Lipopolysaccharide-induced sensitization of adenyl cyclase activity in murine macrophages


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Submitted 11 April 2005; accepted in final form 19 August 2005

Osawa, Y., H. T. Lee, C. A. Hirshman, D. Xu, and C. W. Emala. Lipopolysaccharide-induced sensitization of adenyl cyclase activity in murine macrophages. Am J Physiol Cell Physiol 290: C143–C151, 2006.—LPS is known to modulate macrophage responses during sepsis, including cytokine release, phagocytosis, and proliferation. Although agents that elevate cAMP reverse LPS-induced macrophage functions, whether LPS itself modulates cAMP and whether LPS-induced decreases in proliferation are modulated via a cAMP-dependent pathway are not known. Murine macrophages (RAW264.7 cells) were treated with LPS in the presence or absence of inhibitors of prostaglandin signaling, protein kinases, CaM, G proteins, and NF-κB translocation or transcription/translation. LPS effects on CaMKII phosphorylation and the expression of relevant adenyl cyclase (AC) isoforms were measured. LPS caused a significant dose (5–10,000 ng/ml)- and time (1–8 h)-dependent increase in forskolin-stimulated AC activity that was abrogated by pretreatment with SNS0 (an NF-κB inhibitor), actinomycin D, or cycloheximide, indicating that the effect is mediated via NF-κB-dependent transcription and new protein synthesis. Furthermore, LPS decreased the phosphorylation state of CaMKII, and pretreatment with a CaM antagonist attenuated the LPS-induced sensitization of AC. LPS, cAMP, or PKA activation each independently decreased macrophage proliferation. However, inhibition of NF-κB had no effect on LPS-induced decreased proliferation, indicating that LPS-induced decreased macrophage proliferation can proceed via PKA-independent signaling pathways. Taken together, these findings indicate that LPS induces sensitization of AC activity by augmenting the stimulatory effect of CaM and attenuating the inhibitory effect of CaMKII on isoforms of AC that are CaM sensitive.

In addition to the secretion of cytokines, immune cell proliferation (or the lack thereof) is considered critical to a host’s successful response to sepsis. The elevation of intracellular cAMP by dibutyryl adenosine 3’,5’-cyclic monophosphate or PGE2 has been shown to suppress proliferation of guinea pig peritoneal macrophages (15) and murine bone marrow-derived macrophages (28). Furthermore, Aronoff et al. (8) showed that cAMP inhibition of macrophage function did not activate PKA but instead activated a recently characterized signaling pathway involving the exchange protein directly activated by cAMP-1, Epac-1 (1).

cAMP is synthesized by a nine-member family of mammalian transmembrane adenyl cyclase (AC) isoforms that are differentially regulated in part by Ca2+/CaM or by phosphorylation by diverse kinases, including CaMK, PKA, PKC, raf-1 kinase (9, 29, 30), and tyrosine kinases (25). One well-defined mechanism of increased activity of certain isoforms of AC is “supersensitization” (also termed cAMP overshoot, supersensitivity, superactivation, or heterologous sensitization), which is classically described after chronic activation of G protein-coupled receptors and is thought to be mediated by raf-1 kinase-mediated phosphorylation of AC (9, 29). However, amplified AC responses also have been described after chronic exposure to classic mediators of sepsis, including TNF-α (19, 22) and IL-1β (3, 19). Because the LPS receptor (Toll-like receptor 4, TLR4) has been known to share a common signaling cascade with the IL-1 receptor, we hypothesized that exposure to LPS would sensitize AC activity in a murine macrophage cell line and that LPS-induced cAMP accumulation would induce inhibition of macrophage proliferation.

Thus the effect of LPS on macrophage cAMP levels could modulate two critical events in the macrophages’ responses during sepsis: proliferation and cytokine release. Therefore, we addressed the following questions: 1) Does LPS sensitize AC activity in macrophages? 2) If so, does the mechanism involve a) prostaglandin release, b) phosphodiesterase inhibition, c) kinase-mediated phosphorylation of AC, d) altered expression of AC isoforms, e) CaM/CaMKII regulation of AC, f) NF-κB-mediated gene transcription, or f) new transcription/translation? Finally, we questioned whether the LPS-induced reduction in macrophage proliferation is related to LPS-induced sensitization of AC activity.

