Differential induction of the mitogen-activated protein kinase pathway by bacterial lipopolysaccharide in cultured monocytes and astrocytes

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We recently reported that cyclic AMP (cAMP) specifically inhibits lipopolysaccharide (LPS)-induced interleukin 1 β (IL-1 β) transcription initiation in astrocytic cells but enhances the LPS induction of IL-1 β in monocytic cells. The purpose of this study was to determine how cAMP differentially regulates LPS-induced IL-1 β transcription in these two cell types. Two essential components of the mitogen-activated protein (MAP) kinase signal-transduction pathway, extracellular-signal-regulated kinase (ERK2; p41^{mapk}) and Raf-1, have been shown to be targets of LPS stimulation in other cell types, and therefore may be

linked to the regulation of IL-1 β transcription. In the human astrocytic cell line, U-373MG, LPS was found to strongly activate (and cAMP to inhibit) both ERK2 and Raf-1. In the human monocytic cell line, THP-1, LPS minimally activated ERK2 and did not activate Raf-1. These findings suggest that, in astrocytic cells, elevated intracellular cAMP levels may negatively regulate LPS activation of IL-1 β via the MAP kinase signalling pathway. In contrast, this pathway is not significantly activated by LPS in monocytic cells, thus inhibition by elevated intracellular cAMP levels would not affect IL-1 β transcription.

INTRODUCTION

Bacterial lipopolysaccharide (LPS), a major cell-wall component of all Gram-negative bacteria, is a potent activator of the host immune response which includes induction of the inflammatory mediator, interleukin 1 β (IL-1 β) [1]. The glycosylphosphatidylinositol-anchored monocytic differentiation antigen, CD14, has been identified as a cell-surface receptor for LPS in monocytes [2]. Although CD14 is not present on the cell surface of astrocytes or endothelial cells, a soluble form of CD14 is required for LPS responsiveness in these cells [3]. Although the ability of LPS to interact with CD14 and other soluble serum factors has been extensively characterized, little is known about downstream effector molecules activated as a result of these interactions.

One potential intracellular signalling pathway activated by LPS is the mitogen-activated protein kinase (MAP kinase) pathway [4,5]. Numerous extracellular ligands including epidermal growth factor [6], nerve growth factor [7], phorbol esters [8] and insulin [7] can stimulate the MAP kinase pathway. Downstream members of this pathway include a family of 40–45 kDa serine/threonine protein kinases called extracellularly regulated protein kinases (ERKs) [9]. At least three different ERK family members, including ERK1 (p44^{mapk}), ERK2 (p41^{mapk}) and a recently discovered 38 kDa protein that is similar to the *Saccharomyces cerevisiae* HOG1 gene product, are activated by tyrosine phosphorylation in monocytes after LPS stimulation [4,5,10].

Downstream effector molecules of the MAP kinase pathway and points of cross-talk between this and other signalling pathways have been characterized [9,11]. For example, increased intracellular cyclic AMP (cAMP), via cAMP-dependent protein kinase, blocks activation of the MAP kinase pathway by increasing phosphorylation of Raf-1 (a member of the MAP kinase pathway) [12,13]. Phosphorylation of Raf-1 decreases its affinity for, and prevents its interaction with, Ras, another upstream

proximal member of the MAP kinase pathway [1,13]. This lack of interaction effectively prevents translocation of Raf-1 to the membrane by Ras and subsequent activation of Raf-1 by other as yet unidentified molecules [12–14].

Although monocytes/macrophages are the primary source of IL-1 β in inflammation, LPS induces a similar response in astrocytes [1,15]. Astrocytes present antigen, express major histocompatibility complex class-II molecules and are an important source of IL-1 β in central-nervous-system inflammatory disorders including bacterial meningitis [16–18]. The human astrocytoma cell line used in our studies, U-373MG, retains morphological and physiological properties of primary astrocytes, including the ability to produce IL-1 β in response to LPS stimulation [15]. We recently demonstrated that agents capable of augmenting intracellular cAMP specifically inhibit LPS-induced IL-1 β transcription initiation in astrocytes [19]. The human monocytic cell line, THP-1, in contrast, exhibits increased IL-1 β transcription when stimulated with LPS and agents that increase intracellular cAMP [20]. Taken together, these findings suggest that there are fundamental differences in how cAMP regulates LPS-induced IL-1 β transcription in monocytes and astrocytes. These differences may involve inhibition of an LPS-mediated signal-transduction event, activation of a cAMP-dependent transcription factor or both. Therefore we determined whether LPS could selectively activate (and cAMP could inhibit) the MAP kinase pathway in U-373MG cells but not in THP-1 cells.

