Salicylic Acid and Aspirin Inhibit the Activity of RSK2 Kinase and Repress RSK2-Dependent Transcription of Cyclic AMP Response Element Binding Protein- and NF-κB-Responsive Genes[1]

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Sodium salicylate (NaSal) and other nonsteroidal anti-inflammatory drugs (NSAIDs) coordinately inhibit the activity of NF-κB, activate heat shock transcription factor 1 and suppress cytokine gene expression in activated monocytes and macrophages. Because our preliminary studies indicated that these effects could be mimicked by inhibitors of signal transduction, we have studied the effects of NSAIDs on signaling molecules potentially downstream of LPS receptors in activated macrophages. Our findings indicate that ribosomal S6 kinase 2 (RSK2), a 90-kDa ribosomal S6 kinase with a critical role as an effector of the RAS-mitogen-activated protein kinase pathway and a regulator of immediate early gene transcription is a target for inhibition by the NSAIDs. NSAIDs inhibited the activity of purified RSK2 kinase in vitro and of RSK2 in mammalian cells and suppressed the phosphorylation of RSK2 substrates cAMP response element binding protein (CREB) and I-κBα in vivo. Additionally, NaSal inhibited the phosphorylation by RSK2 of CREB and I-κBα on residues crucial for their transcriptional activity in vivo and thus repressed CREB and NF-κB-dependent transcription. These experiments suggest that RSK2 is a target for NSAIDs in the inhibition of monocyte-specific gene expression and indicate the importance of RSK2 and related kinases in cell regulation, indicating a new area for anti-inflammatory drug discovery. *The Journal of Immunology,* **1999, 163: 5608–5616.**

Sodium salicylate (NaSal)[3] and other members of the nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the expression of genes for TNF-α, IL-1β, IL-6, IL-8, IL-10, and ICAM-1 in monocytes activated by the proinflammatory LPS (1, 2). A potential mechanism that may underlie inhibition of monocyte gene expression by NSAIDs is transcriptional repression due to the perturbation of the activity of the transcription factors NF-κB and heat shock transcription factor 1 (HSF1) (3–5). NaSal and other NSAIDs inhibit the activation of NF-κB and simultaneously activate HSF1 to a DNA binding form at identical drug doses (2, 5). The effect of NaSal on HSF is highly conserved and is observed in human, murine, *Drosophila,* and yeast and is mimicked by at least nine other members of the NSAIDs, indicating an evolutionarily conserved mechanism (4, 6–8). Regulatory mechanisms underlying the transcriptional activation of both HSF1 and NF-κB are similar and involve protein phosphorylation (for review, see Ref. 9). In addition, activation of both factors undergo conversion from latent cytoplasmic forms to a nuclear *trans*-activating factors (reviewed by Refs. 9 and 10). Negative regulation of NF-κB involves the influence of inhibitory subunits, including the protein I-κBα, which binds NF-κB and inhibits nuclear localization (10). Activation of NF-κB by proinflammatory agonists involves the phosphorylation of I-κBα, which targets it for degradation by the proteosome and releases NF-κB to migrate to the nucleus and activate the promoters of target genes (10).

We have examined whether a common signaling pathway involved in the activity of both factors may underlie the effects of NSAIDs on gene expression in human monocytes by studying intermediates involved in the signal transduction cascade that leads to NF-κB activation after exposure of monocytes to LPS. Our previous studies showed that tyrosine kinase inhibitors herbimycin A and AG126, which inhibit LPS induction of tyrosine phosphorylation (11, 12), mimic the effects of NaSal in inducing the binding of HSF1 to its response element and inhibiting the binding of NF-κB to consensus κB elements in primary human monocytes (X. Wang, A. Asea, B. Chu, X. Zhang, and S. K. Calderwood, manuscript in preparation). This suggested the existence of one or more signaling pathways that activate NF-κB and repress HSF1 that are inhibited by exposure to NSAIDs. We therefore examined protein kinases that could potentially function in a signaling cascade downstream of LPS and proximal to HSF1 and NF-κB and found that mitogen-activated protein kinase-activating kinase (MAPKAP K2) and the 90-kDa ribosomal S6 kinase (RSK2) are both inhibited by NaSal at concentrations that activate HSF1 and inhibit the induction of NF-κB to nuclear factors able to bind DNA in in vitro EMSA experiments (X. Wang, A. Asea, B. Chu,

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[3] Abbreviations used in this paper: NaSal, sodium salicylate; CREB, cyclic AMP response element binding protein; HSF, heat shock factor; HSP, heat shock protein; NSAID, nonsteroidal anti-inflammatory drug; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKAP K2, mitogen-activated protein kinase-activating kinase; RSK2, ribosomal S6 kinase 2; Luc, luciferase; Asp, aspirin; Ibu, ibruprofen; ERK, extracellularly regulated kinase; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ATF, activating transcription factor; HA, hemagglutinin; RA, rheumatoid arthritis; PPAR-γ, peroxisome proliferator-activated receptor-γ.

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X. Zhang, and S. K. Calderwood, manuscript in preparation). However, only RSK2 catalyzed the phosphorylation of I-κBα in vitro, suggesting RSK2 as a signal transduction intermediate in the response to LPS, which is targeted by NaSal (X. Wang, A. Asea, B. Chu, X. Zhang, and S. K. Calderwood, manuscript in preparation). We have therefore studied RSK2 as a potential target for NSAIDs in vivo. Here we show that the activity of purified RSK2 is inhibited by a wide spectrum of NSAIDs *in vitro* and that these drugs inhibit the activity of cellular RSK2 in human monocytes and murine fibroblasts. Subsequent studies in vivo indicated that RSK2 overexpression leads to the phosphorylation of at least two target proteins, CREB and I-κBα, on sites crucial for *trans*-activation by CREB and NF-κB and that NaSal inhibits such phosphorylation. As NaSal has been shown previously to repress the promoters of monocyte genes, we examined the potential role of RSK2 in gene transcription and NaSal as a transcriptional inhibitor (2, 13). Inhibition of CREB and phosphorylation in RSK2-transfected cells was correlated with repression of CREB- and NF-κB-responsive promoters.

Materials and Methods

Abs and reagents

pMT2.HA-RSK2 was a gift from Dr. M. E. Greenberg (Harvard Medical School, Boston, MA); pFA-CREB and pFR-Luc were purchased from Stratagene (La Jolla, CA); anti-RSK and p65 Abs, purified PP2A1, and MAPKAP K2 were purchased from Upstate Biotechnology (Lake Placid, NY); anti-CREB and anti-IκBα were purchased from New England Biolabs (Beverly, MA). Chromatographic materials were purchased from Pharmacia (Piscataway, NJ).

