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Lipopolysaccharide Directly Stimulates the Intrapituitary Interleukin-6 Production by Folliculostellate Cells via Specific Receptors and the p38α Mitogen-Activated Protein Kinase/Nuclear Factor-κB Pathway*

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ABSTRACT

Bacterial lipopolysaccharide (LPS) activates the immune system and induces increases in peripheral cytokines, which, in turn, affect the endocrine system. In particular, LPS-induced cytokines stimulate the hypothalamic-pituitary-adrenal axis to increase levels of antiinflammatory-acting glucocorticoids. In the present work, we show that LPS directly stimulates interleukin (IL)-6 release by mouse pituitary folliculostellate (FS) TtT/GF tumor cells and FS cells of mouse pituitary cell cultures. The stimulatory effect of LPS was strongly enhanced in the presence of serum, suggesting that LPS is only fully active as a complex with LPS-binding protein (LBP). Both TtT/GF cells and mouse pituitaries expressed CD14, which binds the LPS/LBP complex, and Toll-like receptor type 4, which induces LPS signals. LPS increased phospoinositol turnover in TtT/GF cells and induced phosphorylation of p38α mitogen-activated protein kinase and the inhibitor (IκB) of nuclear factor-κ B. Nuclear factor-κ B was activated by LPS in TtT/GF cells. Functional studies demonstrated that My4 (an antibody blocking the interaction between LPS/LBP and CD14), SB203580, (a specific inhibitor of p38α mitogen-activated protein kinase phosphorylation), dexamethasone, and the messenger RNA translation inhibitor cycloheximide all inhibited LPS-induced IL-6 production by TtT/GF cells and mouse pituitary FS cells. LPS-induced intrapituitary IL-6 may modulate the function of anterior pituitary cells during bacterial infection/inflammation. (*Endocrinology* **141:** 4457–4465, 2000)

TO DATE, it is well known that the interaction between the immune system and the neuroendocrine system is necessary for the organism to overcome pathological events, like inflammation or infection, in an optimal manner (1). It has become evident that cytokines produced by immune cells affect and modify the hormone secretion of the endocrine cells (2). In turn, hormones modulate the function of immune cells and influence cytokine production (1). In these interactive processes, the activation of the hypothalamic-pituitary-adrenal axis by cytokines is important, because it results in increased levels of antiinflammatory acting glucocorticoids that prevent overshooting reactions (septic shock) of the activated immune system (3).

Lipopolysaccharide (LPS), a component of the outer layer of the cell wall of gram-negative bacteria (4), is a potent activator of the immune system (5). It induces the subsequent release of tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 from macrophages and monocytes (6). Although LPS alone can activate these cells, its stimulatory potency is strongly enhanced when it is coupled to LPS-binding protein (LBP) (7), which is present in serum (8). In monocytes and macrophages, it is known that membrane-bound CD14 (mCD14) binds free LPS or LPS/LBP complex (9). mCD14-negative cell types, like endothelial cells or smooth muscle cells, need soluble CD14, which is present in serum, for an optimal response to LPS (10). The LPS/LBP/CD14 complex subsequently interacts with the Toll-like receptor-4 (Tlr4), a member of the Toll protein family (11, 12). In immune cells, LPS induces the activation of nuclear factor-κB (NF-κB) (13), but the preceding signaling cascade is largely unknown; so far, only the participation of mitogen-activated protein kinase (MAPk) kinase-3 and p38α MAPk (14), or the p42/44 MAPk (15), has been shown.

Cytokines not only represent a lymphocyte message but are produced by pituitary cells themselves (16–18). With respect to IL-6, folliculostellate (FS) cells have been identified as the source of this cytokine within the normal pituitary (19, 20). IL-6 production by FS cells is inhibited by glucocorticoids (21) and stimulated by neuroendocrine factors, such as pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (22–24). LPS and cytokines, like TNF-α and IL-1, also enhance IL-6 production (25–27). There is evidence that the hormone stimulatory potency of the above-mentioned substances is mediated, in part, through the subsequent paracrine action of IL-6, which is a

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potent stimulator of ACTH, GH, PRL, FSH, and LH secretion (16, 17).

We have studied whether essential components needed for the direct action of LPS, such as CD14 and Tlr4, are expressed in the FS TtT/GF mouse pituitary cell line and in mouse pituitaries, and we have characterized the LPS signaling pathway in FS cells.

Materials and Methods

Materials

Cell culture materials and reagents were obtained from Life Technologies, Inc.(Karlsruhe, Germany), Falcon (Heidelberg, Germany), Nunc (Wiesbaden, Germany), Seromed (Berlin, Germany), Flow Cytometry Standards Corp. (Meckenheim, Germany), and Sigma (St. Louis, MO). LPS (*Escherichia coli* serotype 055:B5), dexamethasone, and cycloheximide were purchased from Sigma and PACAP-38 from Bachem AG (Bubendorf, Switzerland). RU486 was obtained from Roussel (Romainville, France); and My4, an antibody blocking the interaction between LPS/LBP and CD14 (28), was from Coulter Technologies (Hialeah, FL). The p38α MAPk phosphorylation inhibitor SB203580 (14) was obtained from Calbiochem (Bad Soden, Germany).

Cell culture

TtT/GF cells were grown in 48-multiwell plates at 37 C and 5% CO_2 in DMEM (pH 7.3) supplemented with 2% FCS, 2.2 g/liter $NaHCO_3$, 10 mM HEPES, 2 mM glutamine, 2.5 mg/liter amphotericin B, 10^5 U/liter penicillin-streptomycin, 5 mg/liter insulin, 5 mg/liter transferrin, 20 mg/liter sodium selenite, and 30 pM T_3. TtT/GF cells were cultured at 37 C and 5% CO_2 until they were confluent. After a monolayer had formed (approximately 70,000 cells per well in 48-well plates) as previously reported, the cells did not grow further (29). The monolayer was washed with PBS, and serum-free culture medium was added for 48 h to wash out any remaining serum. The cells were then washed again with PBS and stimulated as described below.