EXPERIMENTAL

Reagents, antibodies and cell lines

Dibutyl cyclic AMP (Bt₂cAMP; 1 mM) and bacterial LPS (*Escherichia coli* serotype 0127:B8) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The human astrocytoma

Abbreviations used: MAP kinase, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; cAMP, cyclic AMP; Bt₂cAMP, dibutyl cyclic AMP; LPS, lipopolysaccharide; IL-1 β , interleukin 1 β ; FCS, fetal calf serum; MEK 1, MAP kinase kinase or ERK kinase; r, recombinant; MBP, myelin basic protein; CREB, cAMP-response element-binding protein; DTT, dithiothreitol.

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cell line, U-373MG, was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). A clone of the human monocytic cell line, THP-1, was provided by Dr. R. Munford (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). Cells were maintained at 37 °C and 5% CO₂ in RPMI 1640 (Gibco/BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (FCS) (Gibco/BRL), penicillin and streptomycin (Sigma). All cell-culture reagents were demonstrated to be LPS-free by *Limulus* lysate assay [21]. For Raf-1 immunocomplex kinase assays, recombinant (r) MAP kinase kinase or ERK kinase (MEK 1) and catalytically inactive ERK2 were used (obtained from Dr. Melanie Cobb, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). rMEK1 was expressed in pRSETA as a recombinant fusion protein containing a polyhistidine sequence at the N-terminus as described by Boulton and Cobb [22]. The catalytically inactive ERK2 was prepared as described elsewhere [23]. The polyclonal antibodies, Y691 and x837, which recognize both ERK1 (p44^{mapk}) and ERK2 (p42^{mapk}) proteins, were provided by Dr. Melanie Cobb. Polyclonal antibodies against the Raf-1 protein (C-20) and the ERK2 protein (C-14) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Western-blot analysis

Confluent U-373MG and THP-1 cultures grown in medium supplemented with 10% FCS were treated with LPS (500 ng/ml or 10 µg/ml respectively) or LPS and Bt₂cAMP (1 mM) from 5 min to 13 h. Cultures of U-373MG and THP-1 cells were also serum-starved overnight to down-regulate constitutive activity of ERK and Raf-1 phosphotransferases. Cells subjected to serum starvation were either harvested as described or stimulated with 10% FCS for 10 min to induce kinase activity. After stimulation, cells were washed with PBS, harvested by scraping and Dounce-homogenized in buffer A [22]. Homogenates were centrifuged at 500 g for 10 min to pellet nuclei. Nuclei were resuspended in RIPA-H buffer [0.02 M Tris/HCl, pH 8.0, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 0.15 M NaCl, 3 mM MgCl₂, 5 mM EGTA, 1 mM dithiothreitol (DTT), 20 mM *p*-nitrophenyl phosphate, 20 mM β-glycerophosphate, 50 mM NaF, 50 µM sodium vanadate, 10 µg/ml aprotinin, 1 mM leupeptin, 5 mM benzamidine, 1 mM PMSF] and chemically lysed for 10 min at 4 °C with intermittent vortexing. Lysates were centrifuged at 100 000 g for 5 min and the supernatant, containing nuclear proteins, was stored at -80 °C. Supernatants from the 500 g spin (approx. 1 ml volume) were further separated by centrifugation at 100 000 g for 1 h into cytoplasmic (soluble) and membrane (particulate) fractions. Membranes were solubilized in 200 µl of 0.05 M Tris/HCl, pH 7.5, containing 0.15 M NaCl, 0.025 M β-octyl glucoside and 5 mM EDTA for 16 h at 40 °C, to release proteins. For Western blots of Raf-1 protein, solubilized membrane proteins, cytoplasmic extracts and nuclear extracts were separated by SDS/PAGE (12% gels), followed by electrophoretic transfer to nitrocellulose.

