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# Inhibition of LPS-Induced C/EBPδ by Trichostatin A Has a Positive Effect on LPS-Induced Cyclooxygenase 2 Expression in RAW264.7 Cells

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## ABSTRACT

Cyclooxygenase 2 (COX-2) is an important inflammatory factor. Previous studies have indicated that COX-2 is induced with lipopolysaccharide (LPS) treatment. Here, we found that an inhibitor of histone deacetylase (HDAC), trichostatin A (TSA), cannot repress LPS-induced COX-2 but it increased the COX-2 level in RAW264.7 cells. We found no significant difference in NF-κB activation and ERK1/2 phosphorylation, but LPS-induced C/EBPδ expression was completely abolished after TSA treatment of LPS-treated cells. Interesting, reporter assay of C/EBPδ promoter revealed that Sp1-binding site is important. Although there was no alteration in c-Jun levels, but the phosphorylation of c-Jun at its C-terminus was increased dramatically. A DNA-associated protein assay (DAPA) and chromatin immunoprecipitation assay (ChIP) indicated that c-Jun was recruited via Sp1 to the promoter of C/EBPδ after LPS treatment; this recruitment of c-Jun was repressed by TSA. C/EBPδ inhibition by TSA resulted in increased binding of C/EBPα and C/EBPβ to the COX-2 promoter. Therefore, TSA has a positive effect on LPS-induced COX-2 since it decreases the C/EBPδ level by reducing c-Jun recruitment by Sp1 to the C/EBPδ promoter, resulting in increased the recruitment of C/EBPα and C/EBPβ to the COX-2 promoter. J. Cell. Biochem. 110: 1430–1438, 2010. 

**KEY WORDS:** CYCLOOXYGENASE 2; TRICHOSTATIN A; C/EBPΔ; C/EBPα; C/EBPβ

Trichostatin A (TSA) is an antifungal antibiotic that functions as a hydroxamate type histone deacetylase (HDAC) inhibitor by blocking the catalytic site of the substrate of HDACs [Vanhaecke et al., 2004]. TSA is involved in cellular growth arrest, cell differentiation, and apoptosis of malignant cells, it's effect alone or with other anti-cancer drugs was pre-clinically studied in breast, gastric, ovarian, and pancreatic cancers [Marks et al., 2000, 2001; Taddei et al., 2005]. The studies revealed that most tumor suppressor genes are induced, but some others are repressed by TSA treatment [Murphy et al., 1999]. Several recent reports also report that TSA is associated with inflammation, and most of these studies conclude that TSA inhibited inflammation by attenuating NF-κB activation [Usami et al., 2008]. Cyclooxygenase (COX), also known as prostaglandin G/H synthase, is the rate-limiting enzyme in the biosynthesis of prostaglandins from arachidonic acid. Two isoforms derived from distinct genes located on separate chromosomes have been characterized and are referred to as COX-1 and -2 [Reddy et al., 1999]. A third COX isoform produced as an alternate splice variant of COX-1 has recently been identified as COX-3 [Chandrasekharan et al., 2002]. COX-1 is constitutively expressed in most tissues and mediates physiological responses such as the regulation of renal and vascular homeostasis and cytoprotection of the stomach [Hoffmann

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et al., 2006]. In contrast, COX-2 is an inducible enzyme and its pathophysiological role has been linked to inflammation [Kapoor et al., 2005]. Many studies have indicated that COX-2 plays an important role in tumorigenesis [Evans and Kargman, 2004]. Other studies suggest that COX-2 expression is regulated largely at the transcription level by cytokines, growth factors, and tumor promoters [Kujubu et al., 1991; Xie and Herschman, 1996; Newton et al., 1997]. The COX-2 promoter contains a TATA box and many putative transcription factors binding sites, including those of the cyclic AMP response element (CRE), E-box, nuclear factor for interleukin-6, nuclear factor-κB, Sp1, and activating protein-2 [Tanabe and Tohnai, 2002]. A previous study revealed that EGF-induced expression of COX-2 in A431 cells is mediated through the Ras-mitogen-activated protein kinase (MAPK) signaling pathway and subsequent induction of c-Jun after MAPK activation [Chen et al., 2004].

CCAAT/enhancer-binding protein δ (C/EBPδ) is a member of the conserved C/EBP-family of nuclear proteins [Ramji and Foka, 2002]. Thus far, six mammalian C/EBP family members have been identified, including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ [Ramji and Foka, 2002]. Recent studies support that C/EBPδ is related to cell cycle arrest, cell differentiation, and cell fate determination [Hutt et al., 2000]. On the other hand, in adipocyte differentiation models, C/EBPδ is expressed in pre-adipocytes before cells are committed to differentiation, and adipocyte differentiation is defective in C/EBPδ knock-out mouse embryonic fibroblasts [Belmonte et al., 2001]. In addition, in human breast cancer and acute myeloid leukemia, ''loss of function'' alterations have been found in C/EBPδ [Ishii et al., 2005]. Furthermore, previous studies indicate that the transcription factors Sp1, C/EBPβ, and C/EBPδ are important for LPS-induced IL-10 expression [Chiang et al., 2006]. According to these studies, C/EBPδ might have a positive effect on inflammation and a negative one on the subsequent tumorigenesis, but the clear-cut effect in inflammation is still unclear. In this study, we found that C/EBPδ downregulation by TSA has a positive effect on LPS-induced COX-2 expression by increasing the recruitment of C/EBPα and C/EBPβ to the promoter of COX-2 in RAW264.7 cells.

