RESEARCH COMMUNICATION

Salicylate-enhanced activation of transcription factors induced by interferon- γ

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Salicylate enhanced the interferon- γ -dependent activation of two transcription factors in a murine macrophage cell line: signal transducer and activator of transcription (STAT)1 and interferon- γ -responsive factor 1. Salicylate alone did not activate these transcription factors. This enhancement was reflected by increased DNA-binding activities and was the consequence of prolonged tyrosine phosphorylation of these transcription factors following interferon- γ treatment. However, salicylate did not directly inhibit protein-tyrosine phosphatase activity in nuclear extracts of interferon- γ -treated cells. The enhanced activation of

STAT1 resulted in increased induction of mRNA encoding interferon regulatory factor-1. These results not only demonstrate that aspirin and its metabolite salicylate may have pro-inflammatory as well as anti-inflammatory effects but also raise the possibility that new cellular targets may be identified for modulating the Janus kinase–STAT signalling pathway.

Key words: aspirin, interferon regulatory factor-1, macrophage, RAW 264.7, STAT1.

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 (reviewed in [1,2]). However, a growing number of studies has 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 nuclear factor κ B (NF- κ B) by inflammatory stimuli [3–8]. A few exceptions to this finding have recently been noted [9,10], suggesting that there may be some cell-type specificity to the inhibition of NF- κ B. As NF- κ B is only one of many transcription factors induced during inflammation, we sought to determine whether these drugs also affect other transcription factors induced by inflammatory stimuli.

The present study was prompted by our observation that aspirin and salicylate enhanced the induction of inducible nitric oxide synthase (iNOS) mRNA by interferon- γ in the murine macrophage cell line RAW 264.7 [11]. We therefore hypothesized that these drugs may be enhancing, rather than inhibiting, the activity of transcription factors induced by this cytokine. RAW 264.7 cells provide a good model for testing this hypothesis because multiple interferon- γ -inducible transcription factors have been identified in this cell line. We chose to study the effects of salicylate on activation of the transcription factor signal transducer and activator of transcription (STAT)1 because it is involved in induction of iNOS transcription by interferon- γ [12]. STAT1 resides in the cytoplasm of unstimulated cells in a latent inactive form. Treating cells with interferon- γ results in phosphorylation of STAT1 by the Janus kinases 1 and 2 (JAK 1 and 2) associated with the interferon- γ receptor. The phosphorylated

STAT1 forms homodimers and translocates to the nucleus, where it induces transcription of genes containing interferon- γ -activated sequence (GAS) elements (reviewed in [13,14]).

We also included