p38 Mitogen-activated Protein Kinase Regulates Cyclooxygenase-2 mRNA Stability and Transcription in Lipopolysaccharide-treated Human Monocytes*

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p38 mitogen-activated protein kinase (MAPK) is activated by inflammatory stimuli such as bacterial lipopolysaccharide (LPS), interleukin-1, and tumor necrosis factor. We have previously shown that the pyridinyl imidazole SB 203580, which inhibits it, blocks the interleukin-1 induction of cyclooxygenase-2 (COX-2) and matrix metalloproteinase 1 and 3 mRNAs in fibroblasts. Here we explore the role of p38 MAPK in the response of human monocytes to LPS. 0.1 μM SB 203580 significantly inhibited the LPS induction of COX-2 and tumor necrosis factor protein and mRNAs. The activity of MAPK-activated protein kinase-2 (a substrate of p38 MAPK) in the cells was commensurately reduced. Some isoforms of c-jun N-terminal kinase (which is also activated by LPS) are sensitive to SB 203580; the inhibitor had little effect on monocyte c-jun N-terminal kinases up to 2 μM. We investigated the mechanism of inhibition of COX-2 induction. Transcription (measured by a nuclear run-on assay) was 60% inhibited by SB 203580 (2 μM). Importantly, we found that p38 MAPK was essential for stabilizing COX-2 mRNA: when cells stimulated for 4 h with LPS were treated with actinomycin D, COX-2 mRNA decayed slowly. Treatment of stimulated cells with 2 μM SB 203580 caused a rapid disappearance of COX-2 mRNA, even with actinomycin D present. We conclude p38 MAPK plays a role in the transcription and stabilization of COX-2 mRNA.

p38 mitogen-activated protein kinase (MAPK) is a member of the MAPK family and is activated by the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF), by bacterial lipopolysaccharide (LPS), and by a range of cellular stresses (1–5). Although originally characterized as a stress or inflammatory kinase, it is likely to have diverse functions because it is also activated in platelets by thrombin and collagen (6) and in T cells upon activation by various stimuli (7) and is constitutively active in liver (8, 9). Little is known about the physiological functions it controls. One substrate is MAPK-activated protein kinase-2 (MAPKAPK-2) (10, 11), which in turn phosphorylates the small heat shock protein hsp27 (12) and the CAMP-response element binding protein (13). Other putative targets are the MAPK integrating kinase (14, 15) and the transcription factors CHOP (16), myocyte enhancer factor 2C (17), and activating transcription factor 2 (4).

Besides the original p38 MAPK (called α), a closely similar β form has been described (18) as well as two more distantly related enzymes that also contain the TGY motif: stress-activated protein kinase 3 (or p38γ) (19–21) and stress-activated protein kinase 4 (or p38δ) (22–24). The p38α and p38β MAPKs are inhibited by a class of pyridinyl imidazole compounds of which the best characterized is SB 203580 (11). These were first identified as inhibitors of TNF (and IL-1) production by LPS-activated monocytes (25) and were later shown to inhibit p38 MAPK (5, 11). The pyridinyl imidazoles inhibited TNF (and IL-1) protein production with relatively little effect on the levels of mRNA induced, and it was suggested that they worked mainly by inhibiting translation (25, 26). Our laboratory used SB 203580 to investigate the role of p38 MAPK in cellular responses to IL-1 (27). We found that the drug strongly inhibited the induction of cyclooxygenase-2 (COX-2), collagenase (matrix metalloproteinase 1), and stromelysin (matrix metalloproteinase 3) by IL-1 at the protein and mRNA levels within the range of its IC50 for p38 MAPK (0.1–0.5 μM) (27). There was comparatively little inhibition of secondary cytokine production; IL-6 was only inhibited 30–50% (at 1 μM), and IL-8 was unaffected. The inhibition of induction by IL-1 of COX-2 and matrix metalloproteinase in fibroblasts at the mRNA level was in contrast to the inhibition of TNF and IL-1 protein at the translational level in LPS-activated monocytes. However, a recent report showed that LPS induction of COX-2 mRNA was inhibited by SB 203580 in monocytes (28). It was possible that p38 MAPK regulated the expression of different genes at different levels, depending upon the cell type and the nature of the stimulus.

