The Induction of Cyclooxygenase-2 mRNA in Macrophages Is Biphasic and Requires both CCAAT Enhancer-binding protein β (C/EBPβ) and C/EBPδ Transcription Factors*

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Prostaglandins are important mediators of activated macrophage functions, and their inducible synthesis is mediated by cyclooxygenase-2 (COX-2). Here, we make use of the murine macrophage cells RAW264 as well as of immortalized macrophages derived from mice deficient for the transcription factor CCAAT enhancer-binding protein β (C/EBPβ) to explore the molecular mechanisms regulating COX-2 induction in activated macrophages. We demonstrate that lipopolysaccharide-mediated COX-2 mRNA induction is biphasic. The initial phase is independent of de novo protein synthesis, correlates with cAMP-response element-binding protein (CREB) activation, is inhibited by treatments that abolish CREB phosphorylation and reduce NF-κB-mediated gene activation, and requires the presence of the transcription factor C/EBPβ. On the other hand, C/EBPδ appears to be essential in addition to C/EBPβ to effect the second phase of COX-2 gene transcription, which is important for maintaining the induced state and requires de novo protein synthesis. Indeed, both phases of COX-2 induction were defective in C/EBPβ−/− macrophages. Moreover, the synthesis of C/EBPδ was increased dramatically by treatment with lipopolysaccharide and, like COX-2 induction, repressed by combined inhibition of the MAPK and of the SAPK2/p38 cascades. Taken together, these data identify CREB, NF-κB, and both C/EBPβ and -δ as key factors in coordinately orchestrating transcription from the COX-2 promoter in activated macrophages.

Cyclooxygenase-2 (COX-2)1 is a key enzyme catalyzing the rate-limiting step in the inducible production of prostaglandins (PG), and its synthesis can be readily induced in many different cell types in response to a variety of stimuli (1). Although the contrasting biological properties of the different prostanooids make it difficult to define their roles in physiological processes unambiguously, PG secretion by activated macrophages clearly represents an important step in the inflammatory process (2). Indeed, COX-2, as well as COX-1, the isoform responsible for the basal steady state production of PG, represents the main target for nonsteroidal anti-inflammatory drugs, and compounds that can specifically inhibit the inducible but not the basal production of PG (i.e. COX-2 but not COX-1 activity) are being tested for the treatment of chronic inflammatory diseases such as rheumatoid arthritis or ulcerative colitis (3–5).

Many studies have therefore recently focused on the mechanisms regulating inducible COX-2 expression in mononuclear cells. Three main cis-acting elements have been identified on the murine COX-2 promoter that play a role in LPS-mediated induction of COX-2 transcription in macrophages. NF-κB is a transcription factor involved in LPS-mediated induction of many cytokines and inflammatory products, and inhibition of NF-κB activity has been reported to impair COX-2 mRNA induction (6–11). The –138/–130 C/EBP element is generally believed to play an important role in COX-2 promoter induction in macrophages as well as in other cell types, mainly through interactions with the two C/EBP family members C/EBPβ and -δ (9–16). Finally, the overlapping CRE/E-box recognition sequence located at positions –59/–48 appears to be the most generally required promoter element, being essential for both basal and induced COX-2 transcription in most cellular systems analyzed (10, 12, 13, 15–21).

