Role of the Cyclic AMP-Protein Kinase A Pathway in
Lipopolysaccharide-induced Nitric Oxide Synthase Expression in
RAW 264.7 Macrophages

INVOLVEMENT OF CYCLOOXYGENASE-2

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The signaling pathway for lipopolysaccharide (LPS)-
induced nitric oxide (NO) release in RAW 264.7 macro-
phages involves the protein kinase C and p38 activation
pathways (Chen, C. C., Wang, J. K., and Lin, S. B. (1998)
J. Immunol. 161, 6206–6214; Chen, C. C., and Wang, J. K.
(1999) Mol. Pharmacol. 55, 481–488). In this study, the
role of the cAMP-dependent protein kinase A (PKA)
pathway was investigated. The PKA inhibitors, KT-5720
and H8, reduced LPS-induced NO release and inducible
nitric oxide synthase (iNOS) expression. The direct PKA
activator, Bt2cAMP, caused concentration-dependent
NO release and iNOS expression, as confirmed by immu-
nofluorescence studies. The intracellular cAMP concen-
tration did not increase until after 6 h of LPS treatment.
Two cAMP-elevating agents, forskolin and cholera
toxin, potentiated the LPS-induced NO release and
iNOS expression. Stimulation of cells with LPS or
Bt2cAMP for periods of 10 min to 24 h caused nuclear
factor-κB (NF-κB) activation in the nuclei, as shown by
detection of NF-κB-specific DNA-protein binding. The
PKA inhibitor, H8, inhibited the NF-κB activation in-
duced by 6- or 12-h treatment with LPS but not that
induced after 1, 3, or 24 h. The cyclooxygenase-2 (COX-2)
inhibitors, NS-398 and indomethacin, attenuated LPS-
induced NO release, iNOS expression, and NF-κB DNA-
protein complex formation. LPS induced COX-2 expres-
sion in a time-dependent manner, and prostaglandin E2
production was induced in parallel. These results sug-
gest that 6 h of treatment with LPS increases intracel-
lar cAMP levels via COX-2 induction and prostaga-
l glandin E2 production, resulting in PKA activation,
NF-κB activation, iNOS expression, and NO production.

Nitric oxide (NO)† has been identified as an important sig-
aling molecule involved in regulating a wide range of biological
activities in the neural, vascular, and immune systems (1). NO and its metabolites mediate a number of host defense
functions mediated by activated macrophages, including anti-
microbial and tumoricidal activity, implicated in the pathogen-
esis of tissue damage associated with acute and chronic inflam-
ation (2, 3). Macrophages generate NO from the guanidino
moiety of L-arginine via a reaction catalyzed by the inducible
form of nitric oxide synthase (iNOS) (4). iNOS has been iden-
tified in a wide variety of cell types including macrophages,
mesangial cells, vascular smooth muscle cells, keratinocytes,
chondrocytes, osteoclasts, and hepatocytes and can be induced
by many immune stimuli (1, 5). Changes in NO formation in
iNOS-expressing cells usually correlate with similar changes in
iNOS mRNA levels, indicating that a major part of iNOS regu-
lation occurs at the transcription level. The promoter region of
the iNOS gene contains several binding sites for transcriptional
factors, such as nuclear factor-κB (NF-κB) and activator
protein-1, as well as for various members of the CCAAT/en-
hancer-binding protein, activating transcription factor/cAMP-
response element-binding protein, and Stat families of tran-
scriptional factors (6). Of these, the proteins of the NF-κB
family appear to be essential for the enhanced iNOS gene
expression seen in macrophages exposed to the active compo-
ponent of endotoxin, lipopolysaccharide (LPS) (7). In unstimu-
lated cells, NF-κB is retained in the cytoplasm by binding to
IκB but is released by signal induction and translocates to the
nucleus, activating the responsive gene (8). In macrophages,
iNOS induction by LPS requires initiation of gene expression
and de novo protein synthesis over a period of several hours (9).