## MATERIALS AND METHODS

### CELL CULTURE AND TRANSFECTION

RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Grand Island, NY) containing 10% fetal bovine serum, 100 mg/ml streptomycin sulfate, and 100 U/ml penicillin G sodium at 37°C and 5% $CO_2$. Transfection of RAW264.7 cells with pGL2, pGL2-COX-2, pGL2-C/EBPδ plasmids were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with a slight modification. Luciferase assay (Promega, Madison, WI) was performed using a luminometer (model LB9506; Berthod, Bad Wildbad, Germany), according to a method described previously [Liaw et al., 1998]. Each transfection experiment was performed three times and each sample in each experiment was prepared in duplicate.

### WESTERN BLOT ANALYSIS

Total cell lysates were fractionated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotechnologies, Piscataway, NJ) using a transfer apparatus according to the manufacturer's protocols (Bio-Rad Laboratories, Inc., Hercules, CA). After incubation with 5% non-fat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.5% Tween 20) for 1 h, the membranes were incubated with anti-Sp1 (1:3,000; Upstate Biotechnology, Lake Placid, NY), anti-COX-2 (1:1,500; Lab Vision Corporation; Fremont, CA), anti-c-Rel, and anti-p65 (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERK1/2 (1:3,000, Upstate Biotechnology), anti-ERK1/2-P (1:15,000; Cell Signaling, Beverly, MA), anti-C/EBPδ (1:1,000; Santa Cruz Biotechnology), anti-c-Jun (1:1,500; Upstate Biotechnology), anti-c-Jun-c-terminal phosphorylation (p243; 1:250) made by Chen et al. [2007] or anti-actin (1:5,000; Sigma, St. Louis, MO) antibodies at room temperature for 2 h. The membranes were washed for 5 min three times and incubated with a 1:3,000 dilution of horseradish peroxidase-conjugated anti-mouse or -rabbit antibodies (Santa Cruz Biotechnology) at room temperature for 1 h. Blots were washed with TBST three times and developed using the ECL system (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's protocols.

### IMMUNOPRECIPITATION

RAW264.7 cells ($1 \times 10^7$) were washed with PBS. Lysate was prepared using a radioimmune precipitation assay (RIPA) buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton-X100, 0.1% Nonidet P-40, and 10 μg/ml each of MG132, leupeptin, aprotinin, and 4-(2-aminoethyl) benzenesulfonyl fluoride). The supernatant was added with anti-c-Jun (1:1,000 dilution) antibodies (Upstate Biotechnology) at 4°C for 1 h. Protein-A/G agarose beads (30 μl) were added to the lysate, and the mixture was incubated under shaking at 4°C for 1 h. The beads were collected using centrifugation and washed three times with RIPA buffer. Proteins binding to the beads were eluted by adding 30 μl of 2× sample buffer and analyzed using immunoblotting with anti-Sp1 antibodies.

### REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA of cells was isolated with a TRIzol RNA extraction kit (Invitrogen), and 3 μg of RNA was subjected to RT-PCR with SuperScript II (Invitrogen). The primers used for PCR for COX-2 were COX-2-forward: 5'-CTACGAATTCTATACATAATAG-3′, which specifically bound the nucleotide 241–262 cDNA region of COX-2, and COX-2 reverse: 5′-GTATGAAGCTGTGATTTGAGCC-3', which specific bound to the nucleotide 622–601 cDNA region of the COX-2, and for C/EBPδ were C/EBPδ—forward (361–382): 5′-CGCGAACCCGACTGGGGCGACG-3′ and C/EBPδ—reverse (742-721): 5′-GGAGGCTGGCCAGGTCCCGGGA-3′. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′. The PCR products were separated using 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

### TRANSFECTION AND REPORTER GENE ASSAY

Cells ($2.5 \times 10^5$) were seeded on a 3.5-cm dish and reached 40–50% confluence, and the cells were then transfected with plasmids with Lipofectamine 2000 according to the manufacturer's instructions

with a slight modification. We combined 1 μg of pGL2, pGL2-COX-2, pGL2-C/EBPδ luciferase plasmids with 1 μl of Lipofectamine 2000 in 200 μl of Opti-MEM medium without serum and incubated the cells at room temperature for 30 min. The cells were treated by replacing the medium with 2 ml of Opti-MEM medium containing the plasmids and Lipofectamine 2000, and then incubated at 37°C in 5% $CO_2$ for 6 h. After transfection, Opti-MEM was replaced with 2 ml of fresh medium containing 10% FBS, and the cells were incubated for additional 12 h. The luciferase activity in the cell lysate was determined as described previously [Chen and Chang, 2000].

### DNA AFFINITY PRECIPITATION ASSAY (DAPA)

The oligonucleotide 5′-CCCGCCTCCTTGAGGCGGGCCCGGGCGGG-GCGG-3′, localized −82 to −50 bp within the promoter of C/EBPδ, were biotinylated at 5′-terminus and then annealed with their complementary strands. The assay was performed by incubating 1 μg of biotinylated DNA probe with 300 μg of cell extract for 1 h and then incubated with 20 μl of streptavidin–agarose in binding buffer (1 μg of poly(dI–dC), 20 mM HEPES (pH 7.9), 0.1 mM KCl, 2 mM $MgCl_2$, 15 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol) for 1 h. Beads were collected and washed three times with binding buffer containing 0.5% NP-40. Proteins binding to the beads were eluted with 2× sample buffer and separated by SDS–PAGE followed by the immunobloting analysis.

### CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

Cells ($1 \times 10^7$) were cross-linked in 0.5% formaldehyde in PBS for 15 min at room temperature. After cross-linking, cells were washed three times with PBS, and the cell lysate was collected with lysis buffer. The chromatin was fragmented by sonication to an average size of 500 bp. The samples (1 ml) were precleaned with 10 μl of protein A/G agarose containing 1 μg of polydI–dC for 1 h, and then immunoprecipitated with 5 μg of antibodies against IgG and Sp1, C/EBPδ, C/EBPβ, and C/EBPα whom conjugated beads respectively. After 12 h incubation, samples were washed six times, bound proteins were eluted with TE buffer containing 1% sodium dodecylsulfate. Half of samples immunoprecipitated with anti-Sp1 antibodies were used to do the reChIP with the anti-c-Jun antibodies for 12 h. After washing, all of the samples were used to reverse the cross-links at 65°C for 12 h and proteins were digested with proteinase K (0.5 mg/ml) for 2 h at 50°C. DNA was purified by phenol–chloroform extraction and ethanol precipitation in the presence of 1 μg glycine. Immunoprecipitated DNA was analyzed by PCR. The primer sequences for C/EBPδ in PCR analyses were as follows: (5′-ACCAACGCAGGCGAGGGACT-3′ and 5′-CCGGCTCCACAAGGAACTGA-3′), and for COX-2 were 5′-CTGGGTTTCCGATTTTCTCA-3′ and 5′-GAGTTCCTGGACGTGCTCCT-3′.Statistical Analysis

All data from three separate experiments were expressed as mean ± SD. Comparisons among multiple groups were performed using one-way ANOVA with appropriate post hoc tests, whereas comparisons between two groups were achieved using Student's *t*-test (StatView 5.01; SAS Institute). $P \leq 0.05$ was considered statistically significant.

## RESULTS

### POSITIVE EFFECT OF TSA ON LPS-INDUCED COX-2 EXPRESSION IN RAW264.7 CELLS

Previous studies have reported that COX-2 is induced by LPS treatment [Feng et al., 1995]. Herein, we used the HDACs inhibitor, TSA, to pre-treat Raw264.7, and then treated with LPS (Fig. 1). Luciferase activity driven by the COX-2 promoter shows that TSA only could not affect luciferase activity significantly, and LPS could induce about fivefold. After TSA treatment of LPS-treated cells, we

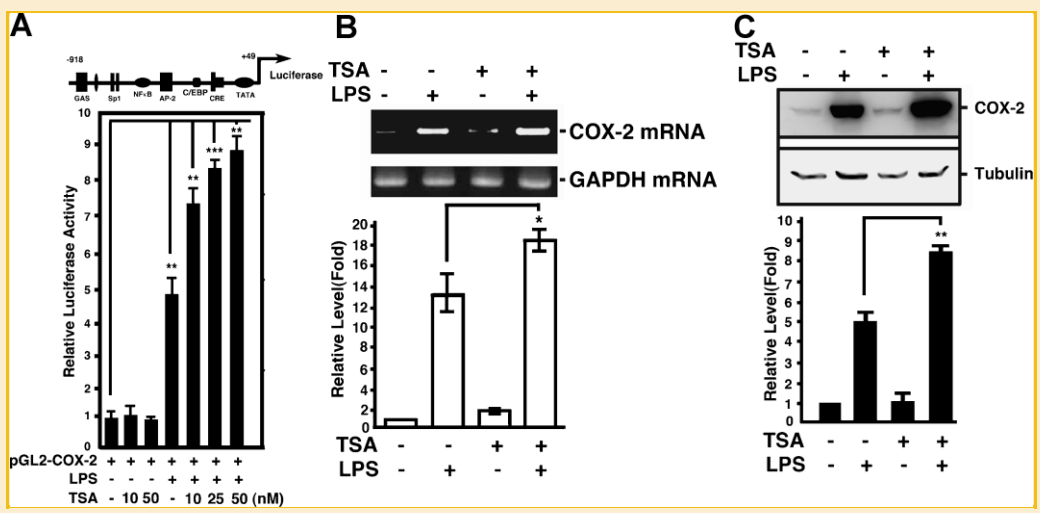


Fig. 1. Effect of TSA on LPS-induced gene expression of COX-2—RAW264.7 cells were transfected with pXC918 containing the COX-2 promoters. After 6 h transfection, cells were treated with 100 ng/ml LPS and 10, 25, 50 nM TSA. After 12 h incubation, cells were harvested with lysis buffer to do the luciferase activity assay (a). RAW264.7 cells were treated with 100 ng/ml LPS and 100 ng/ml LPS/50 nM TSA for 4 h, total RNA was extracted to do the RT-PCR with the coding region of COX-2 shown in Materials and Methods Section as the primers (b), and treated cells were also then harvested with lysis buffer to do the Immunoblot assay of anti-COX-2 antibodies (c). The signals in (b) and (c) were quantified by software, *Scion Image*, individually, and then normalized with the control. All of the experiments were carried out three times independently at least, and then quantified. Statistical significance ($^{***}P < 0.001$; $^{**}P < 0.005$; $^{*}P < 0.01$) between with or without TSA treated cells was analyzed by Student's *t*-test.