We and others have previously shown that stimulation of macrophages with LPS elicits the activation of the classical mitogen-activated protein kinase (MAPK) cascade and the homologous stress-activated protein kinase 2 (SAPK2/p38 pathway (7, 22, 23). Moreover, combined suppression of these pathways utilizing the small cell-permeant inhibitors PD 98059 (24, 25) or U0126 (26), which specifically inhibit the activation of the MAPK kinase-1, together with SB 203580, a specific inhibitor of SAPK2/p38 activity (27), resulted in the coordinated inhibition of LPS-stimulated CREB/ATF1 phosphorylation and COX-2 mRNA and protein induction (28). However, the protein kinase A-mediated phosphorylation of CREB following cell treatment with forskolin did not trigger detectable COX-2 protein induction, suggesting that CREB activation, even if required, is not sufficient to activate COX-2 expression (28). Serendipitously, we have recently observed that COX-2 expression in response to LPS was profoundly impaired in macrophages derived from mice where the transcription factor C/EBPβ was inactivated (29). COX-2 induction could be rescued by transient or stable re-expression of C/EBPβ, suggesting that this factor is required for efficient COX-2 gene tran-
scription in macrophages. Here we explore the kinetics of COX-2 mRNA induction and how it correlates with the induced activities of CREB and C/EBP factors, making use of both the murine macrophage cell line RAW264 and immortalized C/EBPβ−/− or +/+ macrophages. We demonstrate the existence of two waves of COX-2 induction. The first does not involve de novo protein synthesis, correlates with CREB and NF-κB activation, and requires preexisting C/EBPβ, while the second involves newly synthesized C/EBPβ and requires the DNA binding activity of C/EBPβ/C/EBPβ heterodimers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents and antibodies for tissue culture were purchased from Life Technologies (Paisley, UK); forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (Poole, UK); complete protease inhibitor mixture was from Roche Molecular Biochemicals; monoclonal mouse anti-C/EBPβ and polyclonal anti-C/EBPα, -β, -δ, and -ε antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); affinity-purified polyclonal rabbit anti-phospho-CREB and anti-CREB antibodies were from Cell Signaling Technology, Inc. (Beverly, MA); affinity-purified polyclonal rabbit anti-IκBα was from New England Biolabs (Hertfordshire, UK); affinity-purified polyclonal rabbit anti-Ser/Thr phosphorylated histone H1 was from Promega (Southampton, UK); monoclonal mouse anti-C/EBPβ, C/EBPδ, and C/EBPα antibodies were from Novocea, (Ares-Serono, Geneve, Switzerland). LPS was a generous gift from Dr. John Lee (SmithKline Beecham).

**Cell Culture and Stimulation—**RAW264 macrophages were maintained at 37 °C in 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin. 2 h before stimulation, the medium was removed and replaced with 2 ml of Dulbecco’s modified Eagle’s medium. The cells were then stimulated with 100 ng/ml LPS or 20 μM forskolin plus 10 μM IBMX for the times indicated in the figure legends. Where indicated, SB 203580 (10 μM) and/or U0126 (10 μM) were added 1 h before stimulation.

The generation of COX-2β−/− and +/+ immortalized macrophages is described elsewhere.2 The cells were maintained at 37 °C in a 5% CO2 atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum containing 2 mM ascorbate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells at ~70% confluence were stimulated with 100 units/ml of interferon-γ for 16 h and with 100 ng/ml LPS for the indicated times.

**Cell Lysis—**After stimulation, the cells were aspirated, and the cells were solubilized in 0.2 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 mM sucrose, 1 mM microcystin-LR, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, and “complete” protease inhibitor mixture (one tablet/50 ml)). The samples were then snap frozen in liquid nitrogen and stored in aliquots at −80 °C until analysis. Protein concentrations were determined using the Coomassie Protein Assay Reagent.

**Nuclear Extracts—**After stimulation, the cells were resuspended, washed three times in Buffer A (10 mM Hepes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 1 mM microcystin-LR, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, and “complete” protease inhibitor mixture), lysed in Buffer B plus 0.1% (v/v) Nonidet P-40 for 5 min on ice, and then sonicated in a 10°C water bath (four 5-s pulses over 4 min). The samples were centrifuged at 13,500 × g for 10 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 using the Coomassie Protein Assay Reagent.

**Immunoblotting Analysis—**Proteins were denatured in SDS, electrophoresed on a 4–12% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. Ponceau S staining was performed in order to ensure equivalent gel loading. Membranes were then incubated with the antibodies described below, which were detected using the enhanced chemiluminescence reagent (ECL). For immunoblotting of C/EBPβ or C/EBPδ, 50 μg of nuclear cell extracts were electrophoresed and immunoblotted using a monoclonal C/EBPβ antibody or a polyclonal C/EBPδ antibody, respectively. For immunoblotting of IκBα, 30 μg of total proteins were electrophoresed and immunoblotted using a polyclonal anti-IκBα antibody. For immunoblotting CREB, cell lysates (50 μg of protein) were electrophoresed and immunoblotted using, respectively, an anti-CREB antibody or an anti-phosphospecific CREB antibody recognizing CREB phosphorylated at Ser133 and ATF-1-phosphorylated at Ser21.