The intracellular signaling pathways by which LPS causes
iNOS expression in macrophages involve a series of events
resulting in the transmission of the signal from the plasma
membrane through the cytoplasm to the nucleus, where iNOS
gene expression is up-regulated. Previous studies have shown
that LPS first binds to LPS-binding protein and then to mem-
brane CD14 and that it also activates phosphatidylinositol-
phospholipase C and phosphatidylcholine-PLC by tyrosine
phosphorylation, thus causing PKC activation (10). Tyrosine
phosphorylation also causes p38 activation (11). These phos-
phorylation processes result in stimulation of NF-κB DNA-
protein binding and the initiation of iNOS expression and NO
release (10, 11). An increase in intracellular cAMP levels is an
important intracellular signaling mechanism involved in the
regulation of gene expression. Certain in vitro studies have
shown that an increase in cAMP levels causes iNOS induction
(12–14), whereas in other studies, increased cAMP levels
caused a reduction in iNOS (15, 16). In the present study, we
explored the intracellular signaling pathway for the LPS-
induced increase in cAMP levels and its involvement in LPS-
stimulated NO production in RAW 264.7 macrophages. The
results show that, after 6 h of treatment, LPS can increase
cAMP levels by induction of cyclooxygenase-2 (COX-2) and

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† The abbreviations used are: NO, nitric oxide; iNOS, inducible nitric
oxide synthase; LPS, lipopolysaccharide; PKC, protein kinase C;
Bt2cAMP, dibutyryl cyclic AMP; PKA, protein kinase A; NF-κB, nuclear
factor-κB; COX, cyclooxygenase; PG, prostaglandin; EMSA, electro-
phoretic mobility shift assay; CTX, cholera toxin; TTBS, Tris-buffered
saline/Tween 20.
formation of prostaglandin E₂ (PG,E₂), resulting in the activation of PKA and NF-κB, iNOS expression, and NO production. The PKA activation pathway explored in this study had a delayed onset (6 h), whereas the previously reported PKC and p38 activation pathways have rapid onsets (10 min) (10, 11).

**EXPERIMENTAL PROCEDURES**

**Materials**—Affinity-purified rabbit polyclonal anti-iNOS antibody was obtained from Transduction Laboratories (Lexington, KY). Dulbecco’s modified Eagle’s medium, fetal calf serum, penicillin, and streptomycin were purchased from Life Technologies, Inc. The NF-κB probe was from Santa Cruz Biotechnology (Santa Cruz, CA). LPS (from Escherichia coli serotype 0127:B8), forskolin, Bt₂cAMP, cholera toxin (CTX), sulfinilamide, and N-(1-naphthyl)-ethylenediamine were from Sigma. KT-5720 and NS-398 were from Calbiochem. H₈ was from Seikagaku (Tokyo, Japan). T₄ polynucleotide kinase was from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-Cox-2 antibody was from Cayman Chemicals (Ann Arbor, MI). Poly(dI-dC), a CAMP enzyme immunosassay kit, horseradish peroxidase-labeled donkey anti-rabbit second antibody, and the ECL detection reagent were from Amersham Pharmacia Biotech. Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from Cappel (Aurora, OH).

**Cells**—RAW 264.7 cells, a macrophage cell line, were obtained from the ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in 12-plate wells (nitrile assay, iNOS and COX-2 expression, and PGE₂ production), on 24-mm glass coverslips in 35-mm dishes (immunoassay staining), in 6-well plates (cAMP assay), or in 10-cm dishes (NF-κB gel shift assay).

**Immunofluorescence Staining**—RAW cells grown on coverslips were treated for 24 h with LPS or Bt₂cAMP in growth medium and then rapidly washed with phosphate-buffered saline and fixed at room temperature for 10 min with 2% paraformaldehyde. After washing with phosphate-buffered saline, the cells were blocked for 15 min with 1% bovine serum albumin in TTBS (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) containing 0.1% Triton X-100, incubated with anti-iNOS antibody (1:100) for 1 h, washed extensively, and stained for 30 min with anti-rabbit IgG-fluorescein (1:2,000). After additional washes, the coverslips were mounted on glass slides using mounting medium (2% N-propyl gallate in 60% glycerol, 0.1 M phosphate-buffered saline, pH 8.0). Optical sections of the immunostained cells were observed and photographed using a Zeiss Axiovert inverted microscope equipped with the photomicrograph digitized integration system (MGDS).

**Preparation of Cell Extracts and Western Blot Analysis of iNOS and COX-2**—Following treatment with LPS or Bt₂cAMP, with or without pretreatment with various inhibitors, the cells were harvested and collected. Cell lysates were prepared and subjected to SDS-polyacrylamide gel electrophoresis using 7.5% (iNOS) or 10% (COX-2) running gels as described previously (10). The proteins were transferred to nitrocellulose, and the membrane was incubated successively with 0.1% milk in TTBS at room temperature for 1 h, with rabbit antibody specific for iNOS or COX-2 for 1 h, and with horseradish peroxidase-labeled anti-rabbit antibody for 30 min. After each incubation, the membrane was washed extensively with TTBS. The immunoreactive band was detected using ECL detection reagent and developed with Hyperfilm-ECL.

