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The Response of Secondary Genes to Lipopolysaccharides in Macrophages Depends on Histone Deacetylase and Phosphorylation of C/EBPβ

Neus Serrat, Carlos Sebastian, Selma Pereira-Lopes, Lorena Valverde-Estrella, Jorge Lloberas, and Antonio Celada

LPS induces the expression of NO synthase 2 (nos2) in macrophages. The expression of this molecule is one of the hallmarks of classical activation. In this paper, we describe that trichostatin A (TSA), which inhibits deacetylase activity, blocks LPS-dependent nos2 expression. TSA specifically inhibits LPS-dependent genes of secondary response, which require new protein synthesis for their induction but not those belonging to the primary response, which do not depend on this process. Deacetylase activity acts at the transcriptional level because RNA polymerase II was not bound after LPS stimulus when we added TSA. A link between the global acetylation caused by HDAC inhibitor and gene promoter recruitment of CDK8 was found. This Mediator complex subunit associates with Med 12, Med13, and cyclin C to form a submodule that is a transcriptional negative regulator. We also found that TSA reduces C/EBPβ phosphorylation without affecting its binding to DNA. Taken together, these results shed light on the molecular mechanisms involved in the transcriptional regulation of LPS-treated macrophages and on how TSA targets critical LPS-induced genes, such as nos2 and inf-α, in inflammatory macrophage response. The Journal of Immunology, 2014, 192: 000–000.

Macrophages are part of the innate immunity system, and they play a critical role in host defense mechanisms. Under the effect of cytokines or bacterial products, such as LPS, macrophages are activated and undergo a series of biochemical, morphological, and functional modifications (1). LPS or Th1-type cytokines, such as IFN-γ, induce proinflammatory activity that we call classical activation (2). This activity is characterized by the expression of NO synthase 2 (NOS2) and the biosynthesis and release of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6. NOS2 is required for the production of NO (3). Sustained NO production endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, or fungi, although it also has deleterious effects on host cells and therefore must be tightly regulated (4, 5).

Transcription is a highly controlled process that requires the orchestration and coordination of many elements involved in the activation of transcription factors with subsequent DNA binding and in the recruitment of general transcription machinery as well as in chromatin remodeling. It is now accepted that there is a gene-specific and timing-dependent order of events that links chromatin structure modifications and transcription activation. Chromatin-modifying enzymes mark histone residues and change nucleosome conformation, allowing the transcriptional machinery to transcribe or repress genes (6). Histone acetyl-transferases (HATs) promote acetylation of histone proteins (particularly H3 and H4), which leads to relaxed chromatin structure and favors transcription. In contrast, the recruitment of histone deacetylases (HDACs) causes deacetylation of histone residues, chromosomal condensation, and gene repression (7, 8). However, microarray analysis of HDAC-deficient cells has shown that deacetylase activity can also be required for the transcription of some genes (9–11). Moreover, in the past decade, a growing number of non-histone acetylated proteins have emerged. Including transcription factors, coactivators, general factors, metabolic enzymes and chaperones, this list provides a myriad of new histone-independent regulatory mechanisms to explain the effects of HATs and HDACs on transcription (12).

HDAC inhibition has a selective effect on innate immune cells which control the function of Th1 effectors (13–15). By microarray analysis, it has been demonstrated that HDAC inhibitors have differential effects on LPS-activated macrophages, acting as repressors or activators of gene expression (13–15). HDAC inhibitors have recently been reported to downregulate the expression of numerous host defense genes of macrophages and dendritic cells stimulated with TLR agonists, thereby increasing susceptibility to bacterial and fungal infections but conferring protection against toxic and septic shock (16). In a previous study, we found that deacetylase activity is necessary for STAT5-dependent GM-CSF functional activity in macrophages (17). Moreover, cell treatment with trichostatin A (TSA), a potent pan-inhibitor of HDACs, prevents gene induction of STAT5 target genes such as Cis, a member of the suppressor of cytokine signaling (SOCS) family.

In this study, we sought to determine the involvement of HDACs in LPS-induced macrophage activation. Recently, LPS-induced genes have been divided into two groups on the basis of their

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The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; HAT, histone acetyl-transferase; HDAC, histone deacetylase; ISRE, IFN-stimulated response element; MKP-1, MAPK phosphatase-1; NC2, negative control 2; NOS2, NO synthase 2; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; TAF, TATA-binding protein–associated factor; TBP, TATA-binding protein; TSA, trichostatin A; TSS, transcription start site; VPA, valproic acid.

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requirement for new protein synthesis. Those belonging to the primary response are generally induced within 1 h of stimulation, whereas the induction of secondary response genes is delayed because of the requirement for new protein synthesis and chromatin remodeling at their promoters (18).

