Inhibition of p90 Ribosomal S6 Kinase-mediated CCAAT/Enhancer-binding Protein β Activation and Cyclooxygenase-2 Expression by Salicylate*

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We have previously shown that salicylate at a pharmacological concentration suppresses CCAAT/ enhancer-binding protein β (CREBβ) binding, thereby reducing cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase expression (Saunders, M. A., Sansores-Garcia, L., Gilroy, D. W., and Wu, K. K. (2001) J. Biol. Chem. 276, 18897–18904; Cieslik, K., Zhu, Y., and Wu, K. K. (2002) J. Biol. Chem. 277, 49304–49310). We postulated that salicylate targets a kinase that phosphorylates and activates CREBβ. Here we report the identification of p90 ribosomal S6 kinase (RSK) as a target of salicylate. Salicylate inhibited RSK in vitro and blocked the activity of RSK2 purified from cells stimulated by phorbol 12-myristate 13-acetate (PMA). Mutation of the RSK-phosphorylation site (T266A) of CREBβ abrogated PMA-stimulated CREBβ binding activity. RSK activation was required for PMA-induced COX-2 transcriptional activation. Salicylate also inhibited Ras and extracellular signal-regulated kinase (ERK) activation induced by PMA. We conclude that salicylate inhibits CREBβ-mediated COX-2 transcriptional activation by blocking RSK activity and Ras signaling pathway.

Salicylic acid (SA)1 is a natural compound produced by diverse plants as a signaling molecule to defend against environmental insults (1, 2). Aspirin is synthesized from SA and is deacetylated to generate SA in human-circulating blood (3, 4), and both aspirin and SA exert anti-inflammatory and anti-neoplastic actions (5, 6). Although the mechanisms by which SA controls various pathophysiological processes are not entirely clear, recent studies have shown that SA controls the activation of pro-inflammatory and pro-proliferative transcriptional activators including nuclear factor-κB (NF-κB), AP-1, cyclic AMP response element-binding protein (CREB), c-Myc, and CCAAT enhancer-binding protein β (CREBβ) (7–11). The effect of SA on transcriptional activation appears to be concentration-dependent. Its inhibition of NF-κB, AP-1, and CREB requires suprapharmacological concentrations generally at 10–20 mM (7–9), whereas its inhibition of CREBβ occurs at pharmacological concentrations at 10−6–10−4 M (11). The mechanisms by which SA exerts differential concentration-dependent inhibition of transcriptional activators remain to be fully elucidated. It has been shown that SA at suprapharmacological concentrations inhibits IκB kinase β, thereby blocking IκB phosphorylation and dissociation from NF-κB (12). As SA at suprapharmacological concentrations has been shown to inhibit a large number of kinases (13), the action on IκB kinase β may not be specific. Our previous work has shown that SA at therapeutic concentrations selectively inhibits CREBβ binding to its cognate DNA binding site and does not perturb NF-κB activation, suggesting a different mode of action (11). However, the mechanism by which it blocks CREBβ activation remains to be elucidated.

CREBβ mediates transcription of several pro-inflammatory cytokines and mediators (for a review see Ref. 14). Work from our and other laboratories has shown that CREBβ binding to its cognate promoter motif is essential for cyclooxygenase-2 (COX-2) transcriptional activation induced by phorbol 12-myristate 13-acetate (PMA), interleukin-1β, and lipopolysaccharide (15–17). Sodium salicylate at 10−5 M selectively inhibited CREBβ binding to COX-2 promoter, thereby suppressing COX-2 expression (11). It is unclear how SA suppresses CREBβ binding. CREBβ is a member of the CREB family of basic leucine zipper transcription factors (for a review see Ref. 18). It forms homodimers as well as heterodimers with other CREB isoforms, and the dimers bind to a specific DNA sequence at the promoter region of the target genes. CREBβ contains an intramolecular autoinhibitory element that hinders its binding to DNA, and phosphorylation at several discrete threonine or serine residues enables CREBβ to bind DNA presumably by releasing the autoinhibitory element (19, 20). It has been reported in in vitro experiments that phosphorylation of CREBβ by extracellular signal-regulated kinase (ERK) pathway, protein kinase A, p90 ribosomal S6 kinase (RSK), or CaM-dependent kinase IV is associated with increased CREBβ binding activity (21–24). The residues phosphorylated by these kinases have been identified to be corresponding to Thr255 (ERK), Thr256 (RSK), Ser268 (protein kinase A), and Ser265 (CaM-dependent kinase IV) in human CREBβ. Little is known about the kinases that activate CREBβ binding activity in response to stimulation by PMA and pro-inflammatory media-

