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Selective inhibition of STAT3 phosphorylation by sodium salicylate in cardiac fibroblasts

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Abstract

The effects of salicylate on the phosphorylation and nuclear translocation of signal transducers and activators of transcription (STATs) induced by interferon-γ (IFN-γ) were studied in rat cardiac fibroblasts as a possible model for the anti-inflammatory effects of salicylate on this signaling pathway. Salicylate inhibited the tyrosine phosphorylation of both STAT1 and STAT3, but had a more pronounced effect on STAT3 activation. Salicylate pretreatment prevented both the nuclear translocation and the DNA-binding activity of STAT1 and STAT3, assessed by immunoblotting and gel shift assays, respectively. In addition to causing phosphorylation at tyrosine residues, IFN-γ also phosphorylated STAT3 and STAT1 at serine 727. Salicylate attenuated both tyrosine and serine phosphorylations of STAT3, and also suppressed extracellular signal-regulated kinase (ERK) activation, implicating the effect of salicylate on ERK as a possible mechanism for attenuating STAT3 activation. The possibility that salicylate might affect signaling cascades by altering the redox state of the cells was examined, and its effects differed from those of other reducing agents. Salicylate did attenuate the effects of hydrogen peroxide on STAT phosphorylation, consistent with a mechanism involving an interaction between salicylate and reactive oxygen species within the cell.

Keywords: Nonsteroidal anti-inflammatory drugs; Signal transducer and activator of transcription; Interferon-γ; Protein phosphorylation

1. Introduction

Salicylate and its acetylated derivative, aspirin, have been used for many years to treat inflammation. For more than two decades, the anti-inflammatory actions of aspirin and salicylate have been attributed primarily to inhibition of prostaglandin synthesis [1,2]. However, a growing number of studies have provided evidence for immunomodulatory effects unrelated to inhibition of prostaglandin synthesis. A major new finding within the past few years was the discovery that aspirin and salicylate inhibit specifically the activation of the transcription factor NF-κB by inflammatory stimuli [3–5]. The inhibition of NF-κB may explain some of the clinically documented anti-inflammatory effects seen with high concentrations of salicylate [6,7]. A few exceptions to this finding have been noted recently [8,9], suggesting that there may be some cell-type specificity to the inhibition of NF-κB. As NF-κB is only one of many transcription factors induced during inflammation, it is possible that other transcription factors induced by inflammatory stimuli are modulated by salicylate.

Previous studies from our laboratory documented that aspirin and salicylate inhibit the induction of iNOS mRNA by cytokines in cardiac fibroblasts [8,10]. These studies suggested that the anti-inflammatory mechanism of salicylate involved inhibition of iNOS transcription and showed that the effect was independent of NF-κB activation. Since the inflammatory response of cardiac fibroblasts, typified by the induction of iNOS, requires the addition of IFN-γ and presumably necessitates activation of the transcription factor STAT [11], we hypothesized that salicylate influences STAT phosphorylation and activation.

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Abbreviations: NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; STAT, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; NSAIDs, nonsteroidal anti-inflammatory drugs; NAC, *N*-acetylcysteine; DTT, dithiothreitol; SIE, *sis*-inducible element; SIF, *sis*-inducible factor; EMSA, electrophoretic mobility shift assay; ROS, reactive oxygen species; JAK, Janus kinase; PYK2, proline-rich tyrosine kinase 2.

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STAT1 and STAT3 reside in the cytoplasm of unstimulated cells in a latent, inactive form. Treating cells with IFN-γ results in the phosphorylation of STATs by the JAK 1 and 2 associated with the IFN-γ receptor. Upon tyrosine phosphorylation, STAT proteins form homo- or heterodimers through intermolecular interactions between their SH2 domains and phosphorylated tyrosine residues on adjacent molecules. Coupled STATs then rapidly translocate to the nucleus, where transcription of target genes is initiated [12,13]. In the case of STAT1 and STAT3, phosphorylation on Ser 727 in addition to tyrosine phosphorylation on Tyr 701 or Tyr 705, respectively, is essential to maximize their transactivation capabilities [14]. Although cytokine-induced tyrosine phosphorylation of STATs can occur independently of serine phosphorylation [15], serine phosphorylation of STAT1 and STAT3 appears to require ERK activity, since expression of dominant-negative ERK 2 suppresses STAT-mediated gene expression via the IFN-γ receptor [16].

Recent work by us using cultured cardiac fibroblasts has shown that salicylate can inhibit the activities of several kinases involved in the signaling cascade initiated by cytokines or angiotensin II [17,18]. The kinases that we found to be influenced by salicylate included c-Src, PYK2, and, ultimately, ERK activation as well. In the present study, we have used IFN-γ-induced activation of STAT1 and STAT3 as an experimental model to examine the effects of salicylate on the JAK-STAT pathway. We found that salicylate attenuated the phosphorylation of both STAT1 and STAT3, with a selective effect on STAT3 phosphorylation of both tyrosine and serine residues under defined conditions, and that the subsequent nuclear translocation and DNA binding of the STATs were impaired as well. The possibility that an alteration in the intracellular redox state produced by salicylate pretreatment was responsible for the attenuated kinase activity also was considered.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12), fetal bovine serum, and tissue culture reagents were purchased from Life Technologies Inc. Sodium salicylate, aspirin, indomethacin, ibuprofen, NAC, DTT, and ascorbic acid were from the Sigma Chemical Co. Sodium salicylate, aspirin, NAC, and ascorbic acid were dissolved in the culture medium which was then adjusted to pH 7.4. Indomethacin and ibuprofen were dissolved in ethanol to make 100 mM stock solutions and used at final concentrations of 25 μM. Recombinant rat IFN-γ was purchased from R&D Systems. PD98059 (2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one) was purchased from Calbiochem. [γ-^{32}P]ATP (10 mCi/mL) was purchased from DuPont NEN. Poly(dI:dC/dI:dC) was purchased from Pharmacia Biotech. SIE consensus oligonucleotide and mutant SIE oligonucleotide were purchased from Santa Cruz Biotechnology.

2.1.2. Antibodies

Polyclonal antibodies against phosphoserine (Ser 727) STAT1 and STAT3 were from Upstate Biotechnology Inc.; anti-phosphotyrosine STAT1 (Tyr 701) and STAT3 (Tyr 705) antibodies, anti-STAT1 and -STAT3 antibodies, and phospho-p44/42 mitogen-activated protein kinase (Thr 202/Tyr 204) monoclonal antibody were purchased from New England BioLabs. Supershift polyclonal antibodies against STAT1 and STAT3 were purchased from Santa Cruz Biotechnology.

