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LPS stimulation of RAW264 macrophages triggered the activation of mitogen- and stress-activated protein kinases-1 and -2 (MSK1, MSK2) and their putative substrates, the transcription factors cyclic AMP response element-binding protein (CREB) and activating transcription factor-1 (ATF1). The activation of MSK1/MSK2 was prevented by preincubating the cells with a combination of two drugs that suppress activation of the classical mitogen-activated protein kinase cascade and stress-activated protein kinase/p38, respectively, but inhibition was only partial in the presence of either inhibitor. The LPS-stimulated activation of CREB and ATF1, the transcription of the cyclooxygenase-2 (COX-2) and IL-1β genes (the promoters of which contain a cyclic AMP response element), and the induction of the COX-2 protein were prevented by the same drug combination, as well as by Ro 318220 or H89, potent inhibitors of MSK1/MSK2. Two other transcription factors, C/EBPβ and NF-κB, have been implicated in the transcription of the COX-2 gene. However, PD 98059 and/or SB 203580 did not prevent the LPS-induced increase in the level of the transcription factor C/EBPβ, and none of the four inhibitors used in this study prevented the activation of NF-κB. Our results demonstrate that two different mitogen-activated protein kinase cascades are rate limiting for the LPS-induced activation of CREB/ATF1 and the transcription of the COX-2 and IL-1β genes. They also suggest that MSK1 and MSK2 may play a role in these processes and hence are potential targets for the development of novel antiinflammatory drugs. The Journal of Immunology, 2000, 164: 3018–3025.

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3 Abbreviations used in this paper: MSK, mitogen- and stress-activated protein kinase-1 (MSK1); MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPKAP-K, MAPK-activated protein kinase; SAPK, stress-activated protein kinase; PRAK, p38-regulated activated kinase; IBMX, 3-isobutyl-1-methylxanthine; PKA, cyclic AMP-dependent protein kinase; CRE, cyclic AMP response element; CREB, CRE-binding protein; ATF1, activating transcription factor 1; COX-2, cyclooxygenase-2; ECL, enhanced chemiluminescence; HPRT, hypoxanthine guanine phosphoribosyltransferase.
kinase-2 (MAPKAP-K2) and the closely related MAPKAP-K3 (2). However, neither of these protein kinases are inhibited by concentrations of Ro 318220 that ablate MSK1 activity (1, 3). 

Inflammatory mediators, such as PGs and leukotrienes, and proinflammatory cytokines, such as IL-1 and TNF, are produced in macrophages during bacterial infection by LPS-activated signal transduction pathways. These substances play key roles in mounting the immune responses needed to fight infection, but they are a double-edged sword because their uncontrolled production can be a cause of chronic inflammatory diseases. For these reasons, drugs that are capable of suppressing the production of inflammatory mediators and proinflammatory cytokines are useful in treating these conditions. Here, we have investigated the signal transduction pathways by which LPS induces IL-1β and cyclooxygenase-2 (COX-2), the enzyme that catalyzes the rate-limiting step in prostaglandin and leukotriene synthesis (4). Because the COX-2 (5) and IL-1β (6) promoters both contain a CRE, we wondered whether MSK1 might be present in macrophages and play a role in mediating the induction of COX-2 and IL-1β. If this were the case, then MSK1 could be a potential target for an antiinflammatory drug. In this paper, we show that MSK1, and a closely related isoform (MSK2), are both activated when macrophages are stimulated with LPS. We also show that drugs that suppress the activation or activity of MSK1 and MSK2 prevent the LPS-induced phosphorylation of CREB and ATF1, the transcription of the COX-2 and IL-1β genes, and the induction of the COX-2 protein.

Materials and Methods

Reagents and antibodies for cell culture were purchased from Life Technologies (Paisley, U.K.); PD 98059 from New England Biolabs (Beverly, MA); Ro 318220, H89, and SB 203580 from Calbiochem (Nottingham, U.K.); forskolin and 3-isobutyl-1-methyloxanthine (IBMX) from Sigma (Poole, U.K.); complete proteinase inhibitor mixture from Boehringer (Mannheim, Germany); protease inhibitors were purchased from Roche Applied Science (Mannheim, Germany). Complete proteinase inhibitor mixture from Boehringer (Mannheim, Germany) was added to the cell media. 

