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Inhibition of Nuclear Factor-κB Activation in Mouse Macrophages and the RAW 264.7 Cell Line by a Synthetic Adenyl Carbocyclic Nucleoside

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ABSTRACT. Adenyl carbocyclic nucleosides have potent anti-inflammatory effects on a number of cell types. Notable in this regard is their ability to inhibit the production of tumor necrosis factor-α (TNF-α) by mouse macrophages that have been activated with bacterial lipopolysaccharide (LPS). Because the transcriptional activation of the mouse *TNF-α* gene is highly dependent on κB enhancers, the present study determined whether the synthetic carbocyclic nucleoside 9-[(1*S*,3*R*)-*cis*-cyclopentan-3-ol]adenine (cPA) inhibited LPS-induced nuclear factor-κB (NF-κB) activation in these cells. Stimulation of either mouse peritoneal macrophages or RAW 264.7 macrophage-like cells with LPS led to the appearance of four distinct κB-binding nucleoprotein complexes detected by gel mobility shift assays. Cells treated with 100 μM cPA showed significantly reduced levels of NF-κB activation as evidenced by measurements of nucleoprotein κB-binding activity and diminished κB-dependent transcriptional activation. However, both the LPS-induced degradation of the cytoplasmic NF-κB inhibitor IκBα and the nuclear translocation of the NF-κB p50, p65, and c-Rel peptides were unaffected by treatment of the cells with the nucleoside. These findings suggest that certain adenyl carbocyclic nucleosides inhibit the activation of NF-κB/Rel complexes by a novel mechanism that results in an inhibition of their DNA-binding activities, without blocking their dissociation from IκBα or their nuclear translocation. BIOCHEM PHARMACOL **60**;5:717–727, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. adenyl carbocyclic nucleoside; TNF-α; NF-κB; IκBα

Adenyl carbocyclic nucleosides include compounds consisting of an adenine base 9,1-linked to a substituted cyclopentane or cyclopentene ring. Both naturally occurring and synthetic forms of these compounds have been shown to suppress inflammatory responses *in vivo* [1–3] and inhibit a variety of inflammatory cell functions *in vitro* [1, 4–6]. For example, the synthetic adenosine analog cPA† (the chemical structure of this compound is shown in Fig. 1) inhibits the production of TNF-α by mouse macrophages *in vitro* and protects mice against the lethal systemic inflammatory response to bacterial endotoxic LPS [1, 4]. The compound and several of its enantiomers also inhibit TNF-α mRNA expression in LPS-activated mouse bone marrow-derived macrophages [6] and two macrophage-like cell lines [1]. This was not due to changes in TNF-α mRNA stability, suggesting a direct inhibition of *TNF-α* gene transcription. Although these findings indicate that cPA has potent anti-inflammatory properties, there currently exists no clear understanding of its molecular mechanism of action in macrophages or other inflammatory cells.

The initiation of *TNF-α* gene transcription in LPS-activated mouse macrophages is thought to be regulated by *trans*-acting members of the NF-κB/Rel family of dimeric transcription factors [7–10]. Evidence supporting this conclusion is extensive. κB sequence motifs located both upstream and downstream of the core *TNF-α* promoter bind NF-κB/Rel nucleoprotein complexes comprised of the peptides p50 (NF-κB1), p65 (RelA), and c-Rel (Rel) in activated, but not unstimulated, cells [7–9, 11]. Reporter gene constructs containing several of these κB sequences are transcriptionally active in transfected LPS-stimulated mouse macrophages or macrophage-like cell lines [7–9]. Mutant κB sequences within cloned upstream *TNF-α* gene fragments that lack NF-κB binding activity also lack *trans*-activating activity [8].

Inactive dimeric NF-κB/Rel complexes are sequestered in the cytoplasm of most cells by members of the IκB inhibitor family of polypeptides [12, 13]. Upon cell activation, IκB is phosphorylated and degraded, permitting NF-κB/Rel complexes to translocate to the cell nucleus and bind κB motifs of the sequence GGGRNTYYCC. A number of agents that inhibit NF-κB activation and cytokine gene expression, including glucocorticoids, salicylates,

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† *Abbreviations*: ADO, adenosine; CAT, chloramphenicol acetyltransferase; cPA, 9-[(1*S*,3*R*)-*cis*-cyclopentan-3-ol]adenine; db-cAMP, dibutyryl cyclic AMP; LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor-κB; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; and TNF-α, tumor necrosis factor-α.

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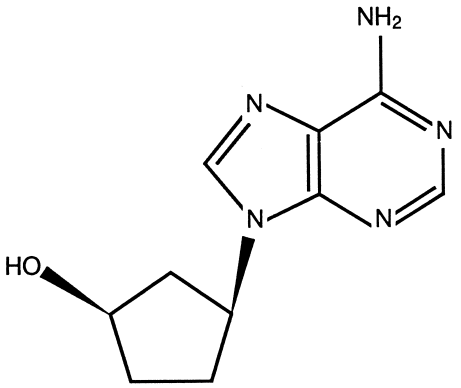

FIG. 1. Chemical structure of 9-[(1S,3R)-*cis*-cyclopentan-3-ol]adenine.

and certain kinase inhibitors, do so by altering signaling pathways at steps proximal to IκB degradation [14]. In turn, this prevents both the release of NF-κB from its inhibitor and its nuclear translocation. Based on these findings, we reasoned that the activation of NF-κB would be a logical target for the action of adenyl carbocyclic nucleosides, and we have characterized the effects of the nucleoside cPA in two different populations of LPS-stimulated mouse macrophages. The results reported here suggest a novel mechanism by which this group of compounds inhibits the expression of pro-inflammatory genes.