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† The abbreviations used are: SA, salicylic acid; CREB, cyclic AMP response element-binding protein; PBS, phosphate-buffered saline; COX, cyclooxygenase; NF-κB, nuclear factor-κB; RSK, p90 ribosomal S6 kinase; DNA, dominant negative; CA, constitutively active; ERK, extracellular signal-regulated kinase; EMSA, electrophoretic mobility shift assay; PKC, protein kinase C; WT, wild type; MOPS, 4-morpholinepropanesulfonic acid; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; DTT, dithiothreitol.
itors in vivo, nor is it known whether SA reduces C/EBPβ binding activity by suppressing any of the kinases. In this study, we tested the hypothesis that PMA stimulates C/EBPβ-mediated COX-2 transcriptional activation by inducing one of the kinases that phosphorylate C/EBPβ and SA blocks the activity of this kinase. To test this hypothesis, we mutated each of the four phosphorylation residues, constructed them into a FLAG expression vector, and determined the binding activity of wild-type (WT) and mutant C/EBPβ to an authentic C/EBPβ enhancer element by streptavidin-agarose pull-down and electrophoretic mobility shift assays. The results show that mutation of the RSK phosphorylation residue (T266A) abrogated PMA-stimulated C/EBPβ binding. Overexpression of a dominant negative (DN) mutant of RSK2 suppressed PMA-induced C/EBPβ binding and COX-2 transcriptional activation. PMA increased RSK activity, which was inhibited by sodium salicylate in a concentration-dependent manner. The results suggest that SA directly inhibits RSK activity.

EXPERIMENTAL PROCEDURES

Materials—Lipofectin, minimum Eagle’s medium, Dulbecco’s modified Eagle’s medium high glucose medium, Opti-MEM I medium and antibiotics were obtained from Invitrogen. Rabbit polyclonal COX-2 antibodies were obtained from Cayman (Ann Arbor, MI). Antibodies against ERK, phosphorylated ERK, and HA were obtained from Cell Signaling Technology (Beverly, MA). C/EBPβ antibodies and Protein A/G Plus-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence solution and BCA reagent for protein assay were obtained from Pierce. SDS-PAGE-ready gels (acrylamide 29:1) were obtained from Bio-Rad. Fetal bovine serum, anti-FLAG antibody, PD98059, PMA, and streptavidin immobilized on 4% agarose were obtained from Sigma. Luciferase assay system and S6 kinase assay kit were purchased from Promega (Madison, WI).

Ras Activation Assay—Ras activation was assayed using a kit from BIOMOL (Beverly, MA). Each experiment was repeated at least three times.

RESULTS

T266A C/EBPβ Mutant Had a Reduced Binding Activity—To determine whether the C/EBPβ phosphorylation residues (Thr265, Thr266, Ser288, and Ser225) are involved in PMA-induced C/EBPβ binding to COX-2 promoter, we mutated each residue individually to alanine, constructed it into a FLAG expression vector, and expressed it in human fibroblasts by transient transfection. Each mutant expressed a similar amount of C/EBPβ proteins as wild type detected with a C/EBPβ antibody (Fig. 1A). Major bands representing full-length and liver-enriched transcription active protein were de-
we treated human fibroblasts that had been transfected with WT and mutant C/EBP under “Experimental Procedures.” The was determined by streptavidin pull-down binding assay as described in three experiments. The represents densitometric analysis of three blots from three separate experiments. We treated with a biotinylated C/EBP probe and streptavidin-agarose beads as described under “Experimental Procedures.” Nuclear extracts prepared from the treated cells were incubated with a biotinylated C/EBP probe and streptavidin-agarose beads as described under “Experimental Procedures.” The upper panel shows a representative Western blot, and the lower panel represents densitometric analysis of three blots from three separate experiments. The error bar denotes mean ± S.E. B, C/EBP binding was determined by streptavidin pull-down binding assay as described under “Experimental Procedures.” The upper panel shows a representative blot, and the lower panel represents densitometry of gels from three experiments. Each bar denotes mean ± S.E.

