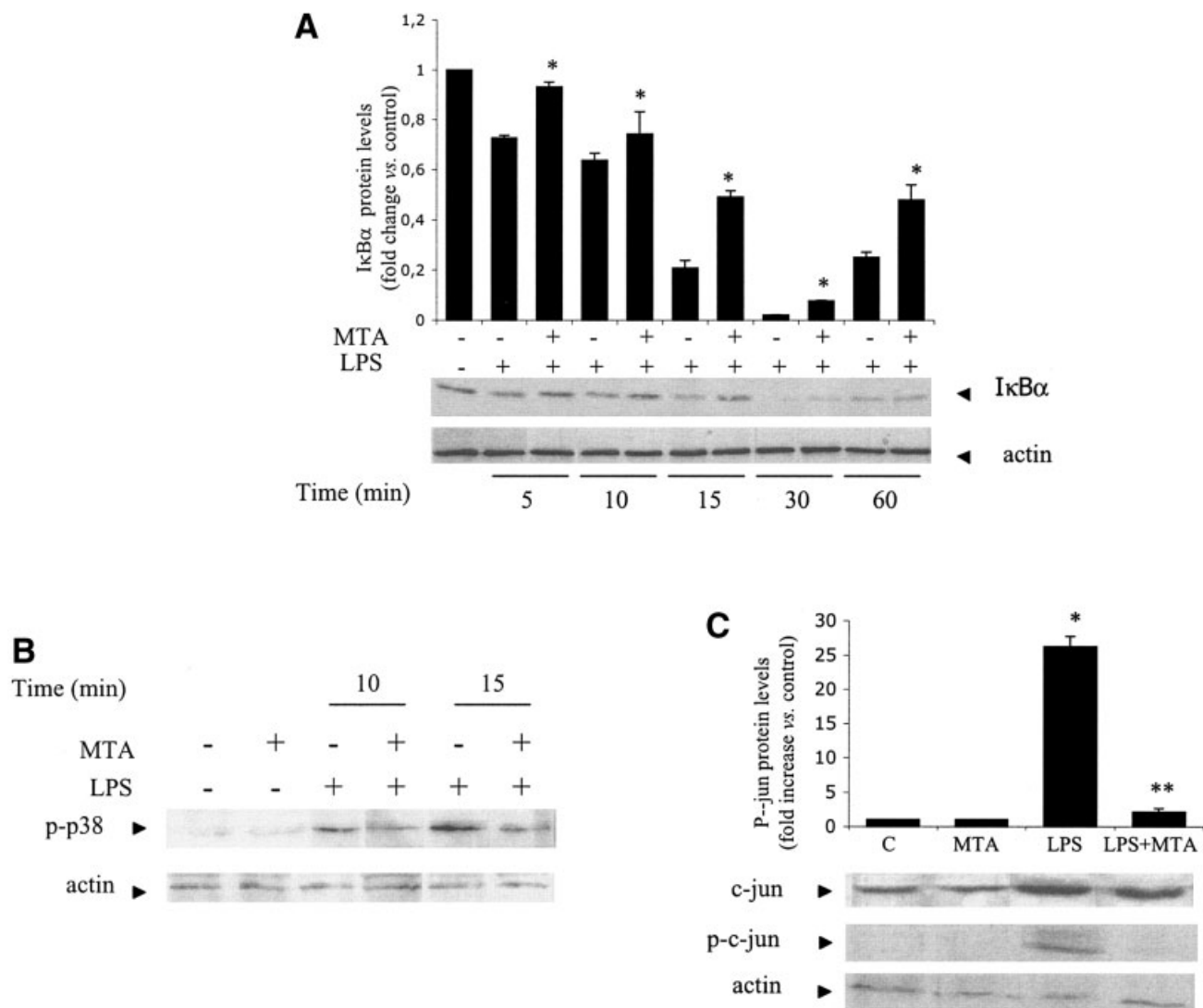


Fig. 6. Effect of MTA on I κ B α protein levels, p38 MAPK, and c-jun phosphorylation in LPS-treated RAW 264.7 cells. Cells were pretreated with MTA and then with LPS for different periods of time. (A) I κ B α protein levels as determined by Western blotting. * P < .05 compared to LPS alone. (B) Phosphorylation of p38 MAPK was monitored using a specific anti-phospho-p38 MAPK antibody by Western blotting. Lower panel shows total cellular contents of p38 MAPK protein. (C) Phosphorylation of c-jun after 1 hour of treatment with LPS was monitored using an anti-phospho c-jun (Ser-63) antibody by Western blotting. * and ** P < .01 compared to control and to LPS, respectively.

cells and alveolar macrophages. Taken together, these effects indicate that MTA can directly modify the cytokine and pro-inflammatory mediators balance towards an anti-inflammatory profile.