MATERIALS AND METHODS

Reagents

Modified RPMI 1640 medium, L-glutamine, and penicillin–streptomycin were obtained from JRH Bioscience, and fetal bovine serum (FBS) was obtained from Atlanta Biologicals. Poly(dI-dC)-poly(dI-dC) was purchased from Pharmacia Biotech, T4 polynucleotide kinase from Promega, DNase I from Boehringer Mannheim, and BSA from New England Biolabs. The western blot chemiluminescent Renaissance® substrate and [γ-^{32}P]ATP were obtained from DuPont-NEN. Rabbit polyclonal Trans-Cruz® antibodies to NF-κB p50, p65, c-Rel, and RelB (sc-114X, sc-109X, sc-70X, and sc-226X, respectively) were from Santa Cruz Biotechnology. Rabbit antibody to a mouse IκBα N-terminal peptide (No. 751) [12] was obtained from Nancy Rice (National Cancer Institute). Rabbit anti-TNF-α was provided by David Morrison (University of Kansas Medical Center). Phenol-extracted *Escherichia coli* LPS (O111:B4) was from List Biologicals. *Staphylococcus aureus* protein A–peroxidase conjugate, TPCK, and ADO were purchased from the Sigma Chemical Co. The carbocyclic nucleoside cPA (M_r 217) was synthesized by David Borcherding of Hoechst Marion Roussel, Inc. The pCAT3-Promoter vector was obtained from Promega. Lipofectamine 2000 (LF2000) and Opti-MEM I Reduced Serum Medium without serum were purchased from Gibco BRL Life Technologies. D-*threo*-[*dichloroacetyl*-1-^{14}C]Chloramphenicol (25 μCi/mL, 55 mCi/mmol) was obtained from Amersham.

Cells and Cell Culture

The mouse macrophage-like cell line RAW 264.7 was obtained from Stephen Russell (University of Kansas Medical Center) and maintained in complete culture medium consisting of RPMI 1640 containing 2 mM l-glutamine, 100 U penicillin/mL, 100 μg streptomycin/mL, and 10% FBS [15]. Thioglycollate-elicited peritoneal exudate macrophages were prepared from 7- to 12-week-old C3HeB/FeJ mice (Jackson Laboratories) as described previously [15]. For gel shift and western blotting experiments, two million or five million cells were plated in 60- or 100-mm Primaria dishes (Falcon), respectively. Both macrophages and RAW 264.7 cells were allowed to adhere overnight, and nonadherent cells were removed by washing the plates prior to each experiment. Cell viability following various treatments was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [16].

Preparation of Nuclear and Cytosolic Extracts

Cellular proteins were extracted by a modification of the methods of Schreiber *et al.* [17]. Briefly, the adherent cells were first washed twice with 5 mL of cold PBS and then removed with the aid of a rubber policeman in 0.5 to 1 mL of hypotonic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$). After centrifugation, the cell pellets were resuspended in 50–200 μL of hypotonic lysis buffer containing 0.5% Nonidet P-40 (NP-40) and gently vortex-mixed for 15 sec. Nuclei were separated from the cytosolic fraction by centrifugation. The supernatant cytosolic fraction was clarified by centrifugation, supplemented with 1/5 vol. glycerol, and stored in small aliquots at −80°. The nuclear pellet was washed in 200–500 μL of hypotonic lysis buffer containing 0.5% NP-40 and resuspended in 10–15 μL of hypertonic extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol) [18]. The samples were incubated for 20 min on ice with intermittent vortex mixing and then centrifuged. The resulting supernatant (nuclear extract) was combined with 1.5 vol. of dilution buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol) and stored in small aliquots at −80°. All lysis and extraction buffers contained protease inhibitors [0.5 mM dithiothreitol (DTT), 5 μg/mL of leupeptin, 5 μg/mL of aprotinin, 10 μg/mL of soybean trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL of pepstatin]. For extracts that were to be analyzed for IκBα content, the extraction and dilution buffers also contained the following phosphatase inhibitors: 1 mM NaF; 1.5 mM $NaMoO_4$; 1 mM β-glycerophosphate; and 0.4 mM $NaVO_4$. Protein concentrations were determined according to Bradford [19] using a Bio-Rad protein dye reagent.

Preparation of Oligonucleotide Probes

Double-stranded oligonucleotides corresponding to two tandem-arranged copies of the κB site 3 (position −510) of

the mouse *TNF-α* gene were synthesized by the Biotechnology Support Facility of the University of Kansas Medical Center and gel-purified in our laboratory. The following sequences were used: CAGGGGGCTTTCCCT (wild-type sequence with core sequence underlined) [7] and CAG GAAGCTTTCCCT (mutant oligonucleotide with mutated residues in bold) [8]. Each oligonucleotide and its complementary strand were end-labeled using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase. Then they were annealed and used as probes in the gel shift assays.

EMSA

Gel shift assays were performed by the following modifications of the methods of Muroi *et al.* [20]. Nuclear extracts (5–10 μg protein) were incubated for 15 min on ice in 20 μL of binding buffer (10 mM Tris–HCl, pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 4% glycerol) containing 20 μg BSA and 2 μg poly(dI-dC). The appropriate ^{32}P-labeled oligonucleotide probe (1–2 × 10^5 cpm) then was added to the reaction mixtures, which were incubated for an additional 20 min at room temperature. For binding competition assays, a 50-fold excess of nonlabeled oligonucleotide was included in the reaction mixture during the first incubation period prior to the addition of the labeled probe. To identify NF-κB/Rel peptides in κB-binding complexes, nuclear extracts were first incubated with the appropriate Trans-Cruz® antibodies for 30 min at room temperature prior to the addition of the probe. Then the reaction mixtures were electrophoresed through a native 6% polyacrylamide gel with 0.5x TBE (45 mM Tris–borate buffer, 1 mM EDTA). Electrophoresis was carried out at 15 V/cm for 1–2 hr at room temperature. The gel was dried and analyzed by autoradiography. Scanning densitometry was performed with a Molecular Dynamics model PDSI scanner, and data were analyzed using ImageQuant® software.

