Selective Suppression of CCAAT/Enhancer-binding Protein \( \beta \) Binding and Cyclooxygenase-2 Promoter Activity by Sodium Salicylate in Quiescent Human Fibroblasts*

Michael A. Saunders, Leticia Sansores-Garcia, Derek W. Gilroy, and Kenneth K. Wu‡

From the Vascular Biology Research Center and Division of Hematology, University of Texas-Houston Medical School, Houston, Texas 77030

The anti-inflammatory actions of salicylates cannot be explained by inhibition of cyclooxygenase (COX) activity. This study demonstrates that sodium salicylate at a therapeutic concentration suppressed COX-2 gene transcription induced by phorbol 12-myristate 13-acetate and interleukin 1\( \beta \) by inhibiting the binding of CCAAT/enhancer-binding protein \( \beta \) to its promoter region of COX-2. By contrast, salicylate did not inhibit nuclear factor \( \kappa B \) (NF-\( \kappa B \))-dependent COX-2 induction by tumor necrosis factor \( \alpha \). The inhibitory effect of sodium salicylate was restricted to serum-deprived quiescent cells. These findings indicate that contrary to the current view that salicylate acts via inhibition of nuclear factor \( \kappa B \) the pharmacological actions of aspirin and salicylates are mediated by inhibiting CCAAT/enhancer-binding protein \( \kappa B \) binding and transactivation. These findings have a major impact on the conceptual understanding of the mechanism of action of salicylates and on new drug discovery and design.

Salicylic acid is arguably the oldest anti-inflammatory drug preparation, and its acetylated form, acetylsalicylic acid (aspirin), is the most commonly used non-steroidal anti-inflammatory drug. The groundbreaking work of Vane (1) demonstrated that non-steroidal anti-inflammatory drugs, such as aspirin, owe their anti-inflammatory effects to the inhibition of prostaglandin synthesis. The last 30 years has seen major developments in prostaglandin biology, not least the identification of cyclooxygenase-2 (COX-2),1 the inducible COX isoform that is believed to play a major role in inflammation (2–4) and tumorigenesis (5–8). Despite such advances, salicylate remains a pharmacological enigma, as it inhibits prostanoid synthesis in intact cells (9–11) but has little effect on purified COX-1 or COX-2 activity (10). Recent studies have suggested that salicylate owes its anti-inflammatory effects to the inhibition of nuclear factor \( \kappa B \) (NF-\( \kappa B \))-mediated gene expression (12, 13). However, inhibition of NF-\( \kappa B \) is exerted only at suprapharmacological concentrations of sodium salicylate (NaS) (>5 mM) with no effects at pharmacological concentrations (14). Salicylate at such high concentrations inhibits numerous cellular kinases nonspecifically (15). Our group recently demonstrated that NaS and aspirin at pharmacologically relevant concentrations equipotently inhibited COX-2 transcription (16) and that the maximal inhibitory effect occurs in cells deprived of serum for 24 h. Because aspirin is rapidly deacetylated and converted to salicylate in vivo its action on COX-2 expression is attributed to salicylate. To elucidate the mechanism by which salicylate suppresses COX-2 transcription we evaluated the effects of NaS, at a representative therapeutic concentration, on COX-2 expression in serum-deprived G0 and serum-driven cycling human foreskin fibroblasts (HFF). The results show that the inhibitory effect of salicylate on phorbol 12-myristate 13-acetate (PMA)- and interleukin 1\( \beta \) (IL-1\( \beta \))-induced COX-2 transcription was confined to G0 cells. Salicylate suppressed PMA- and IL-1\( \beta \)-induced COX-2 promoter activity by inhibiting the binding of CCAAT/enhancer-binding protein (C/EBP) \( \beta \) to its cognate site on the 5′ promoter region. By contrast, salicylate did not inhibit NF-\( \kappa B \) binding to the COX-2 promoter or NF-\( \kappa B \)-dependent COX-2 induction by tumor necrosis factor \( \alpha \) (TNF\( \alpha \)).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HFF were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 \( \mu \)g/ml streptomycin, and 100 units/ml penicillin at 37 °C in 5% CO\(_2\). In all protocols 90–95% confluent HFF were serum-deprived for 24 h to synchronize the cells in the G/G\(_0\) phase before treatment with serum or other stimuli.