MATERIALS AND METHODS

Materials. Cell culture reagents were obtained from GIBCO-BRL (Grand Island, NY). The bicinchoninic acid protein assay reagent was obtained from Pierce (Rockford, IL). [α-32P]ATP (800 Ci/mmol),

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rates, which ranged from 75% to 90%. Data are expressed as the percentage of cAMP accumulation in treated cells relative to that in control cells treated with vehicle.

**Immunoblot analysis.** Whole cell lysates were electrophoresed (10% SDS-PAGE) and immunoblotted using antibodies directed against phospho-CalMII Thr286 (1:1,000 dilution; Upstate Cell Signaling Solutions, Charlottesville, VA) and β-actin (1:2,000 dilution; Sigma). Epitopes were visualized using horseradish peroxidase-conjugated secondary antibodies (1:1,000–1:5,000 dilution; Amersham Biosciences, Arlington Heights, IL) using ECL or ECL Plus (Amersham Biosciences) and developed on light film (BioMax; Kodak, Rochester, NY). Film was developed such that band intensities were within the linear range of film responses, and band intensities were quantified using Quantity One software (Bio-Rad Laboratories). Levels of phospho-CalMII immunoreactivity were normalized to β-actin to control for total protein levels. Data are presented as means ± SE.

**RNA isolation and RT-PCR.** Total RNA was isolated from confluent RAW macrophages in T75 flasks and from whole mouse brain (positive control for all 9 AC isoforms) using the RNAzol RNA isolation reagent (Ambion, Austin, TX) according to the manufacturer’s recommendations. Using the Advantage RT-PCR kit (BD Biosciences, Palo Alto, CA), we reverse transcribed 1 µg of total RNA at 42°C for 1 h in 20 µl of solution, including 200 U of Moloney murine leukemia virus reverse transcriptase, 20 U of RNase inhibitor, 10 µM of each dNTP, 100 µM iRNase K, SN50, SN50M, geldanamycin, and W-7 were added twice in warm PBS and used directly for AC assays.

To investigate the potential role of prostaglandins in LPS-induced AC sensitization, we used an inhibitor of prostaglandin synthesis (10 µM indomethacin added 30 min pretreatment) or prostaglandin receptor antagonists [prostaglandin type E (EP)2 or EP4 antagonists AH6809 or AH23848, respectively, 10 µM, added 30 min pretreatment]. To evaluate the potential role of phosphodiesterase inhibition in the LPS-induced increase in intracellular cAMP, we treated some cells with 10 µM IBMX 30 min pretreatment. To evaluate the potential role of the heterotrimeric protein G, in the LPS-induced sensitization of AC, some cells were pretreated with pertussis toxin (100 ng/ml; 4 h) before LPS exposure and AC measurements.

To investigate the potential role of specific kinases in the LPS-induced AC sensitization, cells were pretreated with Rp-8-BrcAMP (PKA inhibitor, 100 µM, 1 h), Tyrphostin G10-203X (PKC inhibitor, 100 nM, 1 h), Raf-1 kinase inhibitor I (100 nM, 1 h), herbimycin A (tyrosine kinase and NF-κB inhibitor, 1 µM, 1 h), and genistein (tyrosine kinase inhibitor, 50 µM, 1 h). To investigate the potential role of CaM in the LPS-induced sensitization of AC, cells were pretreated with W-7 (CaM antagonist, 25 µM, 1 h).

To investigate the potential requirement for new gene transcription and translation in the LPS-induced sensitization of AC activity, some cells were pretreated with inhibitors of transcription or translation (actinomycin D, 5 µg/ml, 4 h, or cycloheximide, 0.5 µg/ml, 30 min, respectively). To examine specifically the transcriptional factor NF-κB in the LPS-induced sensitization of AC, some cells were pretreated with an inhibitor of NF-κB nuclear translocation (SN50, 20 µM, 1 h) or its inactive control peptide (SN50M, 20 µM, 1 h).