Cells and cell culture

NIH 3T3 mouse embryonic fibroblasts and HeLa S3 human carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA), grown in DMEM containing 10% bovine calf serum (NIH 3T3) or Ham's F-10 plus 10% FCS (HeLa S3), and passaged at a 1:10 ratio using trypsin/EDTA.

Isolation and enrichment of human monocyte

PBMC were isolated from freshly drawn peripheral venous blood (Kraft Family Blood Center, Dana-Farber Cancer Institute, Boston, MA) using the Ficoll-Paque separation technique as previously described (14). Monocytes were enriched from the PBMC fraction by negative depletion using magnetic beads coated with appropriate mAbs (15). Briefly, using a monocyte isolation kit, (Miltenyi Biotec, Auburn, CA), PBMC were treated with FcR blocking agent followed by hapten-Ab mixture containing monoclonal hapten-conjugated CD3 (mouse Ig2A), CD7 (mouse Ig2A), CD19 (mouse IgG1), CD45RA (mouse IgG1), CD56 (mouse IgG2b), and anti-IgE (mouse IgG2A) Abs and incubated for 5 min at 4°C. Cells were washed twice in PBS and incubated in FcR blocking buffer and MACs anti-hapten microbeads. After a 5-min incubation at 4°C, cells were washed and passed through a column attached to a magnet. Unlabeled monocytes ($CD45^+CD14^+$) eluted from the column were >98% pure, as judged by flow cytometry.

Protein extraction immunoprecipitation and Western blot analyses

Proteins were extracted from cells following treatment with ice-cold RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 2.5% deoxycholate, 2 mM EGTA, 1 mM leupeptin, 1 mM aprotinin, 10 mM NaF, and 1 mM PMSF), and samples were cleared by centrifugation at 15,000 × *g* for 20 min at 4°C. For immunoprecipitation experiments, supernatant was carefully removed and incubated with primary Ab for 1–2 h on ice, and immunoprecipitates were collected with protein A-Sepharose beads (Pharmacia) for 30 min at 4°C. The precipitate was washed three times with PBS, boiled in SDS-PAGE sample buffer before electrophoresis on 12% SDS-polyacrylamide gels, and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membrane was blocked by incubation in TBS buffer supplemented with 5% nonfat dry milk (Bio-Rad, Hercules, CA) and 0.1% Tween 20 for 1 h at room temperature, washed three times in TBS buffer, and incubated overnight with the appropriate primary Ab at 4°C. Membranes were washed three times with TBS buffer and incubated with the appropriate alkaline phosphate-conjugated secondary Ab for 1 h at room temperature. Detection of proteins was achieved by the enhanced chemiluminescence system.

Immunoblot assay

Cells were washed three times in ice-cold Dulbecco's PBS and dissolved in Laemmli sample buffer, and samples ere fractionated by 10% SDS-PAGE before immunoblot assay with anti-HSF1 Abs using procedures similar to those described previously (16).

In vitro kinase assays

RSK2 was purified from rabbit muscle, using a preparation scheme described previously, as a 90-kDa polypeptide by SDS-PAGE analysis and was identified by an Ab that recognizes RSK2, but not RSK1 (17). Although free of RSK1 and RSK3, some of the enzymatic activity in the extract could possibly be due to a trace contaminant. However, purified RSK2 contained negligible activity for MAPK, MAPKAP K2, or PKCα activity assessed using enzyme-specific peptides. RSK2 (0.2 U) was incubated for 20 min at 30°C with 0.25 mM peptide substrate (RRRLSSLRA; 95% pure) and 0.5 μCi [γ-^{32}P]ATP (New England Nuclear, Boston, MA) in 25 μl of GNM buffer (60 mM β-glycerophosphate, 30 mM *p*-nitrophenylphosphate, 25 mM MOPS, 15 mM $MgCl_2$, 150 mM ATP, 0.1 mM sodium orthovanadate, 5 mM EGTA, and 1 mM DTT, pH 7.0). Phosphopeptides were isolated on p81 filters (Pierce, Rockford, IL), washed in ice-cold 75 mM phosphoric acid, and assayed by Cherenkov counting. Purified MAPKAP K2 (0.1 U) was assayed with synthetic peptide KKPLNRTLSVASLPGLamide (3) and 0.5 μCi of [γ-^{32}P]ATP (New England Nuclear) as described above for RSK2. To test for cross-reactivity of the purified MAPKAP K2 with RSK2, controls were incubated with 0.1 mM H7, which inhibits RSK2 but not MAPKAP K2 (3). Recombinant HSF1 (13) and GST-I-κBα were incubated (5 μg/reaction) with RSK2 and assayed as described above.

In vivo immunokinase assay

Cells were washed in ice-cold PBS and lysed in MGGE buffer (30 mM 4-morpholinepropanesulfonic acid (pH 7.0), 10% glycerol, 80 mM β-glycerophosphate, 20 mM, EGTA, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, 0.5% Triton X-100, 5 mM DTT, 1.0 mM PMSF, and 1 mM leupeptin). RSK2 was then immunoprecipitated from supernatants after a 15-min spin at 15,000 × *g* using anti-RSK2 antiserum (60 min at 4°C). Immune complexes were collected on protein A-Sepharose beads and washed in GNM buffer (described above), supplemented with 1 mM PMSF, 1 mM leupeptin, and 30 ng/ml of the protein kinase A inhibitor peptide (Sigma). The immune complexes were then assayed by incubation with RSK2 substrate peptide and [γ-^{32}P]ATP (New England Nuclear) as described above (see *In vitro kinase assays*).

Transient transfection and transcriptional reporter gene assay

For transfection experiments, cells were dispensed into six-well plates (Costar, Cambridge, MA) at 2.2×10^5 cells/well and left for 20–24 h before liposome-mediated transfection with reporter plasmids (κB-Luc and pFR-Luc), expression plasmids (pMT2.HA-RSK2 and pFA-CREB), and control (pCMV-*lacZ* expression plasmids) as previously described (18). κB-Luc was made by inserting four contiguous consensus κB elements into the polycloning site of the pGL3-Promoter vector (Promega). All treatments contained the same amount of DNA. Transfectants were incubated overnight in a 2.5% CO_2/air atmosphere. Cell extracts were prepared as described in detail, and luciferase (Luc) and β-galactosidase assays were performed as previously described (19). Reporter gene activity was normalized to β-galactosidase activity, which was used as an internal transfection efficiency control. Results are expressed as the fold increase in activity over the control value.

