Lipopolysaccharide-induced NF-κB Activation and Cytokine Release in Human Alveolar Macrophages Is PKC-independent and TK- and PC-PLC-dependent

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A critical feature of sepsis-induced adult respiratory distress syndrome (ARDS) is the release of cytokines (such as interleukin [IL]-6, IL-8, and tumor necrosis factor [TNF]) from endotoxin (lipopolysaccharide [LPS])-activated alveolar macrophages (AM). Nuclear factor kappa B (NF-κB) is activated in AM from patients with ARDS, and it is essential for the transcription of many cytokine genes. In these studies, we evaluated the regulation of LPS-induced cytokine release and the activation of NF-κB in human AM. We found that the activation of NF-κB and the release of IL-6, IL-8, and TNF from AM exposed to LPS was protein kinase C-independent and tyrosine kinase- and phosphatidylcholine-specific phospholipase C-dependent. We also found that LPS-induced activation of NF-κB was enhanced in AM cultured in serum or in the presence of LPS-binding protein, simulating conditions in the lung that are present in ARDS. In addition, LPS triggered the activation of several different NF-κB complexes in AM, and different forms of NF-κB bound to the IL-6, IL-8, and TNF promoter sequences. These observations suggest that physiologic abnormalities present in the lungs of patients with ARDS facilitate the activation of NF-κB and local release of cytokines. **Carter, A. B., M. M. Monick, and G. W. Hunninghake. 1998. Lipopolysaccharide-induced NF-κB activation and cytokine release in human alveolar macrophages is PKC-independent and TK- and PC-PLC-dependent. Am. J. Respir. Cell Mol. Biol. 18:384–391.**

The adult respiratory distress syndrome (ARDS) is a form of acute lung injury that often results from sepsis. Many factors are involved in the development of ARDS, but one of the early and ongoing characteristics of this disease is inflammation in the lung. These inflammatory responses are triggered, at least in part, by alveolar macrophage (AM)-derived cytokines. Several studies have shown that various cytokines are increased in the lung in patients with ARDS (1–3). Further, the persistence of high cytokine concentrations in the lung has been correlated with ongoing inflammatory injury (2). Although many cytokines are present in patients with ARDS, three cytokines, interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF), have been evaluated in many studies, and they are associated with continued inflammation and poor outcome (1–3).

The regulation of these cytokines in macrophages is controlled, at least in part, at the level of gene transcription. A crucial transcription factor that regulates expression of the IL-6, IL-8, and TNF genes is nuclear factor kappa B (NF-κB). It regulates expression of these genes by binding to specific promoter sequences (4–13). A primary means by which NF-κB is activated to bind to the promoters of these genes is via translocation of the factor from the cytoplasm to the nucleus of the cell. This occurs following phosphorylation and degradation of an inhibitor protein, IκB, which binds NF-κB in the cytoplasm and prevents its translocation to the nucleus (14). It is likely that the regulation of NF-κB in AM after exposure to lipopolysaccharide (LPS) is critical to the inflammatory response that occurs in ARDS. In fact, NF-κB is present in the nucleus of AM in patients with ARDS (15). No prior studies, however, have evaluated how NF-κB is activated in AM after exposure to LPS.

Several studies, using other types of cells, have evaluated second messenger pathways that regulate NF-κB after exposure to LPS (16–26). These studies, as an aggregate, show that NF-κB is not regulated by the same mechanisms in various types of cells. Pathways that have

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Abbreviations: alveolar macrophage(s), AM; adult respiratory distress syndrome, ARDS; electrophoretic mobility shift assay(s), EMSA; mitogen-activated protein kinase, Erk2; interleukin, IL; lipopolysaccharide-binding protein, LBP; lipopolysaccharide, LPS; nuclear factor kappa B, NF-κB; Nonidet P-40, NP-40; phosphatidylcholine-specific phospholipase C, PC-PLC; protein kinase C, PKC; phorbol myristate acetate, PMA; Roswell Park Memorial Institute, RPMI; tyrosine kinase, TK; tumor necrosis factor, TNF.

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been linked to NF-κB activation include protein kinase C (PKC) pathways (16, 17), tyrosine kinase (TK) pathways (16, 18–21), and phosphatidylcholine-specific phospholipase C (PC-PLC) pathways (22–25). We found that NF-κB activation by LPS in AM is PKC-independent and TK- and PC-PLC-dependent. Release of IL-6, IL-8, and TNF was also PKC-independent and TK- and PC-PLC-dependent. In addition, we found that different NF-κB complexes bind to specific promoter sequences in the IL-6, IL-8, and TNF genes. Finally, the ability of LPS to trigger NF-κB activation in AM is enhanced by the presence of serum and LPS-binding protein (LBP), factors that are increased in the lungs of patients with ARDS.