2.2. Cell culture

Rat cardiac fibroblasts were obtained and prepared for experiments exactly as described previously [10,17]. Cells in the sixth passage were used for all experiments. The confluent cells were made quiescent by exposure to serum-free medium for 24 hr before use.

2.3. Western blot analysis

Following treatment of the cells with IFN-γ or other agents, the cells were washed twice with ice-cold phosphate-buffered saline, and solubilized in lysis buffer consisting of 50 mM Tris, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg/mL of leupeptin, and 10 μg/mL of aprotinin. Phosphorylation of STAT1 or STAT3 was measured directly by immunoblotting of the whole cell extracts with the anti-phospho-STAT antibody. Immunoblotting procedures were performed as described previously [17,19]. Images were obtained using a PDI scanner (model 420oe).

2.4. Preparation and analyses of nuclear extracts

Nuclear extracts were prepared from cardiac fibroblasts as reported previously [20]. The STAT3 or STAT1 contained in 20 μg of nuclear extracts was immunoblotted and then detected using anti-phosphotyrosine STAT3 or STAT1 antibody. For EMSA, the oligonucleotide probes for SIE and mutant SIE contained the following sequences: SIE, 5′-CAGTTCCCGTCAATC-3′; mutant SIE, 5′-CAGCCACCGTCAATC-3′. The double-stranded SIE and mutant SIE probes were labeled with [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase. ^{32}P-Labeled probe was incubated for 20 min at room temperature with 5 μg of nuclear proteins and 1 μg of poly(dI:dC/dI:dC) and then it was electrophoresed on a 5% polyacrylamide gel as described previously [8]. The gel was dried and exposed

for autoradiography. For supershift assays, nuclear extracts were incubated with 4 μg of antibodies to STAT3, STAT1, or both in the binding buffer that contained 22.5 μg/mL of poly(dI:dC), 20 mM HEPES, pH 7.9, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 65 mM NaCl for 1 hr at room temperature, and the complexes were resolved by gel electrophoresis.

2.5. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed by one-way ANOVA with StatView version 4.01 (Abacus Concepts Inc.). A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of salicylate on the tyrosine phosphorylation of STAT3 and STAT1

The temporal response of cardiac fibroblasts to IFN-γ stimulation of STAT3 and STAT1 phosphorylation is shown in Fig. 1A. Cells were incubated for the indicated times with 25 U/mL of IFN-γ, and Western blot analysis

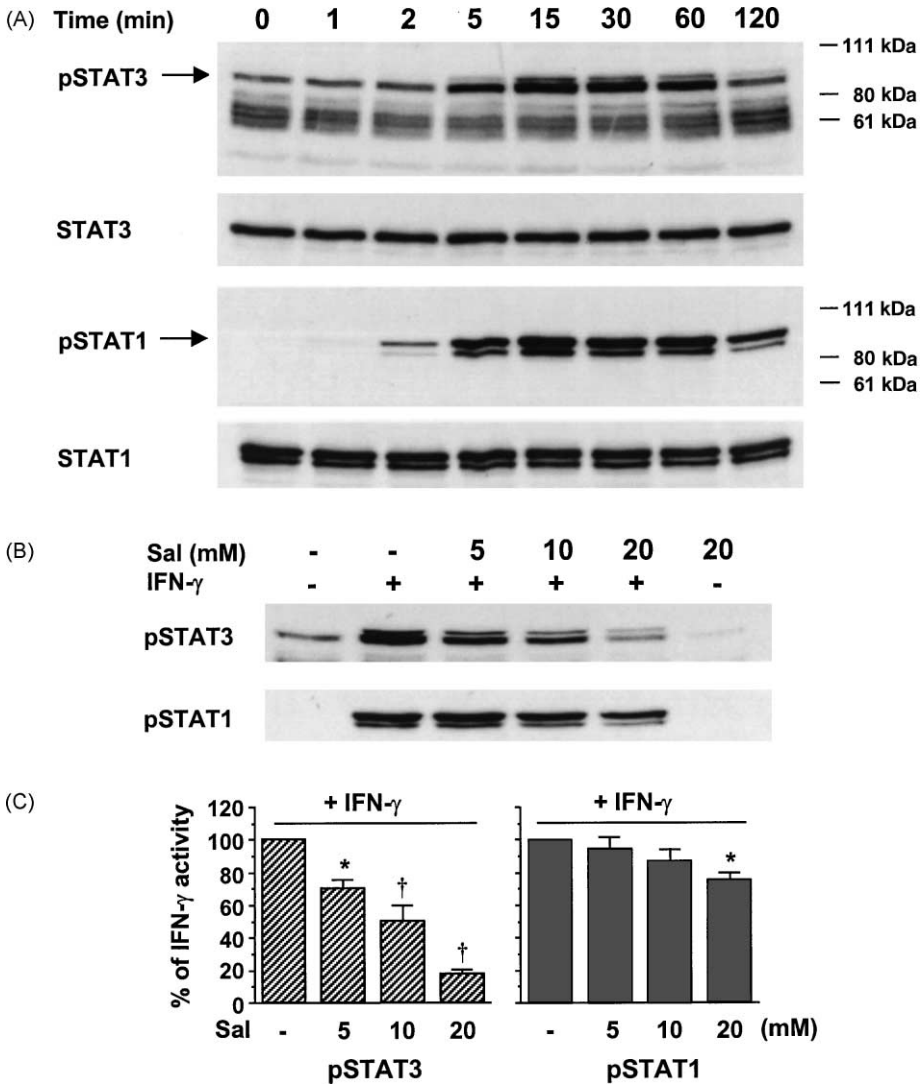

Fig. 1. Effect of salicylate on IFN-γ-induced STAT3 and STAT1 tyrosine phosphorylation. (A) Time course of STAT3 and STAT1 tyrosine phosphorylation after IFN-γ stimulation in cardiac fibroblasts. Cells were treated with IFN-γ (25 U/mL) for the designated time, and then whole cell lysates were analyzed by Western blotting using either anti-phosphotyrosine STAT3 or STAT1 antibody. Total STAT3 and STAT1 protein levels were assessed by re-probing the membrane with anti-STAT3 or -STAT1 antibody. The STAT3 antibody detected protein subunits of 83 kDa (STAT3β) and 92 kDa (STAT3α). The STAT1 protein subunits are 84 kDa (STAT1β) and 91 kDa (STAT1α). (B) Cells were pretreated for 1 hr with the indicated concentrations of salicylate followed by adding IFN-γ (25 U/mL) for 15 min. Phosphotyrosine STAT3 and STAT1 were used as described for panel A. (C) Bar graphs summarizing the effects of salicylate concentration on the densitometric analysis of STAT3 and STAT1 phosphorylation. Data (mean ± SEM) are obtained from three separate experiments. Key: (*) $P < 0.05$ and (†) $P < 0.001$, significantly different compared with IFN-γ treatment without salicylate pretreatment. pSTAT, tyrosine-phosphorylated STAT; Sal, salicylate.