Results

NSAIDs inhibit the activity of purified RSK2 in vitro

We first examined the effects of NSAIDs shown previously to induce HSF1 and repress NF-κB on the activity of RSK2 purified from rabbit muscle. We investigated the ability of the NSAIDs to inhibit the stimulation of phosphorylation of a peptide substrate for RSK2 (Fig. 1*A*). RSK2 activity was inhibited by drugs from a number of classes of NSAID, including the salicylates NaSal and aspirin (Asp); the arylproprionic acid ibuprofen (Ibu); the arylacetic acids indomethacin, sulindac, and diclofenac; the oxicam piroxicam; and the pyrazolidinedione phenylbutazone (Fig. 1*A*). The

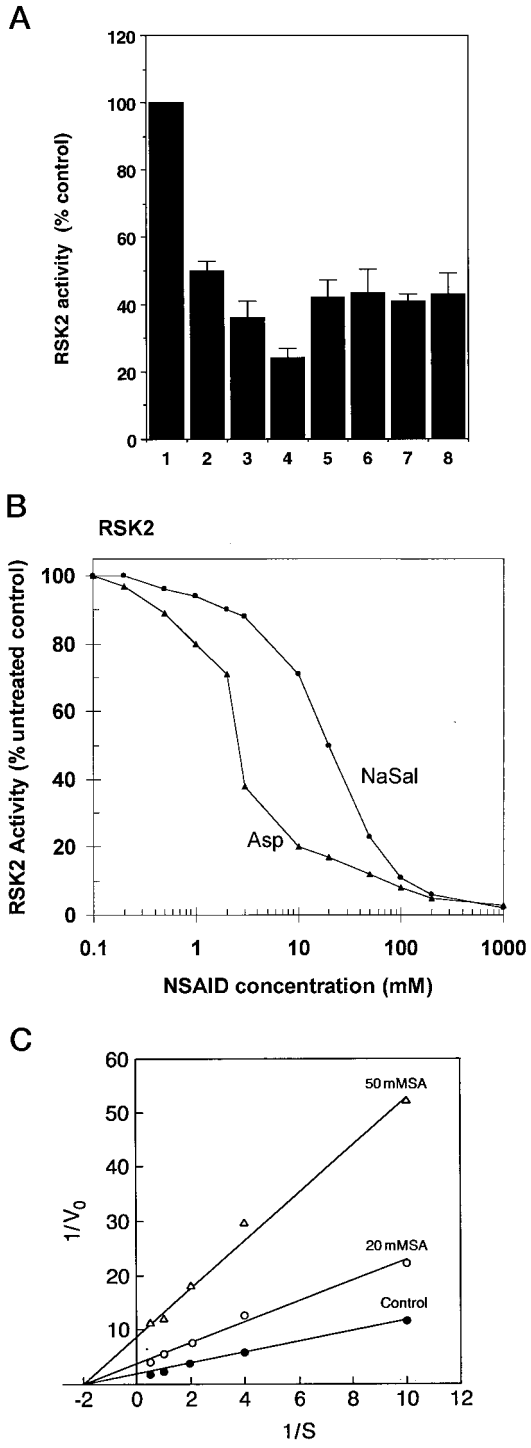

FIGURE 1. Effects of NSAID family members on the phosphotransferase activity of purified rabbit RSK2 in vitro. *A*, RSK2 was incubated without drug (*lane 1*) or with 20 mM NaSal (*lane 2*), 3 mM Asp (*lane 3*), 2 mM Ibu (*lane 4*), 0.3 mM indomethacin (*lane 5*), 0.6 mM sulindac (*lane 6*), 0.3 mM diclofenac (*lane 6*), 1 mM piroxicam (*lane 7*), or 1 mM phenylbutezone (*lane 8*). Experiments were conducted in triplicate as described in *Materials and Methods* and were repeated three times with consistent findings. Mean values are expressed as a percentage of control RSK2 activity in the absence of inhibitors ± SD. *B*, Dose-response curve for the inhibition of purified RSK2 kinase by NaSal (●) and Asp (▲). RSK2 was incubated for 20 min with specific peptide substrate and 0.5 μCi [γ-^{32}P]ATP as described in *Materials and Methods*. *C*, Double reciprocal plot of RSK2 activity against substrate concentration under control conditions with 20 and 50 mM NaSal (SA).

drugs inhibited RSK2 at concentrations (Fig. 1*A*) shown previously to activate HSF1 and to inhibit NF-κB (2, 4). Most of the drugs inhibited RSK2 at concentrations between 0.1 and 1 mM, although Asp (3 mM) and NaSal (20 mM) required higher concentrations (Fig. 1*A*). Even though it required higher concentrations for inhibition of RSK2 activity, we used NaSal in most of the subsequent studies shown here due to its low toxicity in tissue culture (NaSal could be used at concentrations up to 100 mM without significant killing of NIH 3T3 cells). We next went on to examine the dose dependency of RSK2 inhibition by NaSal and Asp (Fig. 1*B*). RSK2 activity was inhibited by 50% at a concentration of 20 mM NaSal, while Asp, which inhibits HSF1 and NF-κB activity at a lower concentration than NaSal (4, 5), was also a more effective RSK2 inhibitor, reducing RSK2 activity by 50% at ~2.5 mM (Fig. 1*B*). Kinetic analysis of RSK2 inhibition was then performed using a double-reciprocal plot of RSK2 activity against the peptide substrate concentration, either with no inhibitor or with 20 or 50 mM NaSal (Fig. 1*C*). The plots do not share a common intercept on the 1/Vo axis characteristic of a noncompetitive inhibitor, indicating that NaSal interacts with RSK2 to cause allosteric inhibition (Fig. 1*C*).

Effects of NaSal on the stimulation of protein phosphorylation by purified RSK2 in vitro

We next examined the effectiveness of NaSal as an inhibitor of the ability of RSK2 to catalyze phosphorylation of protein substrates in vitro (Fig. 2). As the DNA binding activities of NF-κB and HSF1 are modulated by NaSal in cells, we first determined whether HSF1 and I-κBα were phosphorylated by RSK2 in vitro in a NaSal-sensitive manner (5, 6). RSK2 stimulated the phosphorylation of both HSF1 (Fig. 2*A*, *lane 2*) and I-κBα (*lane 4*), and this activity was inhibited by NaSal (Fig. 2*B*, *lanes 3* and *5*) at concentrations that lead to the activation of HSF1 and the inhibition of NF-κB binding to DNA in vivo (20 mM*)*. Because previous studies showed that RSK kinases phosphorylate I-κBα at Ser^{32} and that site-directed mutagenesis at this site blocks the response to activators (20, 21), we determined whether Ser^{32} phosphorylation was inhibited by NaSal and Ibu (Fig. 2*B*). Using Abs specific for I-κBα phosphorylated at Ser^{32}, we found that RSK2 catalyzed the phosphorylation of I-κBα on Ser^{32}, an effect prevented by incubation with NaSal and Ibu (Fig. 2*B*). Recent studies indicate that HSF1 is phosphorylated in vitro by RSK2 on at least three residues (S. K. Calderwood, unpublished observations), which may account for the greater incorporation of ^{32}P into HSF1 compared with I-κBα, which is apparently phosphorylated at only one site (Fig. 2*B*). However the nature of the amino acids phosphorylated in HSF1 after incubation with RSK2 is not yet known. We also investigated the effect of NaSal on phosphorylation of a well-characterized RSK2 substrate protein, the transcription factor cAMP response element binding protein (CREB) (22). Incubation with RSK2 resulted in the phosphorylation of murine CREB protein immunoprecipitated from NIH 3T3 cells, and this effect was inhibited by NaSal (Fig. 2*C*). As CREB is a well-characterized substrate for RSK2, we used this protein as a primary model for examining the effects of NaSal on RSK2 activity in subsequent studies in tissue culture.

