

Endotoxin, but not platelet-activating factor, activates nuclear factor- κ B and increases I κ B α and I κ B β turnover in enterocytes

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SUMMARY

Bacterial endotoxin (lipopolysaccharide; LPS) and platelet-activating factor (PAF) are important triggers of bowel inflammation and injury. We have previously shown that LPS activates the transcription factor nuclear factor (NF)- κ B in the intestine, which up-regulates many pro-inflammatory genes. This effect partly depends on neutrophils and endogenous PAF. However, whether LPS and PAF directly activate NF- κ B in enterocytes remains controversial. In this study, we first investigated the effect of LPS and PAF on NF- κ B activation in IEC-6 (a non-transformed rat small intestinal crypt cell line) cells, by electrophoresis mobility shift assay and supershift, and found that LPS, but not PAF, activates NF- κ B mostly as p50–p65 heterodimers. The effect was slower than tumour necrosis factor (TNF). Both LPS and TNF induce the expression of the NF- κ B-dependent gene inducible nitric oxide synthase (iNOS), which occurs subsequent to NF- κ B activation. We then examined the effect of LPS and TNF on the inhibitory molecules I κ B α and I κ B β . We found that TNF causes rapid degradation of I κ B α and I κ B β . In contrast, LPS did not change the levels of I κ B α and I κ B β up to 4 hr (by Western blot). However, in the presence of cycloheximide, there was a slow reduction of I κ B α and I κ B β , which disappeared almost completely at 4 hr. These observations suggest that LPS causes slow degradation and synthesis of I κ B α and I κ B β and therefore activates NF- κ B via at least two mechanisms: initially, through an I κ B-independent mechanism, and later, via an increased turnover of the inhibitor I κ B. NF- κ B activation precedes the gene expression of iNOS (assayed by reverse transcription–polymerase chain reaction), suggesting that LPS up-regulates iNOS via this transcription factor.

INTRODUCTION

Lipopolysaccharide (LPS), a component of the Gram-negative bacterial membrane, plays a central role in septic shock. Systemic injection of LPS to rats causes shock and

intestinal injury.¹ Many of the *in vivo* effects of LPS are probably mediated by endogenous cytokines and lipids. Our previous work has shown that platelet-activating factor (PAF), a pro-inflammatory phospholipid produced in the intestine during endotoxaemia, is pivotal for the development of bowel injury.¹ The source of endogenous PAF probably includes inflammatory cells as well as intestinal epithelial cells.² Systemic administration of PAF to animals causes shock and bowel injury and the effects of PAF and LPS are synergistic.³ The site of PAF- or LPS-induced bowel injury is usually in the small intestine, particularly the ileum.^{1,3} Most of the adverse effects of PAF *in vivo* are dependent on the activation of polymorphonuclear neutrophils (PMN).⁴ LPS also enhances the transcription of interleukin (IL)-1,⁵ IL-6,⁶ tumour necrosis factor (TNF),⁷ chemokines,⁸ several adhesion molecules,⁹ and inducible nitric oxide synthase (iNOS).¹⁰ It is believed that the gene

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Abbreviations: cPAF, carbamyl-platelet-activating factor; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine saline; I κ B, inhibitor protein κ B; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF- κ B, Nuclear factor- κ B; PAF, platelet-activating factor; PMN, polymorphonuclear neutrophils; TNF, tumour necrosis factor- α .

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expression of these proteins is induced via the activation of the transcription factor nuclear factor- κ B (NF- κ B).¹¹ Nitric oxide production by iNOS has been shown to mediate endotoxic shock,¹² and to contribute to LPS-induced intestinal injury.¹³

NF- κ B has been found to up-regulate the expression of many genes implicated in inflammation. It is present constitutively in the cytoplasm in an inactive state, bound to inhibitory proteins (I κ B α , β , γ and ϵ are the major isoforms in mammals).^{11,14,15} Upon activation, NF- κ B dissociates from its inhibitor, translocates to the nucleus, binds to κ B-binding sites in target genes such as pro-inflammatory cytokines and stimulates their transcription.^{11,14} The major pathway leading to NF- κ B activation, involves the activation of an I κ B kinase complex which leads to the phosphorylation of I κ B α and to its degradation by the proteasome.¹⁵ There is constitutive activation of NF- κ B in mature B lymphocytes, monocytes, macrophages, thymocytes, and some neurons.¹⁴ In most other cell lines, NF- κ B becomes active only upon stimulation by agents like LPS, cytokines and reactive oxygen species.^{11,14} NF- κ B is a homo- or heterodimer of several proteinic subunits (p50, p65, p52, cRel and RelB in mammals). Only p65, cRel and RelB contain a trans-activation domain and are thought to up-regulate gene transcription.¹¹