was performed with whole cell extracts using antibodies against the tyrosine-phosphorylated forms and the unphosphorylated forms of each STAT. The cells displayed a transient response to IFN-γ stimulation with respect to tyrosine phosphorylation of both STAT proteins, with a maximal effect at 15 min, and reduced phosphorylation evident within 120 min. The total amounts of STAT3 and STAT1 present in the extracts were unchanged at all the time points analyzed (Fig. 1A).

The effect of salicylate on the IFN-γ-induced phosphorylation of STAT3 and STAT1 is shown in panels B and C of Fig. 1. Cells were pretreated with various concentrations of salicylate for 60 min and then incubated for an additional 15 min with IFN-γ. There was a concentration-dependent inhibition of STAT3 phosphorylation by salicylate which was clearly detected even at 5 mM salicylate, whereas phosphorylated STAT1 decreased significantly only in the presence of the highest concentration of salicylate used (20 mM). Salicylate treatment alone, in the absence of cytokine, had no effect on phosphorylated STAT1 but caused a slight decline in phosphorylated STAT3 (Fig. 1B). Total STAT3 and STAT1 protein abundance did not vary in the absence or presence of these salicylate concentrations (data not shown).

To further determine whether salicylate might affect the transient changes in STAT3 and STAT1 phosphorylation produced by IFN-γ, the order of addition of IFN-γ and salicylate was changed so that 10 mM salicylate was added at different times following IFN-γ to test for any potential effect on dephosphorylation. Panels A and B of Fig. 2 show that the disappearance of tyrosine-phosphorylated STAT3 or STAT1 within a 2-hr time frame following 15 min of IFN-γ treatment was unaffected by the presence of salicylate, suggesting that salicylate selectively affects STAT phosphorylation via the action of tyrosine kinases rather than phosphatases on STAT.

3.2. Salicylate inhibition of nuclear translocation and DNA binding of STAT3 and STAT1

Members of the STAT family are latent cytoplasmic transcription factors that, when activated at the plasma membrane by tyrosine phosphorylation, translocate into the nucleus and bind to the target DNA [12,21]. To determine the effect of salicylate on the translocation of STAT3 and STAT1 induced by IFN-γ, cardiac fibroblasts were pretreated for 60 min with different concentrations of salicylate (5–20 mM) and then were stimulated with IFN-γ for 15 min. Measurement of phosphorylated STAT3 and STAT1 in nuclear extracts was made by Western blot analysis with anti-phosphotyrosine STAT3 or STAT1 antibody. As indicated in panels A and B of Fig. 3, IFN-γ treatment caused the anticipated translocation of STAT3 or STAT1 into the nucleus, and salicylate prevented this

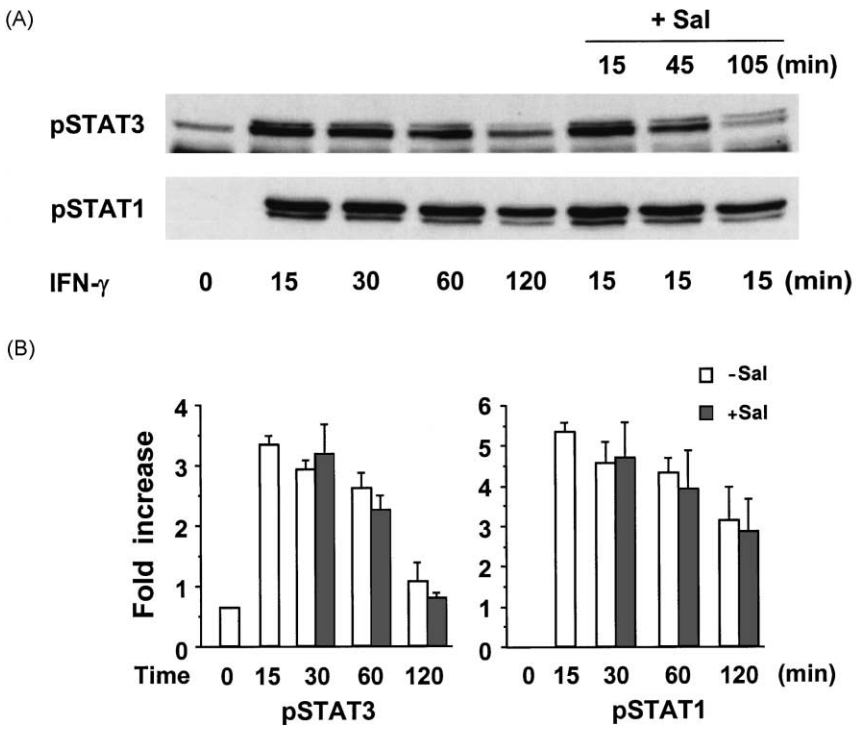

Fig. 2. Effect of salicylate addition when added at different times following IFN-γ addition. (A) Cells were either treated with 25 U/mL of IFN-γ for 15, 30, 60, and 120 min (lanes 2–5) or initially stimulated with IFN-γ for 15 min, followed by the addition of 10 mM salicylate (Sal) for the times indicated (lanes 6–8). Immunoblots of whole cell extracts were probed with anti-phosphotyrosine-STAT3 (pSTAT3) or -STAT1 (pSTAT1) antibody. (B) The bar graphs are the summary of three separate experiments performed as described for panel A when salicylate was added after IFN-γ. No significant difference of STAT3 or STAT1 phosphorylation was observed between the cells without salicylate and those with salicylate at the same time point for IFN-γ treatment.