CAT Reporter Gene Transcription Assay

A double-stranded oligonucleotide containing two tandem copies of the κB site 3 of the mouse *TNF-α* gene (sequence listed above) was inserted between the *Mlu*I and *Bgl*II sites of the pCAT3-Promoter plasmid upstream of the SV40 promoter. This new plasmid was designated pCAT3-κB3-P. Plasmid DNA (4 μg) and LF2000 (10 μL) each were prepared in 250 μL Opti-MEM and incubated for 5 min. Then the DNA was combined with the LF2000, and the mixtures were incubated for 20 min at room temperature immediately prior to adding to cell cultures.

Twenty-four hours prior to transfection, RAW 264.7 cells were subcultured in complete culture medium at a density of 10^6 cells per well in 6-well plates. The medium was then replaced with Opti-MEM I, and the freshly prepared DNA–LF2000 mixtures were added gently to each well. Twenty-four hours later, the cells were stimulated with LPS (10 ng/mL) in the presence or absence of 100 μM cPA or 100 μM ADO. Eighteen hours later, total cellular protein was determined, and CAT enzymatic activity was measured by liquid scintillation counting of *n*-butyryl chloramphenicol as previously described [21]. The CAT activity of each culture was first normalized on the basis of total cellular protein. For each plasmid, the activities of the treated cultures were divided by the mean activity of the cultures that received neither LPS nor test compounds, and the data were reported as fold-activation.

Western Blotting

Nuclear and cytosolic extracts were fractionated by discontinuous SDS–PAGE (10% separating gel) and transferred at 4° to nitrocellulose membranes (0.2 μm; Bio-Rad) in transfer buffer (10% methanol, 25 mM Tris, 192 mM glycine, pH 8.3). Then the membranes were blocked for 1 hr with 22% skim milk (Difco) in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20 (TBS-T), washed with TBS-T, and treated with various anti-NF-κB/Rel or anti-IκBα antibodies, first at room temperature for 1 hr and then overnight at 4°. After washing with TBS-T, the membranes were incubated with a protein A–peroxidase conjugate, washed, and incubated with an enhanced chemiluminescence reagent (DuPont-NEN). Certain membranes were blocked and reprobed after treating with stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) at 50° for 30 min.

Immunocytochemical Localization of NF-κB p65

RAW 264.7 cells were grown in 8-well chamber slides (Nalge Nunc) to near confluence and then activated with LPS (10 ng/mL) in the presence or absence of 100 μM cPA. Thirty minutes later, the slides were washed thoroughly with PBS and fixed in 4% paraformaldehyde at 4° for 30 min. Following a 2-hr wash in cold PBS, immunocytochemical staining was performed as previously described using a peroxidase biotin–streptavidin system [22]. Briefly, cell monolayers were first incubated with a blocking solution containing 5% normal goat serum, 0.5% IgG- and protease-free BSA (Jackson ImmunoResearch Laboratories), 0.1% saponin, and 0.5% cold-water fish skin gelatin (Sigma). This solution was also used to dilute primary and secondary antibodies. Then an avidin–biotin blocking reagent (Vector Laboratories) was applied according to the manufacturer's instructions. Next, cell monolayers were incubated with rabbit anti-NF-κB p65 antibody (SC-109, Santa Cruz) at a concentration of 3 μg/mL at 4° overnight followed by a 1-hr incubation at room temperature. Primary antibody binding was detected by incubating the slides with biotinylated goat anti-rabbit IgG (BioGenex) diluted 1:30. After washing, the monolayers were treated with 1% H_2O_2 in methanol to inactivate endogenous peroxidases. Bound secondary antibody was detected by incubating the slides with a peroxidase–streptavidin conjugate (BioGenex) diluted 1:30, and reaction sites were visualized with diami-

nobenzidine (DAB kit, Vector) according to the manufacturer's instructions. Monolayers incubated with PBS or normal rabbit IgG instead of the primary antibody showed no staining.

TNF Assays

The concentration of TNF-α in 9-hr culture supernatant fluids was measured by the L929 bioassay [16] or by ELISA (Pharmingen) with similar results. The addition of 100 μM cPA had no effect on the detection of recombinant mouse TNF-α in this assay.

RESULTS

Effects of the Adenyl Carbocyclic Nucleoside cPA on TNF-α Production and κB DNA-Binding Activity

To determine whether or not cPA alters the induction of κB DNA-binding activity, gel mobility shift assays were performed with nucleoprotein extracts and a double-stranded synthetic oligonucleotide corresponding to κB site 3 of the mouse *TNF-α* gene [8]. LPS induced a concentration-dependent increase of κB-binding activity in both macrophage and RAW 264.7 cell nucleoproteins with two bands, designated #2 and #3 in Fig. 2A, constituting the predominant species seen at both 15 and 120 min. Nucleoprotein binding to the κB probe was specific in that it was not seen with a labeled mutant κB oligonucleotide in which two A for G substitutions had been made at positions −510 and −511. Similarly, a 50-fold excess of the unlabeled wild-type, but not the mutant oligonucleotide, completely inhibited nucleoprotein binding to the labeled wild-type probe (data not shown).