We had previously shown that NF- κ B is constitutively active at low levels in the small intestine of rats, mainly as p50 homodimers¹⁶ and PAF activates intestinal NF- κ B p50 homodimers *in vivo* within 30 min.¹⁶ Immunohistochemical studies localize NF- κ B in both epithelial cells and lamina propria cells.¹⁶ We recently reported that LPS activates both p50-p50 and p50-p65 dimers; the effect is less rapid and more sustained than that of PAF and is partially dependent on PMN activation.¹⁷ Furthermore, we found that both PAF antagonists and TNF-blocking antibodies decrease LPS effects on NF- κ B activation, suggesting that the LPS effect depends on endogenous PAF and TNF.¹⁷

There have been many *in vitro* studies on the effect of LPS on NF- κ B activation in various cell types.¹¹ Reports on enterocytes are limited to colon carcinoma cell lines. It has been shown that adhesion of certain pathogenic bacteria such as enteroinvasive *Escherichia coli* to Caco-2-BBe cells activates NF- κ B and induces I κ B α degradation.¹⁸ In T84 cells, LPS and non-pathogenic *E. coli* did not activate NF- κ B.¹⁹ However, the effect of LPS on non-transformed small intestinal cell lines has not been examined to date. There has been no study on the direct effect of PAF on intestinal epithelium.

In the present study, we first investigated whether PAF and LPS directly activate NF- κ B in IEC-6 cells, a non-transformed small intestinal crypt cell line, and if so, which dimers are involved. Next, we compared the LPS effect with that of a potent pro-inflammatory cytokine, TNF. We then examined whether the LPS effects on NF- κ B activation are mediated by endogenous PAF, whether PAF has a direct effect and whether PMN potentiate NF- κ B activation. Additionally, we determined the effect of LPS on the expression of iNOS, a known target gene of LPS and

NF- κ B.²⁰ (iNOS was selected for the study because it is known to depend on NF- κ B for transcription: Mutation of NF- κ B binding sites in iNOS gene promoter abolishes its transcription in response to LPS or cytokines.²¹) Lastly, we examined the pathway of NF- κ B activation, by assessing the changes of the inhibitory proteins I κ B following LPS treatment.

MATERIALS AND METHODS

Cell cultures and treatment

IEC-6 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured (1×10^6 cells/flask) for 3–4 days in Dulbecco's modified Eagle's minimal essential medium (DMEM) with high glucose containing 10% fetal bovine saline (FBS) until 95% confluence. Cells (4×10^6 cells/flask) were incubated at 37° in 10 ml fresh DMEM containing 10% FBS and one of the following: (a) PAF (0.5 μ g/ml) (1-*O*-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine; Biomol Research Laboratory, Plymouth Meeting, PA) or carbamyl-PAF (cPAF; a stable PAF analogue) (0.5 μ g/ml); (b) LPS (0.2, 1, 5, 10 μ g/ml; from *Salmonella typhosa* – Difco, Detroit, MI); (c) LPS (1 μ g/ml) + rat peritoneal PMN (1×10^6 /ml); (d) WEB2170 (0.5 μ g/ml), a PAF receptor antagonist (kindly provided by Dr H. Heuer, Boehringer-Ingelheim, Mainz, Germany), for 30 min followed by LPS (1 μ g/ml); (e) LPS (1 μ g/ml) + cycloheximide (50 μ g/ml); (f) cycloheximide (50 μ g/ml); or (g) TNF (10 ng/ml) (R&D Systems, Minneapolis, MN). PMN were obtained as follows: After anaesthesia, a Sprague-Dawley rat was injected intraperitoneally with 10 ml of 10% caseinate (in sterile water). After 4 hr, the peritoneal cavity was lavaged with 10–15 ml sterile Hank's salt solution, centrifuged at 500 g, and the cells were resuspended in DMEM with 10% FBS, counted, and added to IEC cells.