For analysis of Raf-1 or ERK1 and ERK2 protein expression, nitrocellulose filters were blocked with 3% fish gelatin (Sigma) in Tris-buffered saline + 0.05% Tween (Sigma) for 1 h. Nitrocellulose filters were incubated with either anti-Raf-1 (C-20, 1 µg/ml) or anti-ERK1/ERK2 (Y691, 1:5000 dilution) antibodies for 1 h in Tris-buffered saline + 0.05% Tween with 1% fish gelatin. A horseradish-peroxidase-conjugated anti-rabbit IgG was used as secondary antibody (1:5000 dilution) in 1% fish gelatin with Tris-buffered saline + 0.05% Tween for 1 h. Proteins were detected using an enhanced chemiluminescence system

(Amersham, Arlington Heights, IL, U.S.A.) following the instructions of the manufacturer.

ERK immunocomplex kinase assay

Aliquots of U-373MG and THP-1 cytoplasmic extracts that were prepared for Western-blot analysis were also tested for LPS-inducible ERK1 and ERK2 activity. Using either an anti-ERK1 antibody (x837) or an anti-ERK2 antibody (C-14) immunocomplex kinase assays were performed as described elsewhere [21]. Cytoplasmic extracts (100 µl) were incubated with 10 µl of the anti-ERK2 antibody or preimmune serum for 2 h at 4 °C. Immunoprecipitated protein was recovered by sedimentation in a microcentrifuge for 2 min and washed twice in buffer containing 0.24 M Tris/HCl, pH 7.6, and 0.1 M NaCl. Pellets were resuspended in 50 µl of Hepes, pH 8.0. For the assay, an equal volume of 2× reaction buffer was added resulting in final concentrations of 50 µM [γ -³²P]ATP (4400 c.p.m./pmol), 10 mM MgCl₂, 1 mM DTT, 1 mM benzamidine, 0.3 mg/ml myelin basic protein (MBP) and 0.025 M Hepes, pH 8.0. Reaction mixtures were incubated at 30 °C for 30 min. Pansorbin (Calbiochem) immunocomplexes were pelleted and MBP in the assay supernatants was precipitated with 10% trichloroacetic acid and adsorbed on to glass-fibre filters. Counts incorporated into MBP were quantified by liquid-scintillation counting at an efficiency of 55%.

Raf immunocomplex kinase assay

To measure LPS-inducible Raf-1 phosphotransferase activity in U-373MG and THP-1 cells, 10⁸ were cultured for 16 h in 2% FCS followed by 2.75 h stimulation with either LPS (500 ng/ml or 10 µg/ml respectively) or LPS and Bt₂cAMP (1 mM). Cells were washed three times with cold PBS, harvested by scraping and pelleted. Cell pellets were resuspended in 100 µl of RIPA-H buffer and incubated at 4 °C for 10 min with vortexing. Homogenates were sedimented at 12000 g for 10 min. To measure Raf-1 phosphotransferase activity, a coupled immunocomplex kinase assay was performed [24]. The coupled assay is an indirect measure of Raf-1 activity, and relies on the phosphorylation of catalytically inactive rERK2 by rMEK-1 that itself has been phosphorylated by immunoprecipitated Raf-1. Because MEK-1 will undergo autophosphorylation *in vitro*, distinguishing a difference between MEK-1 that has been phosphorylated by Raf-1 and MEK-1 that has undergone autophosphorylation is impossible. However, whereas autophosphorylation of MEK-1 does not activate the enzyme, phosphorylation mediated by Raf-1 will activate MEK-1. Activation of MEK-1 can be detected by measuring its ability to phosphorylate a substrate, ERK2. The ERK2 protein used in this assay was rendered catalytically inactive by substituting arginine for lysine in the kinase domain; therefore ERK2 is unable to undergo autophosphorylation. Either 10 µl of anti-Raf-1 antibody (C-12) or preimmune serum was added to 100 µl of extract for 2 h at 4 °C. Immunocomplexes were recovered by sedimentation in a microcentrifuge for 2 min. Pellets were washed twice with RIPA-H buffer, twice with 0.5 M LiCl/0.1 M Tris/HCl, pH 6.0, then twice with PAN (0.01 M Pipes, pH 7.0, 0.1 M NaCl, 10 µg/ml aprotinin). For the first portion of the coupled assay, Pansorbin pellets were resuspended in 21 µl of PAN containing 4 µl of rMEK-1 (0.5 mg/ml). Reactions were initiated by the addition of 25 µl of a 2× reaction buffer composed of 0.03 M Pipes, pH 7.0, 0.01 M MnCl₂, 100 µM ATP, 20 µg/ml aprotinin and 10–20 µCi of [γ -³²P]ATP. Reaction mixtures were incubated at 30 °C for 30 min. After the