Inhibition of cellular RSK2 activity in primary monocytes stimulated by LPS and in quiescent monocytes induced by mitogens

We next examined the ability of NSAIDs to inhibit cellular RSK2 in vivo. As RSK2 is largely inactive under resting conditions, we examined two cell types in which RSK2 can be induced by upstream signaling cascades (22). We first investigated the effects of

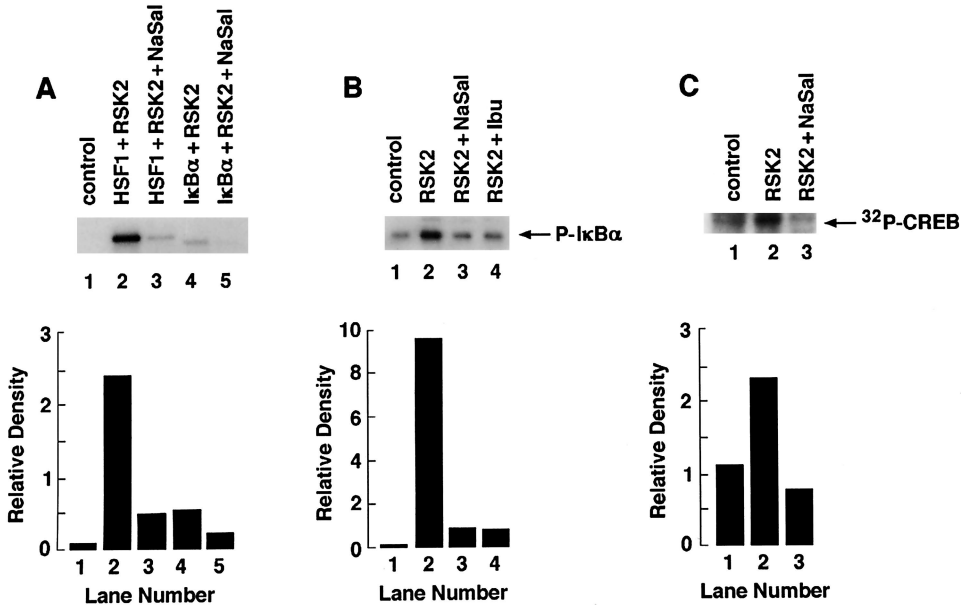

FIGURE 2. Effects of NaSal and Asp on protein phosphorylation by RSK2 in vitro. *A*, Phosphorylation of purified recombinant HSF1 and GST-I-κBα by purified RSK2 without or with 20 mM NaSal (SA). Proteins were incubated with MAPKAP K2 and RSK2 as described in *Materials and Methods*, then quenched in SDS-PAGE sample buffer before analysis by 10% gel electrophoresis and x-ray film autoradiography. *B*, Phosphorylation of purified GST-I-κBα on Ser^{32} in vitro detected by phospho-specific Abs. After incubation, the proteins were analyzed by 12% SDS-PAGE and immunoblot analysis, with an Ab specific for Pi-Ser^{32}-I-κBα as described in *Materials and Methods*. *C*, Effect of NaSal on the phosphorylation of CREB immunoprecipitated from NIH 3T3 cell extracts with anti-CREB antiserum and incubated with RSK2 in vitro. Immunoprecipitated CREB (confirmed by immunoblot analysis) was dephosphorylated with PP2A, then incubated with RSK2 in the presence of 10 nM okadaic acid to inhibit PP2A.

LPS on RSK2 activity in freshly recovered human monocytes isolated from peripheral venous blood (Fig. 3*A*). Exposure to LPS caused an ~2-fold increase in cellular RSK2 activity measured by immunokinase assay, and this increase was inhibited by exposure to NaSal (Fig. 3*A*). Thus, doses of NaSal that inhibit the activity of purified rabbit RSK2 in vitro inhibit cellular RSK2 in human monocytes (Fig. 1, *A* and *B*, and 2*A*). However, these experiments do not indicate whether the effects of NaSal are due to direct interactions of the drug with the enzyme or to indirect effects on upstream signaling, cellular phosphatases, or other regulatory interactions. RSK2 is activated downstream of the extracellularly regulated kinase (ERK) MAPK pathway and is a direct substrate for ERK (23, 24). As NaSal has been shown to inhibit LPS-induced cellular ERK activity, we examined these interactions further using mitogen-activated fibroblasts in which RSK2 is activated downstream of ERK. As NaSal has been shown not to inhibit epidermal growth factor (EGF) stimulation of ERK in fibroblasts and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulation of ERK in PMN, we examined the effects of NaSal and other NSAIDs on RSK2 activated by EGF and TPA in murine fibroblasts. RSK2 activity was strongly stimulated by both TPA (Fig. 3*B*) and EGF (Fig. 3*C*) in NIH 3T3 murine fibroblasts. TPA- and EGF-induced RSK2 activity was inhibited by NaSal, Asp, and Ibu (Fig. 3, *B* and *C*). The possibility that inhibition of RSK2 by NSAIDs may be independent of the effects of ERK activity was confirmed by experiments in which TPA-induced cellular ERK activity was not significantly inhibited by 10 or 5 mM NaSal, 1 mM Asp, or 1 mM Ibu (Fig. 3*D*), concentrations previously demonstrated to drastically inhibit TPA-induced cellular RSK2 activity (Fig. 3*C*), indicating that RSK2 is indeed the primary target for NSAIDs. Interestingly, TPA-induced ERK activity was inhibited by 20 mM NaSal, 3 mM Aps, and 2 mM Ibu (Fig. 3*D*), suggesting the possibility that at high concentrations NSAIDs may target multiple kinases.