tected in WT- and mutant-transfected cells (Fig. 1A). To evaluate the influence of C/EBPβ mutation on its binding activity, we treated human fibroblasts that had been transfected with FLAG-tagged WT or mutant C/EBPβ with PMA (100 nM) for 4 h and nuclear extracts prepared from the treated cells were incubated with a biotinylated C/EBP probe and streptavidin-agarose beads as described under “Experimental Procedures.” FLAG-C/EBPβ bound to the C/EBP probe and streptavidin-agarose beads as described under “Experimental Procedures.” FLAG-C/EBPβ was not detected in vector control, and in the absence of PMA stimulation, only trace of FLAG-C/EBPβ was detected in the complex (Fig. 1B). PMA induced a large increase in FLAG-C/EBPβ binding (Fig. 1B). When compared with the WT, the T266A C/EBPβ level in the complex was reduced by ~50%, whereas the T235A, S288A, or S325A level was not significantly changed (Fig. 1B). To corroborate the binding data from the streptavidin-agarose pull-down assay, we analyzed C/EBPβ binding by EMSA. We have previously shown by EMSA and supershift assays with specific antibodies for each isoform of C/EBP that PMA selectively stimulated complex formation between C/EBPβ and a 32P-labeled probe containing the authentic C/EBP enhancer element sequence (~132 to ~124) of COX-2 promoter (11). The complex was abolished by 50-fold excess of unlabeled probes or a mutated probe (11). We repeated the experiments, and the results were similar (data not shown). We next transfected cells with FLAG-WT, FLAG mutants, or FLAG vector control and analyzed protein-DNA complex by gel retardation. The retarded band of T266A was diminished when compared with that of WT and vector control (Fig. 2), suggesting that T266A may be a dominant negative mutant that inhibits the binding of native C/EBPβ to the probe. Taken together, these results indicate that Thr266 is required for PMA-induced C/EBPβ binding.

RSK Activation Was Required for COX-2 Transcriptional Activation—Because Thr266 is a target of RSK phosphorylation, we reasoned that PMA may induce C/EBPβ binding and C/EBPβ-dependent COX-2 transactivation by activating RSK. Therefore, we evaluated the effect of PMA on RSK activity. Cells transfected with wild-type HA-RSK2 (RSK-WT), mutant HA-RSK2 (RSK-DN), or vector were treated with or without PMA for 15 min. HA-RSK2 was pulled down, and their activities were measured. RSK activity in cells transfected with RSK-WT was increased by PMA by ~2-fold (Fig. 3). Consistent with a previous report (26), RSK-DN retained a basal activity but failed to respond to PMA stimulation (Fig. 3). We next assessed the influence of RSK-DN on COX-2 promoter activity. Cells were transfected with RSK-WT, RSK-DN, or vector control, and COX-2 promoter activity was determined by co-transfection of cells with a luciferase expression vector containing a core COX-2 promoter. RSK-WT augmented PMA-induced COX-2 promoter activity, whereas RSK-DN abrogated COX-2 promoter activity stimulated by PMA (Fig. 4A). Corresponding to the promoter data, RSK-WT enhanced PMA-induced C/EBPβ binding, whereas RSK-DN completely suppressed it (Fig. 4B).

Sodium Salicylate Inhibited RSK Activation—It has been shown that sodium salicylate at pharmacological concentrations inhibits COX-2 transcriptional activation induced by PMA and other pro-inflammatory mediators by blocking C/EBPβ binding to the C/EBP enhancer element at -132/-124.
of human COX-2 promoter (11). To determine whether salicylate may target RSK, we treated HA-RSK2-transfected human fibroblasts with sodium salicylate at increasing concentrations for 30 min followed by PMA for 15 min. RSK2 was immunoprecipitated using a HA antibody, and RSK activity of the precipitated RSK2 was assayed. Sodium salicylate inhibited PMA-stimulated RSK activity in a concentration-dependent fashion, and maximal inhibition was noted at 10^{-5} M (Fig. 5A). To provide direct evidence for inhibition of RSK by SA, we pretreated HA-RSK2-transfected cells with or without PMA for 15 min and isolated RSK2 with a HA antibody. After extensive washing, HA-RSK2 was incubated with sodium salicylate (10^{-5} M) for 30 min and RSK activity was measured. PMA-stimulated RSK activity was suppressed by SA to an extent comparable with that in vivo (Fig. 5B). These results suggest that SA at pharmacological concentrations inhibit RSK activity stimulated by PMA.