Heat shock protein 90 (HSP90) is a protein involved in the receptor complex responsible for LPS signaling. To determine whether LPS-induced sensitization was due to specific activation of the LPS-HSP90 receptor complex, some cells were pretreated with an inhibitor of HSP90 before LPS treatment (geldanamycin, 5 µM, 1 h).

**AC assays.** Basal, forskolin (10 µM)-, or PGE2 (10 µM)-stimulated AC activity was determined by measuring the conversion of [α-32P]ATP to [γ32P]cAMP according to the method of Salomon et al. (23). In brief, AC assays were performed for 15 min at 37°C in a total volume of 150 µl containing 50 mM HEPES, pH 7.5, 2 mM ATP, 0.5 mM cAMP, 2% SDS, and 1 µCi/ml [3H]CAMP. The synthesis of [32P]cAMP was determined by performing sequential column chromatography over Dowex (Bio-Rad Laboratories, Hercules, CA) and alumina (23). Recovery of [3H]CAMP from each column was used to calculate column recovery
was added for the last 4 h. The medium was removed, and cells were washed twice with 500 μl of cold PBS. After being washed twice in 500 μl of ice-cold 10% TCA and rinsed with 500 μl of 70% ethanol, the cells were solubilized in 250 μl of 0.2 N NaOH at room temperature for 15 min. This method allows for the recovery of [3H]thymidine incorporated into the DNA as opposed to measuring the total cellular uptake of [3H]thymidine and is therefore a more sensitive method of cellular (i.e., DNA) proliferation. Incorporated [3H]thymidine was quantified using scintillation counting, and results are expressed as means ± SE.

Cell viability. Cell viability after LPS exposure (100 ng/ml) for 6 or 24 h was measured in triplicate using Trypan blue dye exclusion. After incubation for 5 min with 0.2% Trypan blue in PBS, 300 cells from each group were counted and the cells that had internalized the dye were considered nonviable.

Statistics. Statistical analysis was performed using repeated-measures ANOVA, followed by a Bonferroni posttest comparison using Prism 4.0 software (GraphPad, San Diego, CA). Data are presented as means ± SE, and P < 0.05 was considered significant.

RESULTS

AC activity after LPS treatment of murine macrophages. LPS demonstrated both concentration- and time-dependent sensitization of forskolin-stimulated AC activity. Significant sensitization of forskolin-stimulated AC activity was achieved within 1 h of LPS treatment (100 ng/ml), with peak sensitization occurring at 4 h (Fig. 1A). Sensitization of forskolin-stimulated AC activity occurred with concentrations of LPS ranging from 5 to 10,000 ng/ml with a peak effect at 100 ng/ml (487 ± 85% of control) (Fig. 1B). Therefore, subsequent experiments were performed with 100 ng/ml LPS for 4 h. AC activity decreased to below control levels at 24 h after treatment, presumably because of the cytotoxic effects of prolonged exposure to this concentration of LPS (see Cell viability subheading below).

LPS has been shown to inhibit phosphodiesterase activity (16). Therefore, we questioned whether the increased accumulation of [32P]cAMP was due to LPS-induced inhibition of phosphodiesterase activity as opposed to sensitization of AC activity. Preincubation of murine macrophages with 10 μM IBMX did not affect LPS-induced AC sensitization (data not shown), suggesting that the effects on AC activity and cAMP accumulation that we observed were independent of phosphodiesterase effects.