**Electrophoretic Mobility Shift Assays (EMSAs)—**EMSA were made by annealing single-stranded oligonucleotides with 5′-GATC overhangs. 1 pmol of probe was radiolabeled by filling in with [α-32P]dATP using the Klenow enzyme. The labeled probes were purified on a Sephadex G-50 spin column. Sequences are as follows: C/EBP, 5′-GATCCGCTGCGTCTTGCACACTCAT-3′; HoxA, 5′-GATCAGAGGTCTTCTTTGACGC-3′; CREB, 5′-GATCCTCTATGCGACCTTT-3′. EMSAs were performed with 4 μg of nuclear extract in 20 μl Hepes (pH 7.9), 1 mM EDTA, and 2.5 mM diethiothreitol, containing 3 μg of poly(dI-dC). The complexes were separated by electrophoresis on a 6% (v/v) or 5% (v/v) for CREB or 5% (v/v) for NF-kB and CREB/E-box) polyacrylamide-0.25× Tris borate-EDTA gel. For supershift experiments, 2 μl of polyclonal purified antibody was incubated with nuclear extracts and poly(dI-dC) for 30 min on ice prior to the probe addition. Unlabeled double-stranded oligonucleotide competitors were preincubated at a 50-fold molar excess 10 min prior to the probe addition.

**RT-PCR—**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 50 ng was reverse transcribed using Promega avian myeloblastosis virus reverse transcriptase (5 units/ml) with the oligonucleotides 5′-AAATCCTTGCTGTTCC and TGGGCAAAGAATGCAAACATC) or for HPRT (5′-GGCAAGAAGAGAAGACGG and 5′-GGGCGGGAAGG). 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 Tfl DNA polymerase (5 units/ml), and 1 μg MgSO4. The PCR products showed a band of 515 bp for COX-2, a band of 233 bp for HPRT, and a band of 352 bp for CREB/E-box.

**Statistical Analysis—**Results obtained after densitometric quantifications were analyzed using the two-tailed t test. P value of <0.05 was considered statistically significant.

**RESULTS**

**COX-2 mRNA Induction Is Biphasic—**Phosphorylation of CREB obtained by treatment of RAW264 macrophages with forskolin and IBMX did not trigger detectable accumulation of COX-2 protein after 4 h, suggesting that a distinct factor(s) induced by LPS but not by forskolin is required for COX-2 induction (28). Since, however, forskolin induces a faster and more transient phosphorylation of CREB compared with LPS stimulation, we decided to also analyze COX-2 mRNA induction at shorter time points. Indeed, forskolin was able to trigger an increase of the COX-2 mRNA (1.8-fold) already after 30 min (Fig. 1A, lane 1), corresponding to an increase of COX-2 protein (1.14-fold) by 2 h (Fig. 1A, lane 2). The induction kinetics observed. In agreement with these reports, the quick activation kinetics observed. In agreement with these reports, the quick activation kinetics observed.
increase of COX-2 mRNA induced by 1.5 h of LPS treatment was not affected by pretreatment with the protein synthesis inhibitor cycloheximide (CHX) (Fig. 1B, compare lanes 2 and 4, 8.6  0.33-versus 7.6  0.61-fold induction), which, as already reported (33–35) caused by itself a slight (2.38  0.34-fold) induction (Fig. 1B, lanes 3 and 7). Interestingly, however, CHX pretreatment did abolish COX-2 mRNA induction following a longer treatment with LPS (Fig. 1B, compare lane 6 (21.7  0.64-fold induction) with lane 8 (7.95  0.93-fold induction) and lane 7 (9.77  1.67-fold induction after treatment with CHX alone), suggesting that de novo protein synthesis is involved in the later phases of COX-2 transcription.

Taken together, these results suggest a biphasic activation of the COX-2 gene. The first phase, corresponding to the initial activation, correlates with the kinetics of CREB phosphorylation and does not involve de novo protein synthesis, while the second phase, involved in the maintenance of the induced state, requires the action of some newly synthesized factor(s).