Treatment of macrophages with TSA downregulates the expression of secondary but not primary genes induced by LPS. To determine the molecular mechanisms behind the transcriptional inhibition, we analyzed the expression of nos2 in untreated cells and of nos2 promoter. RNA polymerase II did not bind after LPS stimulation when TSA was present. Surprisingly, we detected a link between global acetylation caused by the HDAC inhibitor and gene promoter recruitment of CDK8. This Mediator complex subunit associated with Med12, Med13, and cyclin C to form a submodule that can act as a transcriptional negative regulator (19, 20). Finally, C/EBPβ binding to nos2 promoter was not affected by TSA treatment, but TSA inhibited p38, which is responsible for the phosphorylation of several molecules. The lack of phosphorylation is probably the signal required for CDK8 recruitment to the Mediator complex and, as a consequence, for the inhibition of nos2 transcription when TSA is present.

Materials and Methods

Reagents

LPS was purchased from Sigma-Aldrich. In several experiments, the results obtained with commercial LPS were compared with highly purified LPS from Salmonella abortus equi, provided by Dr. C. Galanos (Max Planck Institute, Freiburg, Germany) (21). No differences were found between the two. TSA and valproic acid (VPA) were from Sigma-Aldrich. The concentrations of TSA and VPA used did not affect macrophage viability (>92% cell recovery after 24 h). The Abs used were as follows: anti-phospho-C/EBPβ (Thr212) (Cell Signaling Technology); anti-TATA-binding protein (TBP) (Abcam) (ab818); anti-β-actin (Sigma-Aldrich), anti-HDAC1, and anti-acetyl histone H3 (Upstate Biotechnology); and HDAC inhibitor and gene promoter recruitment of CDK8. This Mediator complex subunit associated with Med12, Med13, and cyclin C to form a submodule that can act as a transcriptional negative regulator (19, 20). Finally, C/EBPβ binding to nos2 promoter was not affected by TSA treatment, but TSA inhibited p38, which is responsible for the phosphorylation of several molecules. The lack of phosphorylation is probably the signal required for CDK8 recruitment to the Mediator complex and, as a consequence, for the inhibition of nos2 transcription when TSA is present.

Cell culture

Bone marrow–derived macrophages were isolated from 6-wk-old BALB/c mice (Charles River Laboratories, Wilmington, MA), as described previously (22). After 7 d of culture, a homogeneous population of adherent macrophages was obtained (>99% Mac-1+). To synchronize the cells, when they were 80% confluent they were deprived of M-CSF for 16–18 h before being subjected to the treatments. Animal use was approved by the Animal Research Committee of the University of Barcelona (number 2523).

RNA extraction and real-time RT-PCR

RNA was extracted with Tri Reagent from Sigma-Aldrich, as described previously (26). Insoluble material was removed by centrifugation at 13,000 × g for 8 min at 4˚C. Cell lysates (50–100 µg) were boiled at 95˚C in Laemli SDS-loading buffer and separated by 10% SDS-PAGE (unless otherwise stated). The primers used were of commercial origin (eBioscience).

Western blot analysis

The nonparametric Wilcoxon test for paired differences was used in all statistical comparisons (27). In our experiments, we used bone marrow–derived macrophages, a homogeneous population of primary and quiescent cells. Treatment of these cells with certain cytokines causes several modifications that allow them to develop their functional activities (1).

Chromatin immunoprecipitation assay

This assay was performed as described previously (25). In brief, cells were cross-linked with paraformaldehyde and lysed. Lysates were sonicated on ice, and the size of fragments obtained (between 200 and 1200 bp) was confirmed by electrophoresis. Soluble chromatin was collected and pre-cleared with salmon sperm, and then unspecific IgGs and preimmune serum and protein A–Sepharose were added. After incubation overnight, the samples were centrifuged. The supernatants were then collected, and specific Abs were added. A control was performed with unspecific IgGs. Mixtures were incubated in rotation, and then protein A–Sepharose was added. Beads were collected and washed. Immunoprecipitates were eluted three times. Reversion of cross-linking was performed overnight by heating samples and input controls at 65˚C, and DNA was purified. Real-time PCR was performed as described above. The primers used were nos2 transcription start site (TSS) (−139 to +38 bp); nos2 (IFN-stimulated response element [ISRE] boxes) (−765 to −696 bp); nos2−2.6 kb (−2.600 to 2.494 bp); and nos2 exon 22 (+36,611 to +367,24 bp), IL-1β TSS (−107 to +35 bp), IL-6 ISRE box (−372 to −158 bp), IL-1β TSS (−127 to −48 bp), and nef TSS (−132 to +35 bp). These primers are shown in Table II.

Statistical analysis

The nonparametric Wilcoxon test for paired differences was used in all statistical tests (27).