For primary cell culture, pituitaries from 12-week-old male C57BL/6 mice were enzymatically and mechanically dispersed as previously described for rat pituitaries (30). Dispersed cells were seeded at an initial density of 100,000 cells per well in 48-well plates and cultivated in D-Val-MEM (to suppress fibroblast growth) (30) supplemented with 10% FCS and additives indicated above for TtT/GF cell culture. After an initial attachment period of 48 h, followed by a serum washout period of 48 h, the cells were used for stimulation experiments as indicated.

Stimulation and measurement of IL-6

All stimulation or inhibition experiments were performed in the appropriate culture medium in the presence or absence of FCS (2%), as indicated. Before and after the treatment period, cell viability and numbers were routinely monitored to ensure that these parameters did not change during the experiment. Cell viability was determined microscopically after ethidium bromide/acridine orange staining. Cell numbers were determined with an adapted Coulter counter, as previously described (29).

Mouse IL-6 was measured according to the manufacturer's instructions, by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN), as previously reported (29). The detection limit of the assay was 3 pg/ml. The intraassay and interassay coefficients of variation were 3.4% and 6.4%, respectively.

Determination of cAMP and inositol phosphate

Studies on second messengers were performed with confluent TtT/GF cell cultures that had been cultivated for 48 h in serum-free culture medium before the experiments.

To measure inositol 1-phosphate (Ip_1), Ip_2, and Ip_3, monolayers of TtT/GF cells were incubated in 12-well plates for 20 h in serum-free culture medium containing 2.5 μCi/well myo-^{3}H-inositol (Amersham Pharmacia Biotech, Braunschweig, Germany). The medium containing myo-^{3}H-inositol was removed, and the monolayers were washed 3 times with PBS. The cells were treated with 10 mM LiCl for 15 min and stimulated with LPS for an additional 60 min in the presence (2%) or absence of FCS. The reaction was terminated by the addition of 1 ml ice-cold methanol (96%). Subsequently Ip_1, Ip_2, and Ip_3 values were determined as previously described (31).

cAMP was determined as described by Matsumoto *et al.* (24). TtT/GF monolayer cell cultures were incubated for 24 h, with or without LPS, in the presence (2% FCS) and absence of serum. In the cell culture supernatants, cAMP was measured by RIA (NEN Life Science Products, Cologne, Germany).

RT-PCR for mouse mCD14 and Tlr4

For RT-PCR, total cellular RNA was isolated from TtT/GF cells, mouse anterior pituitary, and mouse spleen by guanidinium isothiocyanate, followed by the phenolchloroform method (32). RT of 1 μg RNA was performed with Superscript-II (Life Technologies, Inc./BRL, Karlsruhe, Germany) for 1 h at 45 C, followed by a denaturation step at 94 C for 1 min. With the complementary DNA (cDNA) template obtained, 35-cycle PCR was performed with specific primers (Table 1). Each cycle consisted of denaturation at 94 C for 1 min, annealing of primers at 60 C for 1 min, and chain extension at 72 C for 1 min. Amplified products were electrophoresed in 1.8% agarose gel and stained with ethidium bromide.

Specific primers to detect murine mCD14 and Tlr4 messenger RNA (mRNA) (Table 1) were constructed according to the published DNA sequences (GenBank accession numbers NM009841 for mouse CD14 and AF185285 for mouse Tlr4).

Determination of phosphorylation of p38α MAP kinase and IκB

Confluent 75-cm^2 culture flasks of TtT/GF cells were given a change of medium (with and without FCS) and then, 2 h later, stimulated with LPS for 15, 30, and 60 min. The cells were washed with ice-cold PBS, lysed with SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% wt/vol SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% wt/vol bromophenol blue], and collected in Eppendorf (Hamburg, Germany) tubes. After the samples were sonicated and heat-treated for 3–5 min at 95 C, 20 μl from each sample were fractionated on a 10% SDS-polyacrylamide gel and then electrotransferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The immunoreactive products on the membranes were detected using PhosphoPlus IκB-α (Ser32), and p38α MAPk (Thr 180/Tyr 182) antibody kits (New England Biolabs, Inc., Schwalbach, Germany). In brief, the membranes were treated with 5% wt/vol nonfat milk protein in TBST [50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween-20] for 1 h at room temperature, and then overnight at 4 C in the primary antibodies diluted 1:1000 in 2.5% nonfat milk protein in TBST. After a 20-min wash in TBST, the membranes were incubated at room temperature for 1 h in a 1:2000 dilution of the hydrogen peroxidase-conjugated secondary antibody in 2.5% nonfat milk protein in TBST. The membranes were washed in TBST and then incubated with LumiGLO for 1 min at room temperature and then imme-

TABLE 1. Primer sequences and cDNA fragment sizes

Target	Primer sequence (5′ → 3′)	cDNA size
mCD14 (mouse)	5′: TAC CGA CCA TGG AGC GTG TGC 3′: GCC CGC ACC GTA AGC CGC T	289 bp
Tlr4 (mouse)	5′: GGG TCA AGG AAC AGA AGC AG 3′: GCT CAT TTC TCA CCC AGT CC	264 bp

diately exposed to Lumi-Film chemiluminescent detection film (Roche Diagnostics GmbH, Mannheim, Germany).