The LPS-mediated Induction of C/EBPβ, but Not of C/EBPβ, Is Suppressed by Treatment with U0126 plus SB 203580—Members of the C/EBP family, and particularly C/EBPβ and -δ, may well be involved in the second phase of COX-2 transcriptional induction, since their synthesis is increased by LPS in a number of cell types (36). We have therefore analyzed their induction following LPS treatment in the presence or absence of the protein kinase inhibitors that abolish COX-2 expression. C/EBPβ is already present in untreated macrophages, but it is increased by 2–3-fold following LPS treatment, peaking at 4 h (not shown). We have shown previously that treatment with PD 98059 and/or SB 203580 did not affect the induction of C/EBPβ triggered by LPS (28). Indeed, U0126 and SB 203580, alone or in combination, were unable to modify the 3-fold LPS-induced increase of all three C/EBPβ isoforms, the full-length protein (FL), the liver-activating protein (LAP), and the liver-inhibitory protein (LIP) (Fig. 2A, upper panel, compare lane 1 with lanes 2–5).

The levels of C/EBPδ were in contrast almost undetectable in untreated RAW264 cells, but they were increased dramatically following LPS treatment (Fig. 2A, lower panel, compare lanes 1 and 2). Strikingly, the LPS-mediated induction of C/EBPδ was almost totally abolished by combined treatment with U0126 plus SB 203580 (Fig. 2A, lower panel, compare lanes 2 and 5) and only slightly decreased by treatment with either compound alone (lanes 3 and 4). This is in marked contrast to what was observed with C/EBPβ, and suggests that C/EBPδ may represent the factor, or one of the factors, whose synthesis is required to maintain COX-2 transcriptional induction.

Treatment with U0126 plus SB203580 Abolishes the Induction of C/EBPβ-C/EBPδ DNA Binding Activities—Next, we analyzed by EMSA the DNA–protein interactions occurring at the level of the −138/−130 C/EBP site from the murine COX-2 promoter. Four differentially migrating complexes could be detected using nuclear extracts from untreated RAW264 cells (Fig. 2B, lanes 1, 2, and 11). Complexes 1 and 2 were induced by 5-fold upon LPS treatment (Fig. 2B, compare lanes 1 and 2 with lanes 3 and 4, and compare lane 11 with lane 13). This increase was reduced by 50% by treatment with either SB 203580 or U0126 alone (Fig. 2B, lanes 5 and 6 and lanes 7 and 8, respectively) and completely abolished by a combination of the two compounds (Fig. 2B, compare lanes 3 and 4 with lanes 9 and 10, and compare lane 13 with lane 14). The inhibition of DNA binding appeared to be specific to the newly induced activities, since the inhibitors did not affect the formation of complexes using extracts from untreated cells (Fig. 2B, compare lanes 11 and 12).

All DNA–protein complexes detected at the level of the COX-2 C/EBP site could be abolished by an excess of unlabeled double-stranded oligonucleotide carrying either the same sequence (self) or the sequence of a known C/EBP binding site from the hemopexin promoter (HpxA) (Fig. 2C, lanes 6 and 7, 13 and 14, and 20 and 21), but not by an unrelated oligonucleotide (not shown). To assess if C/EBP proteins were involved in the formation of the different complexes detected and particularly of induced complexes 1 and 2, we performed supershift experiments using polyclonal antibodies against C/EBPα, -β, -δ, or -ε and nuclear extracts from RAW264 cells either untreated or treated with LPS (Fig. 2C). In extracts from untreated cells, all complexes could be supershifted by anti-C/EBPβ antibodies (Fig. 2C, lane 3) and did not contain any of the other tested C/EBP family members, as confirmed by densitometric analysis of the retarded bands (Fig. 2C, lanes 2, 4, and 5), although the same antibodies could readily supershift complexes obtained with different extracts and probes (data not shown). Upon LPS treatment, still no binding of either C/EBPα or C/EBPε could be detected (Fig. 2C, lanes 9 and 12), while C/EBPβ was involved in the formation of all four complexes, since all, including those induced by LPS, were abolished by specific antibodies raised against this protein (Fig. 2C, lane 10). C/EBPδ, which was absent from the complexes formed using extracts from untreated cells, was in contrast detected as part of the LPS-induced complexes 1 and 2, 40% of which could be supershifted by anti-C/EBPδ antibodies (Fig. 2C, lane 11). Of note, the amount of complexes supershifted could not be increased using more anti-C/EBPδ antibodies (data not shown). Taken together, these data suggest that complexes 1 and 2 only contain C/EBPβ homodimers in untreated RAW264 macrophages, while they are formed partly of C/EBPβ homodimers and partly of C/EBPβ-C/EBPδ heterodimers in LPS-treated cells.