**Determination of Intracellular cAMP Concentrations**—After cells were treated with LPS for 1, 3, 6, 12, or 24 h or with CTX for 24 h or with forskolin for 10 min, the reaction was terminated by aspiration of the growth medium and addition of 0.1 N HCl. The cells were scraped into Eppendorf tubes and the suspensions were centrifuged; the supernatants were then neutralized with 10 N NaOH and assayed for cAMP levels using an enzyme immunoassay kit from Amersham Pharmacia Biotech.

**RESULTS**

**Inhibitory Effect of PKA Inhibitors on LPS-induced NO Production and iNOS Expression and Effect of Bt₂cAMP**—To determine whether PKA was involved in the LPS-induced NO production, the PKA inhibitors, KT-5720 and H₈, were used. When cells were pretreated for 30 min with 1 or 3 µM KT-5720 or with 30 or 50 µM H₈, LPS-induced NO production (Fig. 1A) and iNOS expression (Fig. 1, B and C) were inhibited in a dose-dependent manner. The respective levels of inhibition were 31 or 62% for 1 or 3 µM KT-5720 and 35 or 65% for 30 or 50 µM H₈.

Because both LPS-induced NO production and iNOS expression were inhibited by KT-5720 or H₈, indicating the involvement of the PKA pathway in the LPS effect, the direct PKA activator, Bt₂cAMP, was used. Exposure of RAW cells to
Bt2cAMP for 24 h resulted in both nitrite production (Fig. 2A) and iNOS expression (Fig. 2B) in a dose-dependent manner, with maximum nitrite release (37.2 ± 0.2 nmol/10^6 cells/24 h; n = 3) being obtained using 100 μM Bt2cAMP (Fig. 2A). In the following NO release experiment, the cells were treated with 100 μM Bt2cAMP for 24 h. Under these conditions, either the transcriptional inhibitor, actinomycin D, or the translational inhibitor, cycloheximide, inhibited the Bt2cAMP-induced nitrite production and iNOS expression (data not shown). Bt2cAMP-induced iNOS expression was also demonstrated by immunofluorescence staining; as shown in Fig. 3, iNOS expression was not seen in the basal state (Fig. 3B) but was induced in the cytoplasm after treatment with either LPS (Fig. 3D) or Bt2cAMP (Fig. 3F).

Because the PKA pathway had been shown to be involved in LPS-induced NO production and because Bt2cAMP stimulated NO production, intracellular cAMP levels were measured following LPS treatment. When cells were treated with 1 μg/ml LPS for various times, cAMP levels increased slightly after 3 h (121% of basal), reached a maximum at 6 h (243% of basal), and then declined (161% of basal after 12 h) (Fig. 4A). Following treatment of cells with 1 μg/ml CTX for 24 h or with 100 μM forskolin for 10 min, cAMP levels increased to 292 and 202% of basal, respectively (Fig. 4B).

**Effect of Cyclic AMP-elevating Agents on LPS-induced NO Production and iNOS Expression**—Forskolin or CTX themselves had no effect on nitrite production but enhanced the LPS-stimulated increase in nitrite production and iNOS expression (Fig. 5). Ten or 30 μM forskolin, which had no effect on cAMP levels in RAW cells (data not shown), also had no effect on LPS-induced NO production and iNOS expression, whereas 100 μM forskolin, which increased cAMP levels 2-fold (Fig. 4B), also increased LPS-induced NO production and iNOS expression (Fig. 5). A similar parallel enhancement of the LPS-stimulated increase in NO production and iNOS expression was seen using Bt2cAMP (Fig. 6).

**Kinetics of NF-κB-specific DNA-Protein Complex Formation in Nuclei Stimulated with LPS or Bt2cAMP and the Inhibitory Effect of H8**—The time course of NF-κB activation after treatment with 1 μg/ml LPS or 100 μM Bt2cAMP was studied. Nuclear extracts prepared from RAW cells were assayed for activated NF-κB in an EMSA. As shown in Fig. 7A, NF-κB-specific DNA-protein complex formation increased after treatment with LPS for 1, 3, 6, 12, or 24 h. When cells were exposed to 100 μM Bt2cAMP for 10 min, increased formation of the NF-κB-specific DNA-protein complex was also seen (Fig. 7B), whereas after treatment with Bt2cAMP for 3 or 24 h, the intensity of these complexes decreased but was still stronger than in resting cells (Fig. 7B). The bands in the upper and lower complex were previously identified as the p65/p50 heterodimer and p50/p50 homodimer, respectively (10). After pretreatment of the cells for 30 min with 50 μM H8, the activation of NF-κB-specific DNA-protein complex formation induced following 1, 3, or 24 h of LPS treatment was not affected, whereas that induced following 6 or 12 h of LPS treatment was inhibited, the extent of inhibition being 48% and 28%, respectively (Fig. 8A). The activation of NF-κB-specific DNA complex formation seen after 6 h of LPS treatment was inhibited by H8 in a dose-dependent manner (30, 50, and 75 μM) (Fig. 8B).