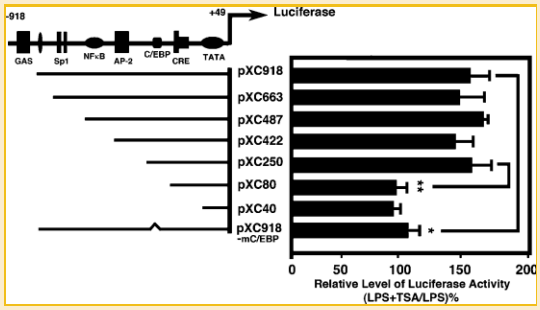


Fig. 2. Response element of COX-2 promoter in LPS treated RAW264.7 cells after TSA treatment—Different truncated promoters of COX-2, pXC918, pXC740, pXC663, pXC487, pXC422, pXC250, pXC80, pXC40, and pXC918-mC/EBP which mutated the binding element, 5′-ttacgcaa-3′ into 5′-ctcagcac-3′, were transfected into RAW264.7 cells individually. After 6 h transfection, cells were treated with LPS and 50 nM TSA. After 12 h incubation, cells were harvested with lysis buffer to do the luciferase activity assay. Statistical significance ($^{**}P < 0.005$) between pXC918 and pXC80, and between pXC918 and pXC918-mC/EBP was analyzed by Student's *t*-test.

found that luciferase activity was increased further in a dose-dependent manner (Fig. 1a). Next, COX-2 mRNA expression was examined after cells were treated with TSA and LPS (Fig. 1b). LPS treatment could induce COX-2 mRNA expression significantly, but TSA only could not. When cells were treated with LPS and TSA, COX-2 mRNA was higher than with LPS treatment alone (Fig. 1b). Finally, the level of endogenous COX-2 was determined in RAW264.7 cells after they were treated with LPS and TSA (Fig. 1c). Results show that endogenous COX-2 expression was induced by LPS treatment but not by TSA treatment alone. However, there was a further increase in the COX-2 level after LPS treatment with TSA, and this increase was consistent with the change in luciferase activity and COX-2 mRNA level. Taken together, these data indicate that COX-2 expression is further induced when LPS-treated RAW264.7 cells are pre-treated with TSA. Next, different truncated promoters of COX-2 were constructed to study which region is important for the TSA response of LPS-induced COX-2 (Fig. 2). Results indicated that there was about 1.5-fold induction in the longer promoter of COX-2 but loss of the TSA response when the C/EBPs-binding region was deleted. Therefore, C/EBPs family members might be important for the TSA-induced increase in COX-2 expression in LPS-treated cells.

## LPS-INDUCED C/EBPΔ IS INHIBITED BY TSA TREATMENT

Previous studies have shown that COX-2 is regulated by C/EBPδ [Cho et al., 2004]. In Figure 1, we found that TSA could further increase the LPS-induced COX-2 level. Therefore, the LPS-induced C/EBPδ level was detected after TSA treatment (Fig. 3). Results show that, indeed, C/EBPδ expression was dramatically induced with LPS treatment, but TSA could completely abolish the increase in C/EBPδ by LPS (Fig. 3a). The RNA level of C/EBPδ was also increased with LPS treatment, but this effect was reversed completely after TSA treatment (Fig. 3b). Luciferase activity also show that the promoter activity of C/EBPδ increased with LPS treatment, but this LPS-induced transcriptional activity was

