Lipopolysaccharide induces 5-lipoxygenase-activating protein gene expression in THP-1 cells via a NF-κB and C/EBP-mediated mechanism

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Serio, Kenneth J., K. Veera Reddy, and Timothy D. Bigby. Lipopolysaccharide induces 5-lipoxygenase-activating protein gene expression in THP-1 cells via a NF-κB and C/EBP-mediated mechanism. Am J Physiol Cell Physiol 288: C1125–C1133, 2005. First published December 29, 2004; doi:10.1152/ajpcell.00296.2004.—We examined induced expression of the 5-lipoxygenase-activating protein (FLAP), which is critical for leukotriene synthesis in mononuclear phagocytes. Prolonged exposure to the bacterial component, lipopolysaccharide (LPS), increased FLAP gene transcription, mRNA expression, and protein expression in the human monocyte-like THP-1 cell line. Activation and inhibition of the NF-κB pathway modulated LPS induction of FLAP gene expression. An NF-κB-mediated mechanism of action was supported by overexpression of dominant-negative IκBα and p50/p65 proteins. EMSA supershift and DNase I footprint analyses revealed that p50 binds to an NF-κB site located in the proximal FLAP promoter, while chromatin immunoprecipitation assays demonstrated that LPS induced binding of p50 but not of p65. Moreover, EMSA supershift analyses demonstrated that LPS induced time-dependent binding of THP-1 nuclear extracts (containing p50) to this promoter region. Mutation of the NF-κB site decreased basal promoter activity and abolished the p50- and p65-associated induction. EMSA supershift analyses also demonstrated that LPS induced binding of THP-1 nuclear extracts [containing CCAAT enhancer binding protein (C/EBP)-α, -δ, and -ε] to a C/EBP site located adjacent to the NF-κB site in the FLAP promoter. We conclude that LPS enhances FLAP gene expression via both NF-κB- and C/EBP-mediated transcriptional mechanisms in mononuclear phagocytes.

monocytes/macrophages; endotoxin shock; inflammation; allergy

LEUKOTRIENES, WHICH ARE LIPID COMPOUNDS that are synthesized via the 5-lipoxygenase (5-LO) pathway, are potent inflammatory mediators involved in asthma, allergic rhinitis, glomerulonephritis, rheumatoid arthritis, inflammatory bowel disease, sepsis, and acute lung injury (15, 25, 30, 31, 37, 40, 46). Important recent studies have implicated 5-LO in the pathogenesis of atherosclerosis (28) and therefore have identified specific 5-LO genotypes in subpopulations with increased risk for atherosclerosis (14). In addition, specific 5-LO activating protein (FLAP) haplotypes have been found to confer an increased risk of myocardial infarction and stroke (18).

The enzyme 5-LO as well as FLAP are required for the synthesis of leukotrienes in intact cells (12). In response to inflammatory stimuli, the localization of the 5-LO enzyme to specific membranes functions to modulate enzymatic activity (26). The 5-LO enzyme catalyzes the oxygenation of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid and its conversion to leukotriene (LT) A4 (LTA4). LTA4 is metabolized to either LTB4 by LTA4 hydrolase or to the cysteinyl leukotrienes (LTC4, LTD4, and LTE4) by LTC4 synthase (41, 42).

FLAP as well as LTC4 synthase, prosta glandin E2 synthase, and the microsomal glutathione-S-transferases are members of a unique class of enzymes termed the membrane-associated proteins in eicosanoid and glutathione metabolism (19). Although FLAP does not exhibit enzymatic activity, it has been demonstrated to possess arachidonic acid binding capabilities (27) and may function as an arachidonic acid transfer protein for 5-LO (1). The FLAP gene spans 31 kb and consists of five exons and four introns (20). A FLAP gene polymorphism in the proximal 5′ untranslated region (5′ UTR) has been identified 94 bp upstream of the transcription start site (22). A hetero- or homozygous poly(A)′ RNA sequence of 21 bp in the proximal FLAP promoter has been reported to occur at a higher frequency in people with asthma (73.2%) than in healthy volunteers (54.9%) (22). In inflammatory cells, FLAP gene expression is known to be induced by dexamethasone, IL-3, granulocyte-monocyte colony-stimulating factor, and TNF-α (8, 35, 36, 39), suggesting regulated expression of this gene.

Recently published findings from our group indicate that the FLAP gene is constitutively expressed in inflammatory cells and mediated via distal (−36 to −28 bp) and proximal (−25 to −12 bp) CCAAT enhancer binding protein (C/EBP) sites located within the first 134 bp of the promoter. This transcriptional regulation of FLAP involves members of the C/EBP family: namely, C/EBP-α, -δ, and -ε (35). In addition, TNF-α induces FLAP gene expression via binding of C/EBP-α, -δ, and -ε to both the proximal and distal FLAP promoter C/EBP sites (35). These findings are consistent with the role of C/EBP proteins in the regulation of cellular differentiation and the inflammatory response (24, 33). Cell-specific gene regulation by C/EBP proteins has been shown to be dependent on interactions with other transcription factors, including NF-κB, Sp1, and Fos/Jun family members (23, 34).