first half of the coupled assay, Pansorbin complexes were sedimented in a microcentrifuge. Supernatant (7 μ l) containing phosphorylated MEK-1 was added to 18 μ l of a reaction mixture containing 5 μ l of catalytically inactive rERK2 (500 μ g/ml), 0.01 M Hepes, pH 7.5, 1 mM ATP, 1 mM DTT, 1 mM benzamidine, 1 mM EGTA, 0.01 M MgCl₂ and 10–20 μ Ci of [γ -³²P]ATP. The ability of rMEK-1 to phosphorylate catalytically inactive rERK2 was assayed at 30 °C for 20 min. Reactions were stopped by the addition of 6 $\times$ SDS sample buffer. The samples were boiled for 5 min, and proteins were separated on SDS/12 % polyacrylamide gels; the gels were dried and subjected to autoradiography. The amount of phosphorylated rERK2 (catalytically inactive) was quantified using an AMBIS optical imaging system (CSPI/Scanalytics, Billerica, MA, U.S.A.).

RESULTS

Detection of ERK protein expression in U-373MG and THP-1 cells

As a first step towards determining whether the ERK signal-transduction pathway might be involved in LPS activation of IL-1 β in U-373MG and THP-1 cells, Western-blot analysis was used to detect expression of ERK1 and/or ERK2 protein in these cells. Both U-373MG and THP-1 cells were found to express ERK2 in abundance (Figure 1). Differences in ERK2 expression were not detected in cells that were either untreated or stimulated with serum (after serum depletion overnight) (Figure 1). ERK2 expression was unaffected in either U-373MG or THP-1 cells treated with LPS from 15 min to 13 h (results not shown). The second protein band migrating at 42–43 kDa recognized by serum Y691 is presumably ERK1, as Y691 recognizes both of these ERK isoforms.

ERK activity in U-373MG and THP-1 cells

To determine whether LPS could activate ERKs in both U-373MG cells and THP-1 cells, an immunocomplex kinase assay was performed. Treatment of U-373MG cells with LPS for 1 h resulted in a 1.5-fold increase in ERK2 activity compared with basal activity. Notable increases in ERK2 activity were not observed until after at least 3 h of LPS stimulation. LPS-induced activation of ERK2 peaked at 6 h (4-fold increase) and declined by 13 h (1-fold induction) (Figure 2a). In THP-1 cells, LPS-stimulated ERK2 activity was maximal at 15 min, with a 1.4-fold increase in activity over that in unstimulated cells. However, ERK2 activity fell rapidly below the amount of activity detected in unstimulated cells by 1 h and remained at this level (or lower)

Figure 1 Detection of ERK1 and ERK2 proteins in U-373MG and THP-1 cells

Cytoplasmic extracts were prepared from cells cultured without serum for 16 h before stimulation with 10% FCS for 10 min (+) or with no further treatment (–). Total cytoplasmic protein (20 μ g) was fractionated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with Y691, an antibody directed against ERK1 and ERK2 proteins. Antibody binding was visualized using an enhanced chemiluminescence detection system. Cytoplasmic extracts from Rat-1 cells were used as a positive control for ERK1 and ERK2 expression. ERK2 is detected as a 41 kDa protein, and ERK1 as a 42–43 kDa protein. The position of the 46 kDa molecular-mass marker protein is indicated on the right.