Determination of transcriptional activity of NF-κB

TtT/GF cells (200,000 cells/well) were transfected with lipofectamine in Optimem medium (Life Technologies, Inc./BRL) using 0.7 mg of the κB/luciferase (κB-LUC) reporter plasmid and 0.3 mg of pRL-Tk, used as second reporter control plasmid (Promega Corp., Mannheim, Germany). After 6.5 h, the medium was changed to DMEM with FCS 2%. After an overnight incubation, the cells were given fresh DMEM containing 2% FCS and were stimulated with LPS. After 6 h, the cells were washed with PBS, and the LUC activity was measured with a dual reporter kit (Promega Corp.). Reporter plasmid for κB sites was provided by Dr. M. Bell (Mayo Clinic, Rochester, MN) and has been described elsewhere (33).

Statistics

Each of the experiments was repeated at least three times. The individual experiments were performed with quadruplicate wells. ANOVA, in combination with Scheffe's test, was used for statistics. The data are expressed as mean ± SE.

Results

Expression of CD14 and Tlr4 in TtT/GF cells and mouse pituitary

Primers specific for mCD14 were used to perform RT-PCR on mRNA extracted from TtT/GF cells, mouse pituitary, and mouse spleen (as positive control). A single cDNA band of a predicted 289 bp was found, indicating that FS cells express mCD14 (Fig. 1A), which is needed for the LPS/LBP complex to bind to the cell membrane.

Another prerequisite of LPS action is the presence of the LPS signal transducer Tlr4 in the corresponding target cell. In RNA preparations of both unstimulated TtT/GF cells and normal mouse pituitary, Tlr4 mRNA expression was detected by RT-PCR. After amplification, a single cDNA fragment of a predicted 264 bp in length was found. An identical cDNA fragment was detected after RT-PCR was performed on RNA preparations of mouse spleen, which were used as a positive control (Fig. 1B).

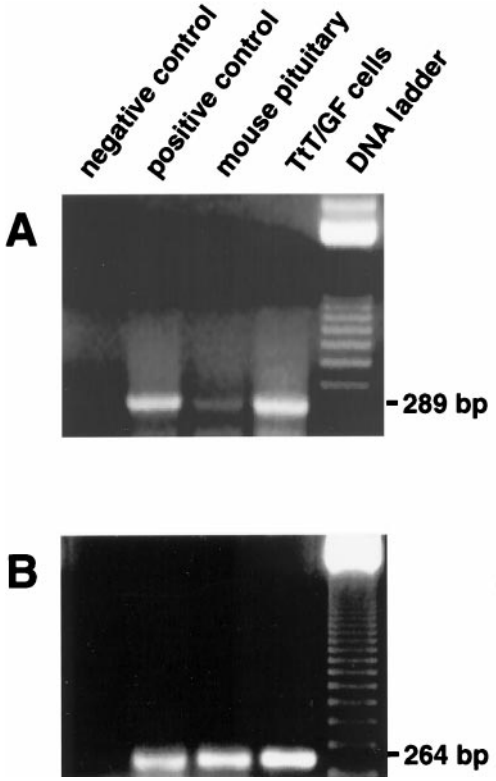

FIG. 1. Detection of mCD14 (A) and Tlr4 (B) mRNA expression by RT-PCR in TtT/GF cells, mouse pituitary, and mouse spleen (positive control). The predicted amplification products (289 bp for CD14 and 264 bp for Tlr4) are not present in negative controls in which either the primers or the cDNAs (results for the latter not shown) were omitted.

LPS stimulation of IL-6 production by TtT/GF cells

TtT/GF cells secreted low amounts of IL-6 in serum-free cell cultures during 32-h time course studies (Fig. 2A). The addition of 2% FCS to the cells induced no further increase in IL-6 production. In the absence of serum, 100 ng/ml LPS significantly stimulated IL-6 production only after 24 h. However, in the presence of 2% FCS, a tremendous stimulation of the IL-6 production was observed in response to LPS (Fig. 2A). IL-6 secretion was significantly elevated after 2 h and linearly increased during a 32-h stimulation period. In dose-response studies, 1 ng/ml LPS, in the presence of serum, significantly stimulated IL-6 production. Maximal stimulation was obtained at a concentration of 100 ng/ml LPS (Fig. 2B). In the absence of FCS, LPS induced a significant increase in IL-6 production only at a concentration of 100 ng/ml LPS, and maximal stimulation was achieved at 1000 ng/ml. Higher concentrations of LPS did not further stimulate IL-6 secretion (data not shown). Studies on the serum-dependency of LPS-induced IL-6 secretion demonstrated that 0.5% FCS was sufficient to induce a significant stimulatory effect of LPS. Saturation of the IL-6-stimulating effect of LPS was achieved at 2% FCS, and no further increase of the stimulatory potency of LPS was observed at higher concentrations of FCS (Fig. 2C). It should be noted that, because FCS is not a clearly defined substrate, variations of the LPS-induced IL-6 secretion were observed with different batches of FCS.

Inhibition of the LPS-stimulated IL-6 secretion in TtT/GF cells

Glucocorticoids have been shown to inhibit the basal release of IL-6 from normal pituitary FS cells; therefore, we have tested the effect of the synthetic glucocorticoid dexamethasone on LPS-stimulated IL-6 secretion in TtT/GF cells. Increasing concentrations of dexamethasone reduced the IL-6 stimulatory effect of 100 ng/ml LPS and completely blocked it at a concentration of 1 μM (Fig. 3). The inhibitory effect of dexamethasone on LPS-induced IL-6 stimulation could be reversed by the glucocorticoid receptor antagonist RU486. This clearly indicates that functional glucocorticoid receptors participate in the regulation of IL-6 secretion in TtT/GF cells.