Cell culture and stimulation

RAW264 macrophages were maintained in a 95% air, 5% CO2 atmosphere and plated at a density of 2 x 106 cells/ml. RAW264 cells were stimulated with LPS, 100 ng/ml for 4 h, and the supernatants were removed, snap frozen in liquid nitrogen, and stored in aliquots at −80°C until analysis. Protein concentrations were determined according to the method described in Ref. 7.

Preparation of nuclear extracts

After stimulation, the cells were resuspended and washed three times in buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, 0.1 mM sodium orthovanadate, 10 mM sodium glycerocephosphate, 2 μM microcystin-LR, 1 mM benzamidine, 0.1% (v/v) 2-ME, and complete proteinase inhibitor mixture), lysed in buffer A plus 0.1% (v/v) Nonidet P40 for 4 min on ice. The lysates were then put on ice at 13,500 x g for 10 min at 4°C. The nuclear pellet was resuspended in buffer B (20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 420 mM NaCl, 1 mM DTT, 0.1 mM sodium o-vanadate, 10 mM sodium glycerocephosphate, 2 μM microcystin-LR, 1 mM benzamidine, 0.1% (v/v) 2-ME, and complete proteinase inhibitor mixture), rotated end over end for 15 min at 4°C, and then sonicated in a 10°C water bath (four 15-s pulses during 4 min). The samples were centrifuged at 13,500 x g for 15 min at 4°C, and the supernatants were removed, snap frozen in liquid nitrogen, and stored in aliquots at −80°C until analysis. Protein concentrations were determined according to the method described in Ref. 7.

Ab production

All Abs were raised in sheep in the Scottish Antibody Production Unit (Carluke, U.K.), and the antisera were purified by affinity chromatography on Ag peptide Sepharose columns. An anti-MSK1 Ab was raised against the peptide FKRANAVIDPLOQHMGVRE corresponding to residues 384–403 of MSK1 (1), and a further Ab was raised against the full length human MSK1 protein. An anti-MSK2 Ab was raised against the peptide RPAVASKGAPRANGLPSS corresponding to residues 753–772 of MSK2.

Immunoprecipitation and assay of protein kinases

MSK1 and MSK2 were immunoprecipitated individually from 0.5 and 1.0 mg cell lysate protein, respectively, using the anti-peptide Abs described above. The immunoprecipitates were washed and assayed at 30°C as described (1). One unit of MSK1 or MSK2 activity was defined as the amount that catalyzes the incorporation of 1 nmol phosphate into the peptide GRPRTSSFAEG in 1 min. MAP kinase-activated protein kinase-1 (MAPKAP-K1, also known as p90RSK) was immunoprecipitated from cell lysates (50 μg protein) with an Ab raised against the peptide RNQSPV LPVGSRSTLQAQRGKIKK corresponding to residues 605–627 of murine MAPKAP-K1b (RSK2 isoform). This Ab immunoprecipitates MAPKAP-K1a (RSK1) as well as MAPKAP-K1b (8). The immunoprecipitates were washed and assayed at 30°C as described (6). One unit of MAPKAP-K1 activity was defined as the amount that catalyzes the incorporation of 1 nmol phosphate into [G245,G246]S (218–249) (a peptide closely related to the C terminus of ribosomal protein S6) in 1 min. MAPKAP-K2 was immunoprecipitated in an manner identical to that for MAPKAP-K1 using an Ab raised against the peptide MTSALATRMDVYEQIK corresponding to residues 356–371 of the human protein. This Ab immunoprecipitates MAPKAP-K2 as well as MAPKAP-K2 (9). MAPKAP-K2 was assayed as described (10), and one unit was the amount of enzyme that catalyzes the incorporation of 1 nmol phosphate into the peptide KKLN RTLsV in 1 min.