Lipopolysaccharide (LPS), a glycosylated component of the gram-negative bacterial cell wall, in conjunction with the LPS binding protein, binds to CD14 and the transmembrane Toll-like receptor 4 on the surface of a variety of cells, including macrophages and neutrophils (4). LPS stimulation of monocytes/macrophages effects the generation of a number of inflammatory mediators, including leukotrienes derived from the 5-LO pathway (9, 44). Recent studies indicate that prolonged LPS exposure downregulates LTB4 synthesis in rat

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alveolar macrophages (9). In addition, studies from our group indicate that prolonged LPS exposure downregulates expression of the downstream LTC4 synthase gene (via a NF-κB-mediated transcriptional mechanism) and the synthesis of cysteinyl leukotrienes in the human monooyte-like cell line THP-1 (44). The role of LPS in the modulation of 5-LO pathway-derived leukotriene synthesis in mononuclear phagocytes suggests an important interaction between bacterial infection and the development of leukotriene-mediated systemic and airway inflammation.

In the present study, we have investigated the role of proximal promoters in mediating the induction of the FLAP gene by LPS in human inflammatory cells. Our findings indicate that LPS induces the binding of NF-κB and C/EBP-α, -δ, and -e to adjacent sites in the proximal FLAP promoter, enhancing FLAP gene expression in mononuclear phagocytes.

MATERIALS AND METHODS

Cell culture. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA). The monocyte-like cell line THP-1 constitutively expresses 5-LO and FLAP and has previously been used as a model to study leukotriene synthetic pathways (36, 43, 44). THP-1 cells were grown at 37°C with 5% CO₂ in RPMI-1640 medium (BioWhittaker, Walkersville, MD) with 10% heat-treated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamicin. The media were changed every 2–3 days for all experiments.

Immunoblot analysis. THP-1 cells were conditioned (at 1 × 10⁶ cells/ml) for 24 h with 10 ng/ml Salmonella minnesota Re595 LPS (Re LPS). Repurified Re LPS was provided by Dr. Theo Kirkland (Department of Veterans Affairs San Diego Healthcare System and the University of California, San Diego, San Diego, CA). LPS was prepared as previously described (21, 50). Immunoblot analyses for FLAP protein were performed using a previously described technique (29, 38).

Northern blot analysis. THP-1 cells were conditioned (at 1 × 10⁶ cells/ml) with 10 ng/ml Re LPS; 10 μM parthenolide (Calbiochem, La Jolla, CA), a NF-κB inhibitor; and/or 10 μM hperoxysvavanum (phen; Alexis Biochemicals, San Diego, CA), a NF-κB activator. After incubation, total cellular RNA was isolated and subjected to electrophoresis on a 1% agarose/2.2 M formaldehyde gel. The RNA was blotted overnight onto a Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA). The blot was probed with a [32P]-labeled, full-length cDNA probe for FLAP, washed under high-stringency conditions, and exposed to autoradiographic film. Loading equivalency and transfer efficiency were assessed by probing with a [32P]-labeled, full-length cDNA probe encoding for β-actin (Clontech, Palo Alto, CA).

Construction of luciferase promoter-reporter constructs. Segments of the FLAP 5′-UTR were prepared as previously described (35) to yield the wild-type −134FLAP-pGL3 and −3.4FLAP-pGL3 constructs. The −3.4FLAP-pGL3 construct was used as a template to perform site-directed mutagenesis of the NF-κB site (located at −43 to −34 bp), using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following primers were used: forward primer, 5′-AATTGTGCGCCGCCCCTCCTACGAAATTG-3′; and reverse primer, 5′-CAATTGTGCGCCGCCCCTCCTACGGGACATTG-3′. Thirty-six cycles of PCR were performed, with each cycle consisting of denaturation at 94°C for 45 s, annealing at 59°C for 30 s, and extension at 72°C for 60 s. Sequences of the wild-type and mutant constructs were subsequently confirmed using the dyeodeoxy chain termination method. The constructs were purified using the EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA).

Transient transfection. THP-1 cells were transiently transfected with 900 ng of either the −134FLAP-pGL3 or the −3.4FLAP-pGL3 promoter-firefly luciferase reporter construct (Promega, Madison, WI) using Effectene transfection reagent (Qiagen) as previously described (35, 43). The cells were transfected in Effectene for 4 h and subsequently incubated (at 1 × 10⁶ cells/ml) for 4 h with Re LPS at 37°C with 5% CO₂ in RPMI-1640 supplemented with 10% FCS.

For IκBα overexpression experiments, DNA mixtures consisted of 450 ng of the −3.4FLAP-pGL3 construct and 450 ng of the wild-type pCMV-IκB or mutant pCMV-IκB expression vector (BD Biosciences/Clontech). The cells were transfected in Effectene for 4 h and incubated (at 1 × 10⁶ cells/ml) for 4 h at 37°C with 5% CO₂ in RPMI-1640 supplemented with 10% FCS.