Sodium Salicylate Suppressed PMA-induced Ras Signaling Pathway—To gain insight into the signaling pathway through which PMA induces RSK-mediated C/EBPβ binding and COX-2 transcriptional activation, we treated fibroblasts with inhibitors of major signaling molecules before the addition of PMA. PMA-induced COX-2 protein expression was suppressed by PKC and MEK-1 inhibitors but not phosphatidylinositol-3 kinase inhibitor (Fig. 6A). These results suggest that PMA-induced COX-2 expression is mediated via the Ras signaling pathway. The effects of DN-Ras and CA-Ras on COX-2 promoter activity were evaluated. Compared with vector control, CA-Ras augmented basal COX-2 promoter activity without enhancing PMA-induced activity, whereas DN-Ras reduced the basal activity as well as abrogated PMA-induced increase in COX-2 promoter activity (Fig. 6B). These results suggest that Ras plays an essential role in regulating COX-2 transcriptional activation. To determine whether salicylate inhibits Ras activation, we measured Ras activity in PMA-treated cells in the presence or absence of sodium salicylate (10^{-5} M). The Ras activity was undetected at basal state and was highly increased after PMA stimulation (Fig. 7A). PMA-stimulated Ras activation was abrogated by salicylate, whereas salicylate had no effect on the basal activity (Fig. 7A). In accord with Ras activation, ERK1/2 were activated by PMA, which was suppressed by sodium salicylate (Fig. 7B). To analyze the possible involvement of a feedback activation of Ras by RSK, we transfected cells with RSK-WT, RSK-DN, and vector control and treated the transfected cells with or without PMA for 15 min. Ras activity in the cell lysates was determined. Neither RSK-WT nor RSK-DN had a significant effect on basal or PMA-stimulated Ras activity when compared with vector control (Fig. 8).

**DISCUSSION**

Results from this study indicate that salicylate inhibits PMA-induced COX-2 transcriptional activation by blocking RSK activation. Several lines of evidence support this conclusion. First, PMA is capable of activating RSK, which is inhib-
ited by a DN-RSK2 mutant. Concordantly, this dominant negative mutant inhibits PMA-induced C/EBPβ binding and COX-2 promoter activity. Second, mutation of the RSK phosphorylation site of C/EBPβ results in a poor activation and binding activity in response to PMA stimulation. Third, salicylate inhibits PMA-induced RSK activation in a concentration-dependent manner in accord with the inhibition of COX-2 protein expression and promoter activity. It was previously reported that sodium salicylate inhibited unstimulated RSK2 only at very high concentrations (20 mM) but was capable of abolishing PMA-stimulated RSK2 at 5 mM (9). Taken together, these results suggest that SA at therapeutic concentrations exerts direct inhibitory action on activated RSK molecules. ERK1/2 activate RSK by docking to the C-terminal region of RSK and phosphorylate several residues at the C-terminal kinase domain and the linker region, thereby activating the N-terminal kinase (30). RSK has been shown to complex with ERK in several cell types (30–32). This raised the possibility that SA may inhibit RSK via inactivation of ERK1/2. However, this is unlikely because ERK1/2 were not reported to be complexed with purified RSK2 (9). A recent study has shown that the complex formation is dynamically regulated. ERK forms complexes with RSK in unstimulated cells but dissociates from RSK following cell stimulation with PMA and other agonists (33). Furthermore, our mutant data indicate that ERK1/2 are not directly involved in C/EBPβ phosphorylation because mutation of ERK1/2 phosphorylation residue (Thr255) did not alter C/EBPβ binding.

