Inhibition of Vascular Smooth Muscle Cell Proliferation by Sodium Salicylate Mediated by Upregulation of p21Waf1 and p27Kip1

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Background—Salicylates may have direct vascular effects by mechanisms that are independent of platelet inhibition.

Methods and Results—We investigated the effect of salicylates on vascular smooth muscle cell (SMC) proliferation in response to platelet-derived growth factor (PDGF) in vitro. Salicylate concentrations of 5 and 10 mmol/L inhibited serum- or PDGF-induced SMC cell count and [3H]thymidine incorporation by 62% and 81%, respectively. There was no evidence of cellular toxicity or apoptosis as determined by trypan blue exclusion and FACS analyses. Because cell cycle progression is regulated by hyperphosphorylation of the retinoblastoma (Rb) protein, we examined the effects of salicylate on Rb hyperphosphorylation. Treatment with salicylate, but not indomethacin, inhibited nuclear factor-κB activation and completely abolished Rb hyperphosphorylation in PDGF-treated SMCs. This effect was associated with a decrease in cyclin-dependent kinase (Cdk)-2 and, to a lesser extent, Cdk-6, but not Cdk-4 activity, without changes in Cdk-2, -4, and -6 and cyclin D and E protein levels. Because Cdk-2 activity is regulated by the Cdk inhibitors p21Waf1 and p27Kip1, we studied the effects of salicylate on p21Waf1 and p27Kip1 expression. Treatment with salicylate prevented PDGF-induced downregulation of p21Waf1 and p27Kip1 but not of the Cdk-4/-6 inhibitor p16Ink4a.

Conclusions—These findings indicate that high doses of salicylates inhibit SMC proliferation by cell cycle arrest at the G1-S phase and suggest a beneficial role for high-dose salicylates in the treatment of vascular proliferative disorders. (Circulation. 2000;102:2124-2130.)

Key Words: aspirin ▪ cells ▪ muscle, smooth ▪ proteins ▪ kinases

The proliferation of vascular smooth muscle cells (SMCs) is a central event in the pathogenesis of vascular lesions, including postangioplasty restenosis, transplant arteriosclerosis, and vein graft occlusion.1 Cellular proliferation is governed by the eukaryotic cell cycle,2 which comprises 4 distinct sequential phases (G0, G1, S, and G2).3 This tightly controlled temporal order is imposed by the sequential activation of a number of serine/threonine protein kinases known as cyclin-dependent kinases (Cdks),4 which hyperphosphorylate the retinoblastoma protein (Rb).3

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In quiescent cells, Rb is present in a hypophosphorylated state that enables it to bind to and sequester members of the E2F family of transcription factors.5 Phosphorylation of Rb at multiple sites induces a conformational change that releases E2F, enabling E2F to activate the transcription of genes required for further cell cycle progression, including those for thymidine kinase, dihydrofolate reductase, edc2, and cyclin A.6,7 The activity of cyclin-Cdk complexes is, in turn, negatively regulated by a number of Cdk inhibitors.8 The known Cdk inhibitors can be grouped into 2 distinct families: members of the INK4 family, which includes p16INK4a, p15INK4b, p18INK4c, and p19INK4d (see References 9 through 12), are specific inhibitors of Cdk-4 and Cdk-6,9,12 whereas p21Waf1/p27Kip1 family members block cell cycle progression by inhibiting cyclin D-, E-, and A-associated Cdk complexes.13

Recent studies suggest that salicylates, when administered at high doses (ie, >1 mmol/L), can exert direct antiproliferative effects. Aspirin and salicylate, in doses ranging between 1 and 10 mmol/L, inhibit cell growth, DNA and protein synthesis, and G1 to S progression in rat glioma and human colon adenocarcinoma cell lines.14,15 Furthermore, high doses of lysinmono(acetylsalicylate), a buffered soluble salt of acetylsalicylic acid, have been shown to prevent SMC proliferation in response to 5% to 10% serum.16 However, the mechanism for this growth-inhibitory effect has not been demonstrated.16 Thus, the purpose of this study was to determine the mechanism by which high-dose salicylates regulate cell cycle progression in human SMCs.