Materials and Methods

Isolation of AM

The use of normal volunteers to obtain AM by bronchoalveolar lavage was approved by the Human Subjects Review Board of the University of Iowa College of Medicine. AM were obtained from normal volunteers who met the following criteria: (*1*) age between 18 and 45 yr; (*2*) no history of cardiopulmonary disease or other chronic disease; (*3*) no prescription or nonprescription medication except oral contraceptives; (*4*) no recent or current evidence of infection; and (*5*) lifetime nonsmoker. The volunteers underwent fiberoptic bronchoscopy and bronchoalveolar lavage in subsegments of the right upper lobe, right middle lobe, and lingula after receiving 0.6 mg atropine delivered intramuscularly and adequate local anesthesia. Each subsegment of the lung was lavaged with five 20-ml aliquots of normal saline, and the first aliquot in each subsegment was discarded. The percentage of AM was determined by Wright-Giemsa stain. The percentage of AM varied from 90 to 98% of the cells.

Expression of NF-κB

We initially determined that the maximal expression (nuclear translocation) of NF-κB occurred 3 h after stimulation with LPS and that the optimal dose of LPS was 1 μg/ml. AM were cultured at 37°C for 3 h in Roswell Park Memorial Institute (RPMI)-1640 medium alone, RPMI medium with 100 ng/ml of LBP (a generous gift from Richard Ulevitch, Scripps Research Institute, La Jolla, CA), or RPMI medium with 5% fetal calf serum. The cells were also cultured in each of these three conditions in the presence or absence of 1 μg/ml *Escherichia coli* serotype 026: B6 LPS (Sigma Chemical Co., St. Louis, MO). In some instances, various inhibitors (Calbiochem, La Jolla, CA) were added 15 min prior to the addition of LPS. The PKC inhibitors were 1 nM staurosporine and 50 nM bisindomaleamide. The TK inhibitors were 40 μM genistein and 10 μM tyrophostin AG 126. The PC-PLC inhibitor was 100 μM D609. After 3 h of exposure to LPS, the cells were washed in phosphate-buffered saline. They were then resuspended in a lysis buffer (10 mM Hepes, 10 mM KCl, 2 mM $MgCl_2$, 2 mM EDTA) for 15 min on ice. Nonidet P-40 (NP-40) (10%) was added to lyse the cells, and the cells were centrifuged at 4°C at 14,000 rpm. The nuclear pellet was resuspended in an extraction buffer (50 mM Hepes, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) for 20 min on ice. After centrifuging at 4°C at 14,000 rpm, the supernatant was stored at −70°C. NF-κB oligonucleotides were labeled with [γ-^{32}P]ATP (New England Nuclear/Dupont, Boston, MA). The consensus oligonucleotide (5′-AGTTGAGGGGACTTTCC-CAGGC-3′) was obtained from Promega (Madison, WI), and the IL-6 (5′-AGTTGAGGGGATTTTCCCAGGC-3′), IL-8 (5′-AGTTGAGTGGAATTTCCCAGGC-3′), and TNFκ3 (5′-AGTTGAGGGGTTTCTCCCAGGC-3′) NF-κB oligonucleotides were purchased from Research Genetics (Huntsville, AL). Electrophoretic mobility shift assays (EMSA) were performed at room temperature for 30 min with 5 μg of nuclear extract protein and the ^{32}P-labeled oligonucleotides in the presence of an incubation buffer (1 M Tris, glycerol, NP-40, 0.1 M $ZnSO_4$, 1 M DTT, 2 M KCl, and 1 M $MgCl_2$) and 1 μg of poly [d(I-C)] (Boehringer Mannheim, Indianapolis, IN). Supershift assays were performed with the addition of p50, p52, p65, Rel B, and c-Rel antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The protein-DNA complexes were separated on 5% polyacrylamide gels at 25 to 45 mA. The gels were subsequently vacuum-dried and exposed to autoradiographic film (Amersham, Arlington Heights, IL) at −70°C for 12 to 48 h.

Expression of Mitogen-activated Protein Kinases

AM were cultured for 15 min at 37°C after stimulation with either 10 ng/ml phorbol myristate acetate (PMA) or 1 μg/ml LPS. PKC inhibitors were used in cells stimulated with PMA, and TK inhibitors and the PC-PLC inhibitor were used in cells stimulated with LPS. The cells were har-

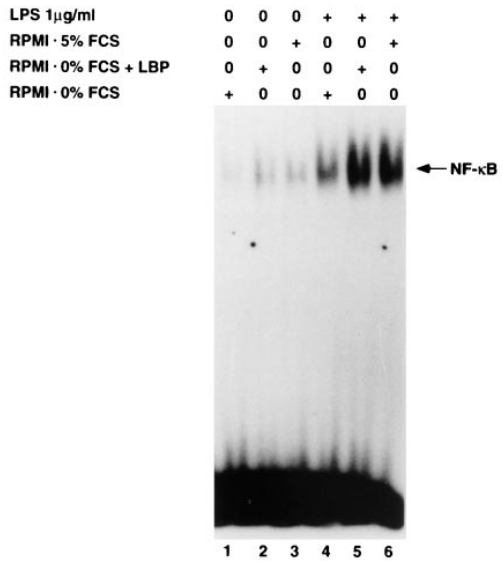

Figure 1. Effect of different culture conditions on the expression of NF-κB: RPMI with no serum, RPMI with LBP (100 ng/ml), and RPMI with 5% serum. In cells cultured without serum (*lane 1*), a small amount of NF-κB activity was present. An increase in NF-κB activity occurred in cells cultured in either LBP or serum alone (*lanes 2* and *3*). LPS significantly increased NF-κB activity in all three conditions (*lanes 4* through *6*), and this effect of LPS was enhanced by both LBP and serum (*lanes 5* and *6*).