Cycloheximide, which inhibits mRNA translation, also suppressed LPS-induced IL-6 secretion. The addition of 1 μM cycloheximide to TtT/GF cell cultures completely blocked the rise of IL-6 in response to 100 ng/ml LPS during a 24-h time course study (data not shown). This indicates that LPS-

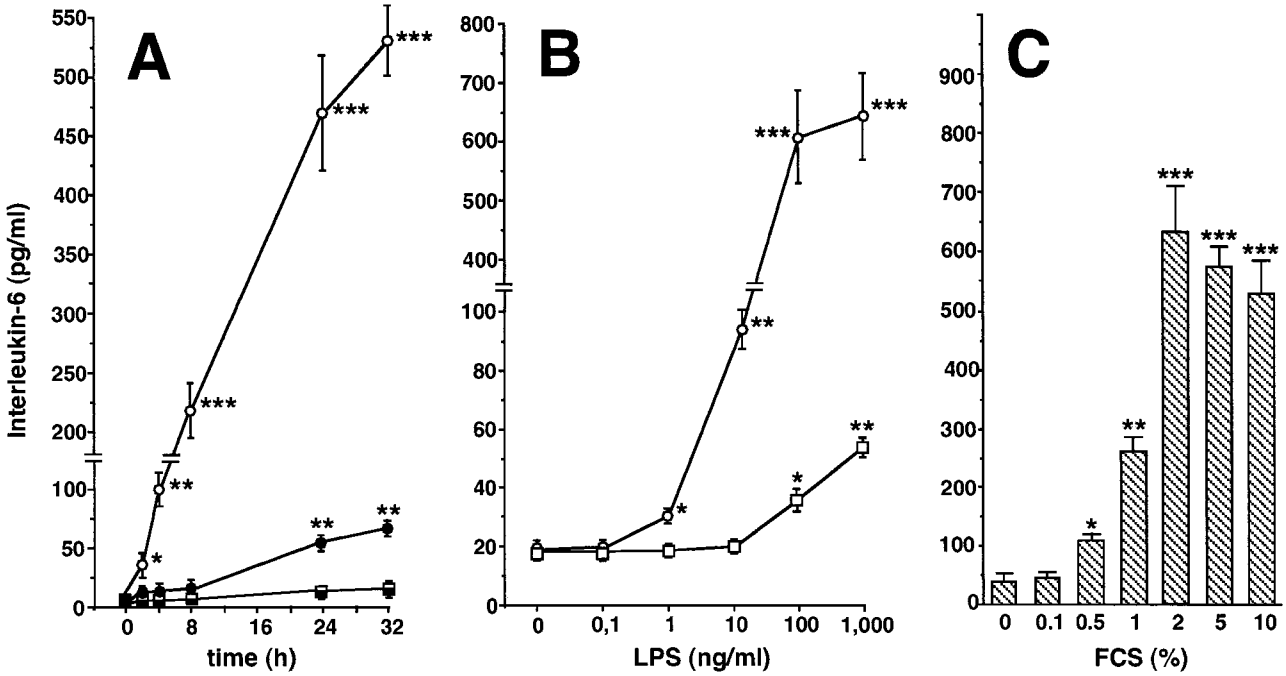

FIG. 2. LPS stimulation of IL-6 secretion by TtG/GF cells. A, Time course of IL-6 release in response to LPS (100 ng/ml) in the absence (*closed circles*) or presence (*open circles*) of 2% FCS. *Closed and open squares* indicate the basal IL-6 release by TtT/GF cells not treated with LPS in the absence or presence of serum. B, Dose-response curves of the IL-6 stimulatory effect of LPS in the absence (*open squares*) or presence (*open circles*) of 2% FCS. C, Effect of LPS (100 ng/ml) on IL-6 secretion at different concentrations of FCS. In B and C, cells were treated with LPS for 24 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs*. basal IL-6 secretion.

stimulated IL-6 production by TtT/GF cells involves *de novo* synthesis of IL-6.

To test whether CD14 is involved in the LPS-induced IL-6 production by TtT/GF cells, the effect of My4, an antibody which blocks the interaction between LPS/LBP complex and CD14 (28), was investigated. Although the antibody preparation itself slightly stimulated IL-6 secretion by TtT/GF cells, My4 dose-dependently inhibited the IL-6 stimulatory effect of LPS (Fig. 4). PACAP-induced IL-6 secretion was not affected by My4, indicating that the antibody specifically blocks LPS-stimulated IL-6 production.

LPS signaling in TtT/GF cells

To study whether classical second messengers are involved in LPS-induced IL-6 production, TtT/GF cells were treated with 100 ng/ml LPS, in the absence or presence of FCS, and alterations in cAMP or inositolphosphate production were determined. LPS slightly, but significantly, enhanced Ip-1 generation under serum-free conditions. In the presence of serum, LPS stimulated Ip-1 production to a greater extent (Table 2). Similar results were obtained for Ip-2 and Ip-3 (data not shown). LPS did not alter cAMP production, both in the presence and absence of FCS. In contrast, PACAP stimulated cAMP production, whereas inositolphosphate turnover was not affected (Table 2).

In immune cells, it has been shown that phosphorylation of either p38α MAPk or p42/44 MAPk is involved in LPS signaling. We found that p38α MAPk was phosphorylated in TtT/GF cells in response to LPS. Under serum-free conditions, LPS slightly induced p38α MAPk phosphorylation; but, in the presence of serum, a strong stimulation was ob-