Preparation of nuclear extracts/electrophoresis mobility shift assay (EMSA)

Cells were washed twice with 4° PBS and scraped with a rubber policeman in 500 μ l of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT)). After 15 min on ice, cells were briefly vortexed after the addition of 25 μ l of Nonidet P-40. Nuclei were collected by centrifugation at 7500 g for 1 min at 4° then washed with 200 μ l of buffer A. Nuclear extracts were obtained by high-salt extraction (incubation in 150 μ l of buffer B (20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA), glycerol 25%, 0.5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF)). Nuclear debris were pelleted by centrifugation, 150 μ l of buffer C (20 mM Hepes, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) were added and the nuclear extracts were stored at -80°. Protein concentration was measured¹⁶ by Bradford's method using a BioRad protein assay kit (BioRad, Hercules, CA). NF- κ B was identified by electrophoresis mobility shift assay (EMSA) using a kit from Promega (Madison, WI), as previously described.¹⁷ Supershift

experiments were performed with anti-p50 (sc-114X), anti-p65 (sc-109X), anti-p52 (sc-298X), anti-c-Rel (sc-272X) and anti-Rel B (sc-226X) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of whole cell extracts and Western blot analysis

After treatment, cells were washed with PBS 4°. Whole cell extracts were prepared as followed: 500 μ l of lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 μ g/ml each of pepstatin, leupeptin, aprotinin) were added to the cell plates and cells were scraped with a rubber policeman. The mixture was sonicated on ice for 30 s and cellular debris were removed by centrifugation at 10 000 *g* for 5 min at 4°.

The cell extracts were diluted 1:1 with Laemmli buffer, boiled for 5 min and electrophoresed on SDS/15% polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane, using 80 mA of current overnight at 4°. Nitrocellulose blots were blocked with 5% dried milk in PBS for 30 min, then incubated with antibodies (Santa Cruz Biotechnology) against I κ B α (C-21, sc-371), I κ B β (C-20, sc-945), I κ B γ (5177C, sc-201; rabbit), I κ B ϵ (1:1000) (G4, sc-7275; mouse) and actin (sc-7210; rabbit) for 2 hr. The membrane was washed with 0.05% Tween-20 in PBS for 30 min, incubated in 1:800 anti-rabbit immunoglobulin G (IgG) antibody or 1:2000 anti-mouse IgG antibody, conjugated with horseradish peroxidase (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and detected using an ECL kit (Amersham).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using the RNA-extracting agent STAT-60 (TEL-TEST, Friendswood, TX), a monophasic solution containing phenol and guanidinium thiocyanate, and stored at -80°. The integrity of each sample was verified by electrophoresis in a 1% denatured agarose gel. cDNA was synthesized using 2 μ g of total RNA, 50 mM Tris-HCl, 75 mM KCl, 2.0 mM MgCl₂, 10 mM DTT, 1.25 mM of each deoxynucleotide-triphosphate (dNTP), 7.5 μ M random hexamer (pdN6), 1 U/ μ l RNasin inhibitor, and 10 U/ μ l Moloney-murine leukaemia virus (M-MLV) reverse transcriptase. The reaction was carried out for 1 h at 37°, followed by heating to 95° for 10 min.

cDNA was amplified on a Thermal Cycler Model 9600 (Perkin-Elmer, Boston, MA) in a total volume of 50 μ l containing 2 μ l of cDNA, 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 0.1 mM of each dNTP, 0.1 U/ μ l of Taq DNA polymerase, and 1.0 μ M of each primer. The primers used were: iNOS primer 1: 5'-ATG GCT TGC CCT TGG AAG TTT CTC-3', primer 2: 5'-TCC AGG CCA TCT TGG TGG CAA AGA-3'; rat β actin primer 1: 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3', primer 2: 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3'.²² The amplification was carried out by 25 cycles (for actin) and 30 cycles (for iNOS) of denaturation at 94° for 45 s, an annealing step at 60° for 45 s, and an extension step at 72° for 1 min. In the final cycle, the 72° step was extended to 10 min. The PCR

products were detected after electrophoresis on a 1.5% agarose gel containing 1:10 000 SYBR green 1.