Results

HDAC inhibition has distinct effects on LPS-induced genes

In our experiments, we used bone marrow–derived macrophages, a homogeneous population of primary and quiescent cells. Treatment of these cells with certain cytokines causes several modifications that allow them to develop their functional activities (1).
nos2 course experiments were performed. Determination of gene expression primary response genes with distinct kinetics, time mined after 6 h of LPS treatment, to exclude that TSA inhibits the secondary genes. Because the expression of the genes was determined after 6 h of LPS treatment, to exclude that TSA inhibits the expression primary response genes with distinct kinetics, time course experiments were performed. Determination of gene expression up to 2 h of LPS treatment showed that TSA blocked nos2, il-6, and il-12 but not il-12p40 or il-1b (Fig. 2). Deacetylase activity is required for LPS-dependent nos2 expression Because of the critical role of NO production in the immune response (3), we determined the mechanism of TSA inhibition using nos2 as model of secondary response gene. To test whether deacetylase activity has any functional relevance in the control of macrophage activation, we treated these cells with TSA, a potent inhibitor of class I and II HDACs, for 2 h before the addition of LPS. Global acetylation caused by TSA had a distinct impact on LPS-induced gene expression. Although il-6, il-12 (p40), nos2, cspg, marco, mx1, and lipg were downregulated, showing that HDAC activity is necessary for their correct expression, tfn-a, il-1b, iff1, sod2, fos, and socs3 were not affected (Fig. 1). Interestingly, the genes unaffected by TSA are primary response secondary genes, whereas those repressed by this inhibitor are secondary genes. Because the expression of the genes was determined after 6 h of LPS treatment, to exclude that TSA inhibits the expression primary response genes with distinct kinetics, time course experiments were performed. Determination of gene expression up to 2 h of LPS treatment showed that TSA blocked nos2, il-6, and il-12 but not il-12p40 or il-1b (Fig. 2). Deacetylase activity is required for LPS-dependent nos2 expression Because of the critical role of NO production in the immune response (3), we determined the mechanism of TSA inhibition using nos2 as model of secondary response gene. To test whether deacetylase activity has any functional relevance in the control of NO production, we treated macrophages with TSA for 2 h before the addition of LPS. Under our experimental conditions, cells subjected to TSA treatment produced less NO in response to LPS than controls (Fig. 3A). To confirm the findings obtained with TSA, we tested the effects of another HDAC inhibitor, namely VPA, a short-chain fatty acid. A significant inhibition of NO production was detected, thus confirming previous observations (16).

NO is generated by the enzyme NOS2, which catalyzes l-arginine conversion to NO and l-citrulline. Macrophages do not express nos2 under basal conditions. After treatment with LPS, nos2 increased in these cells (Fig. 3B). This increase was followed by a rise in protein content (Fig. 3C). An analysis of both NOS2 protein and mRNA showed a marked decrease after TSA or VPA treatment (Fig. 3B, 3C), thereby indicating that the observed reduction of NO is caused by nos2 downregulation. As expected, IL-6 expression was induced by LPS and reduced by TSA or VPA treatment (Fig. 3G). However, the HDAC inhibitors did not affect the induction of TNF-a by LPS (Fig. 3D, 3F). IL-12 is a secondary response gene to LPS, and as expected, both TSA and VPA decreased the induction mediated by LPS (Fig. 3E).

**TSA treatment does not alter the NF-kB pathway**

NF-kB is a transcription factor that has a critical role in inflammation and immune response. NF-kB complexes are retained in the cytoplasm by I-kB inhibitor. In response to LPS, I-kB is phosphorylated, triggering its ubiquitination and proteosomal degradation (29). Free NF-kB then translocates to the nucleus where it binds to target sequences. NF-kB plays a central role in the control of nos2 transcription (30). To detect NF-kB nuclear translocation, we used Abs against the subunit p65 (Rela) because the dimer p50/p65 is the most abundant form of NF-kB. In untreated cells, as expected, most p65 was detected in the cytoplasm and a minor fraction in the nucleus. After LPS stimulation, p65 was located mostly in the nucleus. This distribution was not altered by treatment with LPS and TSA (Supplemental Fig. 1A).

The in vivo binding of p65 to the nos2 promoter was analyzed. This promoter holds two binding sites for NF-kB. The first one is located near the TATA box in an LPS responsive region at −86 and −76 bp (31), whereas the second is more upstream between −972 and −960 bp in the IFN-γ-responsive region. Chromatin immu-