Figure 2 LPS-induced ERK2 activity in U-373MG cells

(a) Kinetics of LPS induction of ERK2. The ability of LPS to induce ERK2 activity in cytoplasmic extracts of LPS-treated U-373MG cells was determined using an immunocomplex kinase assay. To estimate ERK2 activation, ³²P incorporation into MBP was quantified by liquid-scintillation counting at an efficiency of 55%. All time points were assayed in duplicate. Values were normalized to non-specific ³²P incorporation into MBP, as determined by parallel immunoprecipitation with preimmune serum. (b) cAMP-mediated inhibition of ERK2 activity. Cytoplasmic extracts were prepared from cells treated with either LPS or LPS and Bt₂cAMP for 1 h or 1.5 h as indicated. The ability of cAMP to inhibit LPS-induced ERK2 activity was determined by immunocomplex kinase assay as described in (a).

until 13 h of LPS stimulation (Figure 3a). The ability of LPS to stimulate ERK1 activity in U-373MG and THP-1 cells was similarly tested by an immunocomplex kinase assay using an antibody directed against ERK1 (x837). Although a 42–43 kDa protein was detectable by Western-blot analysis using the x837 antibody, ERK1 activity was not inducible by serum, phorbol 12-myristate 13-acetate or LPS in either cell type (results not shown).

Effects of cAMP on ERK activity in U-373MG and THP-1 cells

Elevated intracellular cAMP concentrations can inhibit activation of the MAP kinase in a number of different cell types [12,13]. An immunocomplex kinase assay was therefore used to determine whether augmentation of intracellular cAMP similarly affected LPS-stimulated ERK2 activity in U-373MG and THP-1 cells. Both cell types were treated simultaneously with LPS and Bt₂cAMP for 5 min, 1 h and 1.5 h. In U-373MG cells, elevation

Figure 3 LPS-induced ERK2 activity in THP-1 cells

(a) Kinetics of LPS induction of ERK2. ERK2 activity in cytoplasmic extracts from LPS-treated THP-1 cells was measured as described for U-373MG cells in the legend to Figure 2(a). (b) cAMP-mediated inhibition of ERK2 activity. Cytoplasmic extracts were prepared from cells treated with either LPS or LPS and Bt₂cAMP for 5 min as indicated. The ability of cAMP to inhibit LPS-induced ERK2 activity in these extracts was determined as described for U-373 cells in Figure 2(b).

of intracellular cAMP levels inhibited LPS-stimulated ERK2 activity 5-fold at 1 h and 15-fold at 1.5 h (Figure 2b), but did not affect activity at 5 min (results not shown). In THP-1 cells, however, increased concentrations of intracellular cAMP inhibited all LPS-stimulated ERK2 activity by 5 min (Figure 3b). Basal ERK2 activity in both cell types before LPS stimulation is probably due to the presence of serum in the culture medium. Correspondingly, the reduction in ERK2 activity caused by Bt₂cAMP in cells not treated with LPS is probably a result of suppression of basal serum-induced ERK2 activity.

Localization of Raf-1 protein in U-373MG and THP-1 cells

Recent studies demonstrated that augmentation of intracellular cAMP functions to prevent activation of the MAP kinase pathway by acting on an upstream member of the MAP kinase signal-transduction cascade, Raf-1 [12,13]. Therefore we first sought to determine whether Raf-1 was expressed in U-373MG and/or THP-1 cells. To detect the 74 kDa Raf-1 protein, Western-blot analysis was performed using solubilized membrane

Figure 4 Detection of Raf-1 protein in U-373MG and THP-1 cells by Western-blot analysis

(a) U-373MG or (b) THP-1 cells were maintained without serum for 16 h before stimulation with 10% FCS (+) or with no further treatment (—). Cytoplasmic (C) and membrane (M) fractions were separated by SDS/PAGE and subjected to Western-blot analysis for Raf-1 protein as described in the legend to Figure 1. A polyclonal antibody against Raf-1 was used to detect the 74 kDa Raf-1 protein. Cytoplasmic and membrane extracts from the Rat-1 cell line were used as controls. The position of the 69 kDa molecular-mass marker protein is indicated on the left.

preparations from either serum-starved or serum-stimulated U-373MG or THP-1 cells. Normally, it would be expected that Raf-1 would be located in the cytoplasm in serum-starved cells and the membrane in serum-stimulated cells. Surprisingly, Raf-1 protein was detected in the membrane fraction of both cell types independent of stimulation by LPS or serum (Figure 4 and results not shown).