LPS has been shown to induce the release of prostaglandins in macrophages (6, 20). Prostaglandins also are known to modulate the activity of AC through EP receptors coupled to G proteins in a variety of cell types (7, 33). Therefore, we questioned whether LPS-induced AC sensitization involved prostaglandins in murine macrophages. We used three strategies. 1) We determined whether LPS-induced AC sensitization could be enhanced by acute stimulation with PGE2 (implying functionally coupled EP receptors). 2) We pretreated cells with indomethacin, an inhibitor of prostaglandin synthesis, before exposure to LPS. 3) We pretreated cells with inhibitors of prostaglandin receptors before exposure to LPS, because RAW264.7 cells have been reported to express EP2 and EP4 receptors, both of which couple to the stimulation of AC. LPS enhanced basal, forskolin (10 μM, 15 min), or PGE2 (10 μM, 15 min)-stimulated AC activity (Fig. 2A). However, preincubation with an inhibitor of prostaglandin synthesis, indomethacin (10 μM) for 30 min) did not affect the sensitization of forskolin-stimulated AC activity induced by LPS, and indomethacin alone did not decrease AC activity compared with control (Fig. 2B). Thirty-minute pretreatment with an EP2 antagonist, AH6809 (10 μM), or an EP4 receptor antagonist, AH23848 (10 μM), failed to inhibit the LPS-induced sensitization of AC activity (data not shown). Taken together, these results suggest that the sensitization of AC activity by LPS is not mediated by prostaglandins in these cells.

Role of Gαi in LPS-induced AC sensitization. AC sensitization has classically been described after the chronic activation of Gαi-coupled receptors (31). To determine whether LPS-induced sensitization of AC involved Gi proteins, murine

### Table 1. Adenylyl cyclase isoform-specific primer and GAPDH primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence, from 5’ to 3’</th>
<th>Product, bp</th>
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<tbody>
<tr>
<td>AC1</td>
<td>5’-GCC CCG TGG TGG CTC GAG TGA T</td>
<td>181</td>
</tr>
<tr>
<td>AC1</td>
<td>5’-TGG ACT TGG CCT CTC CAC ACA AAC TGG TAT</td>
<td>418</td>
</tr>
<tr>
<td>AC2</td>
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<tr>
<td>AC3*</td>
<td>5’-CAC GGG ACC CAG CAA T</td>
<td>242</td>
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<tr>
<td>AC3*</td>
<td>5’-GCT GTA AGG CCA CCA TAG G</td>
<td>287</td>
</tr>
<tr>
<td>AC3†</td>
<td>5’-CCA TTT TCT GGG GTC CAA GAA GAG</td>
<td>163</td>
</tr>
<tr>
<td>AC3†</td>
<td>5’-CAA TAT AGG TGG TGC CAA TAG TTT TTA G</td>
<td>313</td>
</tr>
<tr>
<td>AC4</td>
<td>5’-TGA ACC ATG GAC CCG TAG</td>
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<tr>
<td>AC4</td>
<td>5’-TGG ACT GCA ATG TCA TCA G</td>
<td>363</td>
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<tr>
<td>AC5</td>
<td>5’-CTG CTT TGT CTT ATC ATC G</td>
<td>359</td>
</tr>
<tr>
<td>AC6</td>
<td>5’-GAC GCT AAG CAG TAG ATG A</td>
<td>543</td>
</tr>
<tr>
<td>AC7</td>
<td>5’-GAG GCT GGA AGA AAG AGA TTT G</td>
<td>313</td>
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<tr>
<td>AC8</td>
<td>5’-CCG GTC TGA CAG ACT GAT GAA</td>
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<td>AC9</td>
<td>5’-AGC GTA TTC CCA CTA CCT TCC TCC</td>
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<tr>
<td>GAPDH</td>
<td>5’-GGG TGG GTA GTA TGT GGT GGA GTC TAC TGG TGT CTT</td>
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<tr>
<td>GAPDH</td>
<td>5’-CAG ATG CAC GAC GGA CAC ATT GGG GGT</td>
<td>313</td>
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AC, adenylyl cyclase. *Used for detection of AC3 isoform. †Used for semiquantitative RT-PCR.
macrophages were pretreated with pertussis toxin, which inactivates G_{i/o} proteins by ADP ribosylation, but pertussis toxin pretreatment had no effect on the LPS-induced sensitization of AC in murine macrophages (Fig. 3).

**Role of CaM and CaMKII in LPS-induced AC sensitization.** Certain AC isoforms (I, III, and VIII) are known to be stimulated by Ca^{2+}/CaM and inhibited by CaMK isoform CaMKIV (AC I) or CaMKII (AC III) (30). Pretreatment of cells with the CaM antagonist W-7 attenuated LPS-induced sensitization of AC activity (Fig. 4A). This finding suggests that the mechanism of LPS-induced AC sensitization involves CaM and implicates AC isoforms III and/or VIII in these cells.