vested, resuspended in a lysis buffer (1% NP-40, 0.15 M NaCl, 0.05 M Tris [pH 7.4], 100 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 M $NaVO_4$, 10 mM NaFl, and 10 mM sodium pyrophosphate), sonicated, and placed on ice for 20 min. After centrifuging at 14,000 rpm at 4°C for 10 min to remove cellular debris, the lysates were stored at −70°C. Extracellular signal-regulated kinase-2 (Erk2) was immunoprecipitated from the lysates (500 μg) overnight at 4°C with Erk2 antibody (Santa Cruz Biotechnology) bound to Gammabind with sepharose (Pharmacia Biotech, Uppsala, Sweden). The sepharose pellet was placed in a kinase buffer (20 mM $MgCl_2$, 25 mM Hepes, 20 mM β-glycerophosphate, 20 mM *P*-nitrophenylphosphate, 0.1 mM $NaVO_4$, and 2 mM dithiothreitol), and kinase activity was assayed by phosphorylation of myelin basic protein (MBP) using 5 μCi/sample [γ-^{32}P]ATP, 20 μM ATP, and 10 μg/ml MBP (Sigma). After 15 min, the reaction was stopped with the addition of SDS sample buffer, and the samples were heated to 95°C for 5 min to separate the protein from the sepharose. The samples were separated on a 10% SDS-PAGE discontinuous gel at 45 mA. The gels were then vacuum-dried and exposed to autoradiographic film. Western blots were performed simultaneously to ensure equal loading of the samples.

Expression of Cytokines

For these studies, AM were cultured in RPMI medium with 5% fetal calf serum for 24 h in the presence or absence of LPS and with and without inhibitors, as described earlier. The amounts of IL-6, IL-8, and TNF in the supernatant of the cells was measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Statistical Analysis

All of the cytokine measurements are shown as means with the standard error. Statistical comparisons were performed using an unpaired *t* test with a probability value of $P < 0.05$ considered to be significant.

Results

NF-κB DNA Binding Activity

In the absence of LPS, LBP, or serum, a small amount of NF-κB activity was present in AM (Figure 1). Both LBP and serum alone caused a small increase in NF-κB activity. With each of these conditions, LPS caused a significant increase in NF-κB (Figure 1). In absence of LPS, LBP, or serum, only p50 NF-κB was detected (data not shown). Both LBP- and serum-exposed AM had p50/65 NF-κB (data not shown). LPS-stimulated AM contained p50/65 NF-κB in all three (serum-free, LBP, and 5% serum) conditions (data not shown). These initial studies were performed using the consensus NF-κB oligonucleotide. These studies show that LPS increases NF-κB in the nucleus of AM. The studies further show that both LBP and serum (simulating conditions in the lungs of patients with ARDS) enhance this effect of LPS.