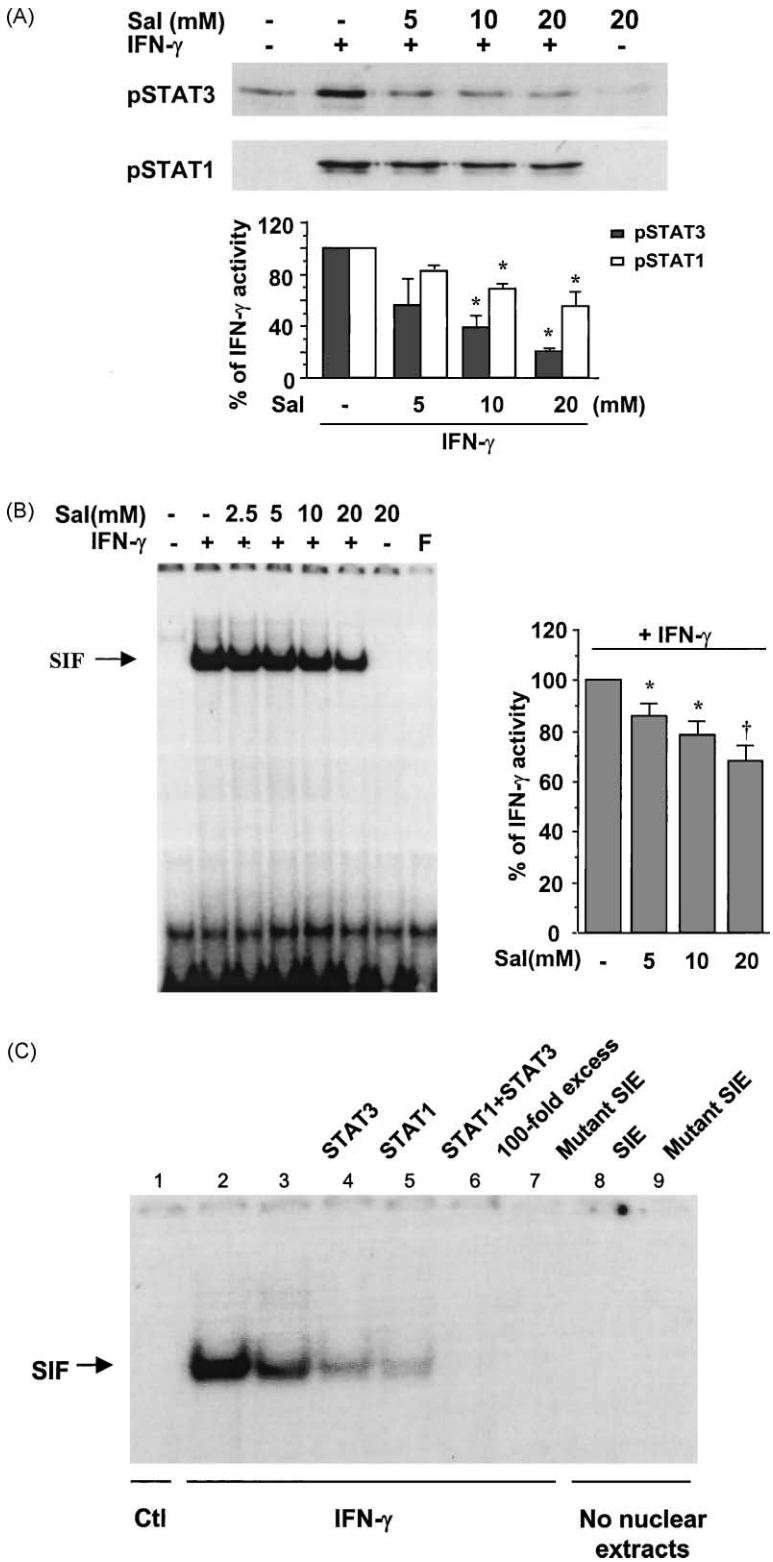

Fig. 3. Salicylate inhibition of STAT3 and STAT1 nuclear translocation and DNA binding induced by IFN-γ. (A) Different concentrations of salicylate (5, 10, and 20 mM) were added 1 hr prior to exposure to IFN-γ (25 U/mL) for 15 min. The last lane represents cells that were treated with only salicylate (20 mM). Nuclear proteins were extracted, and the nuclear translocation of STAT3 and STAT1 was measured with anti-phosphotyrosine STAT3 or STAT1 antibody. (B) The same condition was used to treat the cells as described for panel A. Nuclear extracts were prepared, and DNA binding was measured by EMSA using a ^{32}P-labeled SIE probe. F indicates a reaction with no nuclear extract. The bar graphs in panels A and B present means±SEM from three independent experiments. Key: (∗) $P < 0.05$ and (†) $P < 0.001$, significantly different from IFN-γ-stimulated values, which were arbitrarily set as 100%. (C) Cells were left untreated (Ctl, lane 1) or treated with IFN-γ for 15 min (lanes 2–7). Nuclear extracts were pre-incubated with anti-STAT3 (STAT3, lane 3), anti-STAT1 (STAT1, lane 4), or both antibodies (STAT1 + STAT3, lane 5) before initiating the binding reaction with labeled SIE oligonucleotide. A competition experiment was performed with a 100-fold excess of unlabeled SIE oligonucleotide (lane 6), and a mutant-labeled SIE oligonucleotide was used as a negative control (lane 7). Lanes 8 and 9 indicate ^{32}P-labeled SIE and mutant SIE probes without nuclear extracts. Sal, salicylate.

translocation in a concentration-dependent manner. However, the inhibitory effect of salicylate on phospho-STAT3 translocation was more pronounced than that on STAT1. In untreated cell preparations, a small amount of STAT3 was found in the nuclei, attributable to either basal levels of activation in the absence of stimulus or contamination of the nuclear extracts with extranuclear components.