We have therefore investigated the effects of SB 203580 on the induction of both COX-2 and TNF in monocytes stimulated by LPS and found each to be similarly inhibited at both the protein and mRNA levels. We then went on to show that in the case of COX-2, the p38 MAPK inhibitor impaired transcription and destabilized the mRNA.

Doubts have recently been raised concerning the specificity of SB 203580 (29, 30) because it can inhibit certain isoforms of c-jun N-terminal kinase (JNK), another MAPK family member activated by the same stimuli as p38 MAPK. We have therefore checked the sensitivity of the LPS-activated JNKs in human monocytes to the inhibitor.

EXPERIMENTAL PROCEDURES

Materials—Lymphoprep was from Nycomed Pharma A.S. (Oslo, Norway). Fluorochrome-conjugated anti-CD-14 monoclonal antibodies were from Becton Dickinson (Oxford, United Kingdom). 4-(4-Fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole (SB 203580) was from Calbiochem-Novabiochem, Ltd. (Nottingham, United Kingdom).
LPS from *Salmonella typhimurium* was from Sigma-Aldrich Company, Ltd. (Poole, United Kingdom) and was used at a concentration of 10 ng ml⁻¹. Sheep anti-rabbit MAPKAPK-2 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit antiserum to the C-terminal peptide of p38 MAPK (ISFVFPDLDQEEMES) has been described elsewhere. Rabbit anti-tubulin antibody was from Oxford Biomedical Research, Inc. (Milwaukee, WI). Recombinant human hsp27 was from Bioquote, Ltd. (York, United Kingdom).

A constructing human glutathione S-transferase-c-Jun (1–135) was a kind gift of J.R. Woodgett (Ontario Cancer Institute, Toronto, Canada). A plasmid containing full-length human COX-2 cDNA (from D. Fitzgerald, College of Surgeons, Dublin, Ireland) was used for Northern blots (EcoRI/EcoNI 800-base pair fragment) and nuclear run-ons. Human GAPDH probe for Northern blots was a gift from C. Clarke (the Walter and Eliza Hall Institute, Melbourne, Australia), and human GADPHD (1.2 kb) for nuclear run-ons was from Diane Clarke (the Walter and Eliza Hall Institute, Melbourne, Australia). p38 MAPK (C terminus) and COX-2 (amino acid 187–208) antibodies were from Cell Signaling Technology, Inc. (Lake Placid, NY). The rabbit antiserum to the C terminus of p38 MAPK was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). MAPKAPK-2 immunoprecipitation was performed in an identical fashion, but with 5 ng of sheep anti-rabbit MAPKAPK-2 antibody and protein G beads. Samples were assayed for kinase activity as described above using 1 µg of recombinant human hsp27 as substrate.

### Mono-Clonal Antibodies

Monoclonal antibodies. Monocytes were cultured in the same medium judged by flow cytometry using fluorochrome-conjugated anti-CD-14. Monocytes collected in this way were typically found to be 75% pure as assessed by flow cytometry using fluorochrome-conjugated anti-CD-14 antibodies. In some instances, this purity was increased to 85% to 90% by the addition of a high concentration of sheep anti-rabbit antibody to the culture medium. This antibody was removed by centrifugation before the use of the cells.