Methods

Reagents

DMEM, penicillin, streptomycin, and trypsin were obtained from BioWhittaker. FCS was purchased from Gibco. Sodium salicylate,
acetylsalicylic acid, and indomethacin were purchased from Sigma Chemical Corp. PDGF-BB was purchased from Genzyme. [α-32P]ATP and [3H]thymidine were supplied by New England Nuclear. The antibody detection kit (enhanced chemiluminescence) and protein (polyvinylidine difluoride) transfer membranes were purchased from Amersham Corp.

**Cell Culture**

Human vascular SMCs were isolated from human saphenous veins as described. The cells were characterized by phase-contrast microscopy and staining for SMC-specific α-actin. Cell numbers were determined with a Neubauer chamber hemocytometer, with quadruplicate counts performed for each treatment condition. Cell viability was assessed by trypan blue exclusion as described. Flow Cytometry

Cellular DNA content and the presence of hypodiploid (apoptotic) cells were assessed by flow cytometry as described. The percentage of cells with decreased DNA staining (An), composed of apoptotic cells resulting from either fragmentation or decreased chromatin, of 10,000 cells per experimental condition was counted. As a positive control for apoptosis, SMCs were treated with manumycin A, a known activator of the apoptotic process. Cell debris was excluded from analysis by selective gating based on anterior and right angle scatter.

**[3H]Thymidine Incorporation**

DNA synthesis was assessed by the level of [3H]thymidine incorporation. Cells were seeded in 24-well plates and cultured in growth medium to ~80% confluence. They were then washed twice and incubated in DMEM containing 0.4% FCS for 48 hours for synchronization in G0. Cells were preincubated with the indicated agents for 1 hour. The medium was then replaced with growth medium containing 4 ng/mL PDGF, and the indicated reagents were simultaneously re-added. After 24 hours, [3H]thymidine (10 μCi/mL) was added, and the cells were incubated an additional 24 hours. Incorporated radioactivity in cell lysates was determined with a liquid scintillation counter (Beckman LS 6000IC).

**Western Blotting**

Quiescent blotting of primary smooth muscle cells were treated as indicated, and immunoblotting was performed as described. Immunoblotting was performed with monoclonal or polyclonal antibodies against Rb (14001A), p21waf1 (6951A) (Pharmingen, 1.5 μg/mL), p16ink4 (F-12), p27kip1 (C-19), p53 (DO-1), cyclin A (BF-683), cyclin D (R-124), cyclin E (M-20), Cdk-2 (M-2), Cdk-4 (H-22), and Cdk-6 (C-21) (Santa Cruz Biotechnology, 1 μg/mL).

**Cyclin-Dependent and IκB Kinase Assay**

Cyclin-dependent kinase and IκB kinase complex (IKK) activities were measured as described. Cdk-2, -4, and -6 were immunoprecipitated from 300 mg of total cellular lysates with 3 μg of the corresponding human Cdk-specific antibody (Santa Cruz, M-2, H-22, and C-21, respectively) for 1 hour at 4°C. IKK-α and IKK-β were immunoprecipitated by an IKK-specific antibody that recognizes both isoforms (Santa Cruz). The purified enzymes were then incubated with either full-length GST-pRb or GST–IκB-α fusion protein (2 μg, Santa Cruz) as the substrate in 15 μL kinase buffer (mmol/L: Cdk-2 and IκB-α: Tris 50 [pH 7.6], MgCl2, 10, DTT 1; Cdk-4 and Cdk-6: HEPES 50 [pH 7.5], MgCl2, 10, DTT 1) containing ATP (10 μmol/L), 3 mM of [γ-32P]ATP, β-glycerophosphate (10 mmol/L), NaF (10 mmol/L), p-nitrophenylphosphate (10 mmol/L), NaVO3 (300 μmol/L), benzamidine (1 mmol/L), PMSF (2 μmol/L), aprotinin (10 μg/mL), leupeptin (1 μg/mL), pepstatin (1 μg/mL), and DTT (1 mmol/L) at 30°C for 45 minutes. The reaction was terminated by addition of an equal volume of loading buffer and boiling for 5 minutes. Proteins were separated on 12% SDS-PAGE, and autoradiography of the dried gel was performed at ~80°C. As a control for nonspecific phosphorylation, the IκB-α mutant (Δ32,36: S→T) was used as a substrate.