For p50/p65 overexpression experiments, DNA mixtures consisted of 225 ng of the wild-type or mutant −3.4FLAP-pGL3 construct and 225 ng of pRSV-p50 or pRSV-p65 expression vector or both (the expression constructs pRSV-NF-κB1 (p50) and pRSV-RelA (p65) were obtained from Dr. Gary Nabel and Dr. Neil Perkins (13, 17) through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, Rockville, MD). The cells were transfected in Effectene for 16 h and incubated (at 1 × 10⁶ cells/ml) for 24 h at 37°C with 5% CO₂ in RPMI-1640 supplemented with 10% FCS.

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Measurements were obtained using an Optocomp I luminometer (MGM Instruments, Hamden, CT). Data were normalized to the activity of the promoterless pGL3 Basic vector (Promega).

Nuclear run-on assay. Nuclei were isolated from untreated and LPS-treated THP-1 cells according to a previously described technique (11). Nuclei were resuspended in a solution containing 40% glycerol, 50 mM MgCl₂, and 0.1 mM EDTA and incubated for 30 min at 37°C in the presence of [32P]UTP. Labeled RNA was isolated, and 5 million cpm were hybridized (using a slot blot device) to cDNA for FLAP and β-actin immobilized on Zeta-Probe nylon membranes (Bio-Rad) at 68°C for 12 h. The blot was washed under high-stringency conditions and exposed to autoradiographic film.

Dnase I footprint analysis. Nuclear extracts (11) and the promoter region (35) of the FLAP gene from −134 to +12 bp (with respect to the transcription start site) were prepared as previously described. The probe was labeled on the 3′-end with [32P]ATP using T4 polynucleotide kinase (Promega). Dnase I footprinting was performed in 100-μl reaction volumes with 4 ng (1 × 10⁴ cpm) of labeled probe and 40 μg of nuclear extract. The reaction buffer consisted of 5% glycerol, 10 mM HEPES, 50 mM KCl, 1 mM DTT, 1 μg of poly(dI-dC) (Amersham Biosciences, Piscataway, NJ), and 1 μg of BSA. After a 20-min incubation at room temperature, 1 mM CaCl₂ and 0.5 mM MgCl₂ were added and the reaction product was incubated for 1 min. Dnase I (1 U; Promega) was then added, and the reaction product was digested for 60 s. Next, the reaction was inactivated, and the DNA was extracted using phenol-chloroform followed by ethanol precipitation. The DNA was then analyzed on an 8% PAGE gel and 50% urea gel. A G+A ladder was prepared using the same end-labeled fragment and run on the same gel.

Electrophoretic mobility shift assays. EMSA reactions were performed in 20-μl final volumes under conditions identical to those described for footprinting. The probe was labeled with [32P]ATP using T4 polynucleotide kinase (Promega). Each reaction contained 10 μg of nuclear extract and 3 × 10⁴ cpm of duplexed, labeled nucleotide probe. The reactions were incubated at room temperature for 20 min. Supershift analyses were conducted using H-119 anti-p50 (catalog no. sc-7178; Santa Cruz Biotechnology, Santa Cruz, CA) and C-20 anti-p65 antibodies (catalog no. sc-372; Santa Cruz Biotechnology), which were added 5 min before the radiolabeled probe was added. The samples were electrophoresed on a 5% nondenaturing acrylamide gel containing 1% glycerol. The probes used for EMSAs were as follows: wild-type probe from −52 to −26 bp, 5′-AATTGTGGCGCCGCCCCTCCTACGAAATTG-3′ (containing an NF-κB site from −43 to −34 bp and the distal C/EBP site from −36 to −28 bp); mutant probe from −52 to −26 bp, 5′-AATTGTGGCGCCGCCCCTCCTACGAAATTG-3′.
TCAGAAATGG-3′ (containing a mutant NF-κB site); and wild-type probe from −39 to −20 bp, 5′-ATCTTCAGAAATGTAATGA-3′ (containing only the distal C/EBP site).

**ChIP assays.** Chromatin immunoprecipitation (ChIP) assays were performed using a previously described method (35). PCR was performed using a pair of primers (forward, −134 bp to −118 bp; reverse, −9 bp to −25 bp) that generated a product spanning −134 to −9 bp of the FLAP promoter. PCR was performed for 30 cycles under the following conditions: denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 45 s. The PCR products were electrophoresed through an agarose gel and visualized using ethidium bromide staining.

**Materials.** FCS, penicillin, streptomycin, and gentamicin were obtained from the Cell Culture Facility of the University of California, San Diego. RPMI-1640 medium was obtained from BioWhittaker. All restriction enzymes were obtained from Gibco (Gaithersburg, MD). All synthesized oligonucleotides and primers were obtained from Operon Technologies (Alameda, CA). Autoradiographic film was purchased from Eastman Kodak (Rochester, NY). The Qiagen-tip 500 column was purchased from Qiagen. All other reagents were obtained from Sigma Chemical (St. Louis, MO) and were of the finest grade available.