Phosphorylation of Thr286 of CaMKII is indicative of kinase activation, which is known to inhibit the function of CaMK-sensitive isoforms of AC. LPS treatment dramatically decreased the phosphorylation state of CaMKII in the presence of 300 μM orthovanadate (Fig. 4, B and C). These findings are consistent with the removal of an inhibitory effect of CaMKII on susceptible isoforms of AC and may mechanistically account in part for LPS-induced AC sensitization.

**Role of protein kinases on LPS-induced AC sensitization.** In addition to CaMK regulation of AC isoforms, multiple other kinases are known to regulate certain AC isoforms, including PKA (26), PKC (32), raf-1 kinase (9), and tyrosine kinases (25). Therefore, we pretreated cells with inhibitors of several classes of protein kinases in an attempt to identify any additional mechanisms of LPS-induced sensitization of AC. Pretreatment with the PKA inhibitor Rp-8-BrcAMP, the PKC inhibitor GF-109203X, or a raf-1 kinase inhibitor did not affect LPS-induced AC sensitization (Fig. 5A). In contrast, pretreatment...
ment with the tyrosine kinase inhibitor herbimycin A, but not
with the tyrosine kinase inhibitor genistein, significantly re-
versed the increase of AC activity by LPS (Fig. 5B).

Role of NF-κB in LPS-induced sensitization of AC activity. In
addition to its inhibition of tyrosine kinases, herbimycin A
has also been reported to inhibit NF-κB activation and block
NF-κB-driven gene expression in T lymphocytes (13). Because
many of the effects of LPS are known to be mediated via
NF-κB, we determined whether NF-κB-dependent gene tran-
scription was involved in LPS-induced sensitization of AC.

Pretreatment of cells with SN50, a cell-permeable peptide
inhibitor of NF-κB, completely attenuated the LPS-induced
sensitization of AC (Fig. 6A). The inactive control peptide
SN50M had no effect on LPS activation of AC (data not
shown). Furthermore, pretreatment of cells with either the
transcription inhibitor actinomycin D or the translation inhib-
itor cycloheximide also attenuated the LPS-induced increase in
AC activity (Fig. 6, B and C).

Role of HSP90 in LPS-induced AC sensitization. Recently,
HSP90 has been reported to be a member of a receptor complex
associated with TLR4 and plays an important role in
the specific activity of LPS’s binding to the TLR4 receptor in
macrophages (27). Therefore, we used an inhibitor of the
TLR4-HSP90 receptor complex to determine whether LPS-
induced AC sensitization is mediated by specific binding of
LPS to the TLR4-HSP90 receptor complex. Pretreatment with
geldanamycin, a specific inhibitor of HSP90, significantly
attenuated the LPS-induced sensitization of AC activity (Fig. 7).

Detection of nine AC isoforms using type-specific primers.
RT-PCR using total RNA from whole mouse brain yielded
products of expected sizes for each of the nine known mam-
alian transmembrane AC (positive controls) (Fig. 8). RT-
PCR using total RNA from RAW264.7 cells demonstrated
products of expected sizes for AC III–AC IX but not for AC I
or AC II (Fig. 8).

Effect of LPS on CaM-sensitive AC isoform mRNA. To
determine whether LPS was upregulating mRNA encoding
specific AC isoforms, which in turn accounted for enhanced
total AC activity, we used RT-PCR to quantitate mRNA.
Because inhibition of CaM attenuated LPS-induced sensitiza-
tion of total AC activity, we focused on AC isoforms known to
be regulated by CaM (30) and shown in the present study to be
expressed in RAW macrophages (i.e., AC isoforms III and
VIII). We first established RT-PCR conditions that allowed for

Fig. 4. A: forskolin (10 μM)-stimulated AC activity in cultured RAW264.7
cells after 4-h LPS treatment (100 ng/ml) in the presence or absence of W-7
(25 μM; 1 h before LPS) (n = 4 within each experiment; values determined in
triplicate). Data represent means ± SE. ***P < 0.001 vs. control. ##P < 0.01
vs. LPS. B: Western blot analysis of phospho-CaMKII expression in cultured
RAW264.7 cells after 4-h LPS treatment (100 ng/ml) in the presence or
absence of Na3VO4 (300 μM; 15 min before LPS). Results representative of 3
experiments are shown. C: relative band intensities of phospho-CaMKII from
3 separate immunoblots. The effect of each treatment on phospho-CaMKII
expression is expressed as the percentage of phospho-CaMKII levels in the
positive control. Values are means ± SE. *P < 0.05.