Immunoblotting

Proteins were denatured in SDS, electrophoresed on a 10% SDS/polyacrylamide gel, transferred to nitrocellulose membrane, immunoblotted with the Abs described below, which were detected with the ECL reagent. For immunoblotting of MSK1, MSK1 was first immunoprecipitated with the anti-peptide Ab and then immunoblotted using the Ab raised against the full length protein. For immunoblotting of C/EBPβ, nuclear cell extracts were prepared as described above and immunoblotted with a C/EBPβ-specific Ab. For immunoblotting of COX-2 and CREB, cell lysate (30 μg protein) was electrophoresed and immunoblotted with anti-COX-2 Ab (1.0 μg/ml) and phospho-specific Ab recognizing CREB phosphorylated at Ser152 and ATF1 phosphorylated at Ser47.

Reverse transcriptase-PCRs

Total RNA was prepared from LPS-stimulated or control RAW264 cells using the RNeasy Mini Kit according to the manufacturer’s protocol. Total RNA was measured and 100 ng were reverse transcribed using Promega avian myeloblastosis virus reverse transcriptase (5 U/ml) with the oligonucleotides GTTGGATACAGGCCAGACTTTGTTG and GAGGGTAG CAGGAACTTAAAGGG (coding for the IL-1β gene), and CAGCAAACTTCTCCTGTTCGC and TGGCAGGAATATGCAAACATC (coding for the COX-2 gene). Conditions for PCR
amplification of the resulting first-strand DNA template were 94°C denaturing for 30 s, 60°C annealing for 1 min, 68°C extension for 1 min, 30 cycles using thermostable Taq DNA polymerase (5 U/ml), and 1 mM MgSO4. The PCR products showed a single band of 515 bp for COX-2, a single band of 260 bp for IL-1β, and a single band of 352 bp for HPRT.

Results
The LPS-induced activation of MSK1 and MSK2 is mediated by two different MAP kinase cascades

To measure MSK1 and MSK2 activities in macrophage extracts, we first raised Abs capable of immunoprecipitating these protein kinases specifically. The specificity of the MSK1 Abs was established by the finding that immunoprecipitation was abolished by preincubating the Ab with the MSK1 peptide immunogen, but not the MSK2 peptide immunogen (Fig. 1A). Moreover, after immunoprecipitation with MSK1-peptide Ab, an immunoreactive band comigrating with authentic MSK1 was detected by immunoblotting with an Ab raised against the full length protein. This band was not detected in the immunoprecipitates if the MSK1 peptide Ab was first preincubated with the peptide immunogen or if the Ab was replaced by control IgG (Fig. 1B). Similarly, the immunoprecipitation of MSK2 was abolished by preincubation of the MSK2 Ab with the MSK2 peptide Ag, but not the MSK1 peptide Ag (Fig. 1A). Using these Abs, the activities of MSK1 and MSK2 were found to be negligible in unstimulated macrophages but greatly elevated after stimulation with LPS. Activation peaked 30 min after stimulation with 100 ng/ml LPS and 60 min after stimulation with 10 ng/ml LPS and declined thereafter (Fig. 1, C and D). Little activation was observed after stimulation with 1 ng/ml LPS.

LPS activates both the classical MAPK cascade and the SAPK2a/p38 pathway in macrophages as shown by the activation of MAPKAP-K1 (also called p90 RSK) and MAPKAP-K2, respectively (11). The activation of both enzymes is transient, peaking after 30–60 min in serum-deprived cells before declining to near basal levels after 2 h (11). These results were confirmed in the present study. Further control experiments showed that, as expected, the activation of MAPKAP-K1 was prevented by PD 98059 (Fig. 2A) or U0126 (data not shown) (12), which suppress the MAPK cascade by preventing the activation of MAPK kinase 1, but not by SB 203580 (Fig. 2A) a specific inhibitor of SAPK2a/p38. Conversely, SB 203580 prevented the LPS-induced activation of MAPKAP-K2 (Fig. 2B), whereas PD 98059 (Fig. 2B) and U0126 (data not shown) had no effect. These results establish that PD 98059/U0126 and SB 203580 are specific inhibitors of particular signaling pathways in macrophages.