**Data analysis.** Data are expressed as means ± SE in all circumstances in which mean values are compared. Data were analyzed using an unpaired Student’s t-test or ANOVA with Dunnett’s multiple comparisons test with InStat software, version 2.03 (GraphPad Software, San Diego, CA). Differences were considered significant when \( P < 0.05. \)

**RESULTS**

**LPS induces FLAP protein accumulation in THP-1 cells.** To determine the effect of LPS on FLAP protein accumulation, THP-1 cells were conditioned for 24 h with 10 ng/ml Re LPS. Total protein was extracted and subjected to immunoblot analysis. In the presence of LPS, FLAP protein expression was induced 4.8-fold (Fig. 1A).

**LPS induces FLAP mRNA accumulation in THP-1 cells.** To determine the effect of LPS on FLAP mRNA accumulation, THP-1 cells were conditioned for 24 h with 10 ng/ml Re LPS. Total RNA was extracted and subjected to Northern blot analysis. In the presence of LPS, FLAP mRNA expression was induced 4.9-fold (Fig. 1B and C).

**LPS induces FLAP promoter activity and gene transcription.** To determine the effect of LPS on the FLAP promoter activity, THP-1 cells were transiently transfected with either the −134FLAP-pGL3 or the −3.4FLAP-pGL3 construct and conditioned for 4 h with 10 ng/ml Re LPS. The cells were harvested and assayed for firefly luciferase activity. In the presence of LPS, FLAP promoter activity was induced with both the −134FLAP-pGL3 construct (23.4 ± 0.9 vs. 17.5 ± 1.0-fold increase in luciferase activity, normalized to pGL3 Basic) \((n = 4 \text{ experiments}; \ P < 0.01)\) (Fig. 2B) and the −3.4FLAP-pGL3 construct (46.1 ± 3.2 vs. 22.5 ± 1.5-fold increase in luciferase activity, normalized to pGL3 Basic) \((n = 4; \ P < 0.001)\) (Fig. 1D and E), compared with control.

To determine the effect of LPS on the transcription of FLAP mRNA, nuclear run-on assays were performed using nuclei isolated from radiolabeled, untreated, and LPS-treated THP-1 cells. In the presence of LPS, FLAP gene transcription was increased at 2 and 12 h, with gene transcription decreasing to baseline levels by 24 h (Fig. 1F).

**Bisperoxyvanadium (phen) upregulates FLAP mRNA in a time-dependent manner.** To determine whether pharmacologic activation of the transcription factor NF-κB is capable of inducing FLAP gene expression, THP-1 cells were conditioned with the NF-κB activator bisperoxyvanadium (phen) (10 μM) for up to 24 h. The activation of NF-κB by bisperoxyvanadium (phen) increased FLAP mRNA accumulation 13.5-fold at 16 h compared with control \((n = 3; \ P < 0.05)\) (Fig. 2A and B).

**Inhibition of NF-κB activation blocks the increase in FLAP mRNA induced by LPS.** To determine whether LPS induction of FLAP mRNA is mediated by NF-κB, THP-1 cells were preconditioned for 2 h with the NF-κB activation inhibitor parthenolide (at 10 μM), followed by the addition of 10 ng/ml Re LPS for an additional 12-h incubation. LPS alone induced a 2.6-fold increase in FLAP mRNA compared with control \((n = 2)\). In the presence of parthenolide, LPS induction of FLAP mRNA was inhibited compared with LPS alone (0.7 vs. 2.6 densitometric units, normalized to control; \(n = 2\) ) (Fig. 2, D and E).
wild-type and mutant I
FLAP mRNA (C)

Fig. 2. Modulation of NF-κB influences FLAP mRNA accumulation in THP-1 cells. A: representative Northern blot probed for FLAP and β-actin. THP-1 cells were conditioned for up to 24 h with the NF-κB activator bisperoxyvanadium (phen; 10 μM). B: densitometric analysis of Northern blot for FLAP mRNA relative to β-actin mRNA and normalized to control. Bisperoxyvanadium (phen) treatment resulted in 13.5-fold induction of FLAP mRNA as early as 16 h. Data represent means ± SE; n = 3; *P < 0.05. C: representative Northern blot probed for FLAP and β-actin. THP-1 cells were preconditioned for 2 h with the NF-κB activation inhibitor parthenolide (10 μM), followed by the addition of 10 ng/ml Re LPS for an additional 12-h incubation. D: densitometric analysis of Northern blot for FLAP mRNA relative to β-actin mRNA and normalized to control. LPS alone induced a 2.6-fold increase in FLAP mRNA (n = 2). In the presence of parthenolide, LPS induction of FLAP mRNA was inhibited (n = 2).

C and D). Parthenolide alone exhibited a twofold increase in FLAP mRNA (n = 2).