**Northern Analysis**—RNA isolation and Northern analysis was performed as outlined previously (16). In brief, 25–30 \( \mu \)g of RNA isolated from HFF was fractionated on 1% agarose and was transferred to a positively charged nylon membrane. As a COX-2 probe, agarose gel-purified, full-length, 1.9-kilobase COX-2-cDNA was used. Hybridization and detection by autoradiography were performed according to a procedure reported previously (16).

**Western Blot Analysis**—Whole cell lysates were prepared by lysing HFF with phosphate-buffered saline, pH 7.4, containing 0.1% Triton X-100, 0.01% EDTA, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM pepstatin A, and 0.2 mM leupeptin. Lysates were centrifuged at 13,000 rpm for 10 min. The supernatants were boiled for 5 min with equal volumes of 2x\_ gel loading buffer (100 mM Tris, 10% \( \beta \)-mercaptoethanol, 20% glycerol, 4% SDS, 2 mg/ml bromophenyl blue). Nuclear extracts were prepared by a method described previously (16). 10–50 \( \mu \)g of whole cell lysate or nuclear extract was applied to a 12% SDS polyacrylamide minigel using the Laemmli buffer system and transferred to a nitrocellulose membrane. Non Specific IgGs were blocked with 5% nonfat dried milk containing 1 mg/ml globulin-free bovine serum albumin before being incubated with an antibody against COX-2 (Cayman Chemical) or C/EBP isoforms (Santa Cruz Biototechnology). Protein bands were detected using enhanced chemiluminescence.

**Electrophoretic Mobility Shift Assay (EMSA)**—AP-2 and NF-\( \kappa B \) oligonucleotides were obtained from Promega. C/EBP\(_{(-)}\)oligonucleotides

*This work was supported in part by National Institutes of Health Grants NS-23327 and HL-50675 (to K. K. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
†To whom correspondence should be addressed: Vascular Biology Research Center and Division of Hematology, University of Texas-Houston Medical School, 6431 Fannin St., Houston, TX 77030. Tel.: 713-500-6801; Fax: 713-500-6812; E-mail: Kenneth.K.Wu@uth.tmc.edu.
‡To whom correspondence should be addressed: Vascular Biology Research Center and Division of Hematology, University of Texas-Houston Medical School, 6431 Fannin St., Houston, TX 77030.

1 The abbreviations used are: COX, cyclooxygenase; C/EBP, CCAAT/enhancer-binding protein; HFF, human foreskin fibroblast; NaS, sodium salicylate; NF-\( \kappa B \), nuclear factor \( \kappa B \); PMA, phorbol 12-myristate 13-ace
tate; IL, interleukin; TNF\( \alpha \), tumor necrosis factor \( \alpha \); FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay.

Received for publication, December 11, 2000, and in revised form, February 6, 2001

Published, JBC Papers in Press, March 16, 2001, DOI 10.1074/jbc.M011147200

Printed in U.S.A.
FIG. 1. Cell cycle-dependent effect of NaS on COX-2 mRNA (A and D) and COX-2 protein (B, C, and E) induced by PMA, IL-1β, or TNFα in HFF cells. Serum-starved HFF (0 h) were treated with 2.5% FBS, which drives the cells into the cell cycle. 30 min before time points determined by flow cytometry to best represent each phase of the cell cycle, cells were washed and incubated with fresh serum-free medium.
were synthesized by Sigma-Genosys based on the human COX-2 promoter (17). Wild type, 5′-GCTTACGGAATTCTTTTAAAGG-3′; mutant, 5′-GCGactaAATTTTTTTAAGG-3′. The complimentary oligonucleotides were annealed and purified following the manufacturer’s protocol. Each probe was end-labeled with γ32P/ATP using T4 kinase (Amerham Pharmacia Biotech). EMSA was perfomed by incubating 5 μg of nuclear extract with a labeled probe (10,000 cpm, ~10fmol) in a binding buffer containing 15 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA, 12% glycerol, 5 μM of bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiorthreitol, and 1.5 μg of poly(dI-dC). To assess the specificity of DNA protein binding up to a 100-fold molar excess of unlabeled wild type or mutant oligonucleotide was added. For supershift experiments C/EBP isoform-specific antibodies (Santa Cruz Biotechnology) were applied to the mixture for 30 min at room temperature. The mixture was applied to a 4% polyacrylamide gel and electrophoresed at 150 V for 90 min, and the complex was detected by autoradiography.