We next asked whether binding of C/EBPβ, C/EBPδ, or both was affected by U0126 and SB 203580. As expected, all residual DNA–protein complexes obtained upon LPS treatment in the presence of U0126 and SB 203580 could still be supershifted by...
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Fig. 2. Effect of different protein kinase inhibitors on the LPS-mediated induction of C/EBPβ and δ synthesis and C/EBP DNA binding activity in RAW264 macrophages. A, cells were incubated for 1 h in the presence or absence of SB 203580 (SB) and/or U0126 (U) and then stimulated for 4 h with or without 100 ng/ml LPS in the continuous presence or absence of the inhibitors. Nuclear extracts were prepared and immunoblotted with an anti-C/EBPβ antibody (upper panel) and, with Ponceau S staining of each filter prior to densitometric and statistical analysis of three independent experiments. Differences between lanes 1 and 2 of the upper and lower panels (p < 0.003) and between lanes 2 and 5 of the lower panel (p = 0.001) were found to be statistically significant. B, nuclear extracts prepared as in A were analyzed by EMSA using the −198/−130 C/EBP site from the murine COX-2 promoter as a probe. The arrows and numbers on the left indicate the different DNA-protein complexes detected. Densitometric and statistical analysis of complexes 1 and 2 from three independent experiments was performed. The differences between lanes 1 and 2 of the upper and lower panels (p < 0.003) and between lanes 1 and 3 and 4 (p = 0.04) and between lanes 3 and 4 and lanes 9 and 10 (p = 0.03) were found to be statistically significant. C, nuclear extracts were prepared and analyzed as in B. Where indicated, polyclonal antibodies directed against different C/EBP isoforms (C/EBPα, -β, -δ, or -ε) were also included in the incubation mix. For competition experiments, a 50-fold molar excess of one of the following unlabeled oligonucleotides was used: −138/130 COX-2 C/EBP site (self) or C/EBP site from the hemopexin promoter (HpxA). Densitometric and statistical analysis of complexes 1 and 2 from three independent experiments was performed. The differences between lanes 1 and 3 (p = 0.0005), between lanes 8 and 10 (p = 0.0001), between lanes 8 and 11 (p = 0.02), and between lanes 15 and 17 (p = 0.004) were found to be statistically significant.

anti-C/EBPβ antibodies (Fig. 2C, lane 17). Interestingly, as confirmed by densitometric analysis, no supershift with anti-C/EBPβ antibodies could be detected anymore after treatment with the inhibitors (Fig. 2C, lane 18), suggesting that the DNA binding activity involving C/EBPβ/C/EBPδ heterodimers may represent the main target for U0126 and SB 203580 action.

DNA Binding to the CRE/E-box and NF-κB Elements of the COX-2 Promoter Is Not Impaired Following U0126 and SB 203580 Treatment—To verify if the inhibition of DNA binding by treatment with U0126 and SB 203580 is specifically limited to the C/EBP proteins among the factors playing a role in COX-2 gene transcription, we have examined the pattern of DNA-protein complexes forming at the level of the two other main cis-acting elements involved in the LPS-inducible activation of the COX-2 promoter. EMSA experiments were therefore performed using the CRE/E-box element located at positions −59/−48 and the −402/−392 NF-κB binding site as probes and nuclear extracts from RAW264 cells either untreated or treated with LPS in the presence or absence of a combination of U0126 and SB 203580 (Fig. 3).