LPS induction of FLAP promoter activity is inhibited by wild-type and mutant IκBα overexpression. To determine whether the LPS induction of FLAP mRNA involves a NF-κB-mediated mechanism, THP-1 cells were transiently cotransfected with the −3.4FLAP-pGL3 construct and an expression vector for wild-type IκBα (pCMV-IκBα) or a persistently active mutant IκBα (pCMV-IκBM) and subsequently conditioned for 4 h with 10 ng/ml Re LPS. The cells were harvested and assayed for firefly luciferase activity. LPS again induced FLAP promoter activity compared with control (14.0 ± 0.7 vs. 6.5 ± 0.2 fold increase in luciferase activity, normalized to pGL3 Basic) (n = 3; P < 0.01) (Fig. 3A). The overexpression of wild-type IκBα abrogated the effect of LPS on FLAP promoter activity, inhibiting it to near control values (6.0 ± 0.1 vs. 6.5 ± 0.2 fold increase in luciferase activity, normalized to pGL3 Basic) (n = 3; P = NS) (Fig. 3A). The overexpression of mutant IκBα only partially inhibited the effect of LPS on FLAP promoter activity compared with control values (8.5 ± 0.2 vs. 6.5 ± 0.2 fold increase in luciferase activity, normalized to pGL3 Basic) (n = 3; P < 0.05) (Fig. 3A). Overexpression of wild-type IκBα protein alone had no significant effect on FLAP promoter activity compared with control (7.7 ± 0.5 vs. 6.5 ± 0.2 fold increase in luciferase activity, normalized to pGL3 Basic) (n = 3; P = NS) (Fig. 3A), while overexpression of mutant IκBα protein modestly induced FLAP promoter activity compared with control (9.4 ± 0.6 vs. 6.5 ± 0.2 fold increase in luciferase activity, normalized to pGL3 Basic) (n = 3; P < 0.01) (Fig. 3A).

NF-κB component proteins, p50 and p65, increase FLAP promoter activity. To determine the independent function of each of the NF-κB component proteins, p50 and p65, THP-1 cells were transiently cotransfected with the wild-type −3.4FLAP-pGL3 construct (or the NF-κB site mutant −3.4MutFLAP-pGL3 construct) and p50 and p65 expression vectors. The cells were subsequently incubated for 24 h, harvested, and assayed for firefly luciferase activity. Overexpression of both p50 and p65 resulted in a 2.1-fold increase in FLAP promoter activity using the wild-type −3.4FLAP-pGL3 construct, compared with the activity of the wild-type construct (n = 3; P < 0.01). Overexpression of p50 and p65 proteins alone had minimal upregulatory effects on FLAP promoter activity (Fig. 3B). Constitutive activity of the −3.4MutFLAP-pGL3 construct (containing a mutation at the NF-κB site located between −43 to −34 bp) was decreased to 65% of the activity of the wild-type construct (n = 3; P = NS) (Fig. 3B).

Mutation of the NF-κB site within the −3.4MutFLAP-pGL3 construct abolished the p50- and p65-associated induction of FLAP promoter activity (Fig. 3B).

Purified p50 protein binds to the FLAP promoter. To determine whether the NF-κB component protein p50 binds to the proximal region of the FLAP promoter, DNase I footprint analysis was performed using a wild-type probe from −134 to +12 bp. Purified p50 protein binds to the FLAP promoter region corresponding to the NF-κB site at −43 to −34 bp (Fig. 4A, lane 3).
NF-κB component protein p50 binds to the promoter NF-κB site. To determine whether purified p50 protein is capable of binding to the NF-κB site in the proximal FLAP promoter, EMSAs were performed with wild-type and mutant double-stranded oligonucleotide probes corresponding to the region of the FLAP promoter that demonstrated binding in the DNase I footprint assay (Fig. 4A). In the presence of purified p50, the EMSA probe from −52 to −26 bp containing the wild-type NF-κB site located at −43 to −34 bp exhibited a shifted band (Fig. 4B, lane 2). A supershifted band was observed using an antibody against p50 (Fig. 4B, lane 3). In the presence of purified p50, the EMSA probe from −52 to −26 bp containing the mutant NF-κB site exhibited no binding (Fig. 4B, lanes 5 and 6). These data indicate the binding of p50 to the NF-κB site (located at −43 to −34 bp) in the proximal FLAP promoter.