In other mammalian cells tested, MSK1 can be activated by either MAPK/ERK or SAPK2a/p38. We therefore used PD 98059 and SB 203580 to assess the contribution of these MAP kinase family members to the LPS-induced activation of MSK1 and MSK2 in macrophages. These studies revealed that concentrations of PD 98059 that completely suppress the activation of MAPKAP-K1 (Fig. 2C) only inhibited the activation of MSK1 and MSK2 partially (Fig. 2, C and D). Similarly, MSK1 and MSK2 were only inhibited partially by SB 203580 (Figs. 2, C and D) at concentrations that completely blocked the activation of MAPKAP-K2 (Fig. 2B). In contrast, the activation of MSK1 and MSK2 was almost completely suppressed if macrophages were incubated in the presence of both PD 98059 and SB 203580 (Fig. 2, C and D). Similar results were obtained in additional experiments in which U0126 replaced PD 98059 (data not shown). These observations demonstrate that the LPS-induced activation of MSK1 and MSK2 is mediated by two different MAP kinase cascades.

The LPS-induced phosphorylation of CREB and ATF1 is mediated by two different MAP kinase cascades

Two putative physiological substrates for MSK1/MSK2 are the introduction transcription factors CREB and ATF1. As shown in
The LPS-stimulated induction of COX-2 and IL-1β were obtained in additional experiments in which U0126 replaced PD 98059 (Fig. 3). Similar results were obtained in three additional experiments. As in A, except that the macrophages were incubated for 1 h in the presence or absence of 50 μM PD 98059 (PD) and/or 10 μM SB 203580 (SB), or 5 μM Ro 318220 (Ro), and then stimulated for 1 h with or without 100 ng/ml LPS in the continued presence or absence of inhibitors. MSK1 (C), MSK2 (D), MAPKAP-K1 (A), and MAPKAP-K2 (B) were then assayed in the absence of any drug after immunoprecipitation from the lysates. The results are presented as mean ± SEM for two determinations from two separate dishes. Similar results were obtained in two additional experiments.

Fig. 3A. LPS induced the phosphorylation of CREB at Ser133 and the phosphorylation of ATF1 at Ser63. Like the activation of MSK1 and MSK2, the phosphorylation of CREB and ATF1 peaked after 1 h and declined thereafter. Similarly, the LPS-induced phosphorylation of CREB and ATF1 was partially inhibited by SB 203580, partially inhibited by PD 98059, and completely inhibited in the presence of both drugs (Fig. 3B). Similar results were obtained in additional experiments in which U0126 replaced PD 98059 (data not shown).

The LPS-stimulated induction of COX-2 and IL-1β is mediated by two different MAP kinase cascades

The COX-2 promoter contains a CRE (5). We therefore decided to examine whether the signaling pathways that mediate the LPS-stimulated induction of this enzyme are the same as those required to activate CREB. The COX-2 protein was present at low levels in unstimulated macrophages but was strongly induced 2 h after exposure to LPS. Induction was maximal after 4 h and maintained for at least 8 h (Fig. 4A). The induction of COX-2 was partially inhibited by SB 203580, partially inhibited by PD 98059, and almost completely suppressed in the presence of both drugs (Fig. 4B). Similar results were obtained in additional experiments in which U0126 replaced PD 98059 (data not shown).

We also studied the effect of LPS on the level of COX-2 mRNA. LPS strongly induced COX-2 mRNA, and this was sustained for at least 8 h (Fig. 5A). The induction of COX-2 mRNA was only slightly suppressed by SB 203580, slightly suppressed by PD 98059, but almost completely suppressed in the presence of both inhibitors (Fig. 5B). Similar results were obtained in additional experiments in which U0126 replaced PD 98059 (data not shown).

Thus, the effects of the three drugs on LPS-stimulated COX-2 mRNA and on the synthesis of the COX-2 protein were similar.