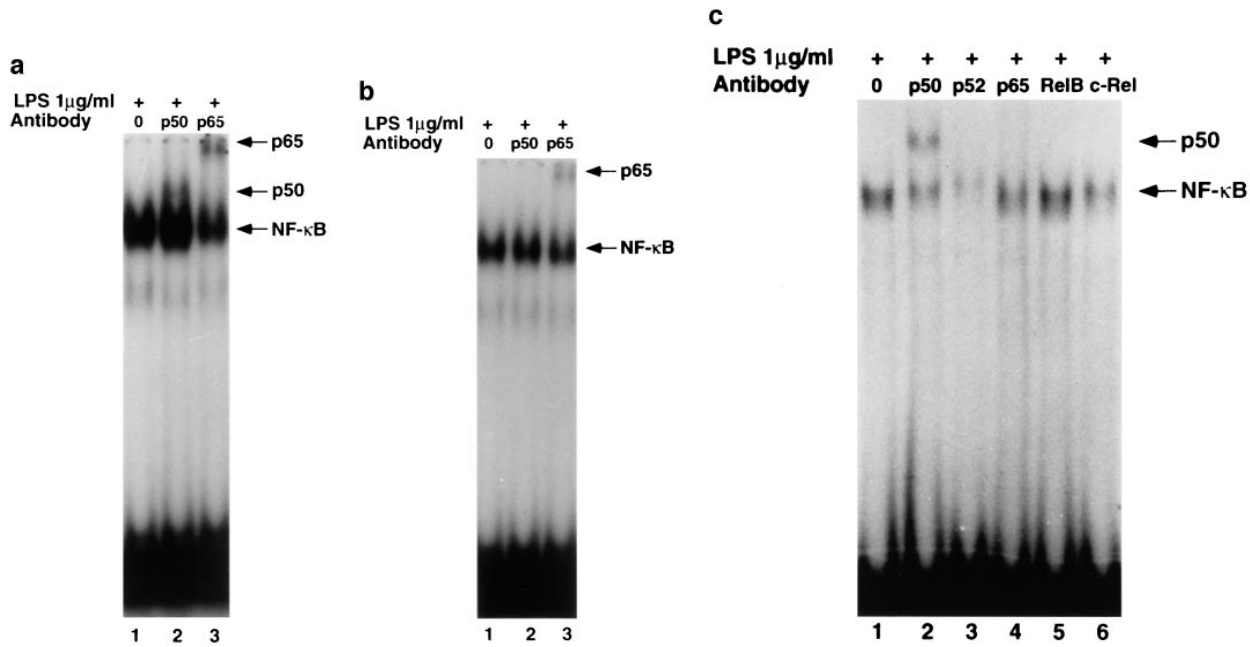

Figure 2. NF-κB binding to the IL-6, IL-8, and TNF promoter sequences. (*a*) NF-κB generated in AM exposed to LPS binds to the IL-6 NF-κB sequence (*lane 1*). Both p50 (*lane 2*) and p65 (*lane 3*) proteins of NF-κB bind the IL-6 sequence. (*b*) NF-κB generated in AM exposed to LPS binds to the IL-8 NF-κB sequence (*lane 1*). No p50 protein was detected (*lane 2*); however, a p65 protein was clearly present (*lane 3*), suggesting the presence of a p65/65 NF-κB that binds to the IL-8 sequence. (*c*) NF-κB generated in AM exposed to LPS binds to the TNFκ3 NF-κB sequence (*lane 1*). A p50 protein (*lane 2*) was clearly detected, but no other antibody resulted in a supershift, suggesting the presence of a p50/50 NF-κB or an interaction of p50 NF-κB with another protein.

NF-κB Binding to Specific NF-κB DNA Sequences

It has previously been shown that specific NF-κB complexes exhibit varying affinities for sequences in the promoter regions of cytokine genes (4–13). Therefore, we used specific NF-κB DNA-binding sequences from the IL-6, IL-8, and TNF promoters for these studies. In LPS-stimulated AM, both p50 and p65 NF-κB proteins bound to the IL-6 sequence, a p65 NF-κB protein bound to the IL-8 sequence, and a p50 NF-κB protein bound to the TNF sequence (Figures 2a through 2c). We did not detect p52, Rel B, or c-rel proteins in these assays. These findings suggest that different NF-κB complexes are generated by LPS in AM and that specific NF-κB complexes are likely used for the transcription of these cytokine genes.

LPS-induced NF-κB Is Independent of PKC Activity

To determine if PKC plays a role in the activation of NF-κB in AM, the cells were cultured in the presence or absence of staurosporine, a nonspecific PKC inhibitor, and bisindomaleamide, which is a relatively specific PKC inhibitor. LPS-induced NF-κB in AM was not inhibited by bisindomaleamide (Figure 3), and similar results were obtained with staurosporine (data not shown). To confirm that these PKC inhibitors were active, we performed Erk2 *in vitro* kinase assays in cells stimulated with PMA. We found that PMA-induced Erk2 kinase activity in AM was significantly reduced by PKC inhibition with staurosporine (data not shown). These observations strongly suggest that LPS-induced activation of NF-κB in AM occurs independently of PKC.

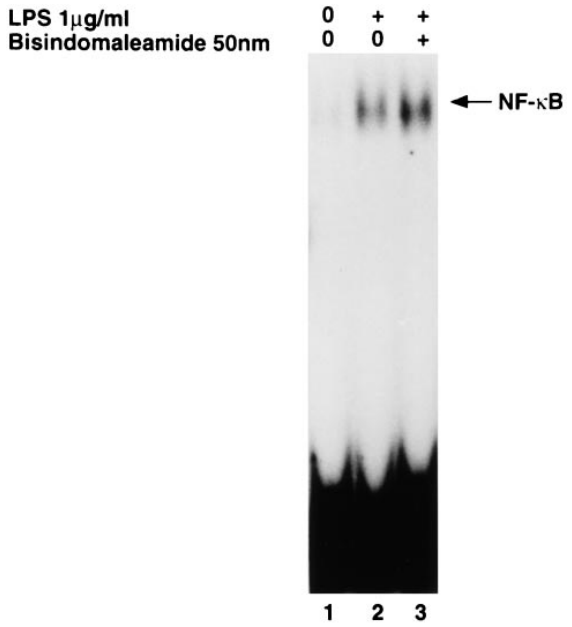

Figure 3. NF-κB activity in AM cultured with the PKC inhibitors in 5% serum. A small amount of NF-κB activity was present in cells not exposed to LPS (*lane 1*). There was a significant increase in NF-κB activity in AM exposed to LPS (*lane 2*), and this effect was not reduced in the presence of bisindomaleamide (*lane 3*).