NaSal inhibits the phosphorylation of RSK2 substrates in NIH 3T3 cells

We next examined the effects of exposure to NaSal on phosphorylation of intracellular CREB, a well-characterized RSK2 substrate in murine NIH 3T3 fibroblasts. We investigated the phosphorylation of CREB at a critical residue, serine 133, within the transcriptional activation domain that is phosphorylated in vivo by RSK2 (22). Exposure to TPA markedly increased CREB phosphorylation on Ser^{133}, and this effect was partially reversed by exposure to 1 mM NaSal and completely inhibited by 10- and 20-mM concentrations of the drug (Fig. 4*A*). Total CREB protein levels, as determined by immunoblot, remained constant. The Ser^{133}-specific Ab used here also cross-reacts with ATF-1 phosphorylated at a serine residue conserved within the *trans*-activation domain of CRE binding proteins (25). As with CREB, TPA-induced ATF-1 phosphorylation is inhibited by 20 mM NaSal (Fig. 4*A*).

To examine more specifically the effects of NaSal on RSK2 activity in vivo, we cotransfected cells with expression vectors containing epitope (HA)-tagged murine RSK2 and a GAL4-CREB hybrid gene encoding the activation domain of CREB (the N-terminal 147 aa of GAL4 fused to aa 1–280 of CREB). We aimed to distinguish the effects of RSK2 on CREB expressed in the transfectants from wild-type CREB in untransfected cells using the GAL4-CREB chimera as a reporter of phosphorylation; GAL4-CREB contains Ser^{133} from the wild-type CREB protein. RSK2 expression led to the phosphorylation of the GAL4-CREB chimera on Ser^{133} (Fig. 4*B*, *lane 6*), an effect that was increased by exposure to serum (*lane 7*) and was inhibited by 20 mM NaSal (*lane 8*). Relative levels of phospho-GAL4 CREB are indicated in the densitometer scan in Fig. 4*C*. Also evident in the immunoblot are native phospho-CREB and phospho-ATF-1, the level of each of which was activated by serum exposure (Fig. 4*B*, *lane 7*) and inhibited by NaSal (Fig. 4*D*, *lane 8*). Fig. 4*D* shows the relative

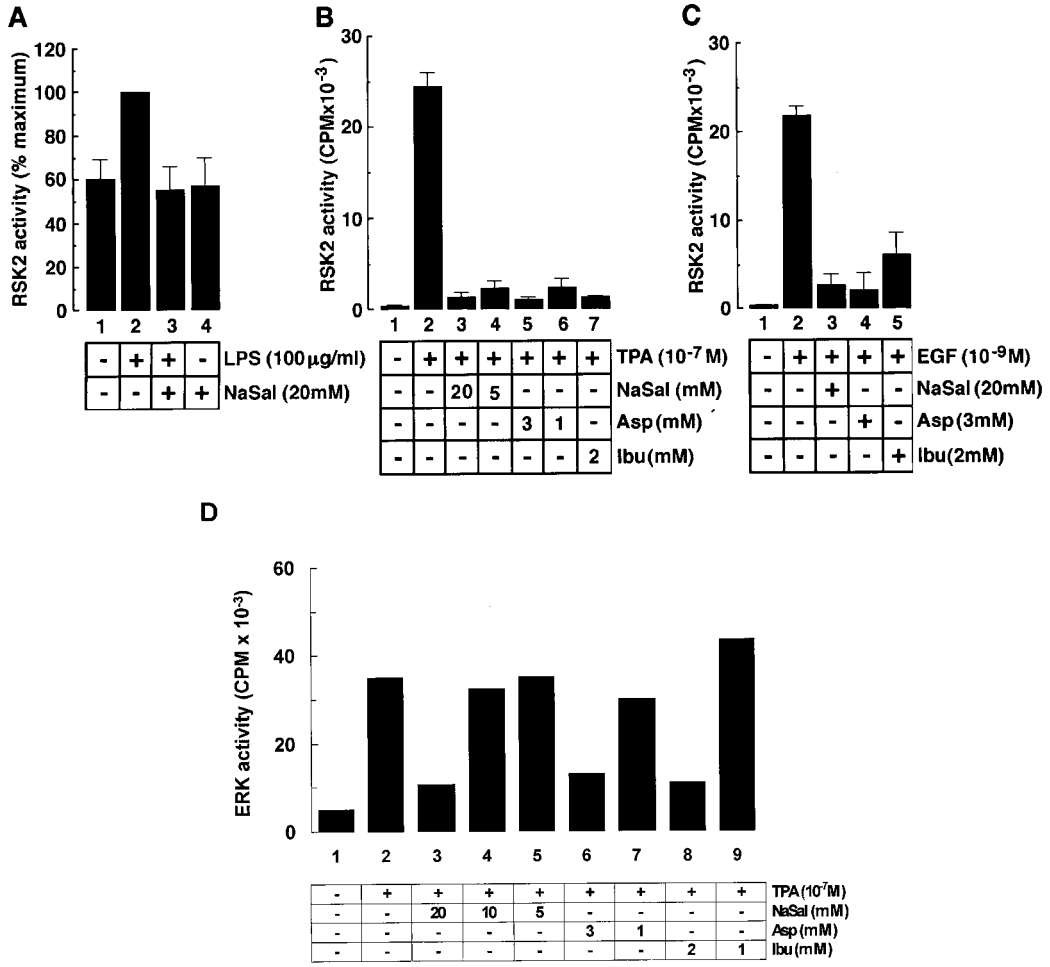

FIGURE 3. Effects of NSAIDs on the activity of cellular RSK2 and ERK in vivo. *A*, Inhibition of LPS-induced RSK2 activity in human monocytes in vivo. Freshly recovered human monocytes (10^7) isolated from peripheral venous blood and enriched by negative depletion using magnetic bead separation were treated with culture medium (control) or 20 mM NaSal for 20 min at 37°C, followed by a further 10-min incubation with LPS (10 μg/ml) or culture medium (control). Extracted proteins were immunoprecipitated, and the immune complexes were then assayed by incubation with RSK2 substrate peptide and [γ-^{32}P]ATP. Bars represent RKS2 immunokinase activity (percentage of maximum). Experiments were conducted three times, and the mean ± SD are shown. *B*, Inhibition of TPA-activated RSK2 activity in quiescent fibroblasts. NIH 3T3 cells were serum starved for 48 h and then treated with TPA (10^{-7} M) either without drug or with NaSal, Asp, or Ibu as shown. Bars represent RKS2 immunokinase activity in counts per minute. Experiments were conducted three times, and the mean ± SD are shown. *C*, Inhibition of EGF-activated RSK2 activity in quiescent fibroblasts. NIH 3T3 cells were serum starved for 48 h and then treated with EGF (10^{-9} M) either without drug or with NaSal, Asp, or Ibu as shown. Bars represent RKS2 immunokinase activity in counts per minute. Experiments were conducted three times, and the mean ± SD are shown. *D*, Inhibition of TPA-activated ERK in quiescent fibroblasts. NIH 3T3 cells were serum starved for 48 h and then treated with TPA (10^{-7} M) either without drug or with NaSal, Asp, or Ibu at the concentration indicated. Bars represent ERK immunokinase activity in counts per minute. Results are a representative of three independently performed experiments with similar results.