### Preparation of Human Peripheral Blood Monocytes

### Isolation and Culture of Human Monocytes—

For each experiment, human peripheral blood monocytes were freshly prepared from the blood of healthy adult donors. Mononuclear cells were prepared by Ficoll-Hypaque centrifugation on a Lymphoprep (Nycomed, Oslo, Norway) gradient. Pooled mononuclear cells were typically 90% pure (judged by trypan blue exclusion) and 80% purity with 5–10% monocytes. The pooled mononuclear cells were washed three times with 1% heat-inactivated fetal calf serum in PBS and cultured salmon sperm DNA. Blots were washed three times for 30 min at 37 °C with 2× SSC and 10× SSC and 0.1% SDS at 50 °C, and then washed twice for 10 min in 2× SSC and 0.1% SDS at 50 °C, and then washed twice for 10 min in 2× SSC and 0.1% SDS at 37 °C. Blots were then washed three times for 30 min at 37 °C with 2× SSC and 0.1% SDS at 50 °C, and then washed twice for 10 min in 2× SSC at 37 °C. Blots were then washed three times for 30 min in 2× SSC and 10 µg ml⁻¹ RNAse A at 37 °C, followed by two washes in 2× SSC and 0.1% SDS at 37 °C. Signals were quantified in a phosphorimager.

**Northern Blot—**Monocyte total RNA (4 µg) was electrophoresed on denaturing formaldehyde/1% agarose gels with 0.41 M formaldehyde (32). RNA was capillary transferred to a Hybond N membrane according to Sambrook et al. (33) and fixed by UV cross-linking. cDNA probes were labeled with [α-32P]dCTP (2000 Ci mmol⁻¹) and hybridized to slot blots (plasmids cloned in pBluescript and UV-damaged N membrane) in 50% formamide, 5× saline sodium phosphate EDTA, 2.5× Denhardt’s solution, 0.1% SDS, and 0.2 mg ml⁻¹ salmon sperm DNA. Blots were washed twice for 10 min in 2× SSC and 0.1% SDS at 37 °C, washed for 30 min in 2× SSC and 0.1% SDS at 50 °C, and then washed twice for 10 min in 2× SSC at 37 °C. Blots were then washed three times for 30 min in 2× SSC and 10 µg ml⁻¹ RNAse A at 37 °C, followed by two washes in 2× SSC and 0.1% SDS at 37 °C. Signals were quantified in a phosphorimager.

**Western Blot—**Cell lysates (total protein, 50 µg) were electrophoresed on 10% SDS-polyacrylamide gels (31) and blotted on polyvinylidene difluoride membranes. Membranes were treated according to the instructions of the Enhanced Chemiluminescence kit. 

**TNF-α Enzyme-linked Immunosorbent Assay—**TNF-α enzyme-linked immunosorbent assay was performed as described by Foey et al. (34).

### RESULTS

**Induction of COX-2 Protein and mRNA by LPS Is Inhibited by the p38 MAPK Inhibitor SB 203580—**Monocytes were untreated or pretreated for 1 h with increasing concentrations of SB 203580. They were then stimulated for 4 h with LPS (with the inhibitor remaining present on the pretreated cells). Cells were then immunoblotted for COX-2 protein (Fig. 1A), or RNA was extracted and Northern blotted for COX-2 mRNA (Fig. 1B). SB 203580 inhibited both protein and mRNA in a similar manner.
Inhibition of induction of COX-2 and TNF by SB 203580 in LPS-treated human monocytes. Monocytes were treated with SB 203580 at the doses indicated for 1 h and then left untreated or treated with LPS for an additional 4 h. A, Western blot of monocyte lysates for COX-2 protein. B, Northern blot of monocyte RNA for COX-2 mRNA. C, monocytes were treated as described in A, but were treated with LPS for 16 h and Northern blotted for TNF mRNA. See “Experimental Procedures” for details.

TNF Protein and mRNA Induced by LPS Are Both Inhibited by SB 203580—Monocytes were treated for 1 h with SB 203580 or left untreated and further treated for 16 h with LPS or left untreated. Secreted TNF protein, which was measured by an enzyme-linked immunosorbent assay, was inhibited in a dose-dependent fashion by SB 203580 (42% at 0.1 μM and 67% at 1 μM). TNF mRNA showed a sensitivity to the inhibitor that was similar to that seen for the protein (Fig. 1C). In this experiment, LPS stimulation increased the levels of GAPDH mRNA. LPS is a very strong monocyte activator, and some batches of cells show up-regulation of housekeeping genes. TNF protein and mRNA showed similar sensitivities to the inhibitor at a concentration of 2 μM (55% and 72%, respectively) in two separate experiments with different batches of cells using a 4-h LPS stimulation (data not shown).