**RESULTS**

**NaS Inhibited PMA- or IL-1β-induced COX-2 Expression Only in Serum-starved Quiescent Fibroblasts**—We recently reported that NaS and aspirin at therapeutic concentrations (10−7 to 10−3 M) equipotently suppress IL-1β- and PMA-induced COX-2 transcription in serum-deprived endothelial cells and fibroblasts (16). To determine whether the inhibitory effect of salicylate is cell cycle-dependent, we performed experiments in HFF that were first serum-deprived for 24 h followed by the addition of 2.5% FBS to drive the cell cycle. This fibroblast model has been extensively used to characterize G0 cells, as well as cells in various phases of the cell cycle (18). Our cell cycle analysis was consistent with previous reports in that over 90% of HFF in medium deprived of FBS for 24 h were in G0, and after 2.5% FBS treatment cells entered into S phase at 16 h, and over 50% of cells were in S phase at 24 h (19). At several time points, chosen by flow cytometry to best represent the individual phases of the cell cycle, 0 h (G0), 4 h (G1), 24 h (S), and 42 h (G2/M), cells were pretreated with a representative therapeutic concentration of NaS (10 μM) for 30 min before adding PMA (100 nM), IL-1β (1 ng/ml), or TNFα (1 ng/ml). COX-2 mRNA and protein expression were determined 2 and 4 h post-treatment by Northern and Western blot analysis, respectively. NaS inhibited COX-2 mRNA (Fig. 1A) and protein levels (Fig. 1B) induced by PMA in serum-starved G0 cells, but the inhibitory action was no longer apparent at 4, 24, and 42 h after 2.5% FBS treatment. Similarly, IL-1β-induced COX-2 protein expression was inhibited by NaS in serum-deprived G0 cells but not in cells treated with 2.5% FBS for 24 h (Fig. 1C).

In contrast, TNFα-induced COX-2 protein expression was not inhibited by NaS in G0 or S phase cells (Fig. 1C). In subsequent experiments, the inhibitory effects of NaS on PMA-induced expression of COX-2 at earlier time points after 2.5% FBS addition was evaluated. NaS inhibited COX-2 mRNA and protein by ~50% in G0 cells. However, after only 1 h of exposure to 2.5% FBS, NaS was no longer able to inhibit COX-2 mRNA and protein (Fig. 1, D and E). We have previously shown that NaS and aspirin inhibited PMA- or IL-1β-induced COX-2 promoter activity conferred by a core promoter fragment −891/+9 (16). Here we show that salicylate suppressed COX-2 promoter activity in a G0-restricted manner (Fig. 2). As NaS is only effective in G0 cells all subsequent experiments to determine its mechanism of action were performed in the absence of serum unless otherwise stated.

**NaS Inhibited C/EBP but Not the NF-κB-mediated COX-2 Promoter Activation**—The cis acting elements in the COX-2 promoter region that are required for the transcriptional inhibition by salicylate were determined using fragment −891/+9 and its deletion or site-directed mutants (17) cloned into pGL3 and its deletion mutants until the active therapeutic concentration of NaS (10 μM) is no longer able to inhibit COX-2 mRNA and protein (Fig. 1, A and B). We have previously shown that NaS and aspirin inhibited PMA- or IL-1β-induced COX-2 promoter activity conferred by a core promoter fragment −891/+9 (16). Here we show that salicylate suppressed COX-2 promoter activity in a G0-restricted manner (Fig. 2). As NaS is only effective in G0 cells all subsequent experiments to determine its mechanism of action were performed in the absence of serum unless otherwise stated.

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not PMA or IL-1β (Fig. 3E). Mutation of NF-κB sites also had no effect on salicylate inhibition of PMA- or IL-1β-induced COX-2 promoter activity (Fig. 3E). Taken together these results suggest that the C/EBP site at −132/−124 is critically involved in PMA- and IL-1β-induced COX-2 expression and salicylate suppression. NF-κB sites at −447/−438 and −222/−213 are critical for TNFα-induced COX-2 expression consistent with reported results (20). However, neither NF-κB site is involved in promoter induction by PMA and IL-1β or suppression by salicylate.