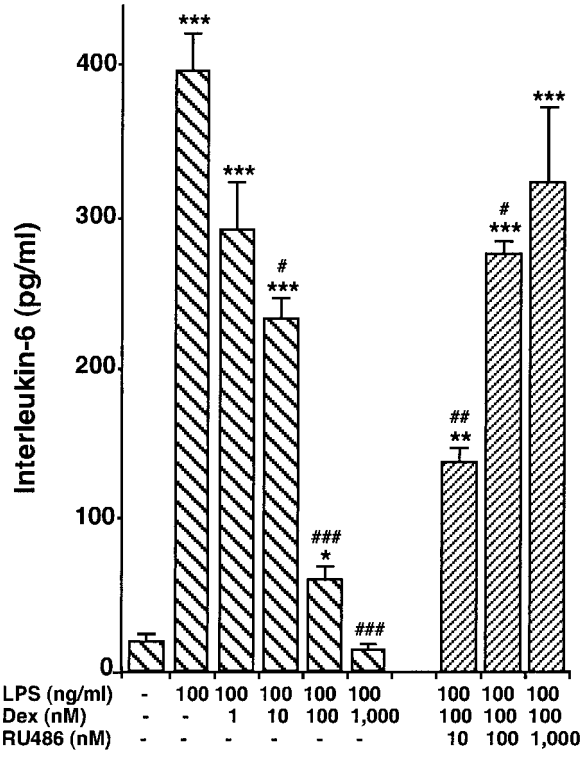

FIG. 3. Inhibition of LPS-induced IL-6 secretion by dexamethasone (Dex) and its reversion by RU486 in TtT/GF cells treated for 24 h with the stimulants. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs*. basal IL-6 release; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ *vs*. LPS-stimulated IL-6 secretion.

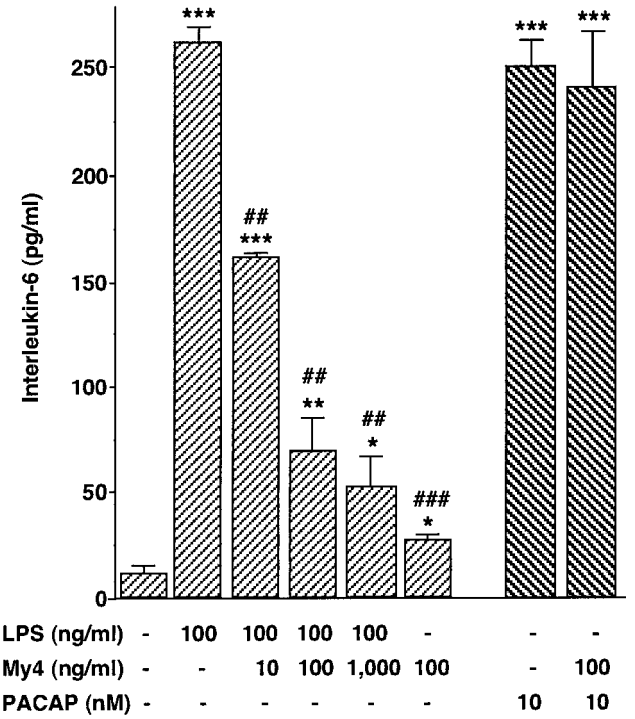

FIG. 4. Suppression of LPS-stimulated IL-6 production of TtT/GF cells by My4, an antibody blocking the interaction between CD14 and the LPS/LBP complex. The IL-6 stimulatory effect of PACAP is not affected by My4. Cells were treated for 24 h with the indicated substances. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* basal IL-6 secretion; ##, $P < 0.01$; ###, $P < 0.001$ *vs.* LPS-stimulated IL-6 secretion.

TABLE 2. Effect of LPS on inositol 1-phosphate and cAMP turnover in TtT/GF cells

Stimulation conditions	IP-1[a]	cAMP[a]
Serum-free	100 ± 4	100 ± 6
Serum-free + LPS (100 ng/ml)	129 ± 7[b]	112 ± 11
Serum-free + PACAP-38 (10 nM)	112 ± 6	437 ± 34[c]
2% FCS	320 ± 48[c]	379 ± 34[c]
2% FCS + LPS (100 ng/ml)	543 ± 41[c,d]	356 ± 27[c]
2% FCS + PACAP-38 (10 nM)	357 ± 29[c]	925 ± 83[c,d]

[a] Values are expressed as percent of second-messenger production under serum-free conditions.
[b] $P < 0.05$ *vs.* serum-free.
[c] $P < 0.01$ *vs.* serum-free.
[d] $P < 0.01$ *vs.* 2% FCS.

served (Fig. 5A). Time course studies demonstrated the appearance of phosphorylated p38α MAPk within 15 min, and maximal stimulation was achieved at 30 min (Fig. 5B). To demonstrate the involvement of phosphorylated p38α MAPk in LPS-induced IL-6 secretion, SB203580, a specific inhibitor of p38α MAPk phosphorylation (14), was tested for its suppressive effect on LPS-stimulated IL-6 production. Treatment of TtT/GF cells or mouse pituitary cell cultures with SB203580 completely abolished the LPS-induced IL-6 production (Fig. 6).

In LPS-responsive cells, the transcription factor NF-κB is activated downstream of p38α MAPk. Stimulation of TtT/GF cells with 100 ng/ml LPS serum-dependently induced phosphorylation of IκB (inhibitor of NF-κB) within 15 min; maximal phosphorylation was achieved after 60 min of incubation (Fig. 7A). Phosphorylation of IκB was only slightly induced under serum-free conditions (data not shown). After IκB phosphorylation, the IκB/NF-κB complex (in which NF-κB is transcriptionally inactive) dissociates, and NF-κB becomes transcriptionally active (34). To demonstrate the latter, TtT/GF cells were transiently transfected with a κB-LUC reporter plasmid responsive to transcriptionally active NF-κB. High basal levels of LUC activity indicate that the cells were efficiently transfected with the κB-LUC construct and that the constitutive basal κB activity is high. Nevertheless, the LUC activity of the reporter plasmid was significantly induced by LPS in TtT/GF cells (Fig. 7B).