Inhibition of p38 MAPK by SB 203580 in LPS-treated Monocytes—Fig. 2A shows the activity of p38 MAPK immunoprecipitated from lysates made from cells at various times after the addition of LPS. Activity increased 6-fold by 10 min and then returned to resting levels after 1 h. It was important to compare the degree of inhibition of p38 MAPK activity with COX-2 mRNA induction. Inhibition of p38 MAPK in monocytes by treatment with SB 203580 (which is a reversible inhibitor) was measured by immunoprecipitating its substrate, MAPKAPK-2, from cell lysates and assaying the latter’s activity on hsp27. Monocytes were pretreated with the inhibitor for 20 min or left untreated, and cells were then treated with LPS for 10 min (Fig. 2B, left panel). Monocytes were also treated with LPS for 4 h and either treated with the inhibitor for the final 30 min of stimulation or left untreated (Fig. 2B, right panel). MAPKAPK-2 was immunoprecipitated from the lysates. At both time points, i.e. at the peak of p38 activation at 10 min and after it had returned to resting levels at 4 h, the MAPKAPK-2 activity was reduced by about 60% in cells treated with 0.1 μM and by 80% in cells treated with 1 μM. The degree of inhibition of the MAPKAPK-2 correlated well with the effect on COX-2 gene expression (Fig. 1A and B).

Effect of SB 203580 on JNK Activity in LPS-treated Monocytes—Certain forms of JNK, particularly the β splice variants of JNK-2, are sensitive to micromolar concentrations of SB 203580 (29, 30). We chromatographed lysates of monocytes that had been treated with LPS for 20 min on a Mono-Q anion exchanger and measured JNK activity in the fractions eluted with a 0–0.5 M NaCl gradient (Fig. 3A). As is characteristic of the enzyme in lysates of activated cells (29, 35), two major peaks were eluted. These were tested for sensitivity to SB 203580 (note that p38 MAPK itself elutes after the JNK peaks at about 0.4 M NaCl (2, 3)). The activity of the first peak (which contains the short ~46-kDa JNKs) was unaffected by the inhibitor at concentrations up to 10 μM (Fig. 3B, left panel). The activity of the second peak (which contains the full-length ~54-kDa JNKs) was unaffected by 0.1 μM but was significantly inhibited (24%) at 2 μM (Fig. 3B, right panel). The data shown in Fig. 3B are means of three experiments on three different batches of monocytes. Because the peaks contain similar amounts of JNK activity, we estimate that total JNK activity might be inhibited by 10–15% in cells treated with 2 μM SB 203580. The inhibition of COX-2 expression by SB 203580 is therefore consistent with its effect on p38 MAPK and not on JNK.

Transcription of COX-2 Is Inhibited by SB 203580—The effect of p38 MAPK blockade on both the protein and mRNA levels of COX-2 could be due to decreased transcription and/or reduced mRNA stability. We tested for an effect on transcription by performing nuclear run-on assays. Monocytes were left untreated or pretreated for 1 h with 2 μM SB 203580 and then further incubated for 4 h in the presence or absence of LPS. Nuclei were isolated as described in “Experimental Procedures,” and COX-2 transcription was measured by nuclear run-on assay. Fig. 4A shows a representative experiment. COX-2 transcription was stimulated 12-fold, and this was inhibited by 74% in nuclei from cells treated with 2 μM SB 203580. Data from three independent experiments are combined in Fig. 4B, which shows an average 5-fold stimulation of COX-2 transcription by LPS with a mean inhibition of 60% by 2 μM SB 203580. The inhibitor had a similar effect when the run-on assay was performed after only a 30-min (rather than a 4-h) stimulation with LPS (data not shown). These results strongly suggested that inhibiting p38 MAPK interfered with the transcription of COX-2.