As expected from knowledge that the proteins involved can bind constitutively to DNA, binding to the CRE/E-box element was already detected in extracts from untreated cells (Fig. 3A, lane 1) and increased by 1.9- or 1.3-fold, respectively, upon 1.5 or 4 h of LPS treatment (Fig. 3A, compare lanes 1, 3, and 5). Treatment with the inhibitors enhanced by 2-fold the binding prior to LPS treatment (compare lanes 1 and 2), but this phenomenon was not investigated further. In contrast, binding to the NF-κB element, which was not detected in extracts from untreated cells (Fig. 3B, lanes 1 and 2) was strongly increased by treatment with LPS for 1.5 h (Fig. 3B, lane 3) and decreased by 50% after 4 h (Fig. 3B, lane 5). Importantly, binding to
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Fig. 2. Effect of protein kinase inhibitors on the binding to other COX-2 promoter sites and on the induction of IκBα. Nuclear extracts from RAW264 macrophages either untreated or treated as in Fig. 2A were analyzed by EMSA using the −59/−48 CRE/E-box (A) or the −402/−392 NF-κB site (B) from the murine COX-2 promoter as a probe. F, free probe. Densitometric and statistical analysis of two independent experiments was performed. In B, the differences between lanes 3 and 4 or lanes 5 and 6 (p < 0.001) were statistically significant. C, RAW264 macrophages were incubated for 1 h in the presence or absence of SB 203580 (SB) and/or U0126 (U) and then stimulated for 1 h with or without LPS in the continuous presence or absence of the inhibitors. Cell lysates were prepared, and 30 μg of total protein were analyzed by immunoblotting using an anti-IκBα antibody. Equivalent gel loading was assessed by Ponceau S staining of each filter prior to immunostaining. Densitometric and statistical analysis of three independent experiments was performed. The differences between lanes 1 and 2 of the upper panel (p = 0.005) and of the lower panel (p = 0.004), but not between lanes 1 and 3 of both panels, were found to be statistically significant. B, nuclear extracts prepared as in A were analyzed by EMSA using the −198/−130 COX-2 site from the murine COX-2 promoter as a probe. Densitometric and statistical analysis of complexes 1 and 2 from three independent experiments was performed. The difference between lanes 1 and 2 and lanes 3 and 4 (p = 0.02) was statistically significant. C, nuclear extracts obtained from forskolin (F) plus IBMX treatment were analyzed as in B and, where indicated, were preincubated with polyclonal antibodies directed against different C/EBP isoforms (C/EBPα, β, δ, or ε). The arrows and numbers on the left indicate the different DNA-protein complexes detected. Densitometric and statistical analysis of three independent experiments was performed. The difference between lanes 1 and 3 (p = 0.005) was statistically significant.

neither element was inhibited by U0126 and SB 203580 treatment.

Remarkingly, LPS-induced nuclear NF-κB DNA binding activity was both increased and prolonged by treatment with the inhibitors as shown in Fig. 3B by comparing lane 3 with lane 4 (4- versus 8-fold induction) and lane 5 with lane 6 (2- versus 3.5-fold induction). Since increased NF-κB activation may be due to impaired IκBα resynthesis after LPS-induced degradation, we have assessed IκBα levels in RAW264 cells both before and after 1 h of LPS treatment, a time when NF-κB-triggered IκBα resynthesis should be completed (37), in the presence or absence of U0126 and/or SB 203580 (Fig. 3C). IκBα levels were indeed reduced about 4-fold in the presence of both inhibitors (Fig. 3C, compare lanes 2 and 5).

Neither C/EBPβ nor C/EBPδ Is Induced by Forskolin Treatment—If C/EBPδ and/or C/EBPβ are indeed the factors whose synthesis is required to effect the second phase of COX-2 transcriptional induction, they should not be induced by forskolin treatment, since this only triggers transient COX-2 mRNA induction that is extinguished before the newly synthesized factors could start accumulating. Indeed, the levels of C/EBPβ as detected by Western blot were only increased slightly (2.1-fold) following forskolin treatment in comparison with the much stronger 5.5-fold induction obtained with LPS (Fig. 4A, upper panel). Even more strikingly, forskolin treatment completely failed to induce C/EBPδ in contrast to the dramatic 22.3-fold induction triggered by LPS treatment (Fig. 4A, lower panel). In agreement with these findings, forskolin could only trigger a weak (1.4-fold) increase of C/EBP DNA binding activ-
COX-2 mRNA induction but not LPS-induced CREB/ATF-1 phosphorylation is defective in the absence of C/EBPβ. A, C/EBPβ+/+ and −/− macrophages were left untreated or stimulated for the indicated times with LPS. Total RNA was extracted at each time point, and mRNA encoding COX-2 and HPRT (internal control) was determined using RT-PCR. Densitometric and statistical analysis of two independent experiments was performed as in Fig. 1. At each time point, differences between C/EBPβ+/+ and −/− samples were statistically significant (p < 0.002). B, cells were left untreated or stimulated for 30 min or 1 h with 100 ng/ml LPS, and 30 μg of total protein were immunoblotted with anti-phospho-CREB/ATF-1 antibody or CREB antibody. Equivalent gel loading was assessed by Ponceau S staining of each filter prior to immunostaining. Densitometric and statistical analysis of three independent experiments was performed, and phospho-CREB (p-CREB) values were normalized against total CREB values. The differences between lanes 1 and 2 or lanes 4 and 5 (p < 0.004) were found to be statistically significant. No significant difference was detected between lanes 2 and 5 and between lanes 3 and 6.