Fig. 5. A: forskolin (10 μM)-stimulated AC activity in cultured RAW264.7
cells after 4-h LPS treatment (100 ng/ml) in the presence or absence of protein
kinase inhibitors. Rp-cAMP (Rp) (100 μM), GF-109203X (GF) (100 nM), or
Raf-1 kinase inhibitor I (Raf) (100 nM) was added 1 h before 4-h LPS
treatment. B: forskolin (10 μM)-stimulated AC activity in cultured RAW264.7
cells after 4-h LPS treatment (100 ng/ml) in the presence or absence of tyrosine
kinase inhibitors. Herbimycin A (H; 1 μM) or genistein (G; 50 μM) was added
1 h before 4-h LPS treatment (n = 4 within each experiment; values deter-
mined in triplicate). Data represent means ± SE. ***P < 0.01, ****P < 0.001
vs. control. ###P < 0.001 vs. LPS.
the relative quantitative assessment of mRNA for AC III and VIII. Figure 9A shows a representative gel image used to evaluate relative band intensities for AC III and GAPDH. Band intensities did not plateau before cycle numbers 32 and 19, respectively, which were the cycle numbers chosen for subsequent semiquantitative RT-PCR experiments. Four-hour treatment with LPS (100 ng/ml) did not upregulate mRNA expression of either AC III or AC VIII (Fig. 9B).

**Proliferation effects of LPS, forskolin, IBMX, or activator of PKA.** LPS treatment (100 ng/ml, 6 h) decreased [3H]thymidine incorporation in murine macrophages (66.0 ± 3.2% of control, \( n = 4 \); \( P < 0.001 \)). In addition, increases in intracellular cAMP with forskolin or IBMX also decreased [3H]thymidine incorporation in these cells (62.3% ± 2.7% or 44.5% ± 0.5 of control, \( n = 4 \); \( P < 0.001 \)).

**Fig. 8.** Representative RT-PCR products using isoform-specific primers of AC I–AC IX in total RNA from RAW264.7 cells (RAW) and whole mouse brain. Total RNA (1 \( \mu \)g) was used in each RT-PCR. Neither mRNA encoding AC I nor mRNA encoding AC II was detected in RAW264.7 cells, despite the fact that they were detected in whole mouse brain. mRNA encoding AC III–AC IX was detected in total RNA extracted from both RAW264.7 cells and whole brain.
We demonstrated both time- and concentration-dependent effects of LPS on subsequently measured forskolin-stimulated AC activity. This finding is consistent with the observation that intracellular cAMP levels were increased by LPS treatment in RAW264.7 cells (6). LPS previously was shown to stimulate prostaglandin release (20) and to inhibit phosphodiesterase activity (16), two mechanisms that could indirectly account for elevated intracellular cAMP levels after LPS treatment. These mechanisms were excluded in the present study by the demonstration that 1) pretreatment with indomethacin to block prostaglandin synthesis had no effect on LPS-induced sensitization of AC activity, 2) prostaglandin receptor antagonists selective for the EP2 and EP4 receptors (AH6809 and AH23848, respectively) failed to affect the LPS-induced sensitization of AC, and 3) pretreatment with the phosphodiesterase.

respectively, n = 4 each; P < 0.001) (Fig. 10A). Furthermore, direct activation of PKA with Sp-8-BrcAMP decreased proliferation of murine macrophages (46.8 ± 2.8% of control, n = 4; P < 0.001) (Fig. 10A). Combined application of forskolin and LPS additively decreased proliferation compared with the effect of a single agent (Fig. 10A). Thus LPS or activation of the AC-cAMP-PKA cascade at several levels was capable of inhibiting macrophage proliferation. We next questioned whether LPS-induced reduction in proliferation was mediated through a similar pathway mediating increased AC activity.