### Table I. Primers used for real-time RT-PCR of mRNA

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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>cspg</td>
<td>5'-ACTAACCACCCTAATGCACTATCGAG-3'</td>
<td>5'-ACCTTTCCAGACAGAGAAGGCTT-3'</td>
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<td>fos</td>
<td>5'-AAATGGAAGACGCTGTCGAGGA-3'</td>
<td>5'-CCTTTCGAGTTCTGCTTCTCT-3'</td>
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<td>il-12</td>
<td>5'-GAACAGCAGAGCACGAGAATA-3'</td>
<td>5'-AATGGAAGAAGAGTAAGAATTGC-3'</td>
</tr>
<tr>
<td>il-1b</td>
<td>5'-TGGCGCTCAGGAGAAAGAGAA-3'</td>
<td>5'-CAGCGTTGTCGCTTTGCT-3'</td>
</tr>
<tr>
<td>il-6</td>
<td>5'-CCACAGAGCCACGAGAATGATGGA-3'</td>
<td>5'-ACCTCAAGAGACAGAGAATG-3'</td>
</tr>
<tr>
<td>iff1</td>
<td>5'-AGAGTGCATTATCCCGCAGACACTT-3'</td>
<td>5'-CCATATTCCAGGCTGCAACCA-3'</td>
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<td>mx1</td>
<td>5'-GACACATAGGGTCCTCCTGACCAA-3'</td>
<td>5'-AGACTTGGCTTTTCTGAAAAAGC-3'</td>
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<tr>
<td>nos2</td>
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<td>5'-CGTACCGATGACTGTTGAAAT-3'</td>
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<tr>
<td>socs3</td>
<td>5'-ATGCGTCACCCACAGACAGTTG-3'</td>
<td>5'-TCCACAGAAATACCTGCTTCT-3'</td>
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<td>sod2</td>
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<td>5'-GTCATCTCTTGCACAAACTCTGAC-3'</td>
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<td>tfn-a</td>
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<td>5'-CACCTGGGTTGTGTTGCTCACG-3'</td>
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<tr>
<td>cxc11</td>
<td>5'-Gcaccacaaccagcaagctgata-3'</td>
<td>5'-Tgtcagaaccagcgctttca-3'</td>
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<tr>
<td>M12β</td>
<td>5'-CAGCAACAGCGTCTTGATG-3'</td>
<td>5'-AGCCAGCCGAGCTAATCAT-3'</td>
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### Table II. Primers used for ChIP assay

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<th>Gene (Sequence Position)</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<td>nos2 TSS (−139 to +38 bp)</td>
<td>5'-TGAATAGATGCACTAATGCAACAC-3'</td>
<td>5'-GGCCAGAGCTCTGAGTCTGTGCTCTTCAAC-3'</td>
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<tr>
<td>nos2 ISRE max (−765 to −606 bp)</td>
<td>5'-TACCCCACACCAACTCGAGAAGTCA-3'</td>
<td>5'-GTTGGCGTCAGTACAGGGCTT-3'</td>
</tr>
<tr>
<td>nos2 −2.6 kb (−2,600 to −2,494 bp)</td>
<td>5'-CAGTCTGGCCTCAAGTGGTACT-3'</td>
<td>5'-GGCACCAGCGCTTTCTGAACT-3'</td>
</tr>
<tr>
<td>nos2 exon 22 (+36,611 to +36,724 bp)</td>
<td>5'-CAGTCTGGCCTCAAGTGGTACT-3'</td>
<td>5'-GGCACCAGCGCTTTCTGAACT-3'</td>
</tr>
<tr>
<td>il-1β TSS (−107 to +35 bp)</td>
<td>5'-CTGCGAGGAGTCCACAGATGCAA-3'</td>
<td>5'-CCAAAGAAGAATTTGCAACG-3'</td>
</tr>
<tr>
<td>Il-1β ISRE box (−372 to −158 bp)</td>
<td>5'-CTGCGAGGAGTCCACAGATGCAA-3'</td>
<td>5'-CCAAAGAAGAATTTGCAACG-3'</td>
</tr>
<tr>
<td>il-6 TSS (−127 to +48)</td>
<td>5'-CACCCACGCCACCTCTCAGGAAG-3'</td>
<td>5'-GCAGAGGGACACCTCAAGGCG-3'</td>
</tr>
<tr>
<td>tfn-a TSS (−132 to +35)</td>
<td>5'-CCGCTTCTCCCTTACAGGATC-3'</td>
<td>5'-CCTTCTGCTAGTCTCTGGCTG-3'</td>
</tr>
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</table>
noprecipitation (ChIP) experiments showed an increase in p65 binding after the LPS treatment. This increase was not modified by TSA (Supplemental Fig. 1B). The specificity of the reactions was checked using unrelated Abs. Amplification of the c-jun coding region also was used as a control of nonspecific immunoprecipitation. Data were expressed as relative immunoprecipitation related to the level of c-jun amplification in each sample. In subsequent experiments in which we used this technique, similar controls were performed. These results suggest that nos2 down-regulation by TSA is not due to an effect on NF-kB.

**TSA treatment blocks nos2 transcription by preventing RNA polymerase II recruitment**

To determine the effect of TSA treatment on transcription, we tested RNA polymerase II recruitment on the nos2 promoter. ChIP experiments showed that, as expected, LPS-activated macrophages induced the binding of RNA polymerase II to the TSS (Fig. 4A). However, when we used the HDAC inhibitor together with LPS, this binding did not occur, thereby pointing to the inhibition of transcription. Similar results were found when we used the promoter of il-6 (Fig. 4A). As examples of genes unaffected by TSA treatment, we used il-1β and tfn-α. In these cases, LPS induced an increase in RNA polymerase II binding to the promoter. This increase was unaffected when we added TSA to the LPS-treated macrophages (Fig. 4B). These results emphasize the specific nature of TSA actions.