Translocation of activated STAT homo- and heterodimers to the nucleus results in the formation of a complex of STAT proteins termed SIF [12,21,22]. Fig. 3B is an EMSA result showing the effects of salicylate pretreatment on the interaction between SIF and an oligonucleotide containing the consensus sequence with which the proteins specifically interact, designated SIE. IFN-γ treatment induced a clear gel shift within 15 min, presumably due to the binding of SIF to SIE. In the presence of salicylate, this DNA binding was reduced significantly with increasing salicylate concentrations. To analyze in more detail the components of the protein–DNA interaction measured by EMSA, specific antibodies against either STAT3 or STAT1 were added to nuclear extracts from the cells treated with IFN-γ. As shown in Fig. 3C, no mobility shift was observed with the mutant SIE probe (lane 7) and the addition of higher concentrations of unlabeled SIE competed with the SIF complex (lane 6), confirming the specificity of binding to SIE. Supershift assays revealed that incubation of the DNA–protein complex with anti-STAT3 antibody partially inhibited the formation of the SIF complex (lane 3), but incubation with either anti-STAT1 antibody (lane 4) or with both anti-STAT1 and -STAT3 antibodies (lane 5) produced a progressively stronger inhibition of the formation of the SIF complex. In numerous experiments conducted under a variety of experimental conditions, we were unable to detect a supershifted band of protein bound to oligonucleotide when the polyclonal antibodies to STAT3 and STAT1 were added. This would indicate that the antibodies prevented the binding of STAT3 and STAT1 to DNA rather than interacting with the bound complex.

3.3. Comparison of salicylate with other NSAIDs in preventing STAT3 and STAT1 activation

We compared the effects of salicylate, aspirin, indomethacin, and ibuprofen on the tyrosine phosphorylation of STAT3 and STAT1. The cells were incubated without or with these drugs for 60 min prior to a 15-min exposure to IFN-γ. Whole cell lysates were run on SDS-polyacrylamide gels, and immunoblotting was performed with anti-phosphotyrosine-STAT3 or -STAT1 antibody. As shown in Fig. 4A, treatment of the cells with either salicylate or

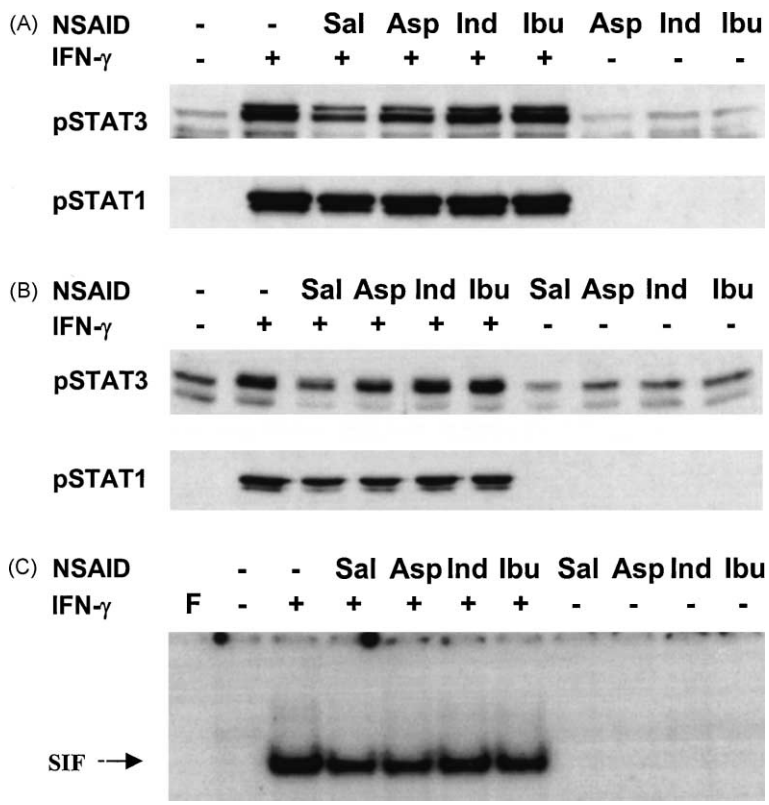

Fig. 4. Comparison of the effects of salicylate with those of other NSAIDs on STAT phosphorylation, nuclear translocation, and DNA binding. Cells were pretreated with 10 mM salicylate (Sal), 10 mM aspirin (Asp), 25 μM indomethacin (Ind), or 25 μM ibuprofen (Ibu) for 1 hr and then were either stimulated with IFN-γ (25 U/mL) for 15 min or left unstimulated. Whole cell lysates (A) or nuclear extracts (B) were analyzed by Western blotting for tyrosine phosphorylation of STAT3 and STAT1. (C) DNA binding activity was measured by EMSA with a ^{32}P-labeled SIE probe. F indicates a reaction with no nuclear extract.

aspirin suppressed IFN-γ-induced phosphorylation of STAT3, but only weakly affected STAT1, while the two other NSAIDs, indomethacin and ibuprofen, had no effect on STAT3 or STAT1 phosphorylation. The concentrations of salicylate (10 mM) and aspirin (10 mM) were based on the inhibitory concentrations on STATs from this study and on NF-κB as reported previously [3], while those of indomethacin (25 μM) and ibuprofen (25 μM) were based on the effective pharmacological serum concentrations of these agents required for their anti-inflammatory properties [23]. Aspirin, indomethacin, and ibuprofen by themselves had no effect on the phosphorylation of STAT3 and STAT1 (Fig. 4A), nor did these NSAIDs affect total STAT3 or STAT1 protein levels (data not shown). Next, the nuclear translocation and DNA binding activity of STAT3 or STAT1 were analyzed by Western blotting and EMSA, respectively. The cells were treated as described above (Fig. 4A), and then nuclear extracts were prepared. Panels B and C of Fig. 4 indicate that salicylate and aspirin selectively inhibited STAT3 and STAT1 translocation and DNA binding activity, consistent with the phosphorylation profile of STAT3 and STAT1 shown in Fig. 4A.