**Results**

**Effect of Salicylate on SMC Proliferation**

SMCs incubated in growth medium alone grew exponentially through day 10, plateauing on day 12 (Figure 1). In a concentration-dependent manner, addition of salicylate to the medium inhibited SMC proliferation. Although 0.1 mmol/L salicylate did not affect cell number compared with controls, 1 mmol/L salicylate led to a reduction in SMC growth beginning at day 4 and resulting in a 33±4% growth inhibition at day 10 (P<0.01). Treatment with 5 mmol/L salicylate completely inhibited SMC proliferation over the course of the experiment (P<0.01 compared with controls).

To assess the reversibility of this growth-inhibitory effect, cells incubated in the presence of 5 mmol/L salicylate were washed on day 4, and the medium was replaced with salicylate-free growth medium. As shown in Figure 1B, removal of salicylate from the medium led SMCs to re-enter the growth phase at a rate comparable to that of controls.

**Effect of Salicylate on Cell Viability**

Salicylate (1 to 5 mmol/L) did not significantly affect SMC viability compared with controls over the first 6 days of treatment (Figure 2A). Even at day 9, when a statistically significant difference between salicylate (5 mmol/L) and controls could be detected, the proportion of salicylate-treated cells showing positive dye uptake was not greater than 7%. Morphologically, salicylate-treated cells did not show any

Figure 1. Effect of salicylate on SMC proliferation. A, Subconfluent quiescent SMCs were cultured in growth medium (10% FCS) alone or in presence of salicylate (Sal, 0.1 to 5 mmol/L). B, SMCs were cultured in growth medium alone or in presence of salicylate (Sal, 5 mmol/L). On day 4 (arrow), cells were washed and medium was replaced with salicylate-free growth medium.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were prepared and electrophoretic mobility shift assays were performed as described. The nuclear factor (NF)-κB oligonucleotide used corresponded to the palindromic NF-κB consensus sequence: AGTGAGGGGACCTCCCAAG (Santa Cruz). The mutant κB oligonucleotide contained the following sequence: AGTIGAATGGACCTTACAGG.

**Data Analysis**

Band intensities from Western blots, electrophoretic mobility shift assay, and kinase assays were quantified by densitometry with the National Institutes of Health Image program. All values are expressed as mean±SD compared with controls and among separate experiments. For statistical significance, paired and unpaired Student’s t tests were used. For comparison between multiple groups, data were analyzed by ANOVA. A significant difference was taken for P<0.05.
Salicylate is a known inhibitor of cyclooxygenase, which mediates prostaglandin synthesis and its inhibition results in a decrease in SMC proliferation (Figure 1A). In contrast, high doses of indomethacin (50 to 250 μmol/L) resulted in complete suppression of Rb phosphorylation, yielding densitometric measurements equivalent to those of quiescent controls. As with DNA synthesis, the calculated IC50 value of salicylate was in the 2 to 3 mmol/L range.

**Effect of Salicylate on Hyperphosphorylation of Rb Protein**

In quiescent cells, most of the Rb protein was present in the hypophosphorylated state (Figure 3B). Addition of PDGF resulted in a prominent induction of phosphorylation, beginning at 18 hours and reaching a plateau after 24 hours. As with DNA synthesis, Rb phosphorylation was dose-dependently inhibited by salicylate (Figure 3C). As calculated from densitometric measurements, doses of 1, 2, 5, and 10 mmol/L salicylate led to 10±5%, 38±7%, 73±1%, and 77±1% inhibition of Rb hyperphosphorylation compared with PDGF-stimulated controls. Doses >5 mmol/L resulted in complete suppression of Rb phosphorylation, yielding densitometric measurements equivalent to those of quiescent controls. As with DNA synthesis, the calculated IC50 value of salicylate was in the 2 to 3 mmol/L range.