We evaluated the effect of the NF-κB inhibitor SN50 on LPS-induced decreases on [3H]thymidine incorporation. Although SN50 completely blocked the LPS-induced increase in AC activity (see above), it had no effect on LPS-induced decreases of [3H]thymidine incorporation (Fig. 10B), suggesting that LPS-induced reduction in proliferation can occur independently of the effects on the cAMP cascade.

Cell viability. Six-hour pretreatment with LPS (100 ng/ml), forskolin (50 μM), IBMX (500 μM), or Sp-8-BrcAMP (100 μM) did not affect cell viabilities of murine macrophages detected using Trypan blue exclusion. Viability was 96.4 ± 0.5%, 96.7 ± 0.4%, 96.1 ± 0.3%, or 96.4 ± 0.3% compared with control, respectively (n = 3; P > 0.05). In contrast, longer treatment (24 h) with 100 ng/ml LPS reduced the viability of murine macrophages to 33.6 ± 2.7% of control (n = 4; P < 0.001).

**DISCUSSION**

The primary findings of the present study are that LPS induced sensitization of CaM-regulated AC isoforms and decreased proliferation in a murine macrophage cell line, RAW264.7. Sensitization of AC activity, but not decreased proliferation by LPS, was NF-κB dependent, despite the finding that elevation of cAMP itself decreased proliferation.

These findings suggest that both cAMP-dependent (activated by forskolin, IBMX, or Sp-8-BrcAMP) and cAMP-independent pathways (activated by LPS) are capable of attenuating proliferation of macrophages.
ase inhibitor IBMX did not change the magnitude of subsequently measured LPS-induced sensitization of AC activity.

Okonogi et al. (17) reported that LPS inhibited PGE$_2$- or forskolin-stimulated cAMP accumulation by increasing phosphodiesterase activity but that membranes prepared from LPS-treated peritoneal macrophages exhibited similar PGE$_2$-stimulated AC activity. However, they used thioglycolate broth-elicited peritoneal macrophages, and they investigated brief periods of PGE$_2$- or forskolin-stimulated cAMP accumulation after 1 h of LPS treatment. Thus their study differed from the present study with regard to 1) the origin of the macrophages studied, 2) pretreatment with LPS for 1 vs. 4 h, and 3) the duration of effector stimulation (PGE$_2$ or forskolin for 2 or 5 min, respectively, compared with 15-min stimulation). However, in our study, inhibition of phosphodiesterases did not alter the LPS-induced sensitization of AC. Thus the peritoneal macrophages studied by Okonogi et al. were phenotypically different from the murine macrophages used in the present study with regard to prostaglandin and phosphodiesterase signaling, which likely accounts for the differences found in AC sensitization.

Isoforms of AC are known to be differentially stimulated or inhibited by a diverse array of intracellular signals, including G protein α- or βγ-subunits, Ca$^{2+}$ concentration, CaM, and multiple isoforms of protein kinases, including PKA, PKC, raf-1 kinase (9, 29), and tyrosine kinases (25). Therefore, we attempted to block the LPS-induced sensitization of AC by pretreating cells with selective inhibitors, including W-7 (CaM inhibitor), Rp-8-BrcAMP (PKA inhibitor), GF-109203X (PKC inhibitor), raf-1 kinase I inhibitor, genistein (tyrosine kinase inhibitor), and herbimycin A (tyrosine kinase and NF-κB inhibitor).

W-7 attenuated LPS-induced AC sensitization, which suggested that CaM-sensitive AC isoforms likely play an important role in sensitization induced by LPS. Three isoforms have been reported to be CaM sensitive, namely, AC I, AC III, and AC VIII (30). Among these isoforms, we found evidence for mRNA expression of AC III and AC VIII in RAW264.7 cells. In addition, there is evidence that CaM can indirectly inhibit AC III via activation and phosphorylation of CaMKII, which phosphorylates AC III, thereby inhibiting its activity (30). In the present study, LPS decreased the phosphorylation of CaMKII, the active form of this enzyme. Thus LPS reduction in CaMKII activity could remove a negative regulatory effect on a susceptible AC isoform, resulting in increased AC activity.