Nucleoproteins prepared from peritoneal macrophages showed maximum κB-binding activity at two distinct time points, 60 and 300 min, following LPS stimulation (Fig. 2B). This bimodal response, which also has been reported by Jeong *et al.* [23], occurred somewhat earlier in RAW 264.7 cells (Fig. 2C). Importantly, the addition of cPA together with LPS led to a reduction in the DNA-binding activity of nucleoproteins recovered from both cell types. The effect was most apparent during the later phase of this response, but significant inhibition also was seen at the early time points and affected both EMSA bands. cPA did not inhibit protein synthesis or the steady-state level of nucleoproteins in resting or activated cells. In a total of seven such experiments, the inhibition of LPS-induced NF-κB activation by cPA correlated well with the ability of the compound to inhibit TNF-α production assessed by either bioassay or ELISA. Thus, 100 μM cPA inhibited TNF-α production an average of 84% (determined by bioassay) and inhibited the 30-min band 2 and band 3 in EMSA, determined by scanning densitometry, by 67 and 51%, respectively. Treating either peritoneal macrophages or RAW 264.7 cells with 100 μM cPA alone had no effect on cell viability, nor did it induce TNF-α production or activate κB-binding activity. Likewise, the direct addition of cPA at concentrations ranging from 50 to 300 μM to nucleoproteins from activated cells did not affect their ability to bind the κB oligonucleotide probe.

TNF-α itself induces NF-κB in a number of cell types [24–27] and can co-activate mouse macrophages [4, 28, 29]. Because cPA is a potent inhibitor of TNF-α production by macrophages [1, 3, 6], we next determined whether or not the depletion of TNF-α from the cultures with a neutralizing antibody resulted in an effect on NF-κB activation similar to that shown in Fig. 2. When RAW 264.7 cells were incubated in the presence of anti-TNF-α at a concentration sufficient to neutralize all detectable cytokine, the pattern of LPS-induced NF-κB activation was unaffected (Fig. 3). This indicated that the ability of cPA to inhibit LPS-induced TNF-α production was not sufficient to explain its inhibitory effect on NF-κB activation.

Polypeptide Composition of Nucleoprotein Complexes That Bind to the TNF-α κB Probe

To verify that the nucleoproteins that bound the TNF-α κB probe were indeed NF-κB complexes, protein extracts were treated with antibodies specific for the NF-κB/Rel peptides p50, p65, c-Rel, and RelB and then analyzed by EMSA. Antibody to p50 completely blocked the formation of band 2 when nucleoproteins were prepared from RAW 264.7 cells either 30 or 180 min after LPS activation (Fig. 4A). In contrast, neither anti-p65 nor anti-c-Rel inhibited band 2. The formation of band 3 was inhibited partially by either anti-p50 or anti-p65, while anti-c-Rel had a lesser effect. A combination of anti-p50 plus anti-p65 (Fig. 4B) completely inhibited both complexes, whereas the addition of anti-c-Rel to either anti-p50 or anti-p65 did not significantly change the pattern of κB-binding activity from that observed with either anti-p50 or anti-p65 alone. Anti-RelB did not detectably alter the binding between RAW 264.7 cell nucleoproteins and the κB probe, when used either alone or in combination with the other antibodies (data not shown). Virtually identical results were obtained with peritoneal macrophages and suggested that band 2 consisted primarily of p50 homodimers, whereas p50–p65 heterodimers predominated in band 3. However, we cannot exclude the possibility that p65 homodimers and/or p65 complexed, for example, with c-Rel were also present in band 3. Overall, these data indicate that cPA inhibited the expression of both p50–p65 and p50–p50 nucleoprotein complexes from LPS-activated cells that could bind to the TNF-α κB probe.

Effects of cPA on κB-Dependent Transcriptional Activation

To determine whether κB-dependent transcription was inhibited by cPA, either a κB-containing promoter-reporter construct (pCAT3-κB3-P) or a control plasmid lacking κB sequences (pCAT3-Promoter) was transfected into RAW 264.7 cells. The cells were then activated with