**HDAC inhibition aborts preinitiation complex formation after TBP binding**

RNA polymerase II requires transcription factors to recognize target promoters. TFIID is composed by the TBP complexed with at least 10 TBP-associated factors (TAFs) (32). TFIID, through TBP and TAFs, makes a key contribution to promoter recognition and binds to distinct DNA elements present in the core promoter. TFIIB binds to the TFIID–promoter complex through both DNA recognition and direct contacts with TFIID. The assembly of TFIIB is an absolute requirement for the recruitment of RNA polymerase II, which enters the preinitiation complex prebound to TFIIF. In promoters containing TATA box, such as nos2, TBP recognition is sufficient to initiate preinitiation complex formation and activate transcription (33). To assess the influence of TSA in preinitiation complex formation, we performed ChIP experiments with Abs against general transcription factors. The activation of macrophages with LPS induced an increase in the binding of the TBP not affected by the TSA treatment to the nos2 promoter (Fig. 5B). Because HATs and HDAC activities control DNA accessibility to transcriptional machinery, this result rules out that chromatin structure impairs preinitiation complex nucleation. As expected, the binding of TFIIB was induced by LPS treatment. However, the concomitant treatment with TSA blocked the binding of TFIIB to the TBP.

**FIGURE 1.** TSA does not downregulate all the genes induced by LPS in macrophages. Macrophages were cultured in the presence of LPS (10 ng/ml) alone or with TSA (20 nM) for 18 h, and the expression was analyzed by real-time PCR. Each point was performed in triplicate, and the results are shown as mean ± SD. All assays are representative of at least four independent experiments. *p < 0.01 in relation to the controls when all the independent experiments had been compared.

**FIGURE 2.** TSA downregulates secondary response genes but not primary response genes induced by LPS in macrophages. Macrophages were cultured in the presence of LPS (10 ng/ml) alone or with TSA (20 nM), and gene expression was analyzed by RT-PCR. Each point was performed in triplicate, and the results are shown as means. All assays are representative of at least four independent experiments.
the complex, and as a result, preinitiation complex formation was aborted after TBP binding (Fig. 5C). As a control, we used the promoter of il-1b. In this case, TSA did not block the binding of TBP or TFIIB to the promoter (Fig. 5D, 5E).

TSA treatment increased the binding of the Mediator module kinase CDK8 to the nos2 promoter

The transcriptional repression associated with HDAC inhibitors has been associated with the recruitment of corepressors and in particular with the induction of Mi-2β, which forms part of the Mi-2/NuRD complex (16). Under our experimental conditions, we did not observe an induction of Mi-2β (Supplemental Fig. 1C). As an alternative hypothesis, we determined the contribution of CDK8 as an inhibitor of the Mediator complex.

Mediator complexes consist of 20–30 protein subunits and weigh ∼2 megadaltons. These complexes are a key group of transcription regulatory multiprotein complexes that interact with transcription factors and transmit active and repressive information to regulate transcription (34). The Mediator complex acts as an integrator of signals and as a molecular bridge between DNA-bound transcription factors and the transcription apparatus, including RNA polymerase II.

The Mediator complex and TFIIB interact with RNA polymerase II, and they are jointly required for recruitment of the latter to the promoter. In fact, the binding of TFIIB to the preinitiation complex depends on the Mediator complex (35). Given that, TFIIB was not incorporated into the preinitiation complex of the nos2 promoter after HDAC inhibition, we hypothesized that CDK8 acts as a transcriptional repressor, as described in other genes (36, 37). CDK8 can regulate transcription by targeting the cdk7/cyclin H subunits of the general transcription initiation factor IIH. This kinase phosphorylates cyclin H, thus repressing the capacity of transcription initiation factor IIH to activate transcription (38). For these reasons, we determined the association of the Mediator complex with the nos2 promoter (Fig. 5A). Combined TSA and LPS treatment increased the binding of Mediator complex module kinase CDK8 to the TSS as well as to the ISRE (Fig. 6). Contrary to what was found with the nos2 promoter, TSA did not increase the binding of CDK8 to the il-1β promoter. Therefore, on the basis of these data, we propose that the increased recruitment of CDK8 on the nos2 promoter could

FIGURE 3. HDAC inhibitors block LPS-induced NOS2 and IL-6 but not TNF-α. (A) Bone marrow–derived macrophages were cultured for 12 h in the presence of LPS alone or with TSA or VPA (20 μM), and NO3 production was then determined. Each point was performed in triplicate, and the results of four independent experiments are shown as mean ± SD. (B) nos2 expression was analyzed by RT-PCR. Each point was performed in triplicate, and the results of four independent experiments are shown as mean ± SD. (C) NOS2 expression was analyzed by Western blot. Similar results were obtained in three independent experiments. (D) and (E) were determined by RT-PCR. Each point was performed in triplicate, and the results of six independent experiments are shown as mean ± SD. *p < 0.01 in relation to LPS when all the independent experiments had been compared.