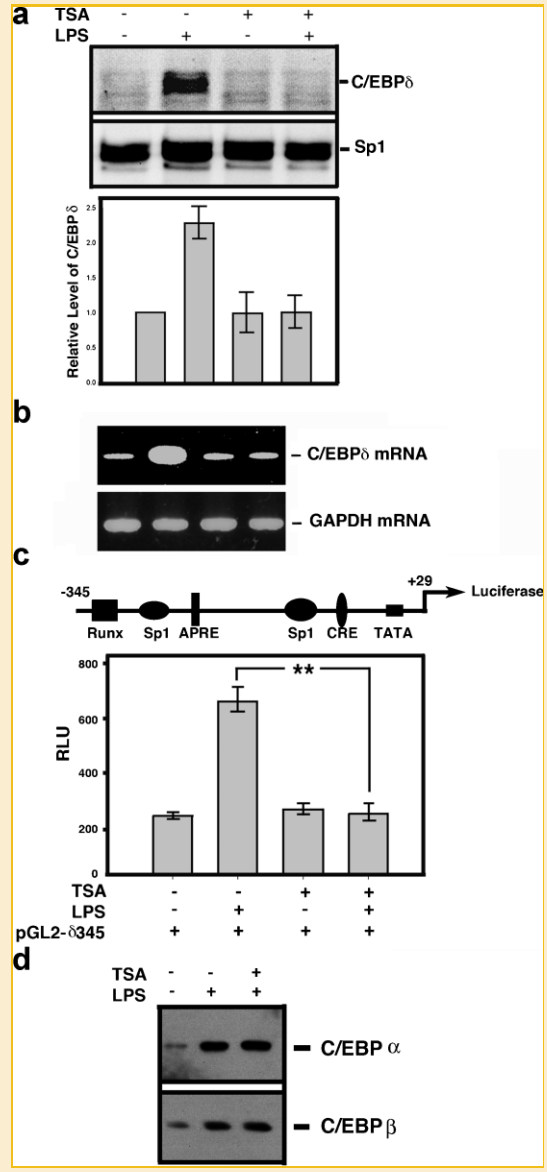


Fig. 3. Effect of TSA on LPS-induced gene expression of C/EBPδ—RAW264.7 cells were treated with LPS and LPS/TSA for 12 h, cells were then harvested with lysis buffer to do the Immunoblot assay of anti-C/EBPδ antibodies (a). RAW264.7 cells were treated with LPS and LPS/TSA for 3 h, total RNA was then extracted from treated cells to detect the level of C/EBPδ mRNA by RT-PCR (b). RAW264.7 cells were transfected with pGL2-δ345 plasmids of C/EBPδ promoters. After 6 h transfection, cells were treated with 100 ng/ml LPS and 50 nM TSA. After 12 h incubation, cells were harvested with lysis buffer to do the luciferase activity assay (c). RAW264.7 cells were treated with LPS and LPS/TSA for 12 h, cells were then harvested with lysis buffer to do the Immunoblot assay of anti-C/EBPα and C/EBPβ antibodies (d). All the experiments were carried out three times independently at least, and then quantified. Statistical significance ($^{**}P < 0.005$) between with or without TSA treated cells was analyzed by Student's *t*-test.

abolished completely after TSA treatment (Fig. 3c). In addition, although C/EBPα and C/EBPβ were induced by LPS, but no significant alternation in their levels was observed after TSA treatment (Fig. 3d). Taken together, these results indicate that TSA could abolish the LPS-induced transcription activity of C/EBPδ,

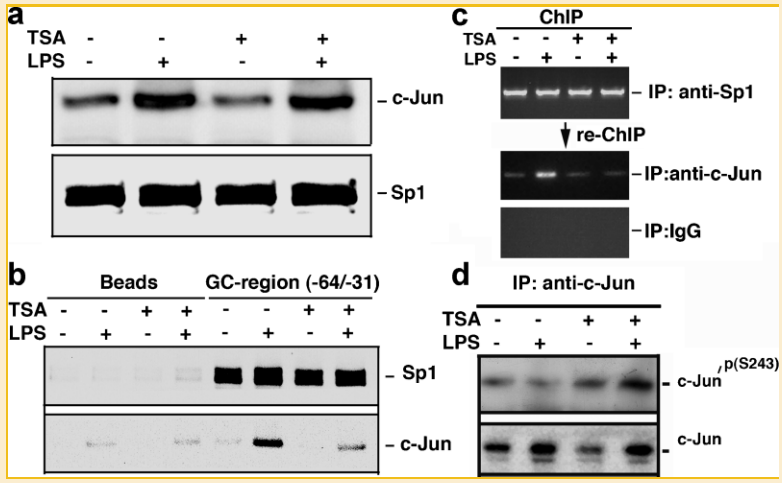


Fig. 4. Inhibition of c-Jun recruited to the promoters of C/EBPδ—RAW264.7 cells were treated with LPS in the presence and absence of TSA for 4 h, and treated cells were harvested with 2× sample buffer to do the Immunoblot of anti-c-Jun and anti-Sp1 antibodies (a). RAW264.7 cells were treated with LPS and LPS/TSA for 4 h, nuclear extracts were extracted to do the DAPA assay with the GC-rich sequence localized within the promoter (−64/−31) of C/EBPδ as the probe, samples were then lysis with 2× sample buffer to do the Immunoblot assay with anti-Sp1 and anti-c-Jun antibodies (b). RAW264.7 cells were treated with LPS and LPS/TSA for 4 h, treated cells were fixed and then sonicated to be use to do the ChIP with anti-Sp1 antibodies, and to do the re-ChIP with anti-c-Jun antibodies. DNA fragments were extracted from ChIP samples, and then assay with PCR to detect the level of promoter of C/EBPδ (c). RAW264.7 cells were treated with LPS and LPS/TSA for 4 h, treated cells were harvested with RIPA buffer to do Immunoprecipitation assay with anti-c-Jun antibodies, the samples were then analyzed with Immunoblots of anti-c-Jun and anti-phospho-c-Jun (Ser243) (d). All the experiments were carried out three times independently at least.

and this might be related to the further induction of COX-2 in LPS-treated RAW264.7 cells after TSA treatment.

### NO SIGNIFICANT DIFFERENCE IN THE LPS-INDUCED ACTIVATION OF NF*K*B IN RAW264.7 CELLS WITH OR WITHOUT TSA TREATMENT

Previous studies have revealed that NF-κB activation leads to IκB degradation, c-Rel and Rel A nuclear accumulation, and ERK1/2 phosphorylation after LPS treatment of RAW264.7 cells [Liu et al., 2007]. In this study, we first checked the c-Rel and Rel A levels in the nuclear extract to detect the activation of the NF-κB pathway after TSA and LPS treatments (Supplementary Fig. 1a,b). Results indicated that c-Rel and Rel A were accumulated significantly after LPS treatment. TSA treatment alone did not cause an increase in the c-Rel and Rel A levels in the nuclear extract and could also not reverse the LPS-induced c-Rel and Rel A accumulation in the nucleus. This data indicates that LPS can activate the NF-κB pathway and this activation is not reversed by TSA treatment. Furthermore, previous reports also revealed that ERK1/2 was activated with LPS treatment [Hwang et al., 1997]. In this study, we detected the signal of phospho-ERK1/2 after cells were treated with LPS and TSA (Supplementary Fig. 1c). The results show that ERK1/2 was phosphorylated obviously after LPS treatment alone, while there was nearly no alteration in the signal of phospho-ERK1/2 induced by LPS after TSA treatment. Taken together, these data reveal that there are no significant alternations in the activation of NF-κB and ERK1/2 after TSA treatment of LPS-treated RAW264.7 cells.