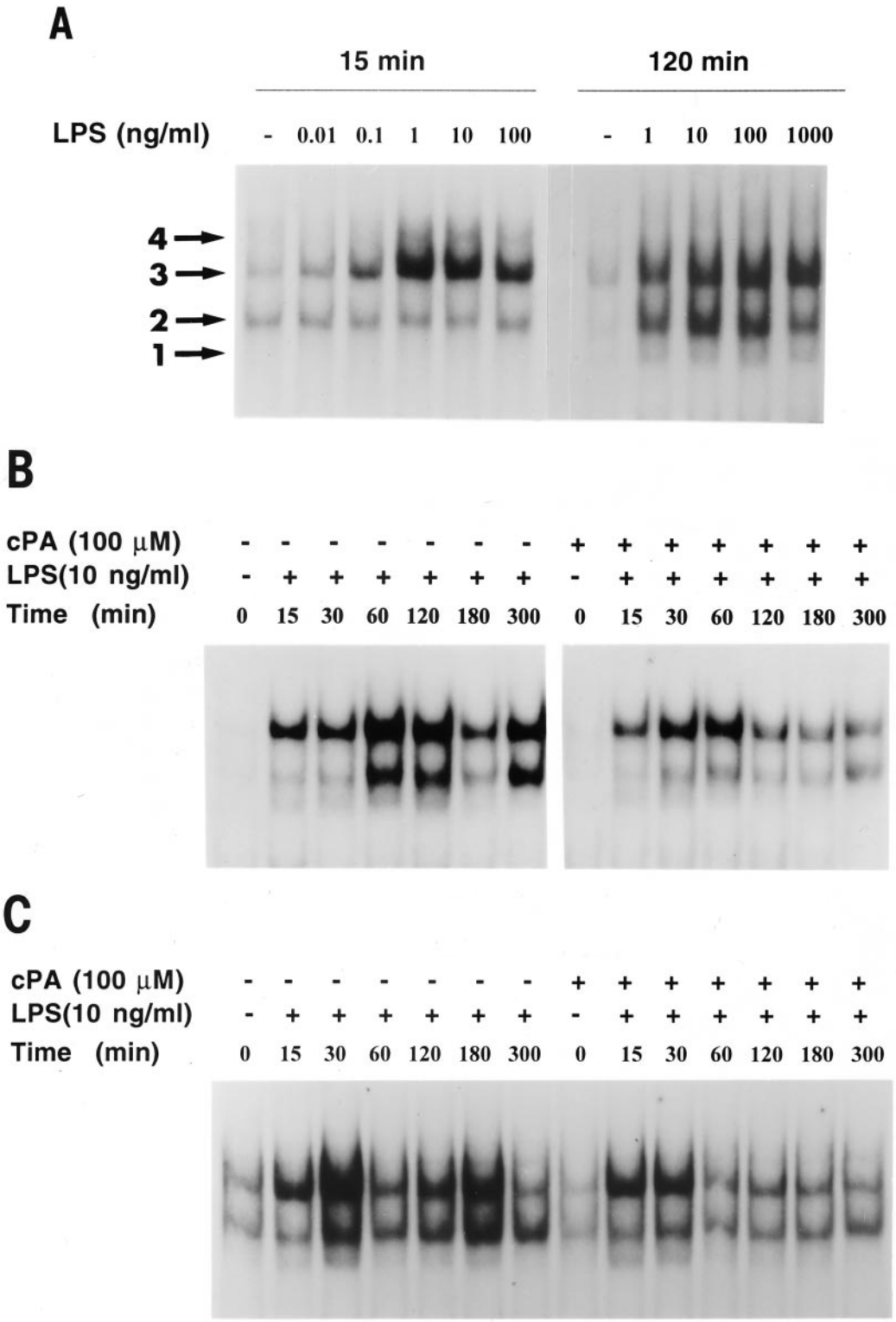

FIG. 2. Effects of cPA on the induction of κB DNA-binding activity in LPS-stimulated mouse RAW 264.7 cells and peritoneal macrophages. (A) Concentration-dependent activation of NF-κB in RAW 264.7 cells. Either 15 or 120 min following activation of cells with the indicated concentrations of *E. coli* LPS, nucleoproteins were extracted and analyzed for κB-binding activity by EMSA using a radiolabeled TNF-α κB probe. This experiment was performed twice with RAW 264.7 cells and once with peritoneal macrophages with similar results. (B) Peritoneal macrophages were activated with 10 ng LPS/mL in the absence or presence of 100 μM cPA, and κB-binding activity was determined by EMSA. The results shown here are representative of three different experiments. In this particular experiment, treatment with cPA resulted in a 71% (band 2) and 68% (band 3) inhibition of κB-binding activity measured at 60 min. (C) RAW 264.7 cells were activated with 10 ng LPS/mL in the absence or presence of 100 μM cPA, and κB-binding activity was determined by EMSA. The results shown here are representative of seven different experiments. Treatment with cPA resulted in a 77% (band 2) and 51% (band 3) inhibition of κB-binding activity measured at 30 min.

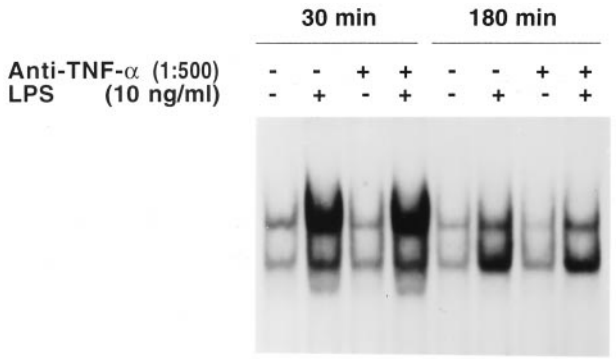

FIG. 3. Role of TNF-α in LPS-induced NF-κB activation in RAW 264.7 cells. Cells were activated with LPS in the absence or presence of excess neutralizing rabbit antibody to mouse TNF-α. The results shown are representative of two experiments.

LPS in the presence or absence of cPA. Adenosine was included as a control for the test compound, and CAT activity for each plasmid was expressed in relationship to the activity of untreated, transfected cells. The results shown in Table 1 are representative of three such experiments and indicate that LPS-induced, κB-dependent transcriptional activation of the CAT reporter gene was inhibited by 100 μM cPA, but not 100 μM ADO.

Degradation of IκBα in LPS-Activated RAW 264.7 Cells

The cytoplasmic inhibitor IκBα negatively regulates LPS-induced κB-dependent transcription in mouse macrophages [30] and is degraded rapidly following cell activation with LPS [31]. Based on scanning densitometry, greater than 95% of cytoplasmic IκBα was degraded within 20 min of exposing RAW 264.7 cells to LPS (Fig. 5), and this was followed by the resynthesis of the inhibitor within 1 hr. In the presence of the protease inhibitor TPCK, IκBα degradation was inhibited markedly. In contrast, when 100 μM cPA was included in LPS-stimulated cultures, there was virtually no change in either the extent of IκBα degradation or its resynthesis compared with that seen in control cells activated with LPS alone.