RESULTS

LPS induces NF- κ B activation, mainly as p50-p65 dimers, in IEC-6 cells

The activation is much slower than the action of TNF. Because our previous studies showed that LPS activates NF- κ B in the rat small intestine *in vivo* and that neutrophils are involved in its action,¹⁷ we sought to examine whether LPS would activate NF- κ B *in vitro* when directly applied to intestinal epithelial cells. We chose to examine IEC-6 cells, a non-transformed rat small intestinal crypt cell line, since the small intestine is the site of predilection of LPS-induced bowel injury.^{1,3} Furthermore, our preliminary results on colonic cell lines showed that LPS does not induce NF- κ B in T84 cells and only minimally in Caco 2 cells, both colon carcinoma cell lines (data not shown). We found that, incubation of IEC-6 cells for 1 hr with LPS activates NF- κ B p50-p50 and p50-p65 (Fig. 1). This effect could be seen with the lowest dose of LPS used (0.2 μ g/ml) and was dose dependent (Fig. 1). Following stimulation with 1 μ g/ml of LPS, activated NF- κ B was detectable (measured by EMSA of nuclear extracts) as early as 20 min, became significant at 30 min, peaked at 60-90 min, and diminished at 2 hr. The early part of the time-course is shown in Fig. 2(a, right panel). Supershift experiments using antibodies against p50, p65, p52, RelB and cRel demonstrate that the induced NF- κ B complexes contain both p50 and p65 (Fig. 2a, right panel), but not p52, cRel or RelB (results not shown). LPS-induced NF- κ B activation occurs much more slowly than TNF-induced NF- κ B activation which is very rapid, and is already strong 10 min following incubation, as shown in Fig. 2(a, left panel) for comparison.

LPS induces iNOS expression in IEC-6 cells

The effect is slower than that of TNF. Because iNOS is an inducible gene dependent on NF- κ B activation, we tested

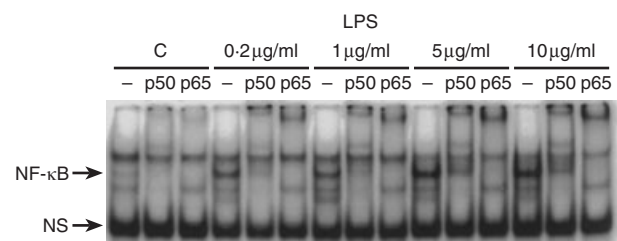


Figure 1. Dose-response of LPS-induced NF- κ B activation. IEC-6 cells (4×10^6 cells) were incubated with either 0.2, 1, 5 or 10 μ g/ml of LPS for 1 hr and the NF- κ B activity of the cell nuclear extracts was assessed by EMSA. NF- κ B activation is seen with the lowest dose of LPS (0.2 μ g/ml) used and the effect is dose dependent. Supershift experiments were done. -, no added; p50, anti-p50Ab added; p65, anti-p65Ab added. (Similar results were obtained in three independent experiments.)