**DISCUSSION**

The induction of the COX-2 promoter in macrophages is complex and involves different promoter elements and transcription factors, but their relative roles are not completely understood. We demonstrate here that induction of the COX-2 mRNA following LPS treatment is biphasic, presumably affected by preexisting transcription factors that become posttranslationally activated in the first phase while requiring the synthesis of a new factor(s) for the second phase.

The initial phase correlates with the activation of the transcription factor CREB, both upon LPS and forskolin treatment. Moreover, both CREB activation and the first phase of COX-2 induction by LPS are insensitive to inhibition of protein synthesis. Notwithstanding the strong correlations between CREB activation and COX-2 induction, recent work proposing that CREB cannot activate the COX-2 promoter in transient transfection assays imposes caution (16), and further work is needed to find out whether CREB is required to initiate COX-2 transcription and which other factors are involved.

NF-κB is a good candidate to be one of these factors. Inhibition of the p38/SAPK pathway (38) and of the MAPK pathway (39), which impairs COX-2 induction, is known to cooperatively repress NF-κB-dependent gene expression, and we show that in RAW264 macrophages treatment with SB 203580 plus U0126 impairs LPS induction, known to be NF-κB-dependent (37). Taken together, these observations suggest that in our system as well NF-κB transactivating capacity may be decreased by treatment with the inhibitors. This decrease might therefore at least in part account for the inhibition of LPS-mediated COX-2 induction triggered by treatment with SB 203580 plus U0126.
Activating protein-1 factors and, in particular, c-Jun have been proposed to play an important role in COX-2 promoter activation both by co-transfection assays and by inhibition studies with a dominant negative form of c-Jun N-terminal kinase (16). Indeed, it has been proposed that it is c-Jun and not CREB that binds to the CRE in the COX-2 promoter. However, the finding that the LPS-induced COX-2 induction is suppressed by a combination of SB 203580 plus PD 98059 cannot be explained by the inhibition of c-Jun N-terminal kinase activation, because the LPS-induced phosphorylation of c-Jun is unaffected by these compounds.3 Likewise, the induction of COX-2 by forskolin cannot be explained by activation of c-Jun N-terminal kinase, since this kinase is not activated by forskolin (40). However, our results do not exclude the possibility that c-Jun N-terminal kinase and c-Jun activity may play a role in COX-2 gene transcription.

Importantly, neither CREB nor NF-κB can be sufficient to both initiate and maintain COX-2 transcription that is still intensely active 3 h after LPS treatment (29), when CREB nuclear localization is already strongly decreased (37). Members of the C/EBP family of transcription factors, and in particular C/EBPβ and -δ, are good candidates to represent the transcription factors whose synthesis is required for the second, CHX-sensitive phase of COX-2 induction. Indeed, their synthesis is induced by LPS in RAW264 macrophages, although C/EBPβ is already present at appreciable levels in unstimulated cells. The finding that forskolin does not significantly increase either C/EBPβ or C/EBPδ levels and that treatment with U0126 plus SB 203580, which abolishes COX-2 induction, also inhibits the

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3 S. Morton and P. Cohen, unpublished results.
LPS-mediated induction of C/EBPβ, hence abolishing the binding of C/EBPβ/C/EBPβ heterodimers to the COX-2 promoter, are consistent with the idea that C/EBPβ and C/EBPβ are essential to mediate the second phase of LPS-mediated COX-2 induction in macrophages.

While C/EBPβ levels are very low in unstimulated cells, C/EBPβ is already present at appreciable levels before LPS treatment and appears to play an obligatory role both in the initiation and in the maintenance of COX-2 gene activation, since both phases are profoundly impaired in C/EBPβ-deficient macrophages. Although C/EBPβ transcriptional activity can be increased by phosphorylation (41, 42), no data are available describing specific phosphorylation events occurring in LPS-treated macrophages and, in preliminary experiments, we could not detect phosphorylation taking place following LPS treatment. We therefore favor the idea that C/EBPβ is already active in unstimulated cells but is either unable to bind to the promoter prior to CREB and/or NF-κB activation or is not sufficient on its own to initiate transcription.