Inhibition of LPS-induced Raf-1 activity by cAMP

To determine whether LPS activates Raf-1 activity and whether elevated intracellular cAMP levels could inhibit LPS-stimulated Raf-1 activity in U-373MG and/or THP-1 cells, a coupled immunocomplex kinase assay was performed. Cells were cultured in 2% FCS for 16 h and then treated with either LPS alone or simultaneously with LPS and Bt₂cAMP. The coupled assay is an indirect measure of Raf-1 kinase activity and relies on phosphorylation of catalytically inactive rERK2 by rMEK-1 that itself has been phosphorylated by immunoprecipitated Raf-1. The amount of phosphorylated rERK2 increased 1.6- and 1.5-fold in U-373MG cells treated with serum and LPS respectively. LPS induction of rERK2 phosphorylation was completely inhibited by augmented intracellular cAMP (Figures 5A and 5C). To ensure that equivalent amounts of Raf-1 were immunoprecipitated before the assay, aliquots from the first half of the coupled assay (phosphorylation of rMEK-1 by Raf-1) were subjected to Western-blot analysis using an antibody against human Raf-1 (Figure 5B). In contrast, rERK2 phosphorylation in THP-1 cells increased 1.6-fold after serum stimulation and did not increase after LPS stimulation. Increasing intracellular cAMP by the addition of Bt₂cAMP to LPS-treated THP-1 cells resulted in reduced rERK2 phosphorylation (Figures 6A and 6C). Western-blot analysis was again used to demonstrate that an

Figure 5 cAMP-mediated inhibition of LPS-induced Raf-1 kinase activity in U-373MG cells

(a) Raf-1 kinase activity was determined by a coupled immunocomplex kinase assay using cell lysates prepared from U-373MG cells maintained in 2% FCS for 16 h before treatment with either LPS or LPS and Bt₂cAMP (2.75 h) or after no further treatment (—). As controls, cells were maintained for 16 h without serum (—FCS) before stimulation with 10% FCS for 10 min at 37 °C (+) or with no further treatment (—). (b) After the first half of the coupled assay, aliquots from each reaction were subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with an antibody against Raf-1 protein to demonstrate that equivalent amounts of Raf-1 were immunoprecipitated before the initiation of the coupled assay. The position of the 69 kDa molecular-mass marker protein is indicated. (c) The amounts of immunoprecipitated Raf-1 protein and rERK2 phosphorylated were determined using an AMBIS optical imaging system. The amount of rERK2 phosphorylation was normalized to the amount of Raf-1 protein immunoprecipitated and is expressed graphically as fold increase in rERK2 phosphorylation.

equivalent amount of Raf-1 protein was present in each reaction (Figure 6B).

DISCUSSION

We recently showed that cAMP enhances LPS-induced IL-1 β transcription initiation in THP-1 cells (CD14⁺ monocytic cells), but inhibits the same process in U-373MG cells (CD14⁺ astrocytic cells) [19]. There is strong evidence supporting the involvement of the MAP kinase signalling pathway in LPS-induced IL-1 β transcription initiation, which led us to investigate the activation

Figure 6 cAMP-mediated inhibition of LPS-induced Raf-1 kinase activity in THP-1 cells

The experiments outlined in the legend to Figure 5 for U-373MG cell lysates were repeated using cell lysates from THP-1 cells with the exception that cells were treated for 15 min with LPS or LPS and Bt₂cAMP.

of this pathway in monocytes and astrocytes. LPS has been shown previously to activate three members of the ERK superfamily, ERK1 (p44^{mapk}), ERK2 (p42^{mapk}) and a newly identified member, p38. In addition, the transcription factors NF- κ B and NF-IL6, which are LPS-inducible and are involved in the induction of IL-1 β gene transcription [25,26], can be activated by Raf-1 and ERKs respectively [27,28]. Furthermore the IL-1 β promoter region also contains at least three canonical AP-1 enhancer elements [26]; c-Fos, a member of the c-Jun/c-Fos heterodimer that binds to the AP-1 enhancer, is also a substrate for ERK phosphorylation [29,30].