LPS-induced NF-κB Is Partially Dependent on TK Activity

To determine if tyrosine kinases play a role in the generation of NF-κB by LPS in AM, the cells were cultured in the presence of genistein, a specific TK inhibitor with broad-spectrum activity, and tyrophostin AG 126, which is a relatively specific TK inhibitor. LPS-induced NF-κB was partially inhibited by both inhibitors (Figures 4a and 4b). To

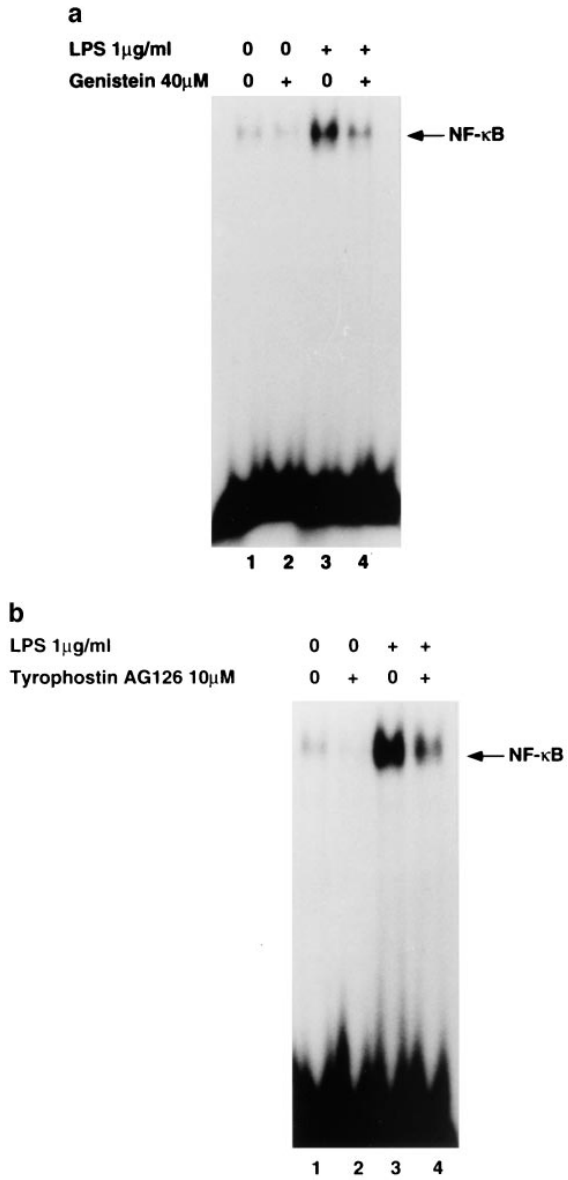

Figure 4. NF-κB activity in AM cultured with various TK inhibitors in 5% serum. (*a*) A small amount of NF-κB was present in cells not exposed to LPS (*lane 1*), and there was no significant change in the activity when genistein was added (*lane 2*). LPS induced a significant increase in NF-κB activity (*lane 3*), and this activity was inhibited significantly with the addition of genistein (*lane 4*). (*b*) In cells not exposed to LPS, there was a small amount of NF-κB activity (*lane 1*), and the addition of tyrophostin AG 126 decreased this activity slightly (*lane 2*). LPS induced a significant increase in NF-κB activity (*lane 3*), and this effect was partially inhibited by tyrophostin AG 126.

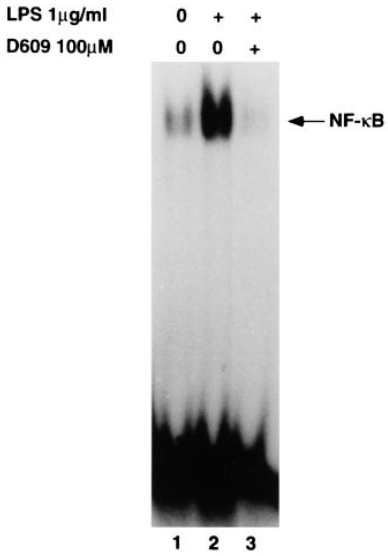

Figure 5. NF-κB activity in AM cultured with D609 in 5% serum. In cells not exposed to LPS, there was a small amount of NF-κB activity (*lane 1*). LPS induced a significant increase in NF-κB activity (*lane 2*), and this effect was almost completely inhibited by D609 (*lane 3*).

confirm that other pathways of tyrosine phosphorylation were inhibited with the inhibitors that we used, we performed Erk2 *in vitro* kinase assays in cells stimulated with LPS. We found that LPS-induced Erk2 kinase activity in AM was significantly reduced to near control levels in the presence of genistein (data not shown). These observations strongly suggest that tyrosine kinase(s) play an important role in the activation of NF-κB in AM exposed to LPS.