Inhibition of p38 MAPK Destabilizes COX-2 mRNA—To examine the stability of COX-2 mRNA in human monocytes, actinomycin D was added to cells after 4 h of LPS treatment, and COX-2 mRNA levels were measured from the time of addition up to 90 min (Fig. 5A). The predominant 4.6-kb and the minor 2.8-kb transcripts decayed at a similar slow rate (t½ = 110 min). Simultaneous addition of SB 203580 (2 μM) and actinomycin D to the cells after a 4-h stimulation with LPS resulted in a rapid decrease (t½ = 30 min) in COX-2 mRNA levels (Fig. 5A). In cells that were not treated with actinomycin D or the p38 MAPK inhibitor, COX-2 mRNA levels were roughly constant for 1 h after a 4-h LPS stimulation (Fig. 5B), showing that a steady state had been reached. The decay observed in cells treated only with actinomycin D indicated that in LPS-treated monocytes, COX-2 mRNA is continually being degraded and transcribed. When SB 203580 (2 μM) alone was added to cells treated with LPS for 4 h, there was a rapid decay of COX-2 mRNA that occurred at a similar rate to that for cells treated with the inhibitor and actinomycin D (Fig. 5B). This
indicated that transcription was not needed for SB 203580 to destabilize COX-2 mRNA.

**DISCUSSION**

We found that the p38 MAPK inhibitor prevented the LPS induction of both COX-2 and TNF protein, and that the degree of inhibition of protein and mRNA for both were very similar. We concluded that there was little evidence for p38 MAPK regulating translation for the expression of both these gene products in primary monocytes. Our additional studies were restricted to COX-2, and these indicated that p38 MAPK is required for optimal transcription of the gene, and that it is needed for stabilization of the mRNA. The importance of this second finding is that it suggests a novel function for p38 MAPK that has not previously been considered.

These conclusions are dependent on the inhibitor being specific for this protein kinase. The original description of SB 203580 suggested that it might be a highly specific inhibitor of p38 MAPK (11). Its IC₅₀ was about 0.5 μM, and it did not affect the activity of a wide range of protein kinases (including JNK and extracellular signal-regulated kinase) and phosphatases up to a concentration of 100 μM (11). However, two recent reports stress that caution is needed in the use of SB 203580 (29, 30). In the first, the β splice variants of JNK-2 were shown to be completely inhibited by 10–20 μM SB 203580 (30). The α splice variants of JNK-2 and JNK-1 were inhibited about 50%, whereas the β forms of JNK-1 were resistant to the drug (30). This raised the possibility that SB 203580 may significantly inhibit JNKs in cells, depending upon the isoforms expressed.
An example of this is a recent report that certain chromatographic forms of cardiac myocyte JNKs are strongly inhibited by 10 mM SB 203580 (29).

To establish that the effects of SB 203580 on COX-2 gene expression were due to the inhibition of p38 MAPK, we compared the suppression of MAPKAPK-2 activity with the suppression of COX-2 protein and mRNA. We also investigated the sensitivity of human myocyte JNKs activated by LPS to the inhibitor. At 0.1 mM SB203580, there was about a 50% inhibition of both COX-2 mRNA and MAPKAPK-2 activity in monocytes, which is consistent with the effects on COX-2 gene expression being due to p38 MAPK blockade.

The LPS-activated JNKs in human monocytes were relatively insensitive to the inhibitor. The second of the two chromatographically distinct forms was inhibited about 10–20% at 1–2 mM SB 203580, whereas the first peak was unaffected. We therefore concluded that the effects of SB 203580 at concentrations up to 1–2 mM were unlikely to be due to the inhibition of JNK isoforms, and the effects on COX-2 gene expression were probably due to the inhibition of p38 MAPK.