Our current findings provide potential mechanisms to explain the opposite effects of cAMP on LPS-stimulated IL-1 β transcription in monocytes and astrocytes. LPS was found to stimulate kinase activity of both ERK2 and an upstream member of the MAP kinase signalling pathway, Raf-1, in U-373MG cells. Furthermore elevation of intracellular cAMP levels dramatically inhibited LPS stimulation of both Raf-1 and ERK2 kinase activity in these cells. In THP-1 cells, however, LPS induction of the MAP kinase signalling pathway was trivial. Therefore, in U-373MG astrocytic cells, stimulation by LPS of the MAP

kinase pathway probably induces IL-1 β transcription that can be inhibited by cAMP at the level of Raf-1. In contrast, in THP-1 monocytic cells, the MAP kinase pathway is not significantly activated by LPS; the negative regulatory effects of elevated intracellular cAMP concentrations on this pathway would not be expected to contribute to inhibition of IL-1 β transcription in these cells. However, another member of the ERK superfamily, p38, is LPS-inducible in murine monocytes and may be a more specific target for LPS stimulation in THP-1 cells [10]. It is not yet known if p38 is affected by increased intracellular cAMP.

Additional important differences between astrocytes and monocytes further suggest that different pathways contribute to LPS induction of IL-1 β transcription in these two cell types. First, the kinetics of LPS-induced IL-1 β transcription differ in U-373MG cells and THP-1 cells. LPS-induced IL-1 β transcription is maximal at 1 h in THP-1 cells but is not detectable in U-373MG cells until 3 h after LPS stimulation [19,26]. Secondly, LPS-induced IL-1 β transcription is dependent on protein synthesis in U-373MG cells but not in THP-1 cells [26,31]. Finally, unlike THP-1 cells, U-373MG cells do not express the glycosyl-phosphatidylinositol-anchored LPS receptor, CD14, but instead rely on a soluble form of the molecule to transduce the LPS signal [3].

In addition to inhibiting the MAP kinase pathway, cAMP can also affect IL-1 β gene transcription via cAMP-dependent-protein-kinase-mediated phosphorylation of cAMP-response element-binding protein (CREB) [32]. Activation of IL-1 β transcription in response to cAMP in monocytes is effected by the binding of phosphoCREB (as a heterodimer with NF-IL6) to the pseudosymmetrical cAMP-response element enhancer motif in the IL-1 β promoter [33]. Dephosphorylated CREB in association with NF-IL6, in contrast, can act as a dominant-negative regulator of LPS-induced IL-1 β transcription in monocytes [33]. Preliminary studies did not demonstrate variation in the amount of phosphoCREB that was detected in either U-373MG or THP-1 cells as a result of treatment with LPS and Bt₂cAMP at different time points. However, the total amount of CREB detected was much greater in THP-1 cells than in U-373MG cells (S. A. Willis and P. D. Nisen, unpublished work). Although phosphoCREB is present in small quantity in U-373MG cells, this amount may be insufficient to augment LPS-induced IL-1 β transcription in U-373 cells compared with THP-1 cells.

Further studies will be required to characterize fully the role of the MAP kinase signalling pathway and its relationship to CD14 and LPS induction of IL-1 β transcription. To date, experiments to confirm directly that the MAP kinase pathway can stimulate IL-1 β transcription have been thwarted by the fact that dominant-negative mutants of Raf-1 are lethal and that reporter gene constructs containing IL-1 β 5'-upstream regulatory sequences do not behave like the endogenous gene (S. A. Willis and P. D. Nisen, unpublished work). Furthermore the exact function of CREB in LPS/Bt₂cAMP-mediated induction/inhibition of IL-1 β transcription in the two cell types remains to be determined.

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