**Effect of Acetylsalicylic Acid and Indomethacin on SMC Cell Cycle**

To determine whether the antiproliferative effect of salicylate is similar to that of acetylsalicylic acid (ASA) and mediated by inhibition of cyclooxygenase, we treated quiescent SMCs with growth medium in the presence of ASA or indomethacin. In a concentration-dependent manner, ASA inhibited SMC DNA synthesis and Rb hyperphosphorylation with an IC50 value equivalent to that of salicylate. (Figure 4A and 4B). In contrast, high doses of indomethacin (50 to 250 μmol/L), an inhibitor of cyclooxygenase, did not suppress thymidine incorporation or Rb hyperphosphorylation (Figure 5A and 5B). Indeed, we observed a small but significant increase in SMC DNA synthesis with indomethacin treatment (18±12% over control, P<0.05).

**Effect of Salicylate on CdkS**

To study the mechanism underlying the inhibition of Rb phosphorylation by salicylate, we measured the effect of salicylate on Cdk-2, Cdk-4, and Cdk-6 activities. When GST-Rb fusion protein was used as substrate, quiescent SMCs had minimal Cdk-2, -4, and -6 activity (Figure 6A). Treatment with PDGF increased Cdk-2 activity after 24 hours compared with controls. In a concentration-dependent man-

**Figure 2. Effect of salicylate on SMC viability and apoptosis.** A, SMCs were cultured in growth medium with and without salicylate (1 to 5 mmol/L). Cell viability was assessed at indicated time points by trypan blue exclusion. Detergent saponin (Sap, 0.1%) was used as positive control. *P<0.05 vs growth medium alone. B, SMCs were stimulated with growth medium (10% FCS) in presence or absence of salicylate (5 and 10 mmol/L) for 24 hours. All cells were subjected to FACS analyses of DNA content. Percentage of apoptotic cells (A0) is indicated above bar encompassing sub-G1 region. Experiments were performed 3 times with similar results. Man A indicates manumycin A.

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features suggestive of cytotoxicity, such as retraction, rounding, or detachment (data not shown). Furthermore, salicylate (1 to 5 mmol/L) did not increase the proportion of floating cells compared with controls. These results are consistent with the ability of salicylate-treated SMCs to resume growth after removal of the compound from the medium (Figure 1B).

**Effect of Salicylate on SMC Apoptosis**

To assess the contribution of apoptosis to a decrease in SMC proliferation, we performed flow cytometry and DNA electrophoresis to evaluate the effect of salicylate on SMC apoptosis. For these experiments, exponentially growing subconfluent SMCs were exposed to salicylate (1 to 10 mmol/L) in growth medium for 24 hours. Evaluation of all SMCs by flow cytometry did not show features of apoptosis. The percentage of hypodiploid cells having a reduced DNA content (ie, sub-G1 or A0) did not exceed 0.5%, even at the highest treatment concentrations (Figure 2B). Similarly, treatment of exponentially growing SMCs with salicylate (1 to 10 mmol/L) did not show degradation of genomic DNA to oligonucleosomal bands, a characteristic marker of apoptosis (data not shown). In contrast, treatment with manumycin A (50 μmol/L), a known stimulus for SMC apoptosis, induced substantial DNA fragmentation.

**Effect of Salicylate on DNA Synthesis**

Compared with untreated controls, salicylate (1 to 10 mmol/L) inhibited PDGF-induced SMC DNA synthesis as assessed by [3H]thymidine incorporation (Figure 3A). Whereas no significant difference was observed with 0.1 mmol/L of salicylate, 1 mmol/L of salicylate inhibited thymidine incorporation by 12±2% (P<0.01), and concentrations of ≥5 mmol/L resulted in >70% inhibition (P<0.005). The calculated IC50 value for salicylate-mediated inhibition of SMC proliferation was 2.4±0.5 mmol/L. This inhibition was apparent as soon as SMC DNA synthesis was detectable (~18 hours after stimulation) and persisted over 96 hours of treatment (data not shown). Taken together, these data suggest that salicylate inhibited cell cycle progression during G1 or at the G1-S transition.