We also observed that MTA inhibits iNOS expression in rat hepatocytes stimulated by a mixture of pro-inflammatory cytokines. The role of NO production in sepsis is controversial, and inhibition of NO production has been shown to have both detrimental and beneficial effects on animal survival and organ damage,⁵ while, as previously mentioned, LPS-induced mortality is attenuated in iNOS-deficient mice.⁴⁰ It seems that excessive NO production, especially when accompanied by reactive oxygen species, may have

detrimental consequences.⁵ Collectively, these effects suggest that the anti-inflammatory action of MTA may be two-fold, first regulating the production of cytokines by inflammatory cells and second attenuating the effect of these cytokines on their cellular targets, such as hepatocytes and macrophages.

MTA is structurally related to adenosine and is able to interact with adenosine purinergic receptors.³⁶ Adenosine receptor agonists are known for their potent anti-inflammatory effects.^{37,43} Signaling of these effects is mainly attributed to the activation of adenosine A_{2A} receptors and the elevation of cAMP levels.³⁷ In this regard, it is known that MTA is able to increase cellular cAMP levels.⁴⁴ Our data showed that MTA effects on the produc-

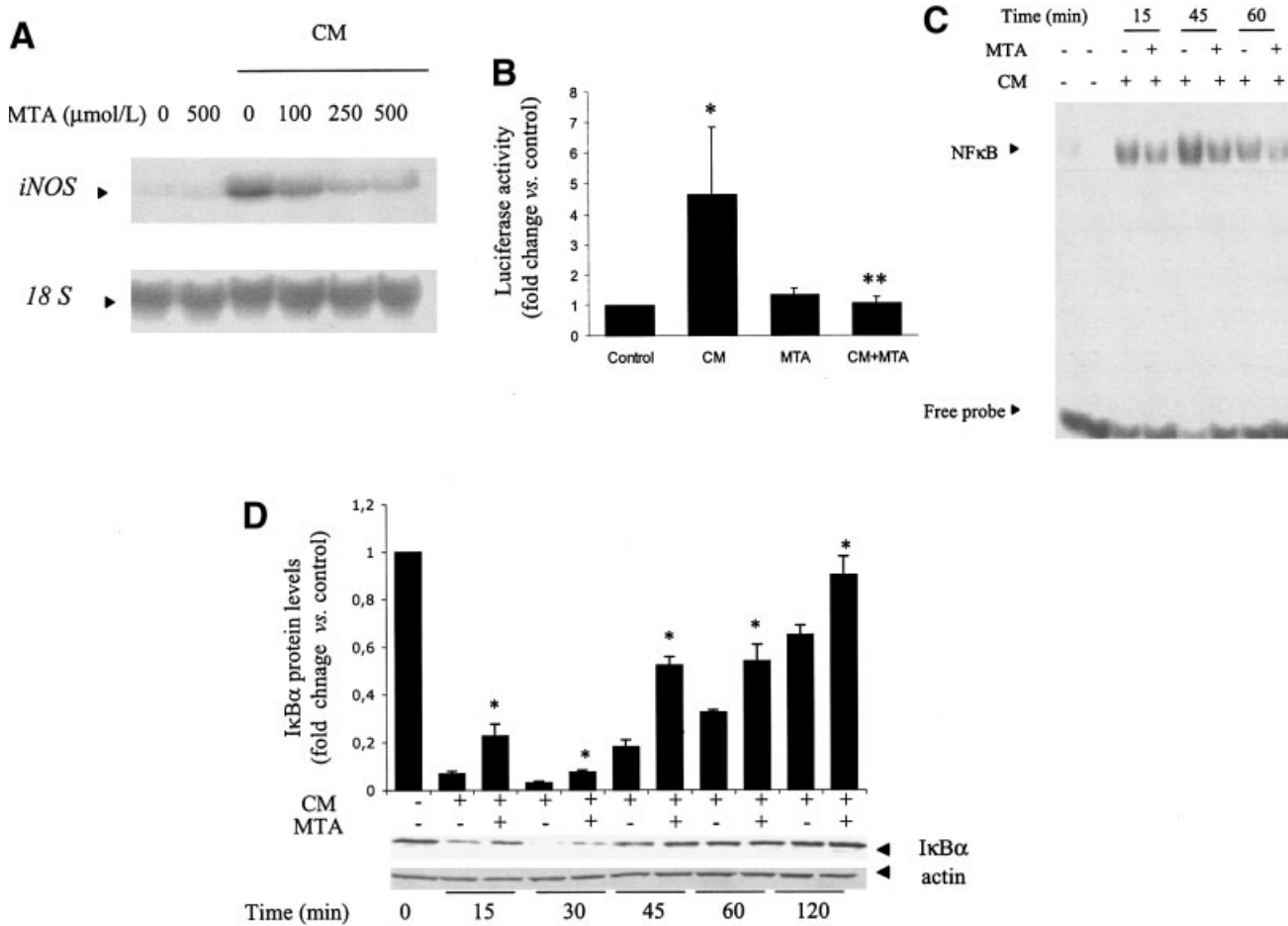


Fig. 7. Effect of MTA on the activation of iNOS gene expression and NFκB transcription factor in rat hepatocytes stimulated with CM. (A) Hepatocytes were pretreated with increasing concentrations of MTA and then stimulated with CM for 3 hours. iNOS mRNA levels were determined by Northern blotting. (B) Hepatocytes were transfected with the pGLNOS-8.3 plasmid harboring the luciferase gene under the control of human iNOS promoter. After transfection, cells were pretreated or not with MTA and then with CM for 24 hours. * $P < .05$ compared to controls, ** $P < .05$ compared to CM. (C) Effect of MTA on CM-induced NFκB DNA binding activity. Nuclear extracts were obtained from hepatocytes treated with CM and MTA as indicated and analyzed by EMSA with a consensus NFκB oligonucleotide. (D) Hepatocytes were treated with CM and MTA as indicated. IκBα protein levels were determined by Western blotting. * $P < .05$ compared to CM alone.