Nuclear Accumulation of NF-κB/Rel Peptides in LPS-Stimulated RAW 264.7 Cells

These latter findings suggested that the ability of cPA to inhibit the activation of NF-κB was not due to changes in the stability of cytoplasmic IκBα, but rather reflected a more direct effect on the behavior of the NF-κB complexes. Treating cells with cPA alone did not affect the cytoplasmic levels of p50, p65, or c-Rel peptides determined by western blotting. To ascertain whether the nuclear translocation of NF-κB/Rel peptides in LPS-stimulated cells was inhibited by the nucleoside, we compared the composition of nucleoproteins from LPS-activated RAW 264.7 cells with those from cells activated in the presence of cPA. The results shown in Fig. 6A demonstrate that the time-dependent accumulation of NF-κB/Rel peptides in the nuclei of LPS-activated cells was similar to the pattern shown by EMSA (Fig. 2C). The nuclear translocation of NF-κB/Rel peptides was virtually the same in control and cPA-treated cells that were activated for various times (Fig. 6A) or with various concentrations of LPS (Fig. 6B). Similar results were obtained when NF-κB p65 nuclear localization was measured by immunocytochemistry (Fig. 6C) rather than by biochemical methods. By contrast, NF-κB peptides did not accumulate in the nuclei of control cells that were activated with LPS in the presence of the

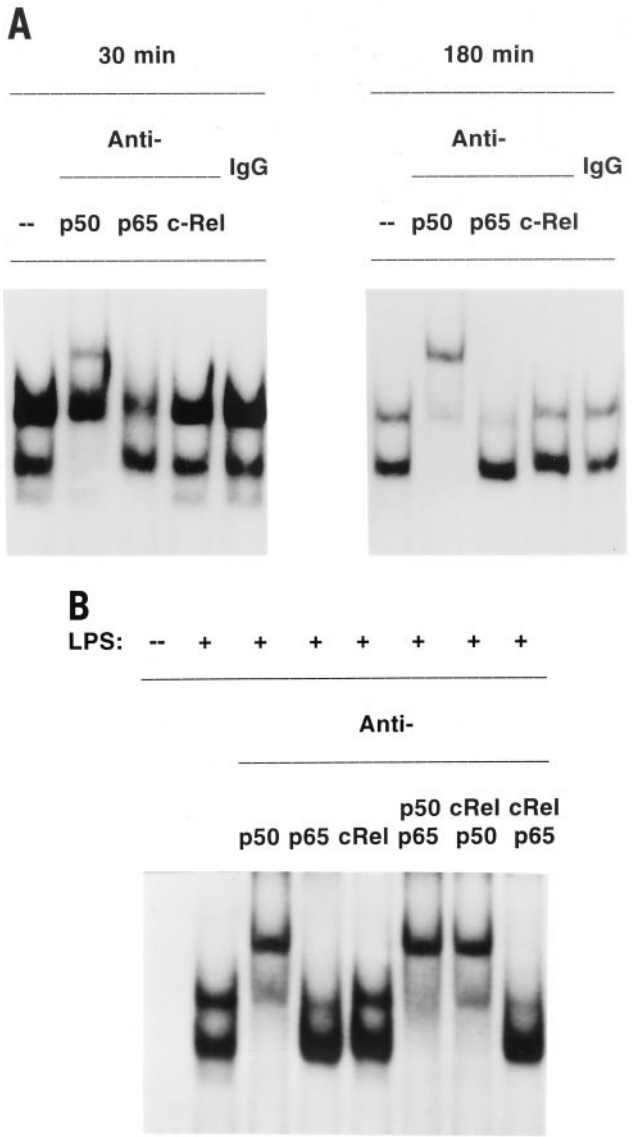

FIG. 4. Identity of NF-κB/Rel nucleoproteins in κB-binding complexes from LPS-activated RAW 264.7 cells. (A) Nuclear extracts were prepared at 30 and 180 min from LPS-activated cells and incubated for 30 min with antibodies to the indicated NF-κB/Rel proteins. Binding to the κB probe was then assessed by EMSA. (B) Nucleoproteins prepared 180 min after activation with LPS were analyzed by supershift EMSA using combinations of antibodies to the indicated NF-κB/Rel proteins. These experiments were repeated with similar results.

TABLE 1. Effect of cPA on LPS-induced, κB-dependent CAT expression in RAW 264.7 cells

	Fold-activation induced by:		
Plasmid	LPS	LPS + cPA	LPS + ADO
pCAT3-Promoter	1.1 ± 0.1	0.8 ± 0.1	ND
pCAT3-κB3-P	4.5 ± 0.8	0.5 ± 0.2*	4.3 ± 0.5

Values are means ± SD for triplicate samples. ND = not determined.
*Significantly different from the LPS group ($P < 0.01$) as determined by Student's *t*-test.

protease inhibitor TPCK (Fig. 6B). These results indicate that cPA inhibited the DNA-binding activity of NF-κB/Rel dimeric complexes without affecting the nuclear translocation of p50, p65, or c-Rel-containing dimers.

DISCUSSION

The ability of certain adenyl carbocyclic nucleosides to inhibit macrophage inflammatory gene expression induced by a variety of stimuli is well established [1, 3, 6, 32]. For example, cPA and several of its structural enantiomers have been shown to inhibit *TNF-α* gene expression in mononuclear phagocytes that were activated with either LPS, opsonized zymosan, interferon-γ + TNF-α, or heat-killed *Listeria monocytogenes* [1, 3, 6, 32]. These findings indicate that adenyl carbocyclic nucleosides share a common immunomodulatory effect on human and mouse cells. However, there exists little information on the mechanism of action of this class of compounds. For this reason, we asked whether cPA altered NF-κB activation in either primary mouse macrophages or the RAW 264.7 macrophage-like cell line. In both cell types, LPS induced the formation of two predominant nucleoprotein complexes (bands 2 and 3) that bound to κB-containing DNA in gel shift assays. These complexes were comprised primarily of p50 and p65, although the existence of other DNA-binding proteins could not be excluded by the present analysis. Importantly, the binding of both complexes 2 and 3 to the κB oligonucleotide was inhibited significantly by treating the cells with cPA at the time of their activation with LPS.