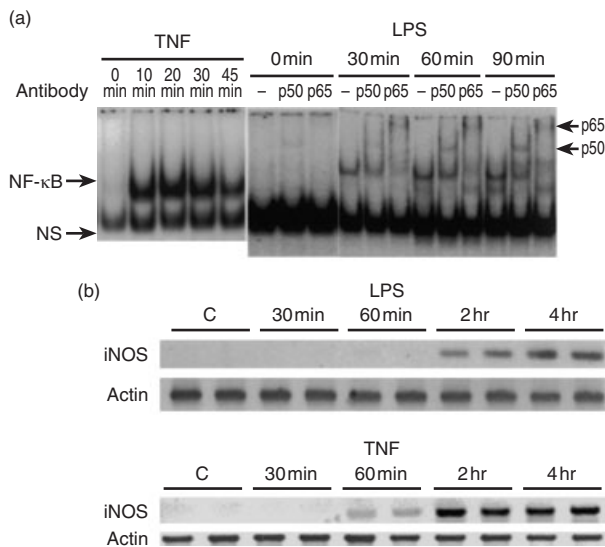


Figure 2. (a) Time-course and subunit composition of LPS-induced NF- κ B activation in IEC-6 cells: comparison with TNF. IEC-6 cells were incubated with 10 ng/ml of TNF or 1 μ g/ml of LPS for various time periods, and NF- κ B activation detected at 0, 10, 20, 30 and 45 min after TNF (left panel) and at 0, 30, 60, 90 min after LPS stimulation (right panel) (arrows, including p50–p50 and p50–p65). In the right panel, a supershift experiment shows that the LPS-induced NF- κ B complex could be supershifted with anti-p50 and anti-p65 antibodies. Similar results were obtained in three independent experiments. (b) LPS and TNF induce iNOS gene expression in IEC-6 cells. IEC-6 cells were incubated for various times with LPS (upper panel) or TNF (lower panel) (two samples for each time-point). RNA was extracted, RT-PCR performed using primers specific for iNOS or actin. PCR products were run on a 1.5% agarose gel and stained with SYBR green I.

the effect of LPS on its expression in IEC-6 cells, and compared it with TNF, using semiquantitative RT-PCR. Unstimulated IEC-6 cells do not express iNOS mRNA (Fig. 2b). iNOS expression in response to LPS and TNF lags behind that of NF- κ B activation: Following LPS, iNOS mRNA becomes detectable only at 2 hr after stimulation, and its level increases further at 4 hr (Fig. 2b). In contrast, TNF-induced iNOS mRNA is already detectable at 60 min and remains strong at both 2 and 4 hr (Fig. 2b).

PMN do not potentiate LPS-induced NF- κ B activation

Because PMN are involved in LPS-induced NF- κ B activation *in vivo*¹⁷ we examined whether PMN would potentiate the LPS effect in enterocytes. Incubation of IEC-6 cells for 30 min with rat PMN (1×10^6 /ml) induces NF- κ B activation (Figs 3b and 4). However, the addition of PMN did not potentiate LPS-induced NF- κ B activation (Fig. 3a). There was a moderate supershift with anti-p50 antibody and a marked supershift with anti-p65 antibody in nuclear extracts of cells treated with LPS, PMN alone or LPS and PMN together (Fig. 3b).

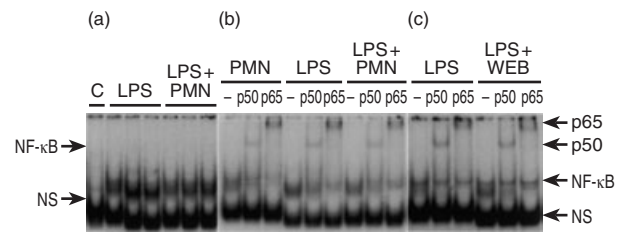


Figure 3. LPS-induced NF- κ B activation in IEC-6 cells is not enhanced by PMN and is not inhibited by PAF receptor antagonists. (a) EMSA showing the lack of difference in the NF- κ B activity between cells treated for 30 min with LPS (1 μ g/ml) alone and LPS plus PMN (1×10^6 /ml). (b) A typical gel with supershift experiments showing no difference in the subunit composition of LPS- and LPS/PMN-treated cells. (c) WEB2170 (0.5 μ g/ml), a PAF receptor antagonist, had no effect on LPS induced NF- κ B activation. Here, a typical supershift experiment is presented. Each experiment was performed at least three times and showed consistent results.

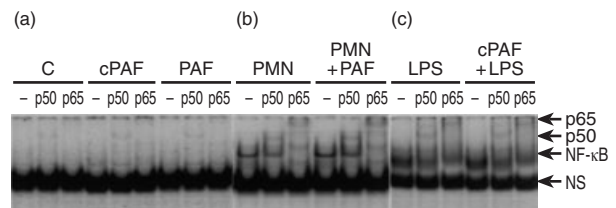


Figure 4. PAF does not activate NF- κ B in IEC-6 cells and does not potentiate LPS-induced NF- κ B activation. EMSA with supershift experiments showing lack of stimulatory effect of PAF (30 min). (a) Lanes 1–3: untreated cells. Lanes 4–6: cells treated with carbamyl-PAF, 0.5 μ g/ml. Lanes 7–9: PAF, 0.5 μ g/ml. Note a low level of constitutively present p50 in unstimulated cells. A typical supershift experiment showing the lack of synergistic effect between PMN and PAF (b) and between cPAF and LPS (c). The same results were obtained in at least five experiments.