tion of TNF-α and IL-10 were preserved in the presence of H89, suggesting that changes in cellular cAMP levels were not behind these actions of MTA. Nevertheless, the interaction of MTA with other adenosine receptors that convey cAMP-independent anti-inflammatory signals, such as the A₃ receptors, cannot be discarded.⁴⁵

In vivo MTA can be metabolized only by MTAP to yield adenine and 5-methylthioribose-1-phosphate. This is the first step in the methionine salvage route, ultimately leading to the conversion of MTA into methionine and subsequently into AdoMet.²¹ AdoMet displays a wide array of cellular effects, observed both in cultured cells and *in vivo*, including the reduction of TNF-α levels and iNOS expression and the stimulation of IL-10 production.^{11,20} Hence, it was important to know to what extent the anti-inflammatory actions of MTA could be attributed to its conversion into

AdoMet. We present evidence suggesting that at least some of these effects of MTA are independent from AdoMet synthesis. The opposite situation, namely the conversion of AdoMet into MTA, is also possible *in vivo*. Our present observations suggest that some of the reported effects of AdoMet related to the modulation of the inflammatory response may be partly mediated through its transformation into MTA.

The molecular bases of MTA effects are likely to be multifaceted. Signal transduction through NF-κB pathway is a critical step in macrophage activation leading to increased TNF-α expression and to the induction of iNOS in inflammatory cells and hepatocytes.^{3,29,46} Moreover, increased NF-κB binding has been inversely correlated with survival in septic patients.⁴⁶ In agreement with previous observations carried out in 70Z/3 cells,⁴⁷ we observed that MTA inhibits the DNA binding activity of

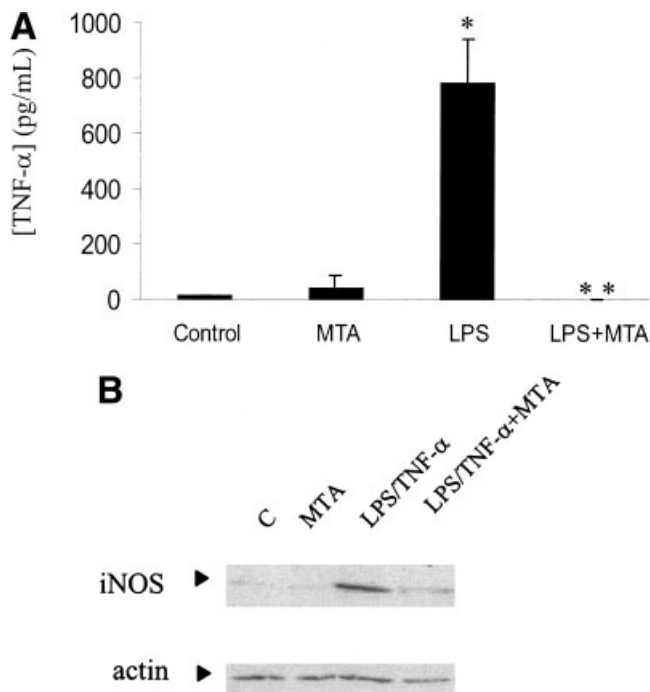


Fig. 8. MTA mediated inhibition of TNF- α production and iNOS expression is independent from its conversion into AdoMet. (A) RAW 264.7 cells were treated with 20 mmol/L cycloleucine for 12 hours and MTA before LPS. TNF- α levels in conditioned media after 6 hours of treatment. * $P < .01$ compared to controls, ** $P < .01$ compared to LPS. (B) A549 cells were pretreated with MTA, and then with LPS plus TNF- α (5 ng/mL) for 24 hours. iNOS protein levels were determined by Western blotting.