We also examined the signal transduction pathways that stimulated the level of the IL-1β mRNA, because the IL-1β promoter also contains a CRE (6). The time course of induction of IL-1β mRNA was similar to that of COX-2 mRNA (Fig. 5A). Interestingly, the induction of IL-1β mRNA was not significantly inhibited by PD 98059, partially inhibited by SB 203580, and completely inhibited in the presence of both drugs (Fig. 5B). Similar results were obtained in additional experiments in which U0126 replaced PD 98059 (data not shown).

In contrast to the level of COX-2 and IL-1β mRNA, the mRNA encoding the housekeeping gene HPRT was unaffected by LPS, PD 98059, and SB 203580 (Fig. 5).

Effect of Ro 318220 on LPS-stimulated proteins

Ro 318220 was originally developed as a protein kinase C inhibitor but is now known to inhibit several other protein kinases with similar potency in vitro, namely MSK1, MAPKAP-K1, p70 S6 kinase, and glycogen synthase kinase-3. In contrast, 20 other protein kinases including those known to be activated by SAPK2/p38 (i.e., MAPKAP-K2 and p38 regulated and activated protein kinase (PRAK) (13)) are unaffected at drug concentrations that ablate MSK1 activity (Refs. 1 and 3; S. Davies, H. Reddy, and P. Cohen, unpublished experiments). To test the specificity of Ro 318220 more rigorously, we first examined whether it affected the activation of the MAPK/ERK and SAPK2a/p38 pathways by LPS. Cells were incubated with Ro 318220 (5 μM) before stimulation with LPS, and the cells then were lysed. MSK1, MSK2, MAPKAP-K1, MAPKAP-K2 were immunoprecipitated from the cell lysates (1) and assayed in the absence of Ro 318220 (Fig. 2). These experiments showed that the LPS-induced activation of all four protein kinases was unaffected by Ro 318220 (5 μM). Thus, Ro 318220 does not inhibit any protein kinase in the signaling pathways leading to the activation of MSK1, MSK2, MAPKAP-K1, and MAPKAP-K2 in these cells.
Because the activation of MSK1 is prevented by SB 203580 plus PD 98059 and the activity of MSK1 is abolished by Ro 318220, any proteins with in vivo phosphorylation that is prevented by SB 203580 plus PD 98059, or by Ro 318220, are candidates to be physiological substrates for MSK1. In the present study, we found that Ro 318220 (5 μM) completely prevented the LPS-induced phosphorylation of CREB at Ser133 and ATF1 at Ser63 (Fig. 3). The same concentration of Ro 318220 also suppressed the LPS-stimulated induction of COX-2 protein (Fig. 4B), COX-2 mRNA, and IL-1β mRNA. In contrast, the mRNA encoding HPRT was unaffected by Ro 318220 (Fig. 5B).

Effect of H89 on LPS-stimulated proteins

H89 was originally developed as a specific inhibitor of PKA but we have recently shown that it is an equally potent inhibitor of several other protein kinases in vitro, namely, MSK1, p70 S6 kinase and Rho-dependent protein kinase α. In contrast, 20 other protein kinases including those activated by SAPK2/p38 (i.e., MAPKAP-K2 and PRAK (13)), as well as protein kinase C were unaffected at drug concentrations that ablate MSK1 activity (S. Davies, H. Reddy, and P. Cohen, unpublished experiments).

To test the specificity of H89 further, we first examined whether it affected the ability of LPS to activate the MAPK/ERK and SAPK2a/p38 pathways. Cells were incubated with 10–50 μM H89 before stimulation with LPS, and the cells were then lysed. MSK1 was immunoprecipitated from the cell lysates (1) and assayed in the absence of H89. These experiments showed that the LPS-induced activation of MSK1 (Fig. 6A) was unaffected by H89 after 1 h. This demonstrated that at the concentrations tested, H89 does not decrease the activity of any protein kinase in these pathways to a level where it would become rate limiting for the activation of MSK1. Therefore, proteins for which phosphorylation in vivo is prevented by SB 203580 plus PD 98059, or by H89, are candidates to be physiological substrates for MSK1. In the present study, we found that H89 suppressed the LPS-induced phosphorylation of CREB at Ser133 and ATF1 at Ser63 (Fig. 6B). The LPS-stimulated induction of the COX-2 protein (Fig. 6C) and the induction of the IL-1β mRNA (Fig. 6D) were inhibited at slightly higher concentrations. In contrast, the mRNA encoding HPRT was unaffected by the same concentration of H89 (Fig. 6D), demonstrating that the drug does not inactivate any essential component of the general transcription apparatus.