FIGURE 4. TSA inhibits nos2 expression at the transcriptional level. Macrophages were cultured for 2 h in the presence of LPS alone or with TSA. ChIP was performed using an Ab against polymerase II (Pol II). The amplified fragments of nos2 or il-6 (A) or il-1β or tnf-α (B) promoter corresponding to the TSS were then determined by quantitative RT-PCR. Each point was performed in triplicate, and the results are shown as mean ± SD. All assays are representative of at least four independent experiments. *p < 0.01 in relation to the controls when all the independent experiments had been compared.
explain the inhibition of RNA polymerase II association with the TSS.

**TSA blocks the LPS-induced phosphorylation of p38**

The treatment of macrophages with TSA increases acetylation of the MAPK phosphatase-1 (MKP-1) (39), and, as result of this acetylation, MKP-1 enhances its interaction with p38, thereby increasing its phosphatase activity and interrupting MAPK signaling and the p38 phosphorylation necessary for nos2 expression. Recently, contradictory data have been published showing that TSA does not affect LPS-induced p38 phosphorylation (16). Under our experimental conditions, TSA significantly reduced p38 phosphorylation but not that of ERK1/2 or JNK (Fig. 7A). Moreover, the inhibition of p38 with SB203580 blocked the LPS-dependent induction of nos2 (Fig. 7B).

C/EBPβ is an intrinsically repressed transcription factor that regulates nos2 (40). This transcription factor is a repressor that becomes an activator through phosphorylation. C/EBPβ interacts with multisubunit Mediator complexes through the CRSP130/Sur2 subunit. This subunit is also associated with the proteins CRSP70 and CDK8, which make a transcriptionally active or inactive Mediator complex, respectively (41). Therefore, C/EBPβ determines differential gene activation through selective interaction with distinct Mediator complexes.

The nos2 promoter contains two CCAAT boxes located in the region of the proximal promoter at −66/−74 bp (next to the NF-κB box) and at −143/−150 bp (31). To determine whether C/EBPβ is involved in the induction of the CDK8 association and the subsequent blocking of RNA polymerase II recruitment, we measured the protein levels of C/EBPβ in cells treated with or without the HDAC inhibitor. Western blot analysis showed that TSA did not modify LPS-induced C/EBPβ expression (Fig. 7B).

We also performed Western blots using specific Abs against phosphorylated C/EBPβ at T188. We assayed several Abs but were unable to achieve a clear image. However, the quantification of the bands in several experiments showed a significant decrease of LPS-induced phosphorylation when TSA was present (Supplemental Fig. 2). Moreover, deacetylase inhibition increased the nuclear localization of C/EBPβ, as determined by immunofluorescence and confocal microscopy (Fig. 8C). A similar cellular localization was observed when cells were treated with the p38 inhibitor SB203580.

**FIGURE 5.** TSA inhibits the binding of TFIIB but not TBP to the nos2 promoter. (A) Schematic representation of the nos2 promoter with the different areas amplified in the ChIP performed assays. Macrophages were cultured for 2 h in the presence of LPS alone or with TSA. ChIP was performed using an Ab against TBP (B, D) or TFIIB (C, E). The amplified fragments of the nos2 and il-1β promoters corresponding to the TSS was then determined by quantitative RT-PCR. Each point was performed in triplicate, and the results are shown as mean ± SD. All assays are representative of at least four independent experiments. *p < 0.01 in relation to LPS treatment when all the independent experiments had been compared.

**FIGURE 6.** TSA induces the binding of the kinase module of Mediator complex CDK8 to the nos2 promoter but not to the il-1β promoter. Macrophages were cultured for 2 h in the presence of LPS alone or with TSA. The fragments of the nos2 promoter amplified are outlined in Fig. 5A. ChIP was performed using an Ab against CDK8. The amplified fragments of the ISRE box and TSS box of the nos2 and il-1β promoters were determined by quantitative RT-PCR. Results are calculated as fold increase of CDK8 binding in the presence or absence of TSA. Each point was performed in triplicate, and the results are shown as mean ± SD. All assays are representative of at least four independent experiments.
Thus, this observation suggests that at least part of the TSA effects are related to defective LPS-induced phosphorylation of C/EBPβ. Because C/EBPβ activation controls the change from CDK8–Mediator to core–Mediator interaction, this could explain the CDK8 enrichment in the nos2 promoter and thus the reduced expression of nos2 after LPS treatment. 

**hdac1 silencing reproduces TSA effects**

To identify the HDAC involved in the LPS-induced expression of NOS2, we used small interfering RNA (siRNA) technology to silence the expression of some class I HDACs (1–3, 8, 10). LPS induced the expression of HDAC1 or HDAC8, but fail to exert this effect when cells were transfected with their respective siRNA (Supplemental Fig. 3A, 3B), thereby showing the specificity of the gene silencing. Inhibition of HDAC1 led to a reduction of LPS-induced NOS2 and IL-6 (Supplemental Fig. 3C). No decrease in HDAC1 levels was observed when we used unspecific siRNA (siGL3), electroporated cells (mock control), or the siRNA against HDAC8 (data not shown). Finally, the siRNA against HDAC1 did not reduce the levels of LPS-induced IL-1β or TNF-α (Supplemental Fig. 3C). These results suggested that HDAC1 is the main deacetylase required for the proper expression of the LPS-induced genes that are sensitive to the effect of TSA.