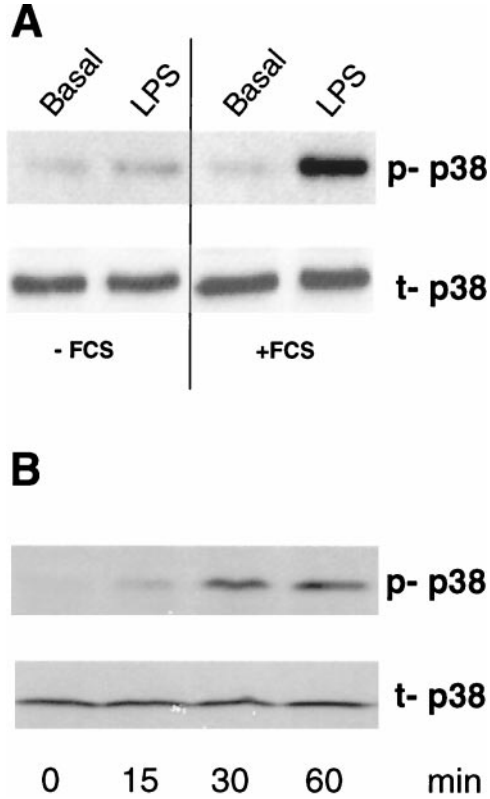

FIG. 5. LPS-induced phosphorylation of p38α MAPk in TtT/GF cells. Specific antibodies were used to detect phosphorylated (p-p38) or total (t-p38) p38α MAPk on Western blots of TtT/GF cell extracts. Phosphorylation of p-38α MAPk was observed in TtT/GF cells stimulated for 30 min with 100 ng/ml LPS in the absence or presence of 2% FCS (A) or in cells that were treated with 100 ng/ml LPS in the presence of FCS for various time periods (B).

Regulation of IL-6 secretion in mouse pituitary cell cultures

To confirm that normal FS cells and TtT/GF cells respond in identical manner to LPS, corresponding stimulation experiments were performed in normal mouse pituitary cell cultures, in which IL-6 is considered to be mainly produced by FS cells (19, 20). IL-6 secretion by mouse pituitary cell cultures was serum-dependently enhanced by LPS. LPS-stimulated IL-6 secretion was suppressed by dexamethasone, My4 antibody, and cycloheximide in an identical manner as in TtT/GF cells (Fig. 8).

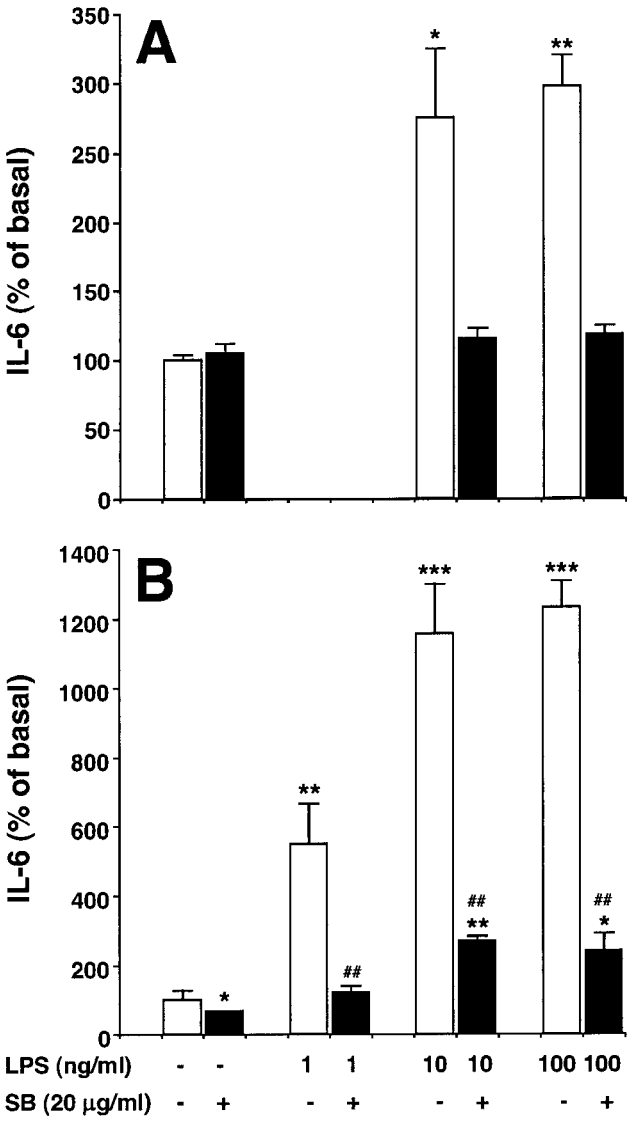

FIG. 6. Effect of SB203580 (SB), an inhibitor of p38α MAPk phosphorylation, on LPS-induced IL-6 secretion. SB (20 μM) inhibited LPS-stimulated IL-6 secretion, both in TtT/GF cells (A) and in mouse pituitary monolayer cell cultures (B). The cells were pretreated with SB for 2 h and then stimulated with LPS for 24 h in the presence of SB. **, $P < 0.01$; ***, $P < 0.001$ *vs.* basal IL-6 (without LPS and SB); ##, $P < 0.01$ *vs.* corresponding LPS-stimulated IL-6 values.