Effect of cyclic AMP elevation on the induction of IL-1β mRNA and COX-2 protein

The in vivo phosphorylation of CREB at Ser133 and ATF1 at Ser63 is not only catalyzed by MSK1 but also by PKA and can therefore be induced by agonists that elevate the intracellular concentration of cyclic AMP, such as forskolin (an activator of adenylate cyclase) and IBMX (an inhibitor of cyclic AMP phosphodiesterase). Stimulation with forskolin plus IBMX increased the phosphorylation of CREB and ATF1 in RAW264 macrophages to a level similar to that attained in the response to LPS. However, the effect was much faster and far more transient (Fig. 7, A and B). The forskolin plus IBMX-stimulated phosphorylation of CREB/ATF1 was unaffected by pretreatment with 5 μM Ro 318220 (Fig. 7B), in contrast to the basal and LPS-stimulated phosphorylation of CREB and ATF1. This is consistent with the finding that MSK1 is inhibited by Ro 318220 in vitro at a 50-fold lower concentration than PKA (Refs. 1 and 3; S. Davies, H. Reddy, and P. Cohen, unpublished experiments).

Interestingly, stimulation of macrophages with forskolin plus IBMX triggered a transient increase in IL-1β mRNA (Fig. 7C). In contrast, forskolin plus IBMX did not stimulate any increase in either COX-2 mRNA (data not shown) or COX-2 protein at either 3 or 6 h (Fig. 7D). Stimulation with forskolin/IBMX did not induce any increase in HPRT mRNA (Fig. 7C), nor did it cause significant activation of MAPKAP-K1 or MAPKAP-K2 (data not shown). These findings are considered further in the Discussion.

Effect of inhibitors of MAP kinase cascades on the LPS-induced induction of C/EBPβ

The transcription factor C/EBPβ is known to play a role in the activation of the COX-2 gene (14), and LPS is reported to increase
the level of this protein and its mRNA in RAW264.7 and J774 macrophages (15). In the present study, we found that the LPS-induced increase in the level of C/EBPβ was unaffected by prior incubation of the RAW264 cells with PD 98059, SB 203580, PD 98059 plus SB 203580, or Ro 318220 (Fig. 8).