**Inhibitory Effect of COX-2 Inhibitors on LPS-induced NO Production, iNOS Expression, and NF-κB DNA-Protein Complex Formation and Induction of COX-2 by LPS**—The fact that the cAMP-PKA pathway had been shown to be involved in LPS-induced NO production and iNOS expression, that LPS caused an increase in cAMP levels after 6 h of treatment, and that H8 inhibited LPS-induced NF-κB-specific DNA-protein complex formation following 6 h of treatment indicated that the...
cAMP formation was a delayed response. To determine whether the increased cAMP levels were because of PG formation produced as a result of COX-2 expression, the COX-2 inhibitors, NS-398 and indomethacin, were used. As shown in Fig. 9A, LPS-induced NO production and iNOS expression were inhibited by 10 μM NS-398 or indomethacin. For iNOS expression studies (B), cell lysates from the nitrate assay were subjected to electrophoresis and Western blotting using iNOS-specific antibody as described under “Experimental Procedures.” *p < 0.05 compared with LPS alone.

Fig. 6. Effect of Bt2cAMP on LPS-induced nitrite release and iNOS expression. A, the cells were incubated for 24 h with the indicated concentrations of Bt2cAMP plus 1 μg/ml LPS; the medium was then removed and analyzed for nitrite. The results are expressed as the mean ± S.E. of three independent experiments performed in triplicate. *, p < 0.05 compared with LPS alone.

Fig. 7. Kinetics of NF-κB-specific DNA-protein complex formation in nuclear extracts of RAW 264.7 macrophages stimulated with LPS or Bt2cAMP. Cells were treated with 1 μg/ml LPS for 1, 3, 6, 12, or 24 h (A) or with 100 μM Bt2cAMP for 10 min or 1, 3, or 24 h (B); then nuclear extracts were prepared and NF-κB DNA-protein binding activity in the extracts was determined by EMSA as described under “Experimental Procedures.” *p < 0.01 compared with the basal level.

Fig. 4. Changes in the intracellular cAMP concentration in RAW 264.7 macrophages following LPS treatment. RAW 264.7 macrophages were treated with 1 μg/ml LPS for the indicated time intervals (A) or with 1,000 ng/ml CTX for 24 h or 100 μM forskolin for 10 min (B). The intracellular cAMP concentration was measured as described under “Experimental Procedures.” The results are expressed as the mean ± S.E. of three independent experiments performed in triplicate. The cAMP concentration in the basal state was 1.88 ± 0.2 pmol/mg protein (n = 3). *, p < 0.05 compared with the basal level.

Fig. 5. Effect of forskolin or CTX on LPS-induced nitrite release and iNOS expression. A, cells were incubated for 24 h with the indicated concentrations of forskolin or CTX plus 1 μg/ml LPS; the medium was then removed and analyzed for nitrite. The results are expressed as the mean ± S.E. of three independent experiments performed in triplicate. For iNOS expression studies (B and C), cell lysates from the nitrate assay were subjected to electrophoresis and Western blotting using iNOS-specific antibody as described under “Experimental Procedures.” *, p < 0.05 compared with LPS alone.
DISCUSSION

The effects of cAMP on iNOS expression have been of increasing interest since the first report that cAMP-elevating agents induced iNOS in cultured vascular smooth muscle cells and that this induction was synergistic with that elicited by inflammatory cytokines (17). Similar effects have also been seen in renal mesangial cells (13, 18) and brown adipocytes (19). Although cAMP alone does not induce iNOS in unstimulated cardiac myocytes, it augments iNOS induction in interleukin-1β-stimulated cells (20).