### TSA REPRESSES THE RECRUITMENT OF C-JUN BY SP1 TO THE PROMOTERS OF C/EBP*Δ*

Since LPS-induced c-Jun plays an important role in COX-2 expression, the effect of TSA treatment on c-Jun level was also studied (Fig. 4a). Data indicated that c-Jun expression was strongly induced with LPS treatment, while treatment with TSA alone had no effect on the c-Jun level, and TSA and LPS-treated cells showed no change in c-Jun level. Next, previous studies have shown that Sp1 plays the role of an anchor protein by recruiting other factors to regulate gene expression [Suske, 1999]. To examine if c-Jun is recruited to the promoter of C/EBPδ through Sp1, the DAPA and chromatin immunoprecipitation (ChIP) assays were carried out (Fig. 4b,c). First, a DAPA assay was carried out, and the result revealed that Sp1 could bind to the promoter of C/EBPδ in the control and LPS-, TSA-, and LPS + TSA-treated cells (Fig. 4b). Furthermore, as in previous studies, c-Jun could be induced and recruited to the promoter through the Sp1 after LPS treatment [Liu et al., 2007], but interestingly, c-Jun could not be recruited via Sp1 to the promoter of C/EBPδ after LPS-treated cells were treated with TSA (Fig. 4c). To confirm this result, a re-ChIP was carried out using LPS-treated cells that were or were not treated with TSA (Fig. 4c). Indeed, data revealed that Sp1 bound to C/EBPδ promoter under all conditions equally and that c-Jun binding to the C/EBPδ promoter occured through Sp1 in LPS-treated cells but not after TSA treatment (Fig. 4c). A previous study indicates that the C-terminal phosphorylation of c-Jun has a negative effect on its interaction with Sp1 [Chen and Chang, 2000]. Therefore, the phosphorylation level of the C-terminal c-Jun was checked in LPS-treated cells that were or were not treated with TSA (Fig. 4d). Data revealed that c-Jun phosphorylation at Ser243 in the C-terminal region declined even after the c-Jun level increased in LPS-treated RAW264.7 cells, but no c-Jun dephosphorylation was found after TSA treatment. In conclusion, no obvious difference was found in c-Jun levels, but an increase in the phosphorylation level of the C-terminal c-Jun and decline in the interaction between Sp1 and c-Jun were observed in LPS-treated cells treated with TSA.

### C/EBPα AND C/EBPβ ARE IMPORTANT FOR THE INCREASE IN THE LPS-INDUCED COX-2 BY TSA

In this study, we have found that C/EBPs-binding element of the COX-2 promoter is important for the positive effect of TSA in the LPS-induced COX-2 level. Therefore, herein we used the C/EBPδ overexpression and silenced various of the C/EBP members, α, β and δ, to more address the relationship between the COX-2 activation by TSA and C/EBP family (Fig. 5a,c). Data indicated that C/EBPδ knockdown could further increase the LPS-induced COX-2 level. Conversely, C/EBPδ overexpression could reverse the COX-2 level induced by LPS. In addition, interestingly, knockdown of C/EBPα and C/EBPβ could obviously decline the transcriptional activity of COX-2 by LPS (Fig. 5a,c). Finally, to more confirm that Sp1 as an anchor protein is necessary for the effect of the TSA on the LPS-induced C/EBPδ transcriptional activity, Sp1 was knockdown or overexpression to determine the transcriptional activity of C/EBPδ (Fig. 5b,c). Based on the Sp1 could be knockdown by shRNA, or over expression by adeno-GFP-Sp1 infection, the increase of Sp1 level could further increase the LPS-induced C/EBPδ activity, and the decline of Sp1 level could decrease the LPS-induced C/EBPδ (Fig. 5b,c).

### TSA DECREASES THE BINDING OF C/EBPΔ BUT INCREASES THE RECRUITMENT OF C/EBPα AND C/EBPβ TO THE PROMOTER OF COX-2 IN LPS-TREATED CELLS

Although we know that LPS-induced COX-2 can be further induced by TSA, we also found that TSA could decline the recruitment of c-Jun through Sp1 to the promoter of the C/EBPδ, but the relationship between the COX-2 upregulation and C/EBPδ downregulation in LPS-treated cells after TSA treatment remained to be clarified. Since C/EBPs family members can compete the same binding element localized in their target genes, we supposed that more α and β might bind to the COX-2 promoter in the absence of δ in LPS-treated cells after TSA treatment. Therefore, to prove our hypothesis, a ChIP assay was carried out with anti-C/EBPα, C/EBPβ, and C/EBPδ antibodies (Fig. 6). Data indicated that C/EBPα, C/EBPβ, and C/EBPδ are recruited to the COX-2 promoter in LPS-treated cells. After TSA treatment, little C/EBPδ is recruited to the promoter, and this might be due to inhibition in its protein level, but conversely, the recruitments of C/EBPα, C/EBPβ, and C/EBPδ to promoter were increased significantly (Fig. 6). Taken together, these data indicate that further induction of COX-2 in LPS-treated RAW264.7 cells after TSA treatment is involved in the reduction of C/EBPδ levels through inhibition of c-Jun recruitment to the C/EBPδ promoter, which results in increased recruitment of C/EBPα and C/EBPβ to the COX-2 promoter in RAW264.3 cells.