**NaS Inhibited C/EBPβ Binding to the COX-2 Promoter**—The 5′ deletion mutation analysis suggested that the region
−193 to −97, containing an AP-2 and C/EBP/NF-IL6 site, is required for PMA- or IL-1β-induced promoter activity, whereas TNFα requires the region −447 to −438 containing a putative NF-κB site for optimal activity. EMSA were performed using an AP-2, C/EBP, or NF-κB probe. AP-2 binding was unaffected by any of the stimuli (data not shown). Concordant with the
reporter assays, IL-1β and PMA but not TNFα increased complex formation between C/EBP probe and nuclear extract proteins (Fig. 4A, left panel, and B). The specificity of binding is demonstrated by the inhibition of the PMA-induced complex formation by a 5- to 100-fold molar excess of unlabeled C/EBP wild type probe but not by a 50-fold molar excess of unlabeled C/EBP mutant probe (Fig. 4A, right panel). NaS significantly reduced C/EBP binding activity induced by PMA and IL-1β (Fig. 4, A and B) but not TNFα (Fig. 4B). A structural analogue 3-OH benzoic acid failed to affect PMA-induced complex formation (Fig. 4C). Hence, the inhibitory effect of salicylate (2-OH benzoic acid) is selective and not common to all substituted benzoic acids. The PMA-induced complex was only supershifted in the presence of an anti-C/EBPβ antibody but was unaffected by antibodies against other C/EBP isoforms (Fig. 5). TNFα and IL-1β but not PMA increased NF-κB binding that was insensitive to NaS inhibition (Fig. 6, left panel). The specificity of binding is demonstrated by the inhibition of the TNFα-induced complex formation by a 50-fold molar excess of unlabeled NF-κB wild type probe (Fig. 6, right panel). C/EBPβ full-length (46 kDa) and C/EBPα (40 kDa) proteins were expressed constitutively in nuclear extract and were not altered by PMA treatment (Fig. 7). The truncated C/EBPβ isoform liver inhibitory protein was barely detectable and was not altered by PMA treatment (data not shown). C/EBPγ, C/EBPδ, and C/EBPε were undetectable (data not shown). To determine whether the increased binding is because of phosphorylation of C/EBPβ, control and PMA-treated nuclear extracts were incubated with alkaline phosphatase (10 units) for 30 min before being applied to EMSA. Alkaline phosphatase treatment reduced the complex formation in both control and PMA-treated HFF cells (Fig. 8).

DISCUSSION

Results from this study challenge the prevailing view that salicylates, including aspirin, exert their therapeutic actions by inhibiting NF-κB-mediated transactivation of proinflammatory genes. Our findings indicate that salicylate at a pharmacological concentration has no effect on NF-κB transactivation induced by TNFα, in contrast to the potent anti-NF-κB effects exerted by suprapharmacological concentrations (>5 mM). Concentrations higher than 5 mM nonspecifically inhibit many cellular kinases (15). This renders NF-κB as an unlikely target for the pharmacological actions of salicylate. We propose that the actions of salicylate are mediated instead by inhibition of C/EBPβ binding and transactivation. Several pieces of evidence support this proposal: 1) deletion or mutation of the C/EBP site abolishes the stimulatory effect of PMA and IL-1β and concomitantly, the inhibitory effect of salicylate; (2) salicylate inhibited the binding of C/EBPβ to the C/EBP site; and (3) salicylate had no effect on TNFα-induced NF-κB binding or COX-2 promoter activity conferred by wild type or C/EBP mutant constructs. C/EBPβ is a pleiotropic transactivator for myriad genes involved in inflammation, cell differentiation, and the immune response (21–27). In this study, we have established the role of C/EBPβ in human COX-2 induction by PMA and IL-1β. C/EBPβ has been reported to be essential for COX-2 induction in murine cells in response to a number of stimuli (28). Thus, salicylate blockade of C/EBPβ-mediated COX-2 expression provides a plausible explanation for the pharmacological actions of aspirin and other salicylate compounds. Furthermore, the action of salicylate may be extended to other C/EBPβ-mediated genes, and as such, our findings have a major impact on the conceptual understanding of the actions of salicylate and on new drug design and discovery.