**Discussion**

In this study, we establish that MSK1 and the closely related MSK2 are present in RAW264 macrophages and that both protein kinases are transiently activated in response to LPS (Fig. 1). The rates of activation and inactivation of these enzymes are similar to those of MAPKAP-K1 and MAPKAP-K2 which are convenient downstream reporters of the activation of the classical MAPK cascade and the SAPK2/p38 pathway, respectively. The LPS-induced activation of MSK1 and MSK2 is partially inhibited by drugs that prevent activation of the classical MAP kinase cascade (PD 98059 and U0126), partially inhibited by SB 203580 (an inhibitor of SAPK2/p38), and inhibited almost completely when macrophages are incubated in the presence of inhibitors of both pathways (Fig. 2). These observations with MSK1 confirm previous work from our unit (1) using other cells and different agonists (see Introduction). However, the present study is the first to report that MSK2 is also activated by both MAPK cascades and is in contrast to another recent study suggesting that MSK2 was solely activated by SAPK2/p38 (16). The activity of MSK2 in macrophages is much lower than that of MSK1, and this is also the case in 293 cells (S. Arthur, M. Deak, and D. R. Alessi, unpublished work from this unit).
Previous work suggested that the transcription factors CREB and ATF1 may be physiological substrates of MSK1 (1) and MSK2 (16) (see Introduction), and the present study is consistent with this hypothesis. Thus, CREB and ATF1 were transiently phosphorylated with kinetics similar to the activation/inactivation of MSK1 and MSK2. Moreover, phosphorylation was prevented when the macrophages were pretreated with inhibitors of both the classical MAP kinase cascade and SAPK2/p38, but inhibition of CREB and ATF-1 phosphorylation was only partial when just one of these signaling pathways was blocked. Furthermore, the phosphorylation of CREB was also prevented by Ro 318220 (Fig. 3B) or H89 (Fig. 6B) at concentrations that inhibit MSK1 (see Introduction). MAPKAP-K2, MAPKAP-K3, and PKA also phosphorylate CREB and ATF1 at Ser\(^{133}\) and Ser\(^{63}\), respectively (2). However, these protein kinases cannot be rate limiting for the LPS-induced activation of CREB and ATF1, because their activities are unaffected in vivo at the concentrations of Ro 318220 used in these experiments, and neither are MAPKAP-K2 and MAPKAP-K3 inhibited by H89 (Ref. 1; S. Davies, H. Reddy, and P. Cohen, unpublished experiments).

Interestingly, the inhibitory effects of SB 203580, PD 98059, Ro 318220, and H89 on the LPS-induced increase in COX-2 mRNA and protein and IL-1β mRNA correlated with their ability to suppress the LPS-induced phosphorylation of CREB/ATF1. Because the genes encoding COX-2 (5) and IL-1β (6) both contain a CRE and the potential importance of the CRE in the transcriptional regulation of these genes is well documented, these observations raise the possibility that MSK1 may play an important role in mediating the increase in COX-2 and IL-1β mRNA. This might be achieved by the phosphorylation of CREB/ATF1 and/or other substrates for MSK1 that have yet to be identified (Fig. 9). These substrates might include other transcription factors or proteins that control the stability of COX-2 mRNA and IL-1β mRNA. The level of the mRNA encoding any protein is clearly a steady state that reflects the relative rates of transcription of the gene and the rate of degradation of its mRNA. Indeed, LPS has been shown to increase COX-2 mRNA stability in human monocytes, as well as to stimulate transcription of the gene. Both effects were inhibited by SB 203580 (17). An SB 203580-sensitive pathway was also reported to regulate the stability of COX-2 mRNA in IL-1-stimulated HeLa cells (18). It will be interesting to examine how all four inhibitors used in the present study affect LPS-stimulated gene transcription and LPS-induced mRNA stability.

One assumption implicit in the model depicted in Fig. 9 is that all four inhibitors used in the present study block COX-2 and IL-1β mRNA through a common pathway mediated by MSK1. Apart from p70 S6 kinase, MSK1 is the only protein kinase of the 25 we have tested that is potently inhibited by both Ro 318220 and H89. However, Ro 318220 and H89 are not exquisitely specific inhibitors. Therefore, despite the correlation between the effects of each of these drugs and those produced by a combination of SB 203580 plus PD 98059, it cannot be excluded that Ro 318220 and H89 suppress COX-2 and IL-1β mRNA production by inhibiting another protein kinase(s) that does not lie downstream of MAPK/ERK and/or SAPK2a/p38. The development of a really specific inhibitor of MSK1/MSK2 and the availability of mice that lack MSK1 and MSK2 will be essential to elucidate their roles in the induction of COX-2 and IL-1β.

The cyclic AMP-elevating agents forskolin and IBMX triggered a much faster PKA-dependent phosphorylation of CREB and ATF1 (Fig. 7A) than that produced by LPS (Fig. 3A). Phosphorylation was maximal after 5 min but had declined to basal levels by 60 min, a time at which LPS-stimulated CREB phosphorylation was still maximal. This is consistent with the smaller and much more transient increase in IL-1β mRNA observed with forskolin plus IBMX (Fig. 7B). However, whether PKA increases the level of IL-1β mRNA by activating CREB/ATF1 or by phosphorylation of additional/other protein(s) is unclear. In contrast to IL-1β mRNA, forskolin plus IBMX did not induce the COX-2 protein (Fig. 7D). This indicates that the activation of CREB alone is insufficient to induce transcription of the COX-2 gene. This is not surprising because the COX-2 gene contains binding sites for a number of important transcription factors, including C/EBPβ and NF-κB.