**Discussion**

The acetylation of nuclear proteins was first detected in histone residues, and this process is considered part of the mechanism that allows DNA to become accessible to the transcription regulatory machinery (42). However, it is now recognized that protein acetylation is a more extended modification, affecting an enormous variety of nuclear and cytosolic proteins. In this paper, we provide evidence that deacetylase activity is required for LPS-dependent
induction of secondary response genes involved in macrophage classical activation, such as nos2, il-12 (p40), mx1, vcan, marco, and il-6. Nevertheless, the requirement of deacetylase activity is not a general mechanism, and TSA does not affect the expression of primary response genes. Although new protein synthesis is required for secondary response genes, it is not needed for the induction of genes belonging to the primary response.

In this study, we have shown that macrophage treatment with TSA or VPA decreases NO production in response to LPS, downregulating NOS2 expression both at the mRNA and protein level. Similar results were obtained with secondary response genes such as il-12 or IL-6 but not with the primary tnf-α at the level of mRNA or protein, thereby confirming the results of Roger et al. (16).

We have also demonstrated that TSA abolishes stimuli-dependent binding of RNA polymerase II to the nos2 or il-6 promoter but not to the promoters of il-1β or inf-α, both primary response genes. Signal-dependent recruitment of RNA polymerase II to promoters of target genes is one of the key regulated steps in inducible gene expression. However, detailed analysis of several model genes and genome-wide studies of polymerase II occupancy have demonstrated that in some cases RNA polymerase II is recruited in the absence of gene expression. Genes that belong to this group have preassembled RNA polymerase II, and they are controlled at the level of transcriptional elongation and mRNA processing through the signal-dependent recruitment of P-TEFb (18, 43). Interestingly, none of the primary response genes analyzed in this study was affected by HDAC inhibitors. This observation suggests that acetylation caused by HDAC inhibition impairs signal-dependent recruitment of RNA polymerase II in secondary genes.

Eukaryotic polymerases require general transcription factors to recognize and bind the TSS. In the nos2 promoter, TBP is bound after stimulation, thereby allowing nucleation of the preinitiation complex. We found that this process is not modified by TSA, because TBP associates with the TSS. This observation suggests that chromatin structure does not interfere with the formation of the preinitiation complex. Nevertheless, we have shown that TFIIB entry to the complex is abrogated in cells treated with TSA. This effect could be related to repressors, such as negative control 2 (NC2), that bind TBP, thus preventing the association with TFIIB (18, 43). Interestingly, NC2 is a complex composed of two subunits Dr1 and Drl, the latest is the repressor effect subunit (46). Although TSA does not increase Dr1 expression (data not shown), Dr1 activity may be regulated by other mechanisms. Therefore, at present, we cannot exclude that TSA represses nos2 expression through NC2 recruitment to the promoter.

It recently has been reported that HDAC inhibitors induce the expression of Mi-2β and the activity of the Mi-2/NuRD complex, which acts as a transcriptional repressor (16). In our experiments, mi2β was not upregulated by TSA or VPA. These contradictory results may be explained by the different experimental designs. We used 10 ng/ml LPS, a concentration that saturates the binding of LPS to its high-affinity receptor CD14 (47), whereas Roger et al. (16) used a different source of LPS at 100 ng/ml, which may have activated CD14-independent signaling pathways.

The association of TFIIB with gene promoters has also been related to the Mediator complex (48). Both TFIIB and the Mediator complex interact with RNA polymerase II and are jointly required for recruitment of the polymerase to the promoter. TFIIB recruitment is dependent on the Mediator complex, whereas the recruitment of the complex is independent of TFIIB. Using the ChIP technique with Abs against Med1 and Med 17 subunits, we determined that the recruitment of the Mediator complex was altered after TSA treatment (data not shown). However, upon TSA and LPS treatment, the CDK8 subunit showed a strong increase in the nos2 promoter but not to that of il-1β. The CDK8 subunit associates with cyclin C, Med12, and Med13 to form a Mediator complex submodule that has been characterized as a transcriptional repressor (20, 36, 37). In vitro assays have shown that the addition of the CDK8 subcomplex shuts down the transcriptional activity driven by several transactivators (38, 49–51). This repression occurs independently of kinase activity. CDK8 acts by preventing RNA polymerase II incorporation into the preinitiation complex on chromatin templates. Using electron microscopy, it was shown that CDK8 binds to the polymerase II–binding cleft of the Mediator complex, where it sterically blocks the interaction with the polymerase (52).