Our investigation of LPS-induced COX-2 in monocytes was prompted partly by the discrepancy between the finding that SB 203580 inhibited COX-2 mRNA induced by IL-1 in fibroblasts (27) and the suggestion that it might be acting as a translational inhibitor of TNF production in monocytes (25). Young et al. (25) reported a 50% suppression of TNF mRNA in monocytes treated with LPS in the presence of 5 mM SKF 86002 (a pyridinyl imidazole very similar to SB 203580) but a 90% inhibition of secreted TNF protein. Further work was carried out in the human monocytic cell line THP 1 (26). In this cell line, TNF protein induced by LPS was inhibited by SKF 86002, whereas mRNA induction was unaffected. Furthermore, TNF mRNA accumulated in a pre-ribosomal compartment (26). It may be that different mechanisms predominate in the immortalized cell line and primary monocytes. Working with SB 203580 at low doses, we have found no discrepancy between the suppression of TNF protein and mRNA. It is possible that p38 MAPK may regulate TNF and COX-2 by similar mechanisms.

We found that p38 MAPK activity is required for the full induction of COX-2 transcription. Analysis of the COX-2 promoter by means of transfection of reporter constructs has implicated several transcription factors in its regulation. These experiments have generally been carried out on transformed cell lines using a variety of stimuli (e.g., phorbol ester, IL-1, TNF, LPS, and growth factors). The transcription factors implicated include NFκB (36), NF-IL-6 (36–38), c-fos (39), signal transducer and activator of transcription 5 (40), and NF-1 (41). A number of reports have emphasized the importance of NFκB in the induction of COX-2 in response to LPS in cells of monocyte-macrophage lineage (42–45). The relative importance of different transcription factors for COX-2 expression in primary monocytes is not known.

It has recently been suggested that the inhibition of p38 MAPK Regulates COX-2 mRNA Stability and Transcription.
MAPK by SB 203580 blocks the activation of NFkB reporter constructs in cells by a mechanism unrelated to either the phosphorylation and degradation of IxB or nuclear translocation and DNA binding of NFkB (46–48). However, in these studies, the response of NFkB-dependent reporter constructs to TNF was diminished less than 50% by 10 μM SB203580 (47). Because we observe stronger effects on COX-2 expression at much lower concentrations of the inhibitor, it seems unlikely that the inhibition of NFkB activity accounts for our findings. However, it is difficult to examine this proposition directly, because the study of events downstream of DNA binding requires the transfection of primary monocytes. The human COX-2 promoter also contains potential binding sites for members of the cAMP response element binding protein activating transcription factor, Ets/ternary complex factor, and myocyte enhancer factor 2 families; activating transcription factor 2 (4), Ets family members (49, 50), and myocyte enhancer factor 2C (17) can all be phosphorylated and activated by p38 MAPK. cAMP-responsive element binding protein (13) is phosphorylated and activated by MAPKAPK-2, which is activated by p38 MAPK. cAMP-responsive element binding protein (13) is phosphorylated and activated by MAPKAPK-2, which is directly downstream of p38 MAPK. These factors all represent possible targets for the transcriptional regulation of the human COX-2 gene by p38 MAPK.

We have found strong evidence that p38 MAPK stabilizes COX-2 mRNA. Although COX-2 mRNA stability was studied in stimulated cells, it is not clear whether LPS actually regulates this, because p38 MAPK activity had returned to resting levels after 4 h of stimulation. It appears that a basal level of p38 MAPK activity plays a housekeeping role in maintaining COX-2 mRNA stability. Destabilization of COX-2 mRNA is also caused by dexamethasone (51). This effect was described in IL-1-stimulated endothelial cells and inhibited by actinomycin D. Thus, the effects of dexamethasone and SB 203580 differ. Sequences that determine the stability of an mRNA have been found in the 5′ untranslated region, the coding region, and the 3′ untranslated region (52). The 3′ untranslated region of COX-2 contains 22 AUUU motifs that are recognized to be important determinants of mRNA instability (51). Both proximal and distal regions have been identified as important in regulating the stability of transfected reporter constructs bearing the 3′ untranslated region in resting cells (53). It is possible that the instability caused by the p38 MAPK inhibitor is mediated through the AU-rich region proximal to the coding region that is contained in the 4.6- and 2.8-kb transcripts, both of which we have found to be stabilized through this protein kinase.

The results of the present study provide evidence for a new role of p38 MAPK in mRNA stabilization. It will be of interest to determine whether this process occurs in other cell types for other genes and to identify the downstream pathway and molecular mechanisms involved.

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