Numerous papers have suggested that NF-κB plays a role in the stimulation of COX-2 gene expression. For example, NF-κB is capable of activating a COX-2 promoter construct in IL-1β-stimulated human pulmonary type II A549 cells (19), and mutation of the NF-κB response element partially inhibited TNF-α-stimulated luciferase activity in transfected mouse osteoblastic MC3T3-E1 cells (20). Moreover, colonic epithelial HT-29 cells infected with an adenoviral vector containing a dominant negative mutant of I-κB prevented the TNF-α-induced increase in COX-2 mRNA (21). However, there is also evidence against a role for NF-κB in COX-2 induction. For example, the antioxidant ammonium pyrrolidinedithiocarbamate or the protein kinase inhibitor tosyllysylchloromethyl ketone completely blocked NF-κB activation in LPS-stimulated J774 macrophages but had little effect on the induction
of COX-2 protein, as judged by immunoblotting, or on the production of PGE\textsubscript{2} (22). Moreover, another proteinase inhibitor, cailpain inhibitor I, blocked the activation of NF-\textkappa B without inhibiting TNF-\textalpha-stimulated induction of COX-2 in rat aortic smooth muscle (23). However, even if NF-\textkappa B plays a role in LPS-stimulated COX-2 gene expression in RAW264 cells, the four inhibitors used in the present study are most unlikely to exert their effects by suppressing the activation of NF-\textkappa B. Thus, SB 203580 does not inhibit TNF-\textalpha-induced NF-\textkappa B activation in L929 fibroblasts (24), and this is also true in RAW264 macrophages (M. Caivano, N. Chapman, and N. Perkins, unpublished experiments). Similarly, PD 98059 does not inhibit LPS-stimulated NF-\textkappa B activation in RAW264.7 macrophages (25) or in IL-1\beta-stimulated human mesangial cells (26). We have also found that neither Ro 318220 (5 \mu M) nor H89 (25 \mu M) affect TNF-\textalpha-stimulated activation of NF-\textkappa B in human embryonic kidney 293 cells or in LPS-stimulated RAW264 macrophages (M. Caivano, N. Chapman, and N. Perkins, unpublished experiments).

The transcription factor C/EBP\textbeta is known to play a role in the activation of the COX-2 gene (14), and LPS was reported to increase the level of C/EBP\textbeta in RAW264.7 and J774 macrophages by stimulating transcription of the gene (15). We confirmed that LPS induces a striking rise in the level of C/EBP\textbeta, and we also found that this increase was not prevented by SB 203580 and/or PD 98059 or by Ro 318220 (Fig. 8). Therefore, the SAPK2p38 and MAPK/ERK pathways do not increase the level of COX-2 mRNA by increasing the amount of C/EBP\textbeta protein. In NIH 3T3 cells (27) and 3T3-L1 preadipocytes (28), C/EBP\textbeta is reported to be activated by phosphorylation of a threonine residue catalyzed by MAPK/ERK and SAPK2p38, respectively. However, LPS does not appear to trigger the phosphorylation of C/EBP\textbeta in RAW264.7 macrophages (15), suggesting that the activation of C/EBP\textbeta by phosphorylation does not underlie the LPS-induced increase in COX-2 mRNA either. In any case, the direct phosphorylation of C/EBP\textbeta by MAPK/ERK and/or SAPK2p38 cannot account for the effects of Ro 318220 or H89 on COX-2 gene transcription, because these MAPK family members (Ref. 3; S. Davies, H. Reddy, and P. Cohen, unpublished experiments), and the signaling pathways that lead to their activation (Fig. 2), are resistant to this drug.

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References