The model depicted in Fig. 7 describes the potential interplay of activated and inducible transcription factors taking place at the level of the COX-2 promoter in macrophages before and after LPS treatment. Although no data concerning promoter occupancy in vivo are available, at least in vitro both the CRE/E-box and the C/EBP elements can be already occupied under unstimulated conditions by preexisting members of the CREB/ATF and C/EBP families, respectively (Fig. 7A). The activation of the MAPK and SAPK pathways by LPS results, among other events, in phosphorylation of CREB and in improved NF-κB activity, and the activation of the IκB kinase complex by MAPK/extracellular signal-regulated kinase kinase-1 leads to phosphorylation and degradation of IκB and hence to the migration of NF-κB to the nucleus. Both phosphorylated CREB and NF-κB are known to recruit histone acetylases such as CBP/p300, which in turn contribute to making the promoter more accessible to transcription factors and help in bridging the transcription factor-CBP complexes to components of the basal transcription machinery (43, 44). These changes are likely to allow preexisting C/EBPβ to bind more stably to its recognition site, adjacent to the CRE. This step is required to initiate transcription (Fig. 7B), perhaps because C/EBPβ can also bind to CBP/p300, thus stabilizing its interaction with the promoter (45). At the same time, LPS also triggers transcriptional induction of the C/EBPβ and α genes through still uncharacterized mechanisms (see below). During later phases of induction, CREB phosphorylation subsides, and NF-κB is sequestered back into the cytoplasm by newly synthesized IκB, but at this stage, more C/EBPβ and newly made C/EBPβ are present and capable of interacting with the promoter (Fig. 7C). Either their increased abundance or, more likely, the availability of C/EBPβ/C/EBPβ heterodimers in addition to C/EBPβ homodimers is able to overcome the need for additional factors binding to the CRE element, perhaps even through direct or indirect interactions with the CRE site itself as proposed previously (10, 15). In addition, other transcription factors able to bind to the CRE site, such as the upstream stimulating factor-1 or -2 and members of the activating protein-1 family, may also come into play either in the initial phases or once the promoter has been activated (13, 18, 21).

The induction of C/EBPβ and α appears to be regulated differentially in RAW264 macrophages. Both genes can be induced by a variety of stimuli in different cell types and particularly by proinflammatory cytokines and LPS (36). In hepatocytes, the transcription factor STAT3 is thought to be involved in activating both genes in response to interleukin-6 (46–48), and C/EBPβ gene transcription has been proposed to be regulated by CREB/ATF factors in hepatocytes as well as in the promonocytic cells U937 (49, 50). In RAW264 cells, C/EBPβ induction appears not to require CREB, since it is not affected by the treatments that abolish CREB activation (28). In contrast, CREB may well be involved in the induction of the C/EBPβ gene in LPS-treated cells, since the same treatments that inhibit CREB phosphorylation also abolish C/EBPβ induction. However, the induction of C/EBPβ also requires distinct as yet unidentified newly synthesized factors, since it is abolished by CHX.

Interestingly, although the relative abundance of C/EBPβ and α appeared to be at least equivalent after LPS induction, C/EBPβ binding to the COX-2 promoter was only observed as part of a heterodimer with C/EBPα. This suggests that C/EBPβ homodimers and/or C/EBPβ/C/EBPα heterodimers may display a higher affinity for this site. Interestingly, no binding of C/EBPβ to this site was detected in C/EBPβ−/− macrophages despite appreciable levels of protein being present, supporting the idea that C/EBPβ may be unable to bind as a homodimer to the COX-2/C/EBPβ site (29). This might also explain why C/EBPβ cannot compensate for the absence of C/EBPβ in the mutant cells. Since C/EBPβ induction is abolished by the same treatments that inhibit CREB activation, we could not establish whether C/EBPβ, in conjunction with C/EBPβ, would be sufficient to bypass the need for CREB and/or NF-κB activation. However, recent data suggest that this might be the case, since co-transfection of C/EBPβ, but not of C/EBPβ, could activate transcription of a COX-2 reporter in the absence of LPS treatment (16).

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