LPS-induced NF- κ B activation is not dependent upon the production of endogenous PAF in IEC-6 cells

We previously found that LPS-induced bowel injury and NF- κ B activation¹⁷ in the intestine is partially blocked by pretreatment with a PAF receptor antagonist, suggesting that this *in vivo* effect of LPS is partly mediated by endogenous PAF. However, in IEC-6 cells, WEB2170 (a PAF antagonist) failed to exert any inhibitory effect on LPS-induced NF- κ B activation in IEC-6 cells (Fig. 3c). WEB2170 did not change the subunit composition of NF- κ B: There were similar amounts of p50 and p65 in both LPS and WEB2170 + LPS treated cells (Fig. 3c).

PAF does not activate NF- κ B in IEC-6 cells, nor does it potentiate the LPS effect on NF- κ B activation

We then examined whether PAF could activate NF- κ B directly in IEC-6 cells, which have been found to have PAF receptors (T. Jilling and M. Caplan, personal communication). Our previous study showed that PAF activates

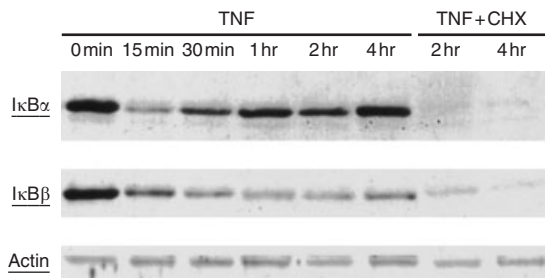


Figure 5. Western blot analysis showing TNF-induced degradation of I κ B α and I κ B β in IEC-6 cells. Cells were treated with: TNF (10 ng/ml) or TNF plus cycloheximide (50 μ g/ml) for various time lengths. At the end of the experiment, total cell lysates were prepared and subjected to immunoblot analysis with anti-I κ B α or anti-I κ B β antibodies. Note the rapid degradation of I κ B α and the slow degradation of I κ B β induced by TNF with resynthesis of both molecules. Cycloheximide (50 μ g/ml) completely blocked the TNF-induced I κ B α and I κ B β resynthesis.

NF- κ B, mainly as p50–p50 homodimers in the intestine of rats.¹⁶ IEC-6 cells have a very low level of constitutive NF- κ B activation, mainly supershifted by anti-p50 antibodies (Fig. 4a). Contrary to *in vivo* experiments, PAF, at the doses (0.5–5 μ g/ml) used, did not cause a significant activation of NF- κ B in IEC-6 cells (Fig. 4a). To rule out the possibility that the absence of PAF effect was caused by its rapid degradation by its degrading enzyme, PAF acetylhydrolase, which is abundant in intestinal epithelium (M. Caplan, personal communication) and in FBS, the experiment was repeated with carbamyl-PAF, a stable PAF analogue. We found that carbamyl-PAF also did not have any stimulatory effect (Fig. 4a). Lastly, based on our previous finding that the PAF effect on intestinal NF- κ B activation *in vivo* is PMN-dependent,¹⁶ we added PAF together with PMN to IEC cells. The resulting NF- κ B activation was similar to that with PMN alone (Fig. 4b).

Because LPS and PAF have synergistic effects in causing systemic inflammation and intestinal injury,³ we examined whether PAF could potentiate the LPS effect on NF- κ B activation. We found that the addition of either carbamyl-PAF (Fig. 4c) or PAF together with LPS (not shown) did not induce more NF- κ B activation than did LPS alone.

TNF induces the degradation of both I κ B α and I κ B β in IEC-6 cells

When IEC-6 cells were incubated with TNF, we found that I κ B α was rapidly degraded within 15 min and progressively resynthesized (Fig. 5). I κ B β levels decreased more slowly, reaching a minimum level at 1 hr and its resynthesis was slower than I κ B α (Fig. 5). The resynthesis of both I κ B α and I κ B β was blocked by cycloheximide (Fig. 5).

LPS increases the turnover of both I κ B α and I κ B β in IEC-6 cells

When analysed by Western blot, I κ B α and I κ B β levels did not significantly decrease after LPS stimulation (Fig. 6).