3.4. Can the effect of salicylate on STAT signaling be explained by its action as a reducing agent?

To determine whether the inhibitory effect of salicylate on STAT phosphorylation was related to changes in the redox state of the cell, studies with both antioxidants and hydrogen peroxide (H_2O_2), a known oxidant, were performed. Figure 5A compares the inhibitory effect of salicylate on IFN-γ-induced phosphorylation of STAT3 or

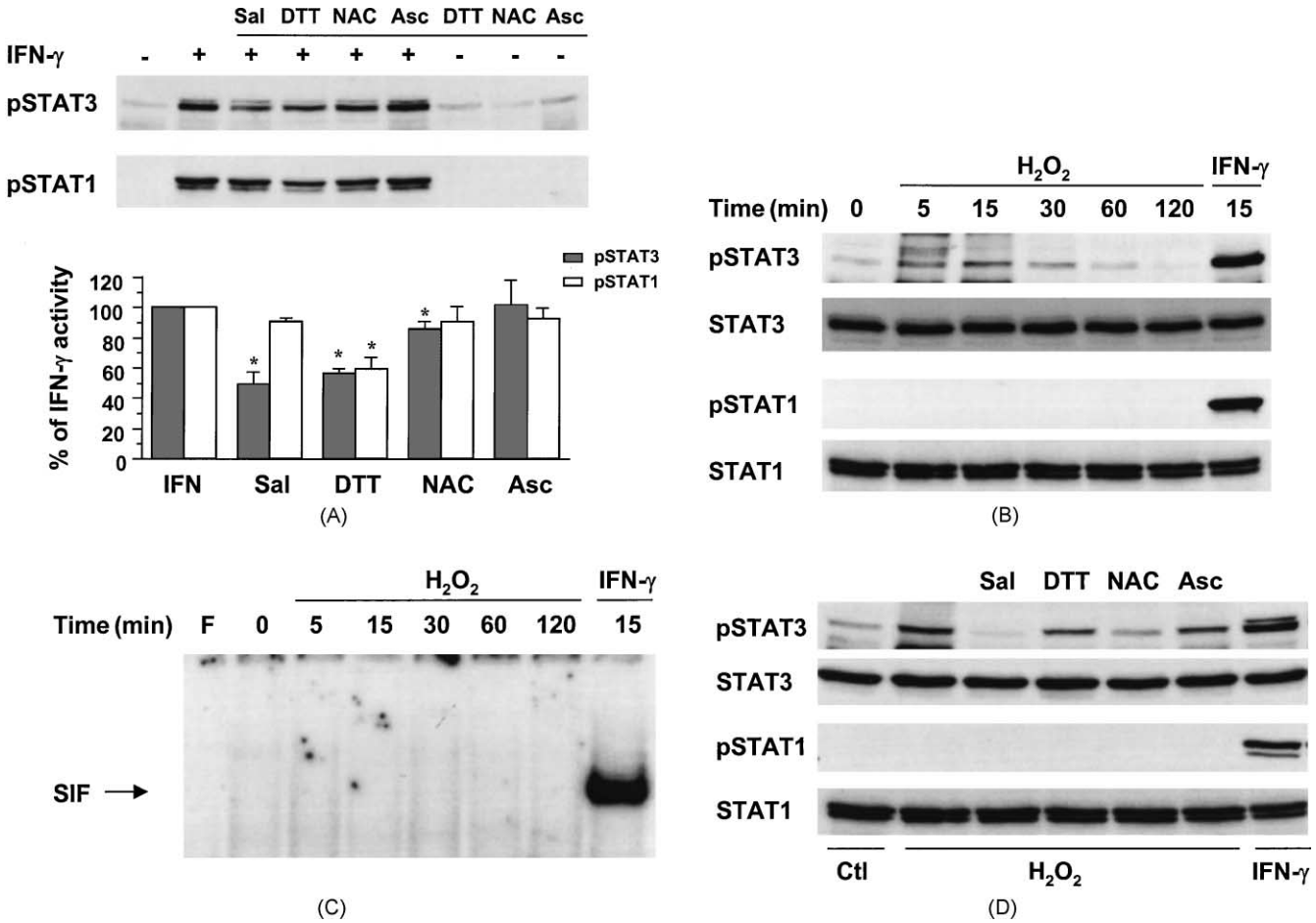

Fig. 5. Possible effect of salicylate on the redox state of cells. (A) Cells were pretreated with 10 mM salicylate (Sal) or 1 mM DTT for 1 hr or with 10 mM NAC or 1 mM ascorbic acid (Asc) for 6 hr prior to exposure to IFN-γ (25 U/mL) for 15 min. Immunoblotting was performed with anti-phosphotyrosine-STAT3 or -STAT1 antibody. The bar graph presents means ± SEM from three different experiments. Key: (∗) $P < 0.05$, significantly different from IFN-γ-stimulated values, which were arbitrarily set as 100%. (B) Cells were treated with either 1 mM H_2O_2 for the designated times or with 25 U/mL of IFN-γ for 15 min, and then cell extracts were analyzed by Western blotting for tyrosine phosphorylation of STAT3 or STAT1. Total STAT3 and STAT1 protein levels were detected by re-probing the membrane using anti-STAT3 or -STAT1 antibody. (C) Cell treatment was the same as described for panel B, nuclear protein was extracted, and EMSA was performed with ^{32}P-labeled SIE probe. F indicates a reaction with no nuclear extract. (D) Cells were either left untreated (Ctl), or were pretreated with 10 mM salicylate (Sal) or 1 mM DTT for 1 hr, or with 10 mM NAC or 1 mM ascorbic acid (Asc) for 6 hr prior to adding H_2O_2 (1 mM) or IFN-γ (25 U/mL) for 15 min. Immunoblotting of phosphorylated STAT3 or STAT1 was performed with anti-phosphotyrosine-STAT3 or -STAT1 antibody. Total STAT3 and STAT1 protein levels were examined by re-probing the membrane using anti-STAT3 or -STAT1 antibody.

STAT1 with other known antioxidants. Comparable inhibition of STAT3 and STAT1 phosphorylation was found when the cells were pretreated with DTT, whereas NAC pretreatment had only a slight effect on STAT3 phosphorylation. The antioxidant ascorbic acid, which lacks a thiol moiety, had no effect on either STAT3 or STAT1 tyrosine phosphorylation.

Because several studies have implicated ROS as signaling intermediates for cytokines [24,25], we utilized H_2O_2, an agent often used to induce ROS within many cell types, to determine whether effects on tyrosine phosphorylation of either STAT3 or STAT1 occurred. As shown in Fig. 5B, following treatment with 1 mM H_2O_2 for various lengths of time the tyrosine phosphorylation of STAT3 reached a peak at 15 min, and returned to a near-basal level by 60 min, whereas the phosphorylation of STAT1 stimulated with H_2O_2 was undetectable throughout the time period studied. By comparison, the activation of STAT1 and STAT3 with IFN-γ was much greater than that produced by H_2O_2. The total amounts of STAT3 and STAT1 present in the extracts were unchanged at all the time points analyzed with H_2O_2 treatment (Fig. 5B). Further examination of the effects of H_2O_2 on protein–DNA interactions, as measured by EMSA, indicated no effect of peroxide on protein binding to SIE (Fig. 5C). The STAT3 tyrosine phosphorylation induced by H_2O_2 was reduced with salicylate pretreatment and also was decreased by the other antioxidants to varying degrees (Fig. 5D). As expected, pretreatment with salicylate and the other antioxidants did not affect STAT1 tyrosine phosphorylation in response to H_2O_2.