LPS-induced NF-κB Is Dependent on PC-PLC Activity

To determine if activation of a PC-PLC plays a role in the activation of NF-κB in AM exposed to LPS, the cells were cultured in the presence of D609, a highly specific inhibitor of PC-PLC. D609 significantly inhibited NF-κB in AM exposed to LPS (Figure 5). We also performed Erk2 *in vitro* kinase assays in cells exposed to LPS to confirm that other pathways linked to PC-PLC activity are inhibited by D609. We found that LPS-induced Erk2 kinase activity in AM is significantly reduced to control levels in the presence of D609 (data not shown). These observations suggest that the activation of a PC-PLC is necessary for the activation of NF-κB in AM by LPS.

Effect of Cytokine Release from AM Stimulated with LPS

To determine if the same second messenger pathways that regulate the activation of NF-κB also regulate cytokine release in AM, we measured the amounts of IL-6, IL-8, and TNF released by AM stimulated by LPS. We found that IL-6, IL-8, and TNF are all increased in the supernatants of the cells exposed to LPS (Figure 6). PKC inhibition with bisindomaleamide did not significantly (IL-6: $P = 0.884$; IL-8: $P = 0.994$; TNF: $P = 0.132$) affect the release of any of these cytokines from AM. Similar results were obtained with staurosporine (data not shown). TK inhibition with genistein caused a significant (IL-6: $P = 0.009$; IL-8: $P = 0.046$; TNF: $P = 0.005$) decrease in the release of all three cytokines. Similar results were found with tyrophostin AG 126 (data not shown). PC-PLC inhibition with D609 significantly (IL-6: $P = 0.002$; IL-8: $P = 0.025$; TNF: $P < 0.001$) decreased cytokine release to levels near control values (Figures 7a through 7c). These observations show that similar pathways are used for the activation of NF-κB and cytokine release in LPS-stimulated AM.

Correlation of NF-κB Activation and Cytokine Release in a Dose-dependent Manner

Although we found that similar pathways are used for both NF-κB activation and cytokine release in LPS-stimulated AM, we also evaluated if the inhibitors used had similar effects on each in a dose-dependent manner. Culture conditions were done as outlined above, but genistein was used at 10 μM and 40 μM and D609 was used at 25 μM and 100 μM. We found that genistein and D609 decreased

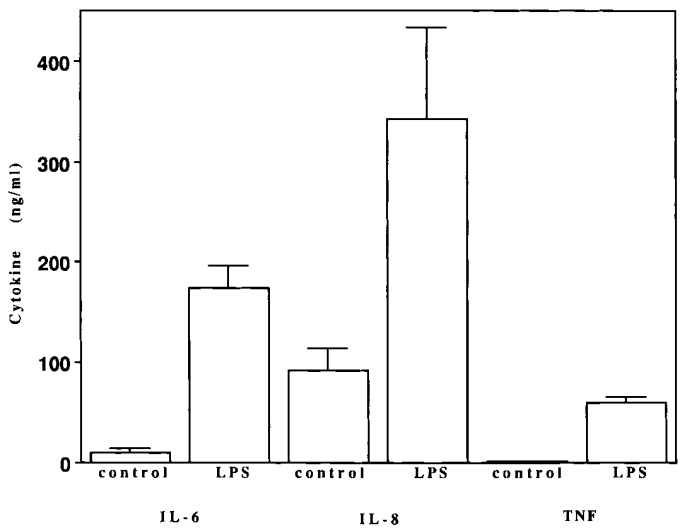

Figure 6. Effect of LPS on the release of IL-6, IL-8, and TNF in AM. All three cytokines were significantly increased in the supernatants of AM exposed to LPS compared with controls.