RSK family proteins comprise three closely related members, RSK 1, 2, and 3, and several distantly related members (for a review see Ref. 34). RSK1 and RSK2 (RSK1/2) are highly homologous and share common functional properties (33). Both RSK1/2 are inhibited by DN-RSK2 (31). By contrast, RSK3 and other members are regulated differently and have different functional properties (30). Thus, it is likely that RSK1 and RSK2 are involved in PMA-induced C/EBPβ activation and are targets of SA. It is unclear how salicylate inhibits RSK1/2 activity. RSK1/2 contain two kinase domains, a C-terminal kinase domain, which is activated by ERK1/2, and an N-terminal kinase domain, which is activated by C-terminal kinase via

FIG. 5. Inhibition of RSK activity by salicylate. A, fibroblasts transfected with HA-tagged WT-RSK2 expression vector were pretreated with indicated concentrations of sodium salicylate for 30 min prior to stimulation with PMA (100 nM) for 15 min. RSK2 was precipitated using a HA-antibody, and RSK activity was measured as described under “Experimental Procedures.” B, RSK2 in lysates of cells treated with or without PMA for 15 min was pulled down, washed, and treated with SA (10⁻⁵ M). Each bar represents the mean ± S.E. of three separate experiments.

FIG. 6. Involvement of MEK-1/2, PKC, and Ras in PMA-induced COX-2 expression. A, fibroblasts were pretreated with each kinase inhibitor for 30 min prior to PMA stimulation for 4 h. COX-2 proteins were analyzed by Western blotting. AG, AG490 (50 μM); LY, LY294002 (50 μM); R, rapamycin (200 nM); SB, SB203580 (2.6 μM); GF, GF109203X (500 nM); PD, PD98059 (100 μM). Lower panel shows corresponding densitometry analysis. Each bar represents mean ± S.E. of three separate experiments. B, fibroblasts were co-transfected with COX-2 promoter vector and CA-Ras, DN-Ras, or vector control. COX-2 promoter activity was measured by luciferase expression. Each bar denotes mean ± S.E. of three separate experiments.
Inhibition of RSK by Salicylate

Salicylate suppressed Ras and ERK activation. A, fibroblasts were treated with sodium salicylate for 30 min followed by PMA for 15 min. C+ denotes positive control wherein cell lysate isolated from unstimulated cells was incubated with GTP (100 μM) for 30 min. C- denotes negative control wherein cell lysate isolated from unstimulated cells was incubated with GDP (100 μM) for 30 min. Ras activity was measured by a method described under “Experimental Procedures.” This blot is representative of three separate experiments. B, salicylate inhibited phosphorylation of ERK but not ERK protein level in fibroblasts stimulated with PMA for 15 min. Salicylate (10−5 M) was given 30 min prior to PMA (100 nM). The upper panel shows a representative Western blot of ERK. Middle panel shows a representative Western blot of phosphorylated ERK (ERK-P), and the lower panel represents densitometric analysis of ERK-P from three independent experiments. Each bar represents mean ± S.E.

Results show that Ras and ERK1/2 are activated by PMA and are essential for PMA-induced COX-2 expression, which are inhibited by SA. Because RSK1/2 has been shown to phosphorylate SOS (37), an upstream signaling molecule of Ras, and thus may regulate Ras activation by a feedback mechanism, we tested the hypothesis that SA indirectly inhibits Ras and ERK1/2 activation via RSK1/2 suppression. Our data did not support this hypothesis, because Ras activation by PMA was not blocked by dominant negative mutant of RSK2. It is possible that SA may act on a signaling molecule upstream of Ras. One possible candidate is PKC, which is essential for PMA-induced COX-2 expression and is known to activate the Ras signaling pathway. PMA is known to activate multiple isoforms of classic and novel PKC isoforms. In human embryonic kidney 293 cells, PMA activates novel PKCs including PKCα, PKCβ, and PKCγ (38). However, it has been shown that salicylate does not inhibit the activity of any of the PMA-activated isoforms (38). Thus, PKC may not be the upstream target. The mechanism by which SA inhibits PMA-induced Ras and ERK1/2 activation remains to be elucidated. Nevertheless, our results indicate that SA inhibits C/EBPβ-mediated COX-2 expression by suppressing RSK1/2 activity via direct and indirect pharmacological mechanisms.

In summary, our results have shown that SA inhibits RSK activation, thereby reducing RSK-induced C/EBPβ binding and transcriptional activation of COX-2. Our results further show that SA blocks Ras and ERK activation, suggesting that salicylate may inhibit other kinases that share active site configuration with RSK1/2. As RSK-related kinases activate a large number of transcription factors including CREB, IκB, Fos, CREB-binding protein, and p300 coactivator (39–41), salicylate may control the expressions of diverse genes important in inflammation and cell proliferation. Therefore, RSK and related kinases may be potentially valuable targets for drug discovery.

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