NF-κB component protein p50 binds to the promoter NF-κB site. To determine whether purified p50 protein is capable of binding to the NF-κB site in the proximal FLAP promoter, EMSAs were performed with wild-type and mutant double-stranded oligonucleotide probes corresponding to the region of the FLAP promoter that demonstrated binding in the DNase I footprint assay (Fig. 4A). In the presence of purified p50, the EMSA probe from −52 to −26 bp containing the wild-type NF-κB site (located at −43 to −34 bp) exhibited a single band (Fig. 4B, lane 2). A supershifted band was observed using an antibody against p50 (Fig. 4B, lane 3). In the presence of purified p50 protein and the wild-type probe resulted in a shifted band (lane 2). Supershift assays performed with p50 protein, anti-p50 antibody, and the wild-type probe resulted in a supershifted band (lane 3). EMSA and supershift assays performed with a probe containing a mutation of the NF-κB site resulted in a loss of both the shifted (lane 5) and supershifted (lane 6) bands. C. THP-1 cells were subjected to chromatin immunoprecipitation. Antibodies against β-actin (as a negative control), p50, p65, and CCAAT/enhancer binding protein (C/EBP)-α (as a positive control) were used. DNA was released from immunoprecipitates and amplified by performing PCR using primers corresponding to −134 to −9 bp of the FLAP gene. In the absence of LPS, neither p50 (lane 3) nor p65 (lane 4) bound to this promoter region. In the presence of 10 ng/ml LPS for 2 h, p50 bound to the promoter region (lane 7) but p65 did not (lane 8). A reaction using C/EBP-α antibody was performed as a positive control (lanes 5 and 7) because this factor binds to the promoter region under constitutive conditions.

ChIP assays demonstrate LPS-induced binding of p50 to the FLAP promoter. To determine whether p50 and p65 are bound to native chromatin in untreated and LPS-treated THP-1 cells, we performed ChIP assays. As a negative control, when the PCR reaction was run in the presence of an irrelevant antibody (β-actin), no product was observed (Fig. 4C, lanes 2 and 6). When chromatin from control cells was immunoprecipitated with antibodies against p50 and p65, no PCR products were generated (Fig. 4C, lanes 3 and 4). When chromatin from LPS-treated cells was incubated with antibodies against p50, but not p65, the appropriate PCR product was generated (Fig. 4C, lanes 7 and 8). When EMSA was performed using a synthetic NF-κB consensus site (5'-CAGAGGGGACTTTCCGAGA-3') and LPS-treated THP-1 cell extract, anti-p65 antibody produced a supershift, confirming appropriate function of this antibody (data not shown). These results indicate...
that LPS induction results in p50, but not p65, binding to this region of the FLAP promoter. Consistent with previously reported constitutive binding of C/EBP-α to the proximal FLAP promoter (35), chromatin immunoprecipitated with an antibody against C/EBP-α resulted in the generation of a PCR product (Fig. 4C, lanes 5 and 9).

LPS induces time-dependent binding of p50 and p65 to the NF-κB site in the proximal FLAP promoter. To determine the effect of LPS exposure on transcription factor binding to the proximal FLAP promoter, EMSAs were performed with a wild-type oligonucleotide probe corresponding to the region from −52 to −26 bp. In the presence of LPS-treated extract from THP-1 cells, the wild-type probe (containing the NF-κB site located at −43 to −34 bp) exhibited a time-dependent increase in binding, with peak binding consisting of a four-band complex occurring at 4 h (Fig. 5A, lanes 1–6). In the presence of 4-h LPS-treated extracts, a supershift of the second band of the complex was observed when EMSA was performed with an antibody against p50 (Fig. 5A, lane 7). No supershift was observed with an antibody against p65 (Fig. 5A, lane 8). Supershift (or competition) of both the first and second bands was observed when p50 and p65 antibodies were used (Fig. 5A, lane 9). These data indicate that LPS induces binding of p50 to the NF-κB site (located at −43 to −34 bp) in the proximal FLAP promoter.

LPS induces binding of C/EBP-α, -δ, and -e to the C/EBP site in the proximal FLAP promoter. To determine the effect of LPS exposure on C/EBP family member binding to the proximal FLAP promoter, EMSAs were performed using THP-1 nuclear extracts after LPS exposure for 4 h. In the absence of LPS treatment, the EMSA probe from −39 to −20 bp (containing the C/EBP site located at −36 to −28 bp but not the adjacent NF-κB site) exhibited a single band (Fig. 5B, lane 1). This finding is consistent with data in our previous report indicating constitutive binding of C/EBP family members to this distal C/EBP site (35). In the presence of LPS-treated extract, this probe exhibited an increased pattern of binding, with supershifts observed using antibodies against C/EBP-α (Fig. 5B, lane 2), C/EBP-δ (Fig. 5B, lane 4), and C/EBP-ε (Fig. 5B, lane 5). No supershift was observed with an antibody against C/EBP-β (Fig. 5B, lane 3). These data indicate that LPS induces binding of C/EBP family members to the distal C/EBP site (located at −36 to −28 bp) in the proximal FLAP promoter. From the data obtained using the shorter −39 to −20 bp EMSA probe, it can be concluded that C/EBP-α, -δ, and -e are components of the LPS-induced four-band complex noted in the EMSA shown in Fig. 5A.