levels of RSK2 in controls and in cells overexpressing HA-RSK2 with and without serum exposure. RSK2 and HA levels increased markedly in the transfectants, and these levels were not altered by exposure to serum or NaSal (Fig. 4, *B* and *C*). Total cellular RSK2 kinase activity determined by immunokinase assay was elevated 2-fold in serum-treated cells and RSK2 transfectants and was markedly increased when RSK2 expression was combined with serum stimulation (Fig. 4*F*, *lane 7*). NaSal inhibited the increases in RSK2 activity observed in RSK2 transfectants with or without serum stimulation (Fig. 4*F*, *lanes 5* and *8*). We also examined the effects of exposure to NaSal on the phosphorylation of I-κBα in cells exposed to TPA and overexpressing RSK2. TPA activated I-κBα phosphorylation of Ser^{32}, and this effect was inhibited by NaSal (data not shown). We also examined the effects of NaSal on I-κBα phosphorylation after treatment with another stimulus, TNF-α, in a TNF-α-responsive cell line (HeLa cells). TNF-α activated phosphorylation of I-κBα on Ser^{32}, and this effect was inhibited by exposure to NaSal (data not shown).

RSK2-dependent activation of CREB- and κB-responsive promoters is inhibited by sodium salicylate

Having examined the effects of NaSal on CREB phosphorylation by RSK2 in vivo, we next attempted to determine whether these effects on CREB phosphorylation translated into changes in transcriptional activation by CREB. We therefore examined the effects of NaSal on the activation of a GAL4-Luc reporter construct by the GAL4-CREB hybrid protein with or without RSK2 expression using the conditions described previously in Fig. 4, *B–F*. Overexpression of RSK2 increased GAL4-Luc activation by the hybrid transcription of GAL4-CREB (3- to 4-fold), an effect that was increased by exposure to serum and inhibited by incubation with NaSal (Fig. 5*A*). Transcriptional activation of GAL4-CREB (Fig.