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![Figure 2](http://circ.ahajournals.org/)

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ner, cotreatment with salicylate inhibited PDGF-induced Cdk-2 activity, leading to almost complete suppression at a salicylate concentration of 10 mmol/L. In contrast, the effects of salicylate on the activities of the cyclin D–associated kinases, Cdk-4 and Cdk-6, were much less pronounced than that on Cdk-2. Salicylate did not appreciably inhibit Cdk-4 activity, even at the highest concentration tested (10 mmol/L). Cdk-6 activity was only partially inhibited by salicylate; its activity was 60% to 70% that of PDGF-treated samples, even in the presence of 10 mmol/L salicylate.

To address whether the inhibition of CDK activities by salicylate was associated with a reduction in cyclin and/or Cdk protein levels, we performed immunoblotting on whole-cell lysates from growth-stimulated SMCs incubated in the absence or presence of salicylate (5 mmol/L). Cyclin D and E protein levels were not affected by salicylate (Figure 6B). Cyclin A was undetectable in quiescent cells and was induced by PDGF stimulation beginning at 18 hours. This induction was completely suppressed by salicylate at all experimental time points. As with cyclins D and E, salicylate did not reduce the expression of Cdk-2, Cdk-4, or Cdk-6 (Figure 6B).

Effect of Salicylate on the Expression of Cdk Inhibitors

Compared with PDGF stimulation, cotreatment with salicylate did not significantly affect p16\textsuperscript{ink4} protein levels over 36 hours of treatment (Figure 6C). In contrast, levels of p27\textsuperscript{kip1} were high in quiescent cells and decreased progressively after PDGF stimulation. Cotreatment with salicylate (5 mmol/L) prevented this PDGF-induced decrease, with an effect predominating between 6 and 18 hours after stimulation. Levels of p21\textsuperscript{waf1} were low in quiescent SMCs. PDGF stimulation led to a transient increase in p21\textsuperscript{waf1} expression at 6 and 12 hours, with a return to baseline levels thereafter. Cotreatment with salicylate (5 mmol/L) increased p21\textsuperscript{waf1} expression over PDGF alone at 6 hours and prevented its decrease at subsequent time points. The expression of p21\textsuperscript{waf1} in salicylate-treated samples was 2.5- to 3.8-fold higher than the corre-
sponding PDGF-treated controls between 24 and 36 hours of stimulation. Because the expression of p21Waf1 is regulated, in part, by the tumor suppressor protein p53, we assessed the effect of salicylate on p53 protein expression. Salicylate (5 mmol/L) markedly upregulated p53 protein levels beginning at 6 hours and prevented the PDGF-induced decline in p53 expression at all subsequent time points (Figure 7).

**Effect of Salicylate on NF-κB Activation**

SMCs possess minimal basal NF-κB activation under tissue culture conditions (Figure 8A). Stimulation with PDGF increased NF-κB activation, which was inhibited by cotreatment with salicylate (5 mmol/L), aspirin (ASA, 5 mmol/L), and indomethacin (Indo, 5 mmol/L) at 24 hours. Salicylate (5 mmol/L) markedly upregulated p53 protein levels beginning at 6 hours and prevented the PDGF-induced decline in p53 expression at all subsequent time points (Figure 7).

Discussion

We have shown that high concentrations of salicylate inhibit SMC proliferation. We find that salicylate inhibited predominantly Cdk-2 activity, whereas it had a lesser effect on Cdk-6 and no effect on Cdk-4 activity. Although a number of Cdks are known to phosphorylate Rb, the suppression of Cdk-2 activity alone may be sufficient to prevent Rb hyperphosphorylation. For example, Cdk-2 can phosphorylate Rb in vitro, and microinjection of Cdk-2–cyclin E complexes induces DNA synthesis when injected into serum-starved human fibroblasts. Furthermore, several reports suggest that inhibition of Cdk-4 and Cdk-6 may not be necessary to that salicylate, but not indomethacin, inhibited PDGF-induced IKK activity in SMCs (Figure 8B). Because the activation of NF-κB is required for SMC proliferation, our results suggest that inhibition of NF-κB may be a partial mechanism by which high doses of salicylate inhibit SMC proliferation.