Herbimycin A, but not genistein, attenuated the LPS-induced sensitization of AC activity. To distinguish herbimycin A’s blockade of tyrosine kinases from its known inhibitory effects on NF-κB, separate experiments were performed with a specific inhibitor of NF-κB nuclear translocation, SN50. SN50, but not its inactive peptide control, was effective in attenuating LPS-induced AC activation. These data suggest that a transcriptional event mediated by NF-κB is required. This finding is not surprising, because a myriad of LPS-induced effects have been attributed to NF-κB-mediated events (18). To further confirm the requirement for new transcription and translation, we performed studies in which murine macrophages were pretreated with actinomycin D and cycloheximide, and both pretreatments blocked LPS-induced sensitization of AC. Sensitization of AC has been described most widely after chronic stimulation of a G$_i$ protein-coupled receptor. Classically, after chronic exposure to an agonist that acutely inhibits AC activity, subsequent stimulation of AC activity results in overshoot, or supersensitization, of AC activity. This G$_i$ protein-required sensitization of AC is pertussis toxin sensitive. Therefore, in the present study, we questioned whether G$_i$ proteins could be intermediates in the LPS-induced sensitization of AC, but pretreatment with pertussis toxin failed to reverse LPS’s effect. Therefore, the cellular mechanisms of AC sensitization by chronic G$_i$ activation vs. LPS exposure are different. Furthermore, AC activity has been enhanced by prior exposure to TNF-α (19, 22) or to IL-1β (3, 19). Similar to our presently reported findings in murine macrophages, IL-1β induced sensitization of forskolin-stimulated cAMP accumulation, which was blocked by cycloheximide (3).

The requirement for new protein synthesis in the LPS-induced sensitization of AC led us to question whether LPS may directly increase AC expression. To determine whether the expression of AC was affected by LPS pretreatment, we quantified mRNA for AC isoforms III and VIII. These specific isoforms were chosen for evaluation because our functional data indicated a role for CaM-regulated AC (30), of which AC isoforms III and VIII were found to be expressed using RT-PCR. We found no evidence for an increase in mRNA encoding AC isoforms III or VIII after LPS treatment, indicating that a change in AC expression is unlikely to account for the increased activity measured.

There is evidence that HSP90 plays an important role in LPS recognition. Byrd et al. (4) reported that HSP90 bound to LPS and mediated the activation of macrophages by LPS. Moreover, a signaling complex of receptors comprising HSP70, HSP90, chemokine receptor 4, and growth differentiation factor 5 has been reported to form after LPS stimulation and is thought to associate with TLR4 to mediate LPS signaling (27). In the present study, geldanamycin, a specific inhibitor of the HSP90 family, attenuated LPS-induced sensitization of AC. This result suggests that LPS’s effect on AC was mediated via specific activation of the LPS receptor complex.

Interestingly, even though blockade of NF-κB completely abrogated LPS-induced AC sensitization, it had no effect on LPS-induced decreased proliferation, despite the fact that direct stimulation of AC with forskolin, elevation of cAMP with IBMX, or stimulation of PKA with Sp-8-BrcAMP was each capable of inhibiting proliferation. Furthermore, combined application of LPS and forskolin caused the additive effect of inhibition of proliferation. Taken together, these results suggest that LPS’s effects on decreased proliferation can proceed through a CAMP-PKA-independent pathway in these murine macrophages.

Cyclic AMP has inhibitory effects on the macrophage immune response, including the production of inflammatory mediators such as TNF-α and interleukins (1, 10, 24). Thus modulation of cAMP levels appears to have an important role in the regulation of innate immune responses. Sensitization of CaM/CaMKII-regulated isoforms of AC could function in an intracellular negative feedback loop to attenuate the LPS-induced release of cytokines or phagocyte function, which in turn could limit the macrophage’s successful response to sepsis.
REFERENCES