this transcription factor in cytokine-stimulated hepatocytes. Inducers of NF- κ B stimulate the phosphorylation, subsequent ubiquitination, and degradation of I κ B α , releasing the NF- κ B proteins that translocate to the nucleus and regulate gene transcription.^{3,29} Now, we show that MTA partially prevents I κ B α degradation and accelerates

its resynthesis. These results contribute to explain the attenuation by MTA of cytokine-induced iNOS promoter activity in cultured rat hepatocytes and the upregulation iNOS and TNF- α in LPS-treated RAW 264.7 macrophages.

MTA is known to have direct inhibitory effects on protein kinases.⁴⁸ We observed that LPS-mediated activation of p38 MAPK and c-jun phosphorylation in RAW 264.7 cells were inhibited by MTA. These signaling pathways play an important role in the activation of TNF- α and COX2 gene expression by endotoxin;^{31,33} consequently, their interference by MTA can significantly contribute to the observed effects of this nucleoside. Finally, it should also be considered that MTA is a well-known inhibitor of protein methyltransferases.⁴⁷ Protein methylation is emerging as a regulatory mechanism in cell signaling, including inflammation-related signaling pathways triggered by LPS,⁴⁹ being in close interaction with protein phosphorylation.⁵⁰ Crosstalk between these two signal transduction mechanisms could be modulated by MTA.

In summary, we have uncovered novel biological effects of MTA supporting a role for this metabolite in the modulation of the acute inflammatory response. Our observations may bear significance to other inflammatory processes, such as CCl₄-induced liver damage in which factors like TNF- α and IL-10 play a role in the progression of the disease, and where MTA administration is beneficial.²⁸ Finally, given the central role that endotoxemia and pro-inflammatory cytokines play in the pathogenesis of alcoholic liver injury and other disease processes such as arthritis, it may be worthwhile to examine the effect of MTA in these diseases.

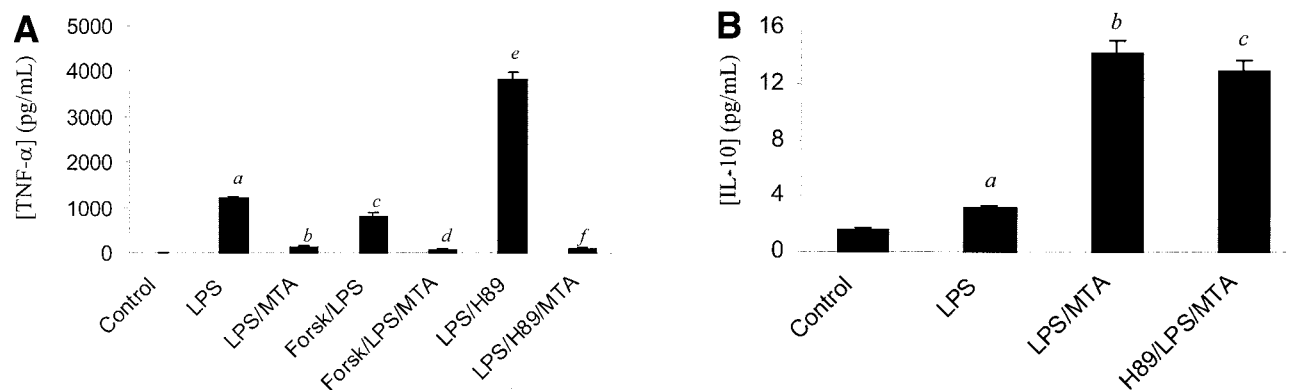


Fig. 9. MTA effects on TNF- α and IL-10 production by RAW 264.7 cells are independent from the cAMP-PKA pathway. (A) Cells were pretreated with MTA, forskolin 1 μ mol/L or H89 1 μ mol/L for 30 minutes, then were stimulated with LPS. TNF- α concentrations in conditioned media 6 hours later. ^a $P < .05$ compared to control, ^b $P < .05$ compared to LPS, ^c $P < .01$ compared to LPS, ^d $P < .01$ compared to forskolin/LPS, ^e $P < .01$ compared to LPS, ^f $P < .01$ compared to LPS/H89. (B) Cells were treated with MTA in the presence or absence of H89 (1 μ mol/L) for 30 minutes and then with LPS as indicated. IL-10 concentrations in conditioned media after 18 hours. ^a $P < .05$ compared to control, ^b $P < .01$ compared to LPS, ^c $P < .01$ compared to LPS.

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