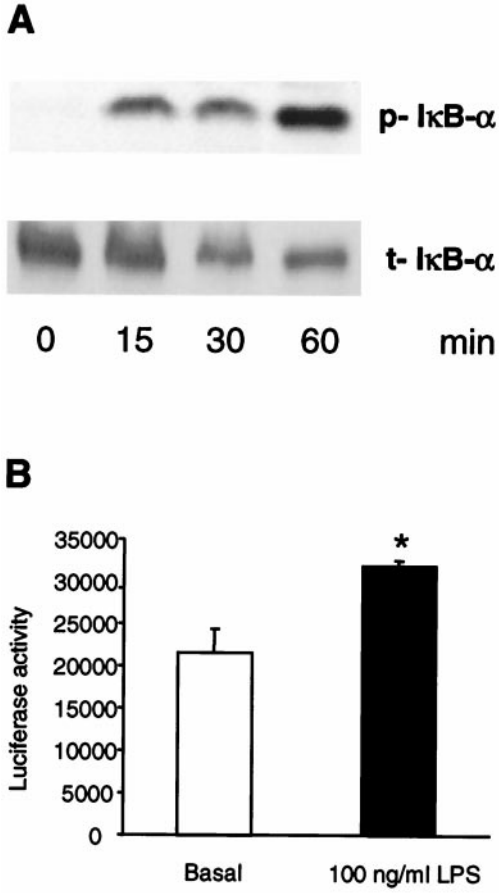

FIG. 7. Phosphorylation of IκB and activation of NF-κB by LPS in TtT/GF cells. A, Specific antibodies were used to detect phosphorylated (p-IκB-α) and total (t-IκB-α) IκB-α on Western blots of TtT/GF cell extracts. Phosphorylation of IκB-α was observed in TtT/GF cells that were treated with 100 ng/ml LPS for the indicated time periods in the presence of 2% FCS. B, Induction of LUC activity by LPS (100 ng/ml) in TtT/GF cells transiently transfected with a κB/LUC reporter plasmid responsive to transcriptionally active NF-κB and cultured in the presence of 2% FCS. The result of one out of three identical experiments is shown. *, $P < 0.05$ *vs.* basal.

Discussion

It is well known that LPS can activate the intrapituitary IL-6 production both *in vivo* and *in vitro*. Because, within the pituitary, IL-6 is produced by FS cells, the IL-6-secreting FS TtT/GF cell line, which exhibits nearly all of the characteristics attributed to normal FS cells (24, 35–37), was used to clarify the mechanism of action of LPS. In the present paper, we have demonstrated, for the first time, that FS cells express mCD14 and Tlr4, which are essential for LPS signal induction. Moreover, the LPS-induced intracellular signaling cascade in FS cells was characterized.

Although free LPS is effective in immune and other cell types, its activity is strongly enhanced when it forms a complex with LBP, which is present in serum (7). We found that LPS-induced IL-6 production by TtT/GF cells was dramatically enhanced in the presence of FCS. The poor IL-6 response to LPS stimulation, reported in previous studies, could therefore be attributable to the serum-free conditions used in these experiments. The cellular binding site for LPS is CD14, which binds free LPS with low affinity and LPS/LBP with high affinity (9). We demonstrated that mCD14 is expressed in TtT/GF cells. However, mCD14 is only anchored in the outer layer of the phospholipid membrane and has no transmembrane domain or intracellular signaling domain (38). Therefore, mCD14 is considered to be necessary only for optimal presenting of the LPS/LBP complex to the LPS signal transducer, which has recently been identified as Tlr4 (12). The latter belongs to the family of Toll proteins, which have been shown to play a central role in immune defense systems in plants, insects, and vertebrates (11). Like most of the Toll proteins, Tlr4 is a type I transmembrane protein with a leucine-rich repeat extracellular domain, and a cytoplasmatic domain homologous to the one of the IL-1 receptor (11).