C/EBPβ is a member of the C/EBP family of basic leucine zipper transcription factors (27). It forms homodimers and heterodimers with other C/EBP isoforms to transactivate or repress promoter activity. There are several C/EBPβ isoforms comprising the full-length and three truncated forms that include liver inhibitory protein, which is considered as a dominant negative mutant of the full-length C/EBPβ (29). In this study, the results indicate that quiescent HFF express predominantly the full-length C/EBPβ. C/EBPα was also detected, but
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... its role in regulating COX-2 expression is unclear. C/EBPβ levels are not altered by PMA, IL-1β, or salicylate. C/EBPβ binding activity has been previously shown to be enhanced by phosphorylation mediated by several kinase pathways such as protein kinase C (30, 31), mitogen-activated protein kinase (32), p90 ribosomal S6 kinase 2 (33), and Ca\(^{2+}\)-calmodulin-dependent protein kinase II (34). These kinases directly or indirectly phosphorylate different threonine or serine residues suggesting multiple mechanisms for activating C/EBPβ. Many of the activities of proinflammatory mediators are mimicked by PMA that activates protein kinase C. We therefore determined whether PMA-induced C/EBPβ binding enhancement was attributable to phosphorylation by treating nuclear extracts with a general phosphatase (alkaline phosphatase), and the results confirm that PMA-stimulated C/EBPβ binding activity depends on phosphorylation of this transactivator. Our results further suggest that it is mediated via a protein kinase C-dependent pathway.\(^2\) It is likely that NaS blocks PMA-induced C/EBPβ binding activity by targeting a downstream kinase in the protein kinase C signaling pathway. Salicylate has been shown to affect several protein kinases that include p90 ribosomal S6 kinase 2 (35), p38 mitogen-activated protein kinase (36), p42/p44 mitogen-activated protein kinase (37), and c-Jun N-terminal kinase (38). These studies were performed using suprapharmacological concentrations of NaS, and as such, these kinases are not likely involved in the specific effects demonstrated in this study. However, a recent report demonstrated that NaS at pharmacological concentrations inhibited PMA-induced phosphorylation of p70 S6 kinase (39). Hence, it is possible that p70 S6 kinase may occupy an important position in mediating C/EBPβ phosphorylation and increasing its binding activity and is the target of salicylate inhibition. This proposal is being investigated in our laboratory.

It is intriguing that the inhibitory action of salicylate on COX-2 transcription stimulated by PMA and IL-1β is confined to serum-deprived fibroblasts. Results from our recent work (19) show that COX-2 is expressed more abundantly in serum-starved cells than serum-treated cells suggesting that COX-2 expression is controlled by an endogenous mechanism that is operative in proliferating cells. Quiescent cells in \textit{vivo} may be the major COX-2-expressing cells and play a key role in the inflammatory response. That salicylate suppresses COX-2 expression only in Gl cells is consistent with its anti-inflammatory actions in \textit{vivo}. However, the mechanism that underlies the restricted efficacy of NaS is not understood. We have excluded the possibility that the lack of effect, after addition of 2.5% FBS, is because of sequestration of salicylate by serum proteins as exposure of NaS to serum prior to its addition to serum-deprived cells did not alter its inhibitory effect (data not shown). However, it is possible that after addition of 2.5% FBS there may be an alteration in the expression of C/EBP isoforms, and this alteration abrogates the inhibitory action of salicylate. Switch of C/EBP isoform expression has been shown to occur in liver cell differentiation (40), but there is no published data on changes in C/EBP isoforms after serum treatment. Certain isoforms of C/EBP, such as liver inhibitory protein and C/EBP homologous protein, bind C/EBPβ to form a heterodimer and inhibits C/EBPβ binding to DNA and transactivation of the promoter (41, 42). Whether these isoforms are regulated in a cell cycle-dependent manner is unknown. G0-restricted salicylate action may be because of an alteration in C/EBPβ phosphorylation in cycling cells. C/EBPβ phosphorylation is probably regulated by coordinated activities of kinases and phosphatases. This delicate balance may be altered by serum or cell cycle progression. C/EBPβ is located in cytosol and translocated into the nucleus after stimulation with proinflammatory cytokines (43, 44). It should be interesting to determine whether this translocation process is altered by serum and cell cycle progression. Understanding why NaS is only effective in G0 cells is critical, as it will potentially provide a target for novel therapeutic compounds with broad implications for the treatment of inflammation, tissue injury, and tumor growth.

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\(^2\) M. A. Saunders and K. K. Wu, unpublished data.
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J. Biol. Chem. 2001, 276:18897-18904.
doi: 10.1074/jbc.M011147200 originally published online March 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011147200

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