**Figure 5.** Effect of indomethacin on SMC DNA synthesis and Rb hyperphosphorylation. A, [3H]thymidine incorporation was measured in PDGF (4 ng/mL)–stimulated SMCs in presence of increasing concentrations of indomethacin (Indo), salicylate (Sal, 5 mmol/L), and aspirin (ASA, 5 mmol/L) at 24 hours. B, Effect of salicylate (5 mmol/L), ASA (5 mmol/L), or Indo (50 mmol/L) on PDGF-induced Rb phosphorylation at 24 hours. **P<0.01 vs PDGF alone. Experiments were performed 3 times with similar results.

**Figure 6.** Effect of salicylate on Cdks and Cdk inhibitors. A, Kinase assays using GST-Rb fusion protein as substrate showing effect of salicylate (Sal, 1 to 10 mmol/L) on PDGF (4 ng/mL)–induced Cdk-2, Cdk-4, and Cdk-6 activity at 24 hours. B, Immunoblots showing time-dependent effect of PDGF (4 ng/mL), salicylate (Sal, 5 mmol/L), or both on cyclin and Cdk protein levels.

**Figure 7.** Effect of salicylate on Cdk inhibitors and p53 protein levels. Immunoblots showing time-dependent effect of PDGF (4 ng/mL), salicylate (Sal, 5 mmol/L), or both on p16Ink4a, p21Waf1, p27Kip1, and p53 levels. Three separate experiments yielded similar results.
arrest cell cycle progression and that inhibition of Cdk-2 alone may be sufficient to achieve cell cycle arrest.27–29 The Cdk inhibitors p16 Ink4, p21 Waf1, and p27 Kip1 are important regulators of cyclin-Cdk complexes.8 Consistent with the role of p16 Ink4 as a selective inhibitor of Cdk-4 and Cdk-6 activity,9,12 p16 Ink4 levels and Cdk-4/-6 activities were relatively unaffected by salicylate. In contrast, salicylate prevented the downregulation of p27 Kip1 after PDGF stimulation and induced p21 Waf1. Although p21 Waf1 and p27 Kip1 have broad inhibitory activity on various cyclin-Cdk complexes, they have greater inhibitory effects on Cdk-2 activity. For example, p27 Kip1 overexpression completely inhibits Cdk-2 activity and cell growth without significant effect on Cdk-4 or Cdk-6 activity.30,31 Similarly, recombinant p21 Waf1 inhibits Cdk-2 but not Cdk-4 activity,32 and p21 Waf1-deficient mouse embryo fibroblasts show increased Cdk-2 but not Cdk-4 activity compared with wild-type cells.33 Thus, our finding that salicylate selectively upregulates p21 Waf1 and p27 Kip1 expression is consistent with its greater inhibitory effect on Cdk-2 compared with that on Cdk-4/-6. Taken together, our data suggest that salicylate reduces SMC proliferation by blocking cell cycle progression from G1 to S phase.

Salicylate has been shown to inhibit the proinflammatory transcription factor NF-κB in part by inhibiting IKK-β, one of the kinases responsible for IκB degradation.34 NF-κB is a member of the Rel family of transcription factors and plays an important role in the regulation of genes involved in inflammation, cell differentiation, and cell growth.35 NF-κB activity is essential for SMC proliferation,24 and increased NF-κB activity has been demonstrated in human atherosclerotic lesions36 and in the rat carotid artery after balloon denudation.37 Therefore, inhibition of NF-κB may mediate some of the antiproliferative effects of salicylate on SMCs. Indeed, treatment of rats with high doses of aspirin (100 mg/kg) prevents NF-κB activation and inhibits neointimal thickening after balloon injury.38 However, the inhibition of NF-κB by salicylate in the present study was relatively modest when compared with the compound’s effect on SMC proliferation. This suggests that additional pathways may be involved in the growth-inhibitory effect of salicylate.

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