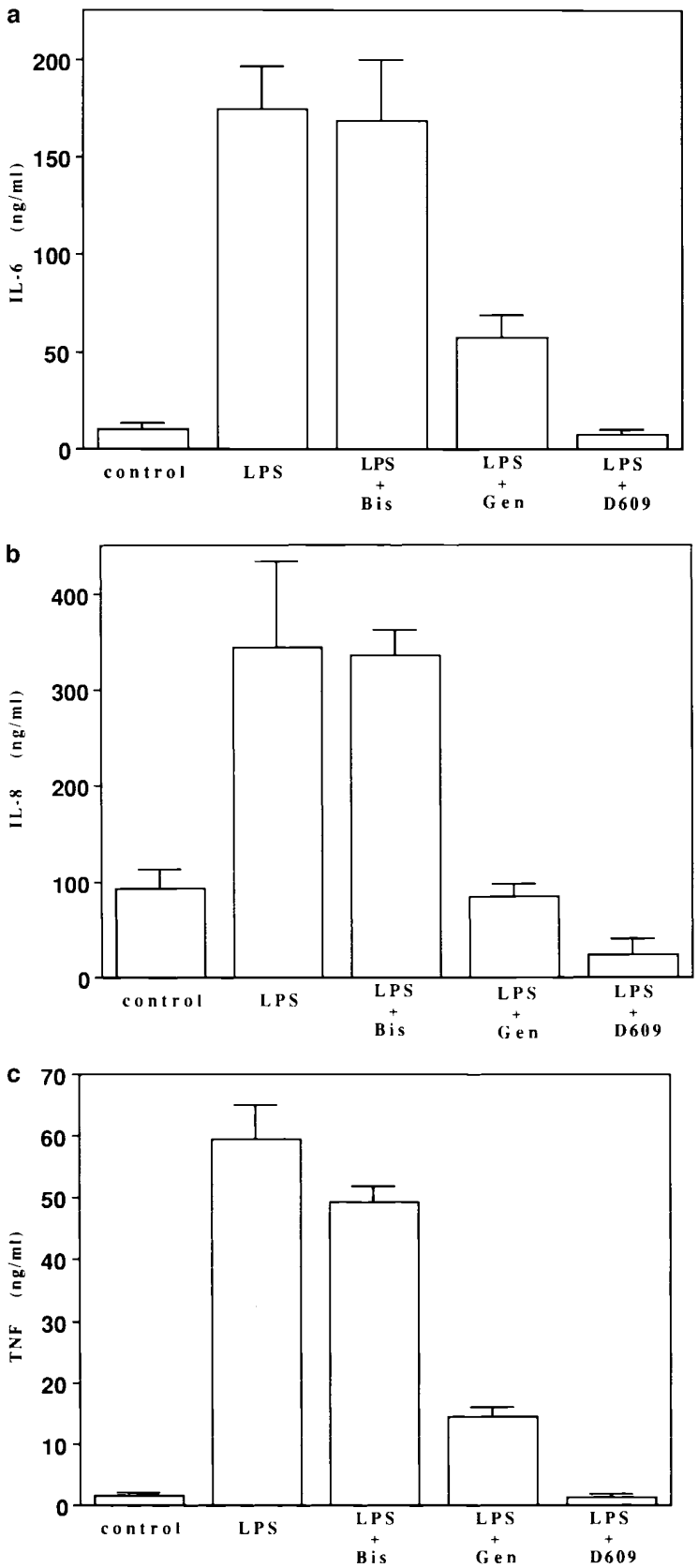

Figure 7. Effect of various inhibitors on IL-6, IL-8, and TNF release in AM in the presence of LPS. (*a*) IL-6 release occurred independently of PKC inhibition with bisindomaleamide. IL-6 release was significantly inhibited by genistein ($P = 0.009$) and by D609 ($P = 0.002$). (*b*) IL-8 release occurred independently of PKC inhibition with bisindomaleamide. IL-8 release was significantly inhibited by genistein ($P = 0.046$) and by D609 ($P = 0.025$). (*c*) TNF release occurred independently of PKC inhibition with bisindomaleamide. TNF release was significantly inhibited by genistein ($P = 0.005$) and by D609 ($P < 0.001$).

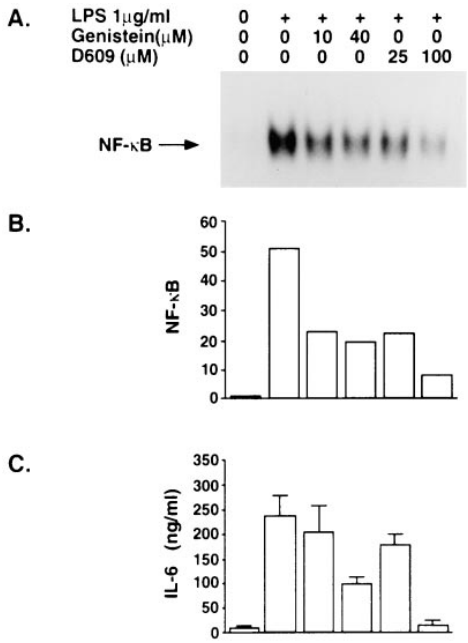

Figure 8. Dose-response curves for NF-κB activation and IL-6 release. (*a*) A small amount of NF-κB activity was present in cells not exposed to LPS (*lane 1*). LPS induced a significant increase in NF-κB activity (*lane 2*), and there was an incremental decrease in this activity with the addition of increasing doses of genistein and D609 (*lanes 3* through *6*). (*b*) Densitometry of the EMSA showing the dose-dependent response of NF-κB to increasing doses of genistein and D609. (*c*) Effect of IL-6 release from LPS-stimulated AM in the presence of genistein and D609. IL-6 release was reduced in a dose-dependent manner with increasing doses of genistein and D609.

both NF-κB activation (Figures 8A and 8B) and cytokine (IL-6) release (Figure 8C) in a dose-dependent manner. These observations further suggest that similar pathways are used for the activation of NF-κB and cytokine release in LPS-stimulated AM.