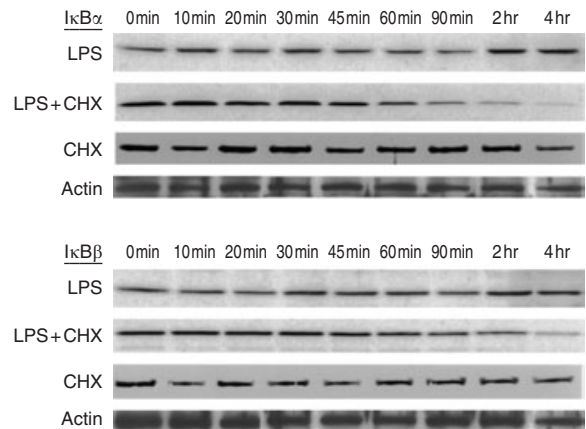


Figure 6. Western blot analysis showing increased turnover of I κ B α and I κ B β in IEC-6 cells after LPS stimulation. Cells were treated with: LPS (1 μ g/ml), LPS plus cycloheximide (50 μ g/ml), or cycloheximide alone, for various time lengths. At the end of the experiment, total cell lysates were prepared and subjected to immunoblot analysis with anti-I κ B α or anti-I κ B β antibodies. Note the absence of I κ B degradation with LPS alone (LPS), and the obvious diminishing of I κ B when cycloheximide was added together with LPS (LPS + CHX). Cycloheximide alone had no effect (CHX) ($N=3$).

However, when cells were treated with both LPS and cycloheximide (to block the synthesis of new I κ B molecules), there was a slow decrease in both I κ B α and I κ B β , which became apparent at 60 min. The I κ B levels continued to drop, and at 4 hr both I κ B α and I κ B β became undetectable (Fig. 6). These results indicate that LPS stimulates I κ B turnover by stimulating its degradation and *de novo* synthesis. Cycloheximide by itself had no effect, as no decrease in I κ B α and I κ B β was noted (Fig. 6). I κ B ϵ was present in whole cell extracts, but its level did not change significantly following LPS and remained unmodified by the addition of cycloheximide (results not shown). I κ B γ was not detected in IEC-6 cells.

DISCUSSION

Bacterial endotoxin induces the expression of cytokines both *in vivo*⁹ and *in vitro*^{5,7} and activates the transcription factor NF- κ B in many cells²³ as well as *in vivo*.²⁴ However, it remains controversial whether LPS could activate NF- κ B in enterocytes although it is well established that these cells could produce many pro-inflammatory cytokines²⁵ in response to LPS. Most studies examining the effect of LPS are limited to colon carcinoma cell lines: It has been shown that the adhesion of *S. dublin*, *S. typhimurium* or of an invasive strain of *E. coli* to Caco-2 cells activates NF- κ B.¹⁸ In another study, enteropathogenic *E. coli* was shown to activate NF- κ B in T84 cells.¹⁹ However, neither non-pathogenic *E. coli* nor LPS is effective in activating NF- κ B in this cell line.¹⁹ Our study is the first to examine the effect of LPS on NF- κ B in a small intestinal cell line and to find a direct activation of NF- κ B by LPS in IEC-6 cells. This finding may be pathophysiologically relevant, because

in necrotizing enterocolitis, a common intestinal inflammatory disease affecting the premature neonate, the ileum is the most common site of inflammation and injury. Furthermore, the intestinal epithelium is constantly exposed to bacterial products such as LPS, and NF- κ B activation and its downstream event, such as iNOS induction, may play an important role in mucosal defence and injury.

In this study, we found that LPS activates NF- κ B within 30 min, peaking between 60 and 90 min; LPS action is much slower than TNF. One possible explanation is that protein synthesis is required for the activation of NF- κ B by LPS. However, our preliminary results suggest that this is not the case, as pretreatment with cycloheximide did not affect the NF- κ B activation by LPS.