In 3T3 fibroblasts, different signaling pathways, including elevation of cAMP, lead to the induction of iNOS by NF-κB mediation (21). In contrast, elevation of cellular cAMP levels has been shown to down-regulate iNOS in LPS- or cytokine-activated astrocytes, hepatocytes, or Kuffer cells (15, 16, 22). In RAW 264.7 macrophages, NF-κB/Rel is positively regulated by the cAMP cascade, thus helping to initiate iNOS gene expression in response to LPS stimulation, and inhibition of adenylate cyclase attenuates LPS-induced activation of iNOS gene expression (12), indicating that, in inducing iNOS expression in these cells, LPS acts by increasing cAMP levels. In the present study, the PKA inhibitors, KT-5720 and H8, inhibited LPS-induced NO release and iNOS expression in a dose-dependent manner, indicating that the LPS effect is indeed related to the cAMP-PKA activation pathway. The cAMP analogue, Bt2cAMP, also increased NO release and iNOS expression; immunofluorescence staining also demonstrated iNOS expression in the cytoplasm. LPS caused a time-dependent increase in cAMP levels that was maximal with 6-h treatment and then declined. In J774 macrophages, the increase in cAMP levels occurs after 6 h of treatment with LPS (23).
and PKA is involved in the LPS-induced activation of junB and NF-κB (24). As previously reported (10, 11), activation of NF-κB-specific DNA-protein complex formation was seen after 10-min to 24-h treatment with LPS, and a similar time course of activation of this complex was seen using Bt2cAMP (Fig. 7B). In contrast with the inhibition seen using PKC or p38 inhibitors (10, 11), when cells were pretreated for 30 min with H8, the NF-κB-specific DNA-protein complex formation seen after 1 h of LPS treatment was unaffected (Fig. 8A). However, the complex formation seen after 6 h of LPS treatment was inhibited by H8, thus correlating with the maximal cAMP level seen after 6 h of treatment (Fig. 4A). Thus, in contrast with the PKC and p38 activation pathways, which are rapid (10 min) (10, 11), the cAMP-PKA activation pathway is a delayed event in LPS-induced NF-κB activation. cAMP may modulate NF-κB activation and iNOS expression via a prostanoid-dependent phosphorylation of the cAMP response element-binding protein (25). In RAW cells, the rapid activation of NF-κB by PKC and p38 pathways, together with the delayed activation of NF-κB by the cAMP-PKA pathway, contributes to the LPS-induced iNOS expression and NO release.

Because the cAMP-PKA activation pathway is a much delayed event (6 h) in LPS-induced NF-κB activation, the mechanism involved in LPS-induced increase in cAMP levels was further explored. Both NS-398 and indomethacin had an inhibitory effect on LPS-induced NO release, iNOS expression, and NF-κB activation, indicating the involvement of COX-2 expression in LPS-stimulated NO release. When the effect of various periods of LPS treatment was studied, no COX-2 expression was seen in unstimulated cells or after 1 h of treatment, but COX-2 expression was seen after 3 h of treatment and continued to increase up to 24 h. COX is a key enzyme in prostanoid synthesis, and it catalyzes the conversion of arachidonic acid to PGH2, which is then metabolized by one- or more terminal synthases to a variety of active prostanoids (26). It possesses both fatty acid cyclooxygenase activity and PG hydroperoxide-dase activity (converting PGG2 to PGH2). COX-2 is a COX isomere that is induced in a number of cells by proinflammatory stimuli and is thought to contribute to the generation of prostanoids at sites of inflammation (27, 28); it is considered to be responsible for high production of PGs (29). PGE2 production following LPS treatment was also measured, and the increases after 1, 3, 6, 12, or 24 h of treatment were, respectively, 2-, 11-, 45-, 85-, and 134-fold of basal levels, paralleling the increase in COX-2 expression. PGE2 acts via receptor-mediated generation of cAMP and activation of PKA (30). As seen in a study on the effects of interleukin-1β on human bronchial smooth muscle cells (31), in the present study, induction of PGE2 synthesis precedes the increase in cAMP, and PGE2 acts as an autocrine factor for adenylate cyclase activation. LPS-induced tumor cell killing in EC4 cells is also because of increased levels of cAMP, and this effect is inhibited by indomethacin (32). In peritoneal macrophages, LPS is reported to act via PGE2 to increase cAMP levels (33).

In summary, in RAW 264.7 cells, LPS increases iNOS expression via a prostanoid- and cAMP-dependent pathway, and this is followed by PKA activation of NF-κB. The increase in PGE2 is because of COX-2 expression. This effect has a more delayed onset (6 h) compared with those involving the PKC and p38 activation pathways (10 min). A schematic representation of the signaling pathway for the LPS-induced NO release in RAW cells is shown in Fig. 11.

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