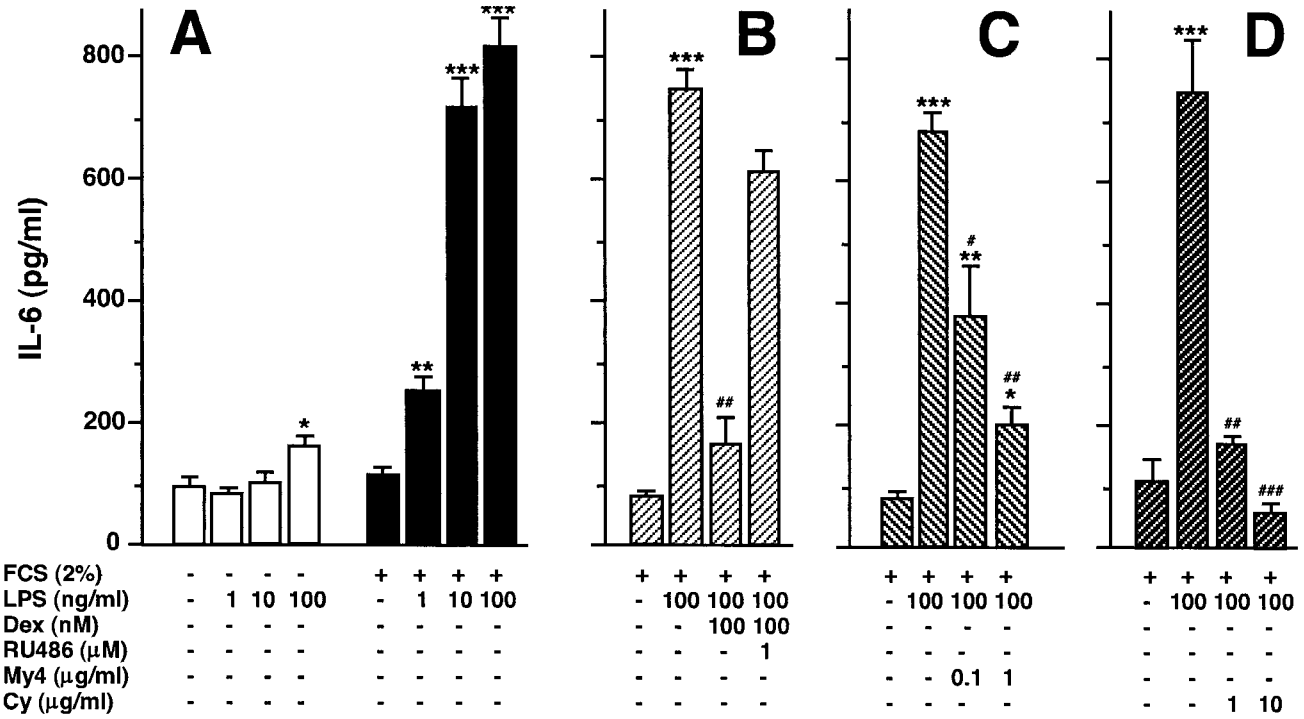

FIG. 8. Regulation of IL-6 secretion in mouse pituitary cell cultures. A, Effect of increasing concentrations of LPS on IL-6 secretion in the absence and presence of 2% FCS; B, inhibition of LPS -stimulated IL-6 secretion by dexamethasone and its reversion by RU486; C, suppression of LPS-induced IL-6 secretion by My4; D, blockade of LPS-stimulated IL-6 by the translation inhibitor cycloheximide (Cy). In all cases, the treatment period was 24 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* corresponding basal IL-6 values; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ *vs.* corresponding LPS-stimulated IL-6 values.

Studies in immune cells have shown that Tlr4 is specific for LPS of gram-negative bacteria, whereas a similar protein, Tlr2, mediates immune effects induced by LPS-negative gram-positive bacteria (39). We demonstrated that Tlr4 is expressed in TtT/GF cells. Thus, both CD14 and Tlr4 (which are essential tools for the action of LPS) are present in TtT/GF cells.

At present, neither the mechanism by which the LPS/LBP/CD14 complex interacts with Tlr4 nor the initial steps of LPS signaling are known in immune cells. We found that, in TtT/GF cells, LPS stimulated the phosphoinositol turnover but did not affect cAMP production. Our findings could be explained by the similarity of the cytoplasmatic domain of Tlr4 with the intracellular domain of the IL-1 receptor. It has been shown that the IL-1-induced IL-6 secretion within the pituitary involves the phospholipid/protein kinase C pathway (40).

In contrast to the initial processes, the final steps of LPS signaling are known. In different types of immune cells, either p38α MAPk (14) or p42/44 MAPk (15) is involved in LPS signal transduction pathways. We demonstrated that, in TtT/GF cells, phosphorylation of p38α MAPk is rapidly induced by LPS. Activated p38α MAPk then phosphorylates IκB of the IκB/NF-κB complex, which induces complex dissociation (34) and the release of the active transcription factor NF-κB. Activation of NF-κB represents the endpoint of LPS signaling in LPS-responsive cell types, and the inducible expression of the IL-6 gene is known to depend on NF-κB (41).

Functional studies confirmed the involvement of some of the above mentioned components or signaling events in LPS-induced IL-6 production in TtT/GF cells. My4, an antibody which blocks the interaction of LPS/LBP complex with CD14 (28), inhibited LPS-induced IL-6 secretion. SB203580, a specific inhibitor of p38α MAP kinase phosphorylation (14), almost completely suppressed IL-6 production in response to LPS. In addition, we have shown that IL-6 stimulation by LPS was blocked by dexamethasone. It needs to be clarified how glucocorticoids inhibit LPS-induced IL-6 production in TtT/GF cells; but, most likely, activated glucocorticoid receptors represent suppressive counterparts of NF-κB and might act as repressors at the level of IL-6 transcription (42). Cycloheximide, which suppresses mRNA translation, almost completely inhibited LPS-induced production of IL-6, suggesting that LPS does not stimulate the rapid release of IL-6 from already existing intracellular stores but induces *de novo* synthesis of the cytokine.

CD14 and Tlr4 expression was also demonstrated in normal mouse pituitary. In primary mouse pituitary cell cultures, LPS serum dependently stimulated IL-6 secretion; the latter was suppressed by My4, SB203580, glucocorticoids, and cycloheximide. The identical findings obtained in TtT/GF and mouse pituitary cell cultures indicate that normal mouse pituitary FS cells, which are the source of IL-6 in mouse pituitary, are the intrapituitary target of LPS, although the cellular localization of CD14 and Tlr4 in normal FS cells needs to be confirmed. In view of the ongoing discussion regarding the origin and function of FS cells (43), our data concerning the expression of mCD14 and Tlr4 suggest that these cells probably represent pituitary tissue-specific,

macrophage-like cells. However, in previous studies, it has been shown that macrophage markers are not expressed in the majority of FS cells but only in a subpopulation of FS cells that are considered to be derived from the lymphoid dendritic cell lineage (44–46). It could be possible that only this latter subset of FS cells participates in the intrapituitary response to LPS and that the TtT/GF cell line was established from this particular subpopulation of FS cells. However, much more work is necessary to clarify whether different FS cell subpopulations with distinct intrapituitary functions exist.

An important stimulator of IL-6 production by FS cells is PACAP (22–24). During infection/inflammation, FS cells are affected *in vivo*, both by LPS and by physiological concentrations of PACAP, which is released into the hypophysial portal blood vessels perfusing the anterior pituitary. Under these conditions, LPS and PACAP might interact to influence IL-6 production. However, in FS cells, the signal transduction pathway and the molecular mechanisms of PACAP-induced IL-6 secretion remain unknown. Future studies on LPS/PACAP interactions would be important to clarify the mechanisms by which IL-6 production is affected in FS cells in (patho-)physiological conditions.

In summary, we have demonstrated the mechanism by which LPS stimulates the production of IL-6 by pituitary FS cells. By its well known stimulatory action on hormone secretion, LPS-induced intrapituitary IL-6 might contribute, in a paracrine manner, to the interactive processes between the endocrine and immune system during acute or chronic inflammation or infection.

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