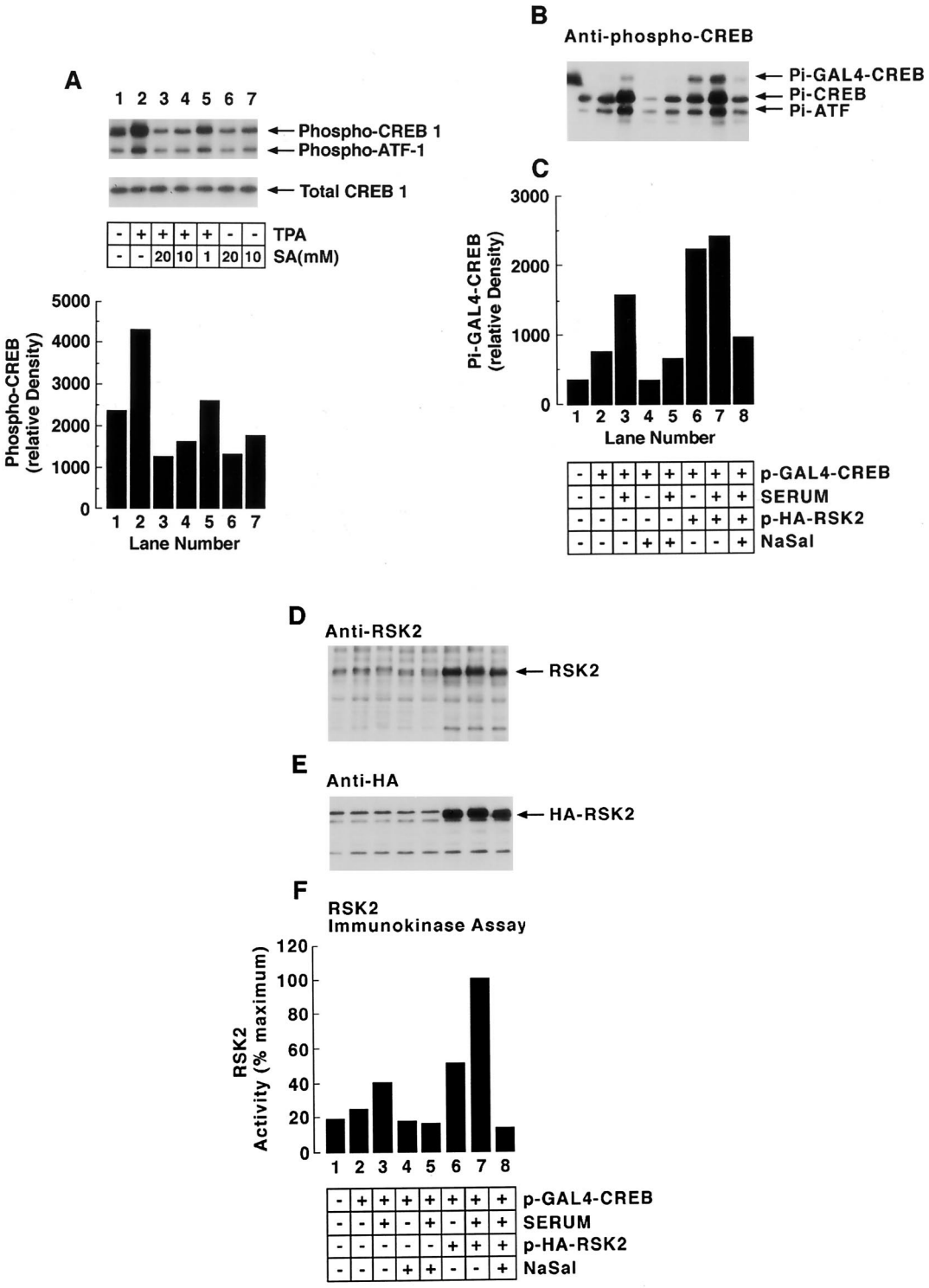

FIGURE 4. Inhibition of RSK2-induced protein phosphorylation by NaSal in vivo. *A*, Quiescent NIH 3T3 cells were exposed to 1 mM TPA for 10 min without or with 15-min preincubation with varying concentrations of NaSal (SA). Extracted proteins were analyzed by 10% SDS-PAGE and immunoblot with Abs specific for phosphoserine 133-CREB or total CREB. Immune complexes were detected with a coupled second Ab-chemiluminescence method. This experiment was conducted twice with consistent results. *B*, Phosphorylation of GAL4-CREB hybrid protein in NIH 3T3 cells overexpressing GAL4-CREB and RSK2. Cells were cotransfected with p-GAL4-CREB (0.5 μg/60-mm dish) and pMT2.HA-RSK2 (5.0 μg) and assayed by immunoblot with anti-phospho-CREB as indicated in *Materials and Methods*. pGAL4-CREB transfectants (*lanes 2–8*) or pMT2.HA-RSK2 cotransfectants (*lanes 6–8*) were incubated for 24 h, then retained as controls (*lane 2*), pretreated for 15 min with 20 mM NaSal (*lane 4*), exposed to 10% serum for 10 min (*lanes 3* and *7*), or treated with NaSal before serum (*lanes 5* and *8*). RSK2 was then assayed by immunoblot with anti-RSK2 Abs. The whole experiment represented in *B–F* was conducted twice with consistent results. *C*, Relative levels of Pi-GAL4-CREB in the incubations in *B* as determined by densitometry. *D*, Relative levels of cellular RSK2 determined by immunoblot with anti-RSK2 Abs. *E*, Expression of NIH 3T3-transfected HA-RSK2 assayed by immunoblot with anti-HA Abs. *F*, Activity of RSK2 immunoprecipitated from cells treated as described above (*B*) and assayed as described in Fig. 1 with the addition of 1 mM PMSF, 1 mM leupeptin, and 30 ng/ml protein kinase A inhibitor peptide to the reactions.

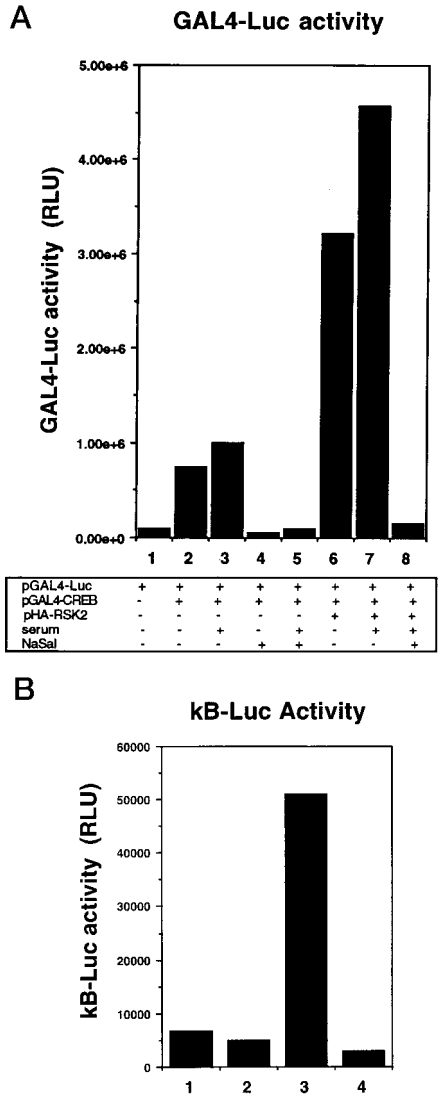

FIGURE 5. Effect of NaSal on the transcriptional activation by RSK2 of a GAL4-CREB hybrid protein and an NF-κB-dependent promoter. *A*, NIH 3T3 cells were transfected with pGAL4-Luc reporter and combinations of GAL4-CREB and RSK2 expression vectors, serum, and NaSal as described in Fig. 4*B*. Transfections, luciferase assay, and β-galactosidase assays were conducted as previously described (2). Controls (*bar 1*) were transfected with empty RSK2 expression plasmid. Results represent relative GAL4-Luc activity. Experiments were conducted three times and yielded consistent results. *B*, NIH 3T3 cells were transfected with κB-Luc plasmid alone (*bar 1*) or with 20 mM NaSal (*bar 2*), RSK2 expression vector (*bar 3*), or RSK2 plasmid plus NaSal (*bar 4*). Extracts were prepared, processed, and assayed as described in *A*. β-Galactosidase activity in controls transfected with pCMV-β-galactosidase alone (*bar 1*) or with 20 mM NaSal (*bar 2*), pMT2.HA-RSK2 (*bar 3*), or pMT2.HA-RSK2 plus NaSal (*bar 4*). Experiments were conducted three times and yielded consistent results.

5*A*) thus paralleled its phosphorylation state (Fig. 4*B*), indicating that activation of CREB in transfectants is repressed by NaSal through a mechanism that may include direct noncompetitive inhibition of the phosphotransferase activity of RSK2.

We further examined the effects of NaSal on RSK2-activated transcriptional activation by NF-κB in cells cotransfected with a κB-Luc promoter-reporter construct and an RSK2 expression vector (Fig. 5*B*). RSK2 expression activated the κB-driven promoter, and this effect was blocked by exposure to NaSal (Fig. 5*B*). The inhibitory effects of NaSal on the CREB- and κB-responsive promoters were specific and were not due to general effects on transcription, as indicated in control experiments showing that the constitutively active CMV immediate-early gene control promoter was neither activated by RSK2 expression nor inhibited by NaSal (Fig. 5*C*). Thus, the effects of NaSal were specific for the activation of transcription by RSK2 (Fig. 5*C*). Transcriptional repression by NaSal is not limited to the experimental CREB- and NF-κB-responsive promoters examined above, and both the *IL1B* and c-*fms* promoters are repressed by NaSal (Y. Xie and S. K. Calderwood, manuscript in preparation).

Discussion

These experiments indicate that NSAIDs inhibit purified RSK2 in vitro at concentrations that lead to inhibition of cytokine gene expression in activated monocytes (Figs. 1 and 2) (2). Although we have not addressed the structural mechanisms involved in RSK2 inhibition by NaSal in any depth, the analysis in Fig. 1 suggests that the drugs can interact directly with the enzyme in such a way as to inhibit catalytic activity. The RSK kinases are unusual in containing two highly conserved kinase domains (26, 27). The N-terminal domain, which is apparently active in phosphorylating exogenous substrates of RSK2 is structurally related to protein kinase C and the catalytic domains of protein kinases A and G (27). The C-terminal domain, which contains sequence similarities with calcium- and calmodulin-activated kinase II and MAPKAP K2, does not directly phosphorylate exogenous substrates but, instead, plays a role in autophosphorylation and apparently is an essential regulatory subunit of RSK2 (27). As NaSal inhibits MAPKAP K2 activity at similar doses as RSK2 inhibition (data not shown), it may be reasonable to infer that NaSal interacts with the C-terminal domain of RSK2 to block a regulatory activity required for RSK2 function. The functional domain in RSK2 inhibited by NaSal may be involved in autophosphorylation, as indicated by the finding that NaSal inhibits RSK2 autophosphorylation in vitro and blocks the mobility shift accompanying activation (which may involve autophosphorylation) in vivo (Fig. 4, *B–E*). However, further studies will be required to determine more precisely the mechanism of RSK2 inhibition by NaSal.