3.5. Effect of salicylate on serine phosphorylation of STAT3 and STAT1

Serine phosphorylation of STATs has been demonstrated previously. A Pro-X-Ser-Pro sequence, which is a recognition site for the mitogen-activated protein kinase ERK, is present at the COOH terminus of STAT1, STAT3, and STAT4, suggesting that ERK is involved in the phosphorylation of these STATs [14]. Since we have shown previously that salicylate can influence ERK activation in cardiac fibroblasts [17], we further tested the possibility that salicylate could selectively inhibit the serine phosphorylation of STAT. Cells were treated with 25 U/mL of IFN-γ for different times, and then cell extracts were used to immunologically detect serine-phosphorylated STAT3 and STAT1 (Fig. 6A). STAT3 was serine-phosphorylated to a limited extent under basal conditions, but enhanced phosphorylation was found with the addition of IFN-γ at 15 min and sustained for nearly 2 hr. The time course of

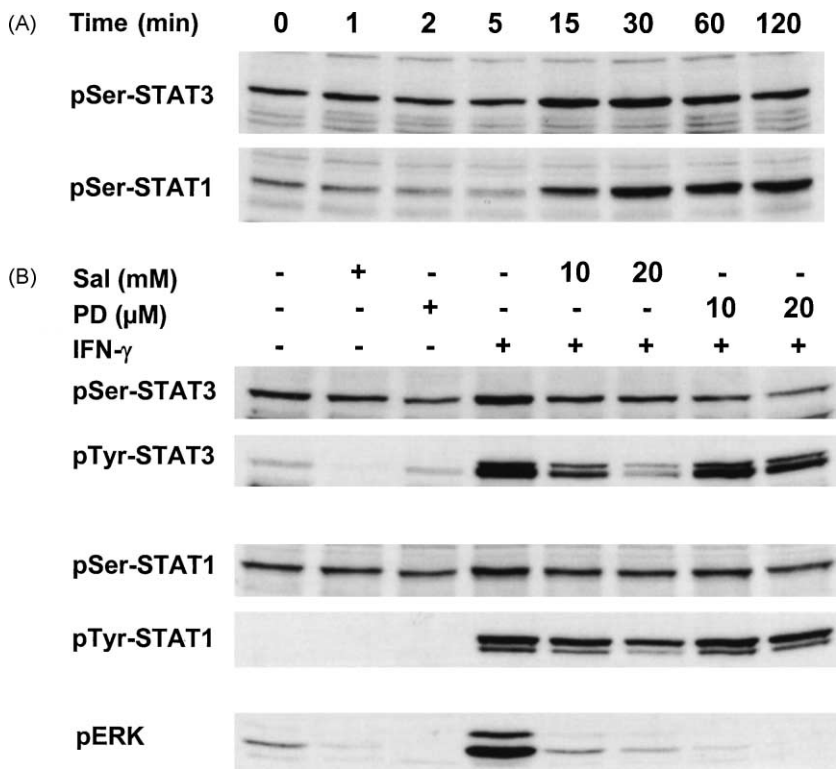

Fig. 6. Effect of salicylate on serine and tyrosine phosphorylation of STAT3 and STAT1, as well as ERK activation. In all experiments, the phosphorylation forms of STAT3 and ERK were determined from whole cell lysates by immunoblotting using either anti-phosphoserine STAT (pSer-STAT3 and pSer-STAT1), anti-phosphotyrosine STAT (pTyr-STAT3 and pTyr-STAT1) antibody, or phospho-ERK (pERK) antibody. (A) Cells were treated with IFN-γ (25 U/mL) for the designated time period, and the serine phosphorylation of STAT3 and STAT1 was measured. (B) Cells were pretreated with either salicylate (Sal, 10 or 20 mM) or PD98059 (PD, 10 or 20 μM) for 60 min, and then IFN-γ was added and extracts were obtained 15 min later. Serine and tyrosine phosphorylations of STAT3 and STAT1, as well as ERK activation, were analyzed.

STAT1 serine phosphorylation was similar to that of STAT3, but maximal activation was at 30 min. When the cells were pre-incubated with salicylate or PD98059, a selective inhibitor of ERK activation, followed by IFN-γ treatment for 15 min, salicylate suppressed both tyrosine and serine phosphorylations of STAT3, and also inhibited ERK activity. PD98059 attenuated serine phosphorylation of STAT3, and abrogated ERK phosphorylation at 10 μM, but it only weakly affected phosphorylation of the tyrosine domain of STAT3 at this concentration (Fig. 6B). The effect of salicylate on serine phosphorylation of STAT1 was similar to that of STAT3, and again the inhibitory effect of salicylate on STAT3 serine phosphorylation was more pronounced than for STAT1 (Fig. 6B).

4. Discussion

In the present study, we characterized the effects of IFN-γ on the phosphorylation and subsequent nuclear translocation of STAT3 and STAT1, and provided data indicating that ERK activation, which also occurred in response to IFN-γ, was required for more complete phosphorylation of both STAT3 and STAT1. We showed that sodium salicylate pretreatment attenuated the phosphorylation of both STATs, but was more selective towards reducing the phosphorylation and activation of STAT3. This effect of salicylate was independent of cyclooxygenase inhibition and could be attributed to its ability to inhibit several kinases, including ERK, perhaps by influencing the intracellular redox state.

IFN-γ activated both STAT3 and STAT1 in cardiac fibroblasts by causing their phosphorylation and subsequent nuclear translocation and DNA binding. This response, which was not documented previously in cardiac fibroblasts, was similar to that described in several other cell types [15,26,27]. The inhibition of this response to IFN-γ by salicylate is a novel observation, although salicylate has been shown to inhibit the phosphorylation cascades associated with activation of other transcription factors, including NF-κB, which often is activated during an inflammatory response and associated with conditions of oxidative stress [28,29]. However, we found no effect of salicylate on the activation of NF-κB by cytokine stimulation in previous studies [8], although we have noted previously that salicylate can reduce the phosphorylation of several kinases known to be activated by cytokines or angiotensin II in the cardiac fibroblast [17,18].