A number of other agents, including glucocorticoids [33, 34], salicylates [35], protease inhibitors [31, 36, 37], and (anti)oxidants [38–45], inhibit NF-κB activation, including that induced by LPS [23, 31, 33, 36, 38, 40, 42–44]. While several of these agents undoubtedly act by more than one mechanism [14, 23, 31, 45, 46], most affect very early stages in the LPS signaling cascade, including the degradation or biosynthesis of IκB. Therefore, it was surprising to find that cPA did not affect IκBα degradation or the nuclear translocation of NF-κB/Rel peptides. These findings indicate that cPA inhibits a step in the process of NF-κB activation, but does not decrease the migration of activated complexes from the cytoplasm to the nucleus.

Sajjadi *et al.* [47] have reported that adenosine and an A3 adenosine receptor agonist inhibit LPS-induced TNF-α production by differentiated human promonocytic U937 cells. The latter compound had no detectable effect on the activation of NF-κB, but did alter the subunit composition of AP-1-binding nucleoproteins. Preliminary experiments have indicated that cPA does not inhibit the AP-1 binding activity of nucleoproteins from LPS-activated mouse macrophages. Likewise, cPA has not been found to change the subunit composition of NF-κB complexes measured by supershift EMSA, although it significantly decreased the quantity of these nucleoprotein complexes that bound to κB-containing DNA.

The extent to which cPA inhibited TNF-α mRNA and protein production strongly correlated with, but generally exceeded, its inhibition of κB-binding activity. This suggested that cPA may also inhibit other transcription factors or the activation of NF-κB/Rel complexes that bind to other TNF-α κB sites [9, 48]. Yao *et al.* [49] recently have shown that optimal transcriptional activation of the human *TNF-α* promoter in response to LPS is dependent upon cooperation between adjacent κB and CRE sites, indicating that the environment in which a κB site functions greatly influences its activity. In this sense, we would not predict

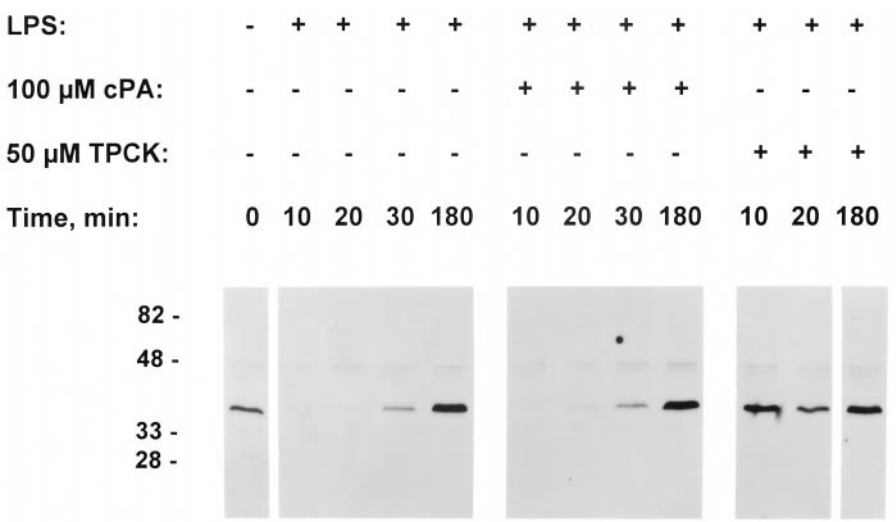

FIG. 5. Effects of cPA on the degradation of IκBα in LPS-activated RAW 264.7 cells. Either medium, 100 μM cPA, or 50 μM TPCK was added to cultures 15 min prior to the addition of LPS (10 ng/mL). The degradation of cytoplasmic IκBα was determined by western blotting at the indicated times, and the results shown are representative of three experiments. The migration of the respective molecular weight markers (× 10^3) is indicated on the left.