Incubation of cells with NSAIDs also leads to the inhibition of intracellular RSK2 activity (Fig. 3). These effects of the NSAIDs on cellular RSK2 may thus reflect direct inhibition of RSK2 activity as observed with purified RSK2 (Figs. 1 and 2). However, the drugs may also act indirectly on regulatory pathways that impact on RSK2 activity in the cell. RSK2 is an inducible kinase activated by the MAPK family members ERK1 and ERK2, which are downstream of a kinase cascade induced by multiple extracellular signals (28). In fact, intracellular ERK activity may be inhibited by NaSal under some circumstances (23, 24). Evidence that argues against ERK as the primary target for NaSal rather than RSK2 in our studies is that intermediate to low concentrations of the NSAIDs NaSal, Asp, and Ibu did not inhibit cellular ERK activity induced by TPA (Fig. 3*D*). In these experiments we observed that high concentrations of NSAIDs caused specific inhibition of TPA-induced cellular ERK activity, indicating the possibility that at these concentrations multiple kinases upstream of RSK2 may also be targets for NSAIDs. Further evidence in favor of RSK2 as the primary target is that the NSAIDs inhibit RSK2 activity induced by EGF and TPA, a treatment that leads to the induction of ERK activity that is not inhibited by NaSal (23, 24). Additionally, both NaSal and Asp inhibit cellular RSK2 at concentrations (5 and 1 mM) that only minimally affect purified RSK2 in vitro (Figs. 1, *A* and *B*, and 2*B*). This discrepancy may have a

number of causes. Firstly, the distribution of the drugs across the cell membrane may not be uniform. The NSAIDs are weak acids that distribute across the plasma membrane according to intra- and extracellular pH (28, 29). The drugs are uncharged at lower pH and rapidly cross membranes, while being charged and relatively immobile across membranes at pH values above 7.0 (28, 29). An additional possibility is that the NSAIDS affect multiple metabolic and regulatory pathways in the cell, and these may have additive/synergistic effects on individual cellular activities such as RSK2. Effects on upstream pathways such as the ERK-MAPK pathway and on the activities of cellular phosphatases, scaffold proteins, or other molecules could be involved in the intracellular effects of the drugs. Inhibition of purified RSK2 by NaSal occurs at concentrations of ~20 mM, well above the range achieved in patients undergoing treatment for inflammatory diseases (for reviews, see Refs. 30 and 31). However, significant inhibitory effects on cellular RSK2 were observed at 5 mM NaSal and 1 mM Asp (Fig. 3*B*). In addition, other members of the NSAIDs that have in common the inhibitory effect on RSK2 were significantly more effective than NaSal and Asp in inhibiting purified RSK2 in vitro (Fig. 1*A*). These, however, remain at the extreme range of concentrations encountered in patients. These findings may be relevant to the behavior of cells in rheumatoid arthritis patients undergoing treatment with high concentrations of NSAIDs (32, 33). Each of the cytokines whose expression by human monocytes was shown previously to be inhibited by NaSal is characteristically found at elevated levels in the synovial fluid of rheumatoid arthritis (RA) patients (2, 34, 35). Such cytokines, which appear to play important roles in the progression of RA, may thus be targets for the NSAIDs used in treatment (31, 35). In support of this conjecture, recent studies have shown that NSAIDs can reduce the concentrations of IL-6 in the synovial fluid of RA patients and inhibit the expression of TNF-α and IL-1β in human synovial membrane explants (16, 36). Our previous findings indicated that NaSal and other NSAIDs inhibit the expression of a wide spectrum of cytokines through inhibition of a number of transcription factors involved in cytokine regulation (2). In one case, NaSal inhibits phosphorylation of CREB at a key site in the transcriptional activation domain and prevents the induction of a CREB-dependent promoter (Fig. 4*A*). As CREB plays a role in cytokine expression, such an effect may impact on NaSal-induced cytokine repression (37). Inhibition of an NF-κB-sensitive promoter may involve a contrasting mechanism, with NaSal inhibiting the phosphorylation of the negative regulatory molecule I-κBα by RSK2, which may prevent NF-κB access to the nucleus (Fig. 2*B*). RSK2 phosphorylates I-κBα on a residue (Ser^{32}) required for a response to NF-κB-activating signals (Fig. 2, *E* and *F*) and is therefore one of the I-κBα kinases that include RSK1 as well as unrelated enzymes (20, 21, 38). Although the contribution of RSK2 to NF-κB activation by proinflammatory signals has not been widely investigated, our studies indicate that prevention of I-κBα phosphorylation on Ser^{32} by NaSal can mediate inhibition of the transcriptional activation of NF-κB (5). In addition, NaSal inhibits the phosphorylation of HSF1, a repressor of cytokine genes, on as yet unknown sites and stimulates its ability to bind DNA, although the precise mechanisms involved in this process are unclear (9, 13, 39). RSK2 is evidently a repressor of HSF1 activation, and NaSal may function to reverse the repression (Figs. 1 and 2*B*). By inhibiting RSK2, NaSal may thus coordinately influence the activity of at least three transcription factors, and its ultimate effects on target promoters may be determined by effects on individual factors or a combination of the factors. As mentioned earlier, however, the NSAIDS appear to have pleiotropic effects on cell regulation, and inhibition of RSK2 may mediate only a portion of the inhibitory effects of the drugs on cytokine gene expression. Recent studies have also shown that three of the NSAIDs bind to the peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear receptor that activates genes involved in adipogenesis but represses monocyte-specific transcription (1). PPAR-γ appears to be capable of repressing both natural promoters, such as the inducible nitric oxide synthase and TNF-α promoters and artificial promoters containing cannonical sites for AP-1, Stat-1, and NF-κB (1). The relative contributions of RSK2 and PPAR-γ or other molecules to cytokine repression by the NSAIDs is, however, not known. In addition, although the effects of NaSal on RSK2 activation occur at concentrations well above those needed for inhibition of prostaglandin H synthase 1 or 2, we cannot rule out a role for prostaglandin H synthase as a necessary, but not sufficient, component in this effect (4). In addition to I-κBα, HSF1, and CREB, other cellular substrates for RSK2 include the ribosomal S6 protein, the G subunit of glycogen synthase, and a number of nuclear proteins, including lamin C, c-*fos*, and p67SRF (22). Preventing the phosphorylation of these proteins may also contribute to the pharmacological actions of the NSAIDs (4–6, 30, 40, 41).

In conclusion, NaSal and other NSAIDs inhibit the 90-kDa RSK2 in vitro and in vivo at concentrations that lead to inhibition of inflammatory gene expression in monocytes. RSK2 stimulates the phosphorylation of a number of transcription factors involved in monocyte gene expression, and NaSal coordinately inhibits CREB phosphorylation and CREB-dependent transcription. Inhibition of signal transduction pathways activated by proinflammatory agonists is thus a promising strategy for the development of anti-inflammatory agents.

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