We have shown previously that LPS induces NF- κ B in rat ileum *in vivo*¹⁷ and that its activation is partly mediated by endogenous PAF production and is partly dependent on PMN.¹⁷ We did not find any involvement of endogenous PAF in LPS-induced NF- κ B activation in IEC-6 cells, nor have we found any direct effect of PAF on these cells. PMN alone did induce NF- κ B activation in IEC-6 cells. The activated NF- κ B detected probably came from IEC-6 cells rather than from the added PMN, because we could not detect NF- κ B activation in nuclear extracts of equal amounts of PMN (not shown). Contrary to our *in vivo* findings, we failed to detect any potentiating effects of PMN on NF- κ B activation by LPS in IEC-6 cells. These observations suggest that, *in vivo*, several mechanisms involving complex interactions of different cell types, contribute to the inflammatory response induced by PAF.

Previous studies have shown that NF- κ B regulates iNOS expression in macrophages^{20,26} kidneys²⁷ and lungs.²⁸ It has also been shown that LPS up-regulates iNOS in the intestine.¹⁰ In rat intestine, nNOS down-regulates iNOS gene expression by inhibiting NF- κ B.²⁹ In this study, we found that the activation of NF- κ B by LPS in IEC-6 cells precedes both TNF- and LPS-induced up-regulation of iNOS gene expression. Furthermore, TNF activates NF- κ B and induces iNOS gene expression faster than LPS. This suggests that NF- κ B mediates LPS- and TNF-induced iNOS gene expression in these cells.

LPS has been shown to induce the loss of I κ B α from NF- κ B complexes in B or pre-B cells.^{30,31} It also causes the loss of both I κ B α and I κ B β *in vivo* in the liver and macrophages³² and *in vitro* in RAW264.7 macrophages.^{32,33} In IEC-6 cells, contrary to our results with TNF, we were unable to detect any significant drop in both I κ B α and I κ B β proteins after LPS stimulation (1 μ g/ml). However, when cycloheximide together with LPS, was added to inhibit new protein synthesis, we observed a decrease in both I κ B α and I κ B β at 60 min, and the levels became nearly undetectable at 4 hr (cycloheximide itself has no effect). These results indicate that LPS induces both the degradation and the *de novo* synthesis of I κ B α and I κ B β , and the net result was an unchanged level of I κ B proteins.

Although our results suggest that both I κ B α and I κ B β play a role in regulating LPS-induced NF- κ B activation in IEC-6 cells, it is puzzling that the decline of these inhibitory proteins lags behind the activation of NF- κ B. Indeed,

it is generally accepted that signal-induced phosphorylation of I κ B α leads to its proteolysis, which results in NF- κ B activation.³⁴ There are two possible explanations to our findings: (a) LPS leads to I κ B–NF- κ B dissociation and subsequent NF- κ B activation, but no I κ B degradation, as it has been rarely reported in the literature,^{35,36} or (b) the initial NF- κ B activation is due to an increase in p105 processing to p50. Changes in I κ B α stability, without significant changes in protein levels have been reported in Hs294T melanoma cells as opposed to normal retinal pigment epithelial (ARPE) cells.³⁵ Other stimuli like TNF and IL-1 β have been shown to induce an early and complete degradation of I κ B α in IEC-6 cells.³⁷ However, the same stimulus (IL-1 β) was shown to produce only delayed and incomplete I κ B α degradation in HT-29 and primary IEC cells.³⁶ In addition, no evidence of I κ B β degradation was found in HT-29 cells by Western blot analysis (C. Jobin and R. B. Sartor, unpublished observations).³⁶ In DLD-1 colon carcinoma cells, PMA activates NF- κ B without the degradation of I κ B α , I κ B β , I κ B γ , or I κ B ϵ and without I κ B–NF- κ B dissociation, through a protein kinase C-dependent pathway, whereas IL-1 β induces I κ B α degradation.³⁸ IEC-6 cells appear different from DLD-1 cells as no detectable I κ B γ was found. I κ B ϵ is present in IEC-6 cells, but there were no significant changes following LPS treatment.

In conclusion, as opposed to what we had found *in vivo* in rat intestine, we did not find any contributing role of endogenous PAF in the induction of NF- κ B activation by LPS in IEC-6 cells. Our study also suggests that LPS activates NF- κ B, and the action is much slower than TNF. Unlike TNF, which causes rapid I κ B degradation, LPS activates NF- κ B probably via at least two mechanisms: initially, through an I κ B-independent mechanism, such as the processing of p105 to p50, and later, via an increased turnover of the inhibitor I κ B.

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