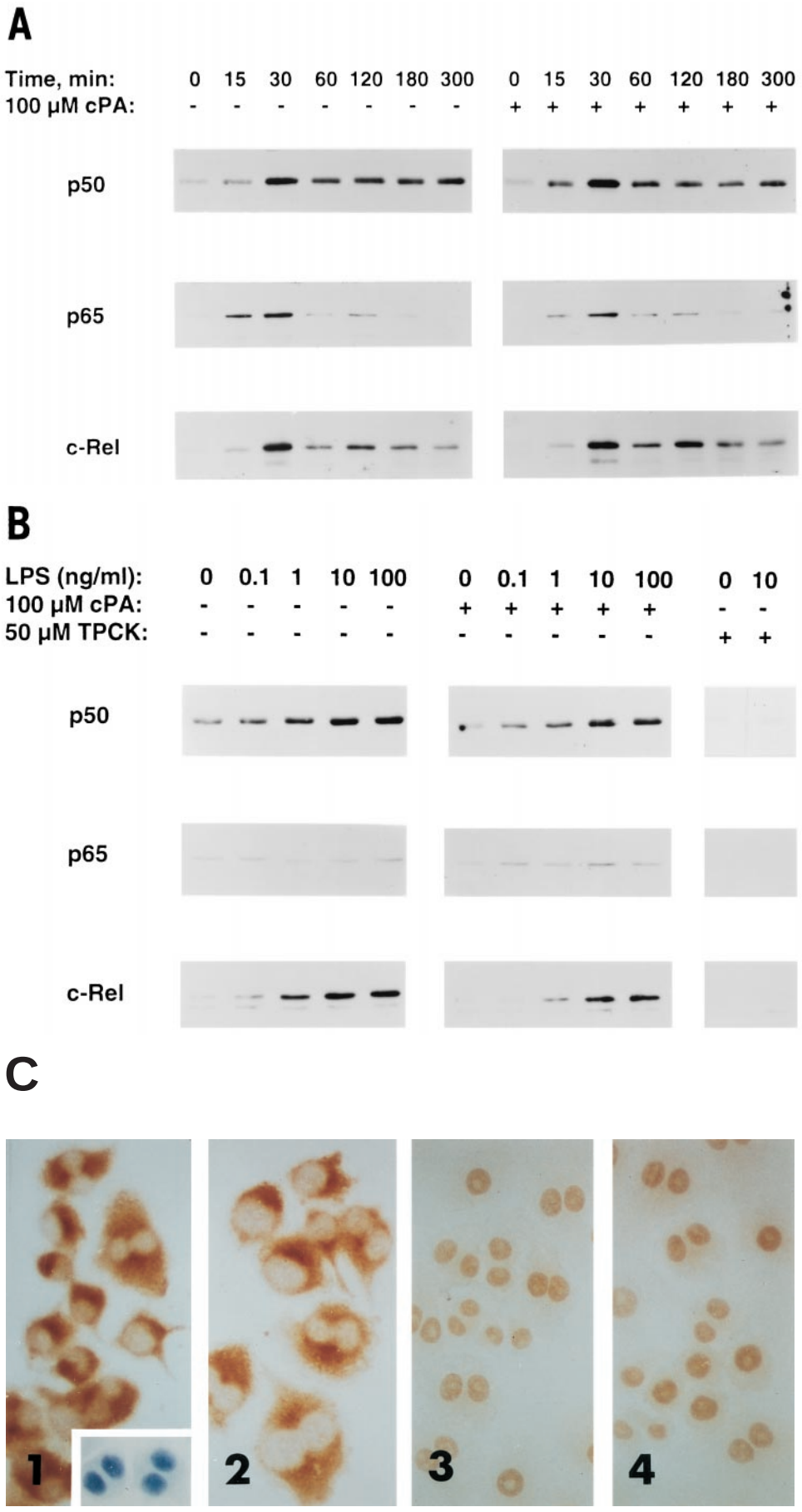

FIG. 6. Effects of cPA on the nuclear localization of NF-κB/Rel peptides in LPS-activated RAW 264.7 cells determined by western blotting. (A) Cells were activated with 10 ng LPS/mL in the absence or presence of 100 μM cPA for the indicated times. Nucleoproteins then were prepared and analyzed with specific antisera for the presence of NF-κB/Rel peptides. (B) Cells were activated with the indicated concentrations of LPS in the presence of medium, 100 μM cPA, or 50 μM TPCK. Nucleoproteins prepared 180 min later were analyzed for the presence of the indicated NF-κB/Rel peptides. Note that p65 peptides were not as abundant in nucleoprotein preparations at this later time point (see panel A). (C) The nuclear localization of NF-κB p65 in activated RAW 264.7 cells was determined by immunocytochemistry after 30 min of culture. Shown here are unstimulated cells (1), cells incubated with 100 μM cPA (2), cells activated with LPS (3), and cells activated with LPS in the presence of cPA (4). The insert in panel 1 shows unstimulated cells counterstained with hematoxylin to show nuclear morphology. Each of these experiments was repeated with similar results.

that cPA would inhibit the expression of all LPS-inducible genes that contain functional κB enhancers. Different κB sites bind distinct NF-κB/Rel complexes and may also require physical contact with other transcription factors that bind adjacent sites for their optimal expression. These complex interactions would not be the same for each LPS-responsive gene. Indeed, cPA did not inhibit the expression of the *inducible NO synthase* or *interleukin-1* genes in mouse macrophages [1, 4, 6, 32], both of which contain κB elements.

Ollivier *et al*. [48] reported that the expression of LPS-induced TNF-α mRNA and κB-dependent transcriptional activation in THP-1 monocytic cells was inhibited by db-cAMP. Treating cells with db-cAMP did not affect the degradation of IκBα, the nuclear localization of NF-κB peptides, the κB-binding activity of nucleoproteins, or the phosphorylation of p65, which can enhance its *trans*-activating activity [50]. These properties differ from the effects of cPA reported here in that cPA did inhibit κB-binding activity. However, like those of db-cAMP, the effects of cPA suggest that the expression of κB-containing LPS-responsive genes can be regulated by processes that are apparent only after activated NF-κB complexes enter the nucleus.

Treating nucleoproteins from activated cells with cPA at concentrations as great as 300 μM (with or without reducing agents) did not affect their κB-binding activity. Thus, cPA does not appear to chemically modify NF-κB/Rel peptides, as has been reported for a number of other inhibitory compounds [29, 51, 52]. Of course, this does not exclude the possibility that metabolites of the compound formed *in vivo* have this property. Reactive oxygen species (e.g. H_2O_2), redox recycling agents that generate oxygen radicals, and nitric oxide donors all have been shown to alter the binding of NF-κB/Rel dimers to κB DNA [38, 41, 53]. In many cases these agents act by causing the oxidation of amino acid residues that are essential for DNA-binding activity [38, 41, 53]. Cysteine-62 of the p50 peptide, which resides within a conserved RxxRxRxxC motif, is particularly sensitive to oxidative inactivation [41, 51]. Cellular oxidative stress may create conditions that favor this form of regulation. Although we do not currently have any evidence that cPA alters the intracellular redox balance within LPS-activated mouse macrophages, the compound does inhibit superoxide production by rat macrophages [5]. This suggests that cPA has the potential to alter cellular redox balance, and studies designed to determine whether this effect might explain decreased NF-κB activation in mouse macrophages are ongoing.

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