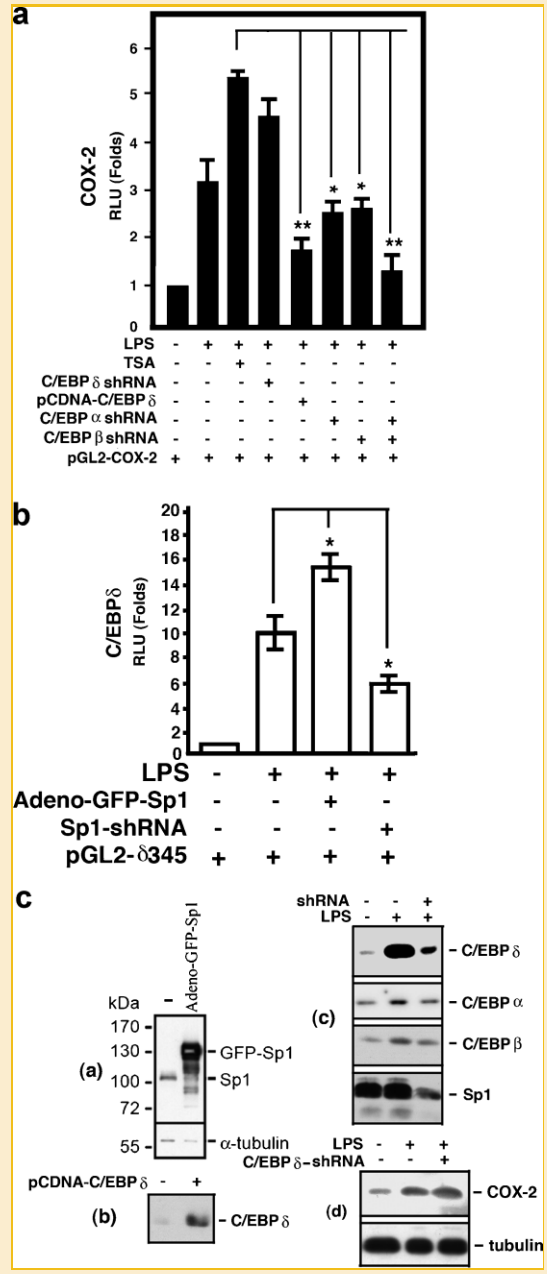


Fig. 5. Effect of C/EBPs family members on the LPS-induced COX-2—The pCDNA-C/EBPδ or shRNAs of C/EBPα, C/EBPβ, and C/EBPδ were co-transfected with Pgl2-COX-2 into RAW264.7 cells individually, and then treated with LPS. Cells were then harvested with RIPA buffer to do the luciferase activity assay (a). Sp1-shRNA was co-transfected with pGL2-δ345 to knockdown Sp1, or adeno-GFP-Sp1 virus infected the RAW264.7 cells after pGL2-δ345 transfection, cells were then treated with LPS, and then harvested with RIPA buffer to do the luciferase activity assay (b). Samples in (a) and (b) were used to do the Immunoblot assay of anti-Sp1, anti-COX-2 and anti-C/EBPα, C/EBPβ, and C/EBPδ antibodies, and tubulin level as an internal control (c).

## DISCUSSION

During the inflammatory processes, large amounts of pro-inflammatory mediators such as prostaglandin E2 (PGE2) are generated by COX-2 [Murakami et al., 1997]. COX-2 can be detected only in certain types of tissues, and it is induced transiently by growth factors, proinflammatory cytokines, tumor promoters, and bacterial toxins. Moreover, COX-2 accumulation has been detected in different tumor types [Dempke et al., 2001; Gately and Kerbel, 2003; Toomey et al., 2009]. Previous studies have indicated that LPS can induce COX-2 expression via activation of NF-κB and upregulation of C/EBPs [Woo et al., 2006; Liu et al., 2007]. Herein, we also found that COX-2 could be induced, and in parallel, NF-κB

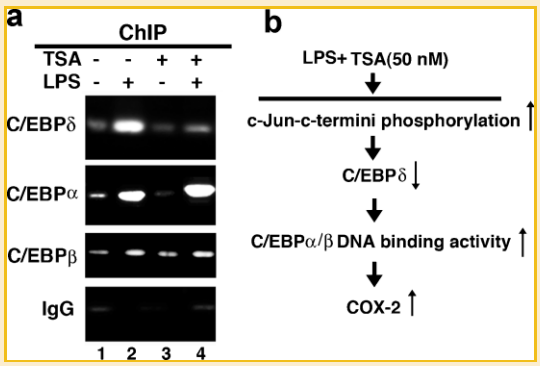


Fig. 6. Effect of TSA in C/EBPα, C/EBPβ, and C/EBPδ in LPS treated cells—RAW264.7 cells were treated with LPS in the absence or presence of TSA for 4 h, and then fixed with 0.5% formaldehyde for 15 min to do the ChIP with C/EBPα, C/EBPβ, and C/EBPδ antibodies, DNA fragments were extracted from the samples for the PCR with the primers shown in Materials and Methods Section design from promoter region of COX-2 gene (a). The schematic diagram shown here illustrates that the inhibition of LPS-induced C/EBPδ by TSA is a positive effect in the transcriptional activity of LPS-induced COX-2 (b).