Discussion

A primary feature of ARDS is a leak of serum proteins (including LBP) onto the alveolar surface of the lung. This disorder is also associated with the activation of NF-κB and the release of cytokines from AM. One of the major causes of ARDS is sepsis. In these studies, we found that the activation of NF-κB in AM by LPS is markedly enhanced by both LBP and serum. LPS-induced NF-κB in AM appeared to be independent of PKC and was dependent on both TK and PC-PLC activity. LPS-induced release of IL-6, IL-8, and TNF from AM also appeared to be independent of PKC and was significantly reduced with both TK and PC-PLC inhibition. We also found that LPS triggered the activation of several different NF-κB complexes, and different forms of NF-κB bound to the IL-6, IL-8, and TNF promoter sequences. To our knowledge, these are the first studies that have evaluated the activation of NF-κB in human AM in response to LPS.

The role of PKC in the activation of NF-κB and the release of cytokines also has not been previously evaluated in human AM, but its role in cytokine release has been evaluated in rat AM (26). In rat AM, LPS-induced TNF production is reduced by staurosporine. Other studies have evaluated the consequence of PKC inhibition on NF-κB activation in other types of cells, including human monocytes (18). Human monocytes do not require PKC activity for NF-κB activation by LPS. Interestingly, in both of these studies, PKC inhibition by staurosporine resulted in reduced TNF release. As staurosporine can alter expression of other second messenger pathways (27), it is not clear if the effect on release of TNF was due to inhibition of PKC. Our studies in human AM strongly suggest that LPS-induced activation of NF-κB and release of IL-6, IL-8, and TNF occurs independently of most PKC isoforms. One caution in interpreting this data is that some PKC isoforms (i.e., PKCζ) are poorly inhibited by the agents used in this study.

The role of TK activity in the activation of NF-κB has not been previously evaluated in human AM. Several studies (16, 18–21), however, have evaluated the role of TK in NF-κB activation and cytokine release in other types of cells. The effect of TK inhibition on LPS-induced NF-κB translocation has been controversial. In human monocytes exposed to LPS, NF-κB activation and cytokine release have been shown to be inhibited by both herbimycin A and genistein (18). In Chinese hamster ovary cells and RAW 264.7 cells, TK inhibitors did not prevent LPS-induced NF-κB translocation (20). A recent study has also shown that LPS-induced NF-κB translocation in THP-1 cells is not inhibited by genistein (21). Several studies have shown that there is increased TK activity after LPS binds to the CD14 cell surface receptor in both monocytes and macrophages (28–30). Tyrosine phosphorylation has also been reported to be linked to inactivation of IκB (31). Our studies strongly suggest that NF-κB translocation and cytokine production in human AM are partially dependent on TK activity.

The role of PC-PLC activity in LPS-induced generation of NF-κB also has not been previously evaluated in human AM. A study, using a murine model, however, reported that the systemic effects of sepsis could be prevented with D609, a specific and potent inhibitor of PC-PLC (32). The role of PC-PLC in TNF-induced activation of NF-κB has been evaluated (22–24). This effect is not due to a diacylglycerol-dependent activation of PKC. Instead, TNF-induced PC-PLC appears to trigger the production of ceramide, which serves as a second messenger downstream of PC-PLC. Ceramide is produced as a result of the activation of an acid sphingomyelinase. The acid sphingomyelinase, which is activated by diacylglycerol, hydrolyzes sphingomyelin to produce ceramide. The ceramide then activates ceramide-activated protein kinase, which phosphorylates IκB on a serine residue, resulting in its degradation (33, 34). This mechanism would explain why D609 inhibited NF-κB and cytokine release in our studies while inhibitors of PKC had no effect. In our studies, D609 was the most potent inhibitor of both NF-κB and cytokine production in human AM stimulated with LPS.

The NF-κB complexes that bind specific cytokine promoters have not been evaluated in human AM. Other

studies have shown, however, that not all NF-κB promoter sequences efficiently bind all forms of NF-κB, and the sequence determines which NF-κB complex(es) will bind. Several studies have reported that a p50/65 NF-κB binds to both the IL-6 and TNF promoters and that a p65/65 NF-κB binds to the IL-8 promoter (4–13). Our findings in human AM exposed to LPS are consistent with these previous studies, with one exception: we could detect only the p50 NF-κB protein in the complex that bound the TNFκ3 promoter.

The enhanced effect of serum and LBP on NF-κB activation in AM is relevant to ARDS. The normal lung alveoli have little exposure to serum. In ARDS, however, there is increased conductance of serum proteins, including LBP, into the lung. We found NF-κB activation to be much greater in AM incubated in serum or with the addition of LBP. NF-κB activation has been previously reported to be activated in AM from patients with ARDS (15). Although NF-κB can be activated in AM in serum-free conditions, its increased activity in AM incubated in serum or LBP simulates conditions present in ARDS, in which there are increased amounts of plasma and proteins (including LBP) in the lung. These observations suggest that the leak of serum proteins into the lung may magnify the level of production of cytokines and, possibly, accentuate the lung injury.

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