The transcription factor C/EBPβ represses the transcription of some genes by recruiting the Mediator complex with the kinase module to promoters (53). According to this model, C/EBPβ phosphorylation might function as a switch that triggers the release of the CDK8 module from the Mediator complex and later induces transcriptional activation. CCAAT boxes have been described in most of the promoters whose genes are downregulated by TSA. In the proximal promoter, nos2 has two CCAAT boxes, and we have found that C/EBPβ binds after LPS stimulus. We have observed that although TSA does not alter DNA binding, it reduces C/EBPβ phosphorylation in Thr188. In fact, it has been described that C/EBPβ phosphorylation in Thr188 by MAPK interacts with the Mediator complex, which also associates with RNA polymerase II and activates transcription (41). Indeed, reduced C/EBPβ phosphorylation may be responsible for the CDK8–Mediator complex binding that prevents RNA polymerase II entrance to the preinitiation complex and as consequence reduces nos2 transcription after TSA treatment.

Furthermore, C/EBPβ is a direct substrate of p38 kinase (54–56). The treatment of macrophages with TSA increases acetylation of the MKP-1 (39), and as result of this acetylation, MKP-1 enhances its interaction with p38, thereby increasing its phosphatase activity and interrupting MAPK signaling and p38 phosphorylation. Our results confirm the observation of Cao et al. (39), who showed that TSA inhibits the phosphorylation of p38 but not that of MAPKs, ERK1/2 or JNK. In addition, we confirm the requirement of p38 phosphorylation for nos2 induction by LPS. These lines of evidence support our hypothesis that the phosphorylation of C/EBPβ is a crucial signal required for LPS-induced nos2 transcription and that TSA, through MKP-1 acetylation, reduces this phosphorylation and abrogates nos2 transcription. The proposed model of TSA inhibition of nos2 is shown in Supplemental Fig. 4.

On the basis of all these data, we can explain why, in the presence of TSA, RNA polymerase II is not recruited to the nos2 promoter or to other secondary LPS response gene promoters and is not affecting its binding to the primary response genes. RNA polymerase II binding appears to be dependent on TFIIB binding to the promoter, and it is abrogated when CDK8 interacts with the Mediator complex in parallel with the decrease in C/EBPβ phosphorylation.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Material

The response of secondary genes to LPS in macrophages depends on histone deacetylase and phosphorylation of C/EBPβ

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SUPPLEMENTAL FIGURE 1. TSA treatment does not alter the NF-κB pathway. (A) p65 was determined by Western blot using cytosolic and nuclear extracts. (B) Chromatin immunoprecipitation was performed using an antibody against p65. The amplified fragment of nos2 was then determined by quantitative RT-PCR. Each point was performed in triplicate, and the results are shown as mean ± SD. (C) TSA does not increase the expression of mi2β induced by LPS in macrophages. Macrophages were cultured in the presence of LPS (10 ng/ml) alone or with TSA (20 nM) for 3 h, and the expression was analyzed by real-time PCR. Each point was performed in triplicate, and the results are shown as mean ± SD. The results are representative of six independent experiments.
SUPPLEMENTAL FIGURE 2. TSA reduces the phosphorylation of C/EBPβ. Macrophages were treated for the indicated times with LPS or LPS and TSA. The phosphorylation of C/EBPβ was determined by Western blot. At the bottom, there is the quantification of 5 experiments expressed as mean ± SD. *p<0.01 in relation to the LPS when all the independent experiments had been compared.
SUPPLEMENTAL FIGURE 3. Silencing of hdac1. (A) Macrophages were transfected with sigl3 (5’AACUUACGCUGAGUACUUCGAdTdT3’), Sihdac1 (5’GUGCUGUGAAGCUUAAUAAUTT3’) or sihdac8 (5’GGACGGUACUACAGUGUCAAA 3’) as described (25). Mock indicates cells electroporated without RNA. We used sigl3 as an unrelated RNA. Silencing efficiency of hdac1 was determined by Western blotting using antibodies against HDAC1. (B) Macrophages were obtained as in (A) and incubated with LPS for 18 h. hdac1 and hdac1 were determined by quantitative RT-PCR. The results are shown as mean ± SD of four independent experiments. *p<0.01 in relation to siGL3 LPS treatment. (C) Inhibition of hdac1 shows a similar effect to that of TSA on secondary and primary response genes induced by LPS in macrophages. The effect of siRNA against hdac1 was tested on the
induction of nos2, il-6, il-1β and tnf-α. The effect of TSA was also shown. Each point was performed in triplicate, and the results are shown as mean ± SD. All assays are representative of at least four independent experiments. *p<0.01 in relation to the controls when all the independent experiments had been compared.

SUPPLEMENTAL FIGURE 4. Schematic representation of the TSA inhibition of LPS-dependent nos2 induction. LPS induces the translocation of NF-kB (p50 and p65) to the nucleus and brings about the p38 phosphorylation, which in turn activates C/EBPβ. This releases the CDK8 complex and allows the Mediator complex to induce the binding of TBP and the Pol II, which will start transcription. By inhibiting the phosphorylation of p38, TSA blocks C/EBPβ phosphorylation. Then the binding of CDK8 to DNA is not released, and the mediator complex is unable to induce Pol II activation and transcription.