and C/EBPs were also induced when RAW264.7 cells were treated with LPS (Figs. 1 and 2 and Supplementary Fig. 1). Since COX-2 induction is involved in inflammation and tumorigenesis, many studies have tried to find ways to repress COX-2 expression [de Souza Pereira, 2009; Sarkar et al., 2007]. One strategy is to inhibit its enzyme activity directly, using inhibitors such as celecoxib, a non-steroidal anti-inflammatory drug (NSAID), and the other is to repress its expression [Stratton and Alberts, 2002]. For example, most inhibitors such as chloromethylketone repress COX-2 induction by modulating NF-κB activity. Therefore, it is well known that NF-κB activity plays a positive role in COX-2 regulation [Maihofner et al., 2003]. In addition, according to the previous studies, C/EBPs upregulation increases COX-2 expression [Kundu et al., 2009]. However, in this study, we found that C/EBPδ could be abolished completely by TSA (Fig. 3); we then checked the expression of its target gene, COX-2 (Fig. 1). Data showed that COX-2 had not decreased; instead, it increased further after RAW264.7 cells were treated with LPS in the presence of TSA. Therefore, we attempted to elucidate the mechanism by which TSA increases the recruitment of the other C/EBP family member, C/EBPα and C/EBPβ (Fig. 6). We found that just C/EBPδ is inhibited by TSA; C/EBPα and C/EBPβ are unaffected (Fig. 3d). Previous studies indicate that C/EBP members bind to the same binding elements of their target genes to regulate gene expression [Ramji and Foka, 2002]. All these members can increase the transcription activity of COX-2 individually, but C/EBPα and C/EBPβ can increase transcription activity more than C/EBPδ. This might be because more transcription activated domains are present within the N-termini of C/EBPα and C/EBPβ proteins than in the N-terminal of C/EBPδ [Ramji and Foka, 2002]. On the basis of these results and those of previous studies, we suggest that C/EBPδ inhibition by TSA facilitates C/EBPα and C/EBPβ binding to the COX-2 promoter to increase its expression. In addition, one recent study also indicates that TSA can repress COX-2 expression by repressing NF-κB activity [Lindstrom et al., 2008]. There could be several reasons for the difference in observations. First, the cell type we used is different. We used the RAW264.7 cells, but others used human primary macrophage cells. Second, the detection method is different. We used the immunoblotting to detect the c-Rel and Rel A levels, while others used ELISA. Third, the time course of TSA treatment differed. We studied the effect of TSA within 12 h, but others have treated cells with TSA for a longer time. Therefore, NF-κB activity may be repressed with long-time treatment with TSA. On checking, we also found that NF-κB activity is inhibited after long-time treatment, but cell survival is poor (data not shown). We also found that C/EBPδ expression is abolished even after long-time treatment with TSA (data not shown). Thus, regardless of short or long time treatment with TSA, inhibition of C/EBPδ by TSA has a positive effect on LPS-induced COX-2 expression. However, inhibition of NF-κB activity by TSA in the late period could reverse the COX-2 induction by LPS.

Furthermore, the second novel finding in this study is clarification of the mechanism of how LPS-induced C/EBPδ expression is abolished by TSA. Previous studies mention C/EBPδ regulation and conclude that NF-κB, Erk1/2, and c-Jun recruitment by Sp1 are important [Liu et al., 2007]. In this study, we found that there is no obvious alteration in the activation of NF-κB and Erk1/2 in LPS-treated cells after TSA treatment (Supplementary Fig. 1). Although, there is also no change in the c-Jun level, the interaction between c-Jun and Sp1 is reduced strongly (Fig. 4). c-Jun could be induced rapidly under many conditions such as growth factors treatment, inflammation, and tumorigenesis [Wang and Chang, 2003]. c-Jun accumulation is important not only for regulation of its target genes through binding to the AP1 site by itself but also through the Sp1 as an anchor protein that recruits it [Chen and Chang, 2000]. We found that the C-terminus of c-Jun is phosphorylated with TSA treatment (Fig. 4). According to previous studies, GSK3β is responsible for the phosphorylation of the C-terminus of c-Jun and PP2A dephosphorylates it [Nikolakaki et al., 1993; Ramirez et al., 2005]. Another recent study also mentions that EGF treatment results in dephosphorylation of the C-terminus of c-Jun, leading to an increase in the interaction between Sp1 and c-Jun [Chen et al., 2007]. Therefore, c-Jun dephosphorylation at the C-terminus by LPS might be related to the interaction between Sp1 and c-Jun, and TSA attenuates this dephosphorylation of the c-Jun C-terminus to prevent the interaction between Sp1 and c-Jun. However, how dephosphorylation of the c-Jun C-terminus can be prevented is still unknown and needs to be clarified. In addition, another report reveals that TSA can inhibit the c-Jun protein level, downregulating COX-2 expression [Yamaguchi et al., 2005]. However, as shown in Figure 1 of that study by the Yamaguchi et al. in 2005, a low dose (50 nM) of TSA might induce, but not repress COX-2 expression. In this study, we found that the c-Jun level did not change in LPS-treated cells treated with TSA. This might be because the cells we used in our experiment were quite different from those used in previous studies. Previous studies used colon and breast cancer cells, while we used mouse macrophages. We also checked the c-Jun level in other human cancer cell lines such as HeLa cells and A549 cells, and found that c-Jun protein levels were not inhibited (data not shown). Since TSA is an HDAC inhibitor, it affects global cell characteristics, such as chromosome packaging. Different cell types might respond to TSA differently.

Furthermore, we used a lower dose of TSA (10–50 nM) than Yamaguchi et al. (100–500 nM). This difference in response hints that the effects of treatment with HDAC inhibitors or their side effects when used in cancer therapy might be very different. Therefore, the details of involved mechanisms need to be clarified.

HDAC inhibitors have been considered candidates of treating cancer, and inflammation generally occurs only during early tumorigenesis. Elucidating the role of TSA in COX-2 expression might be important for tumor therapies of the future.

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