When we compared the effects of the order of salicylate addition relative to IFN-γ, we found that phosphorylation was affected only when salicylate was added prior to the cytokine. The data suggested that salicylate may be involved mainly in the regulation of STAT phosphorylation, but not inactivation by dephosphorylation. Although our experiments do not address this issue directly, other studies have recently implicated phosphatases as potential regulators of STAT phosphorylation in other cell types [30,31]. Furthermore, since the effects of salicylate were not immediate and required pre-incubation prior to the addition of IFN-γ, it seems likely that incorporation of salicylate within the cell may be a necessary event to explain its action.

In response to the stimulation of diverse receptors, all seven STATs (STAT1, 2, 3, 4, 5A, 5B, and 6) described to date are phosphorylated on tyrosine residues; dimerize; and subsequently are translocated to the nucleus where interaction with at least three different DNA consensus sequences occurs for the ultimate regulation of gene transcription [21,32]. We found that salicylate attenuated tyrosine phosphorylation and nuclear translocation of both STAT3 and STAT1 in a concentration-dependent manner, but its effect was more marked on STAT3 than STAT1 and required a lower salicylate concentration. Selective regulation of STAT activation by other agents was also noted. Su and David [33] reported that piceatannol, an Syk/ZAP70-specific kinase inhibitor, selectively inhibits the tyrosine phosphorylation of STAT3 and STAT5, but not that of STAT1 and STAT2. Although the most likely explanation is that the selective effect of salicylate on STAT3 tyrosine phosphorylation is the cause of the reduced nuclear translocation, it is also possible that other alterations induced by salicylate might influence the actual translocation and subsequent binding and retention of phosphorylated STAT3 to the nuclear DNA. The inhibition of DNA binding activity by salicylate as measured by EMSA was not consistent with our previous report [8]. However, in our previous study we used 480 U/mL of IFN-γ to induce the STAT DNA binding, whereas in this study, a considerably lower concentration of IFN-γ (25 U/mL) was used, which nonetheless produced maximal translocation and DNA binding based on the EMSA.

Comparisons between salicylate and other NSAIDs indicated that inhibition of cyclooxygenase was not the major cause of the suppression of STAT phosphorylation. Aspirin could mimic the inhibitory effect of salicylate on STAT3 phosphorylation at a 10 mM concentration, above the range achieved in patients undergoing treatment for inflammatory diseases [23]. The effects of salicylate, which occurred at 5 mM, were consistent with the rather high plasma concentrations of salicylate that are maintained for the treatment of chronic inflammatory states. In addition, local conditions of the inflamed areas may favor the uptake and concentration of salicylate [3]. Thus, the concentration of salicylate sufficient to inhibit STAT activity may be achieved during treatment of inflammatory conditions. However, no inhibitory effect on NF-κB was observed using 5–20 mM salicylate in cardiac fibroblasts [8].

We tested the possibility that salicylate might influence tyrosine phosphorylation of STAT3 through its ability to attenuate the kinases leading to activation of ERK. We had shown previously that several kinases in this pathway,

including PYK2 and c-Src, are affected by salicylate and cause inhibition of ERK activation by agonists such as angiotensin II. Furthermore, we had also demonstrated that ERK is activated rapidly by IFN-γ treatment [17]. Studies from other laboratories showed that STAT3 and STAT1 are also phosphorylated on serine. The major site for serine phosphorylation in STAT1 and STAT3 is residue 727, although additional serine phosphorylation sites have been proposed [34]. It has been reported that ERK2 co-immunoprecipitated with STAT1 in response to IFN-γ and was involved in the regulation of IFN-induced gene expression [16]. It has also been reported recently that ERKs phosphorylate STAT3 on Ser 727 *in vitro* as well as *in vivo* in response to epidermal growth factor (EGF) [35]. Serine phosphorylation of STAT proteins was shown to be required for maximal tyrosine phosphorylation and shown to modulate their DNA binding and/or transcriptional activity [14]. Our results indicate that ERK is necessary for serine phosphorylation of STAT3 in the cardiac fibroblast, but, in contrast, ERK inhibition does not influence tyrosine phosphorylation. Salicylate inhibited the serine phosphorylation of both STAT1 and STAT3, but had a selective effect on STAT3 at concentrations that inhibited ERK as well. Thus, it is possible that salicylate, by inhibiting ERK activation, can modulate the serine phosphorylation of STAT3 more effectively than that of STAT1, and this effect can subsequently influence both the tyrosine phosphorylation of STAT3 and its subsequent translocation and retention within the nucleus.

It is not clear how pretreatment with salicylate might influence IFN-induced STAT activation, although one plausible mechanism is that it may interfere with the phosphorylation of STATs by JAK 1 and 2, but it seems reasonable to assume that changes in the cellular redox state are also involved. Recently, some studies have implicated alterations in the redox state or, alternatively, oxidative stress that regulates JAK-STAT signaling pathways [36,37]. Our data comparing salicylate with other reducing agents, i.e. NAC and DTT, suggest that the inhibition of STAT phosphorylation may be mediated by altering the redox state of the cells. Salicylate has been described to act as a free-radical scavenger in biological systems by selectively inactivating hydroxyl anions [38]. Hence, salicylate may act by scavenging radicals or interfering with radical generation, leading to reduced STAT phosphorylation. The inhibition of NF-κB by salicylate reported in T cells may also be attributable to this property [3,4]. We directly induced oxidative stress by exposing the cells to H_2O_2. This treatment weakly activated the tyrosine phosphorylated STAT3, but not STAT1 in the cardiac fibroblasts. Carballo *et al*. [37] documented that H_2O_2 enhances STAT3 nuclear translocation specifically, since neither STAT1 nor STAT5B underwent translocation to the nucleus under this oxidative condition. Despite the weak effect of salicylate on STAT phosphorylation in the cardiac fibroblast, salicylate pretreatment clearly attenuated H_2O_2-induced tyrosine phosphorylation of STAT3, consistent with the possibility that the drug either directly reacts with ROS, or in some other way changes the redox state within the cell.

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