DISCUSSION

We demonstrate that prolonged exposure to the bacterial component LPS induces FLAP protein and gene expression in the monocyte-like cell line THP-1. Findings from both nuclear run-on and promoter-reporter assays indicate that this increased expression involves transactivation of transcription mediated via elements located within the first 134 bp of the FLAP promoter. In our previous work, this region was found to account for the majority of constitutive promoter activity (35). The predominant LPS-associated transactivating elements within this region are an NF-κB site (located at −43 to −34 bp) in conjunction with an overlapping C/EBP site (located at −36 to −28 bp). This C/EBP site was previously demonstrated to function in the constitutive and TNF-α-induced regulation of FLAP gene expression (35). On the basis of our functional studies using direct pharmacological NF-κB activation, inhibition of NF-κB activation, IκBα overexpression, and p50/p65 overexpression, the LPS-induced upregulation of FLAP promoter activity appears to be mediated via NF-κB acting through the identified site. The binding of p50 to this site is confirmed by DNase I footprinting, ChIP analyses, and EMSA/ supershift analyses. In addition, LPS treatment also results in the binding of C/EBP-α, -δ, and -e to the adjacent overlapping C/EBP site.

FLAP serves an important role in the functioning of the 5-LO pathway of leukotriene metabolism, being required for leukotriene synthesis in intact inflammatory cells (12). The role

Fig. 5. LPS induces binding of p50 and C/EBP family members to sites within the proximal FLAP promoter. A: EMSAs were performed using nuclear extracts from untreated and LPS-treated THP-1 cells and a labeled FLAP promoter segment (from −52 to −26 bp) containing the NF-κB site (located at −43 to −34 bp) and the C/EBP site (located at −36 to −28 bp). LPS-treated extracts exhibit a time-dependent increase in binding to this region, with peak binding consisting of a four-band complex occurring at 4 h (lanes 2–6). Supershift assays performed using 4-h LPS-treated extracts demonstrated that this complex contains p50 (lanes 7 and 9) but not p65 (lane 8). B: EMSAs were performed using 10 μg of nuclear extracts from untreated (lane 1) and LPS-treated THP-1 cells and a labeled FLAP promoter segment (from −39 to −20 bp) containing only the C/EBP site (located at −36 to −28 bp). Supershift assays using LPS-treated extracts (for 4 h) exhibited LPS-induced binding of C/EBP-α (lane 2), C/EBP-δ (lane 3), and C/EBP-ε (lane 5), but not of C/EBP-β (lane 3).
of inflammatory stimuli such as LPS in modulating 5-LO pathway gene expression and product synthesis in monocytes/macrophages is an area of significant recent interest. Studies support the importance of this modulation because prolonged LPS exposure leads to suppression of 5-LO activity that impairs gram-negative bacterial killing in rat peritoneal macrophages (6). In diseases such as asthma and sepsis, dysregulation of leukotriene synthesis in the setting of airway and/or systemic bacterial exposure is likely associated with disease pathogenesis and/or exacerbation. In atherosclerosis, it has also been suggested that bacterial infection may incite local leukotriene-associated inflammatory responses, contributing to the development of the characteristic vascular lesions (45). In an attempt to understand these important links, studies with a focus on the role of prolonged LPS exposure (hours to days) have demonstrated that LPS inhibits LTB4 generation in rat alveolar macrophages but not in human peripheral blood monocytes (9). The mechanism of this suppression has been suggested to involve increased generation of NO. Interestingly, Coffey et al. (9) noted a small increase in FLAP protein expression in rat alveolar macrophages with 1 μg/ml LPS treatment, although the effect of LPS on FLAP mRNA expression was not specifically examined. Our data in the present study provide an important insight into the mechanism of LPS modulation of the FLAP gene in the human THP-1 cell line, demonstrating a significant induction of FLAP mRNA in the presence of a low dose of LPS (10 ng/ml). In similar work with THP-1 cells by our group, prolonged low-dose LPS exposure was shown to downregulate expression of the downstream LTC4 synthase enzyme/gene (and cysteinyl leukotriene synthesis) (44). In contrast to prior work in primary human monocytes suggesting that LPS does not modulate LTB4 synthesis or 5-LO and FLAP protein expression (9), our current studies indicate that LPS is capable of increasing FLAP gene expression in THP-1 cells. Because LPS may serve to phenotypically favor the generation of LTB4 over that of the cysteinyl leukotrienes in THP-1 cells, the net effect of LPS on the synthesis of 5-LO pathway leukotrienes requires further study.

We previously demonstrated that expression of the FLAP gene is regulated in mononuclear phagocytes (35). Constitutive expression of this gene is mediated by C/EBP-α, -δ, and -ε proteins, acting via both distal (located at −36 to −28 bp) and proximal (located at −25 to −12 bp) C/EBP elements in the FLAP promoter (35). In addition, we have shown that TNF-α induces increased C/EBP-α, -δ, and -ε binding to these sites, enhancing FLAP gene expression (35). We now report that FLAP mRNA accumulation is also upregulated by prolonged LPS exposure, demonstrably because of an increase in gene transcription. This is consistent with the known role of LPS in the induction of both C/EBP (2, 3, 7, 10, 24) and NF-κB (48). While the LPS induction of FLAP promoter activity is relatively modest at the measured time points of 4 h (Fig. 1, D and E), we think that an ongoing enhancement of FLAP gene transcription results in the marked accumulation of FLAP mRNA observed at 24 h (Fig. 1, B and C). The more pronounced enhancement of promoter activity observed with the whole 3.4-kb promoter construct suggests that the intact promoter is required for full LPS-induced transactivation, suggesting a role for secondary or tertiary promoter structure. In support of a NF-κB-mediated mechanism of action, we found that overexpression of the IκBα protein, which normally binds to NF-κB, inhibits the increase in FLAP promoter activity, presumably by retaining it within the cytoplasm. Both studies with pharmacological inhibition of NF-κB activation and direct pharmacological NF-κB activation support a role for this transcription factor in mediating the effect of LPS. Interestingly, pharmacological NF-κB activation results in a delayed increase in FLAP mRNA, first appearing between 8 and 16 h. This phenomenon may be related to the bisperoxovanadium (phen) compound inducing some degree of early enhancement of FLAP gene transcription, with accumulation of FLAP mRNA occurring only after a prolonged period of NF-κB activation (at least 8 h with this particular compound). This presumed gradual accumulation of FLAP mRNA is consistent with the modest increase in FLAP promoter activity at early time points (at 4 h), with an associated marked increase in FLAP mRNA observed later (at 24 h). However, differential kinetics of NF-κB activation may occur when one compares activation induced by a pharmacological agent and that induced by LPS. Overexpression of p50 and p65 NF-κB component proteins functions independently to increase FLAP promoter activity, with the most pronounced effect being observed with overexpression of both proteins. Interestingly, p65 overexpression appears to enhance the induction over that observed with the p50 protein alone, suggesting that p65, at least at the high levels of expression in this experimental design, may play a cooperative role with p50 in FLAP gene induction. Mutation of the NF-κB site decreases basal promoter activity (compared with the wild-type construct) and completely abolishes the p50/p65-associated induction, suggesting this is a transactivating domain that is critical and is the only functional NF-κB-responsive element located within the first 3.4 kb of the promoter. Although we acknowledge that other NF-κB consensus sites may exist within the FLAP promoter, the functional studies using site mutation suggest that only the identified site is critical for function in response to p50/p65.

In our previous studies, the proximal FLAP promoter region (which contains the NF-κB site as well as an overlapping distal C/EBP site and a more proximal C/EBP site) exhibited binding of TFH-1 extracts in DNase footprinting assays (35). The present study extends these findings and demonstrates that p50 binds in DNase I footprinting and ChIP assays to the NF-κB site. EMSA and supershift assays demonstrate time-dependent binding of p50 (contained within LPS-treated TFH-1 nuclear

![Fig. 6](http://ajpcell.physiology.org/)
of peak p50 binding at 4 h is consistent with an increase in FLAP promoter activity observed at this time point in transfection assays (Fig. 1, D and E). This binding is consistent with the role of this protein to induce FLAP promoter activity and gene expression. Although p50 homodimers have been well established to exhibit inhibitory effects on gene transcription, particularly in the development of LPS tolerance (16, 32), they may also enhance gene expression in association with other proteins such as Bcl-3 (5, 49). Our findings suggest that p50 transactivates FLAP gene expression in the form of p50 homodimers or in direct interaction with C/EBP family members. In support of the latter mechanism, with comparable levels of exposure, LPS induces binding of C/EBP-α, -δ, and -e to the adjacent, overlapping C/EBP site, accounting for some of the EMSA bands shown in the four-band complex in Fig. 5A. This LPS-induced binding is consistent with our previous report indicating that these proteins transactivate FLAP gene transcription constitutively and in response to TNF-α (35). The combined observation that mutation of either the C/EBP or NF-κB site results in diminished promoter activity and the proximity of the NF-κB and C/EBP sites in the FLAP promoter (35) strongly suggests a potential for cooperative or antagonistic interaction between C/EBP and NF-κB. A cooperative interaction of this nature could allow for signal amplification between similar inflammatory stimuli such as LPS and TNF-α. Such an interaction has been demonstrated to occur in the regulation of the IL-6, IL-8, IL-12, and granulocyte colony-stimulating factor genes (33). In the regulation of the IL-8 gene, antigenic interactions between C/EBP and NF-κB, which bind to adjacent promoter sites, may involve direct protein-protein interactions (47). We propose a model (Fig. 6) that summarizes the roles of LPS and TNF-α in inducing binding of NF-κB and C/EBP family members to the proximal FLAP promoter.

In conclusion, we have demonstrated the induction of FLAP protein and gene expression by LPS in mononuclear phagocytes. The mechanism of this induction involves enhanced gene transcription mediated via the binding of p50 as well as C/EBP family members to the proximal FLAP promoter. Data demonstrating induction of this gene fit into an important body of evidence indicating that prolonged LPS exposure functions to modulate expression of 5-LO pathway genes, possibly altering the leukotriene synthetic capacity of mononuclear phagocytes. These results begin to elucidate the role of bacterial components in modifying lipid mediator synthesis and attenuating microbial killing by monocytes/macrophages. These findings may have significant implications for understanding the pathogenesis of conditions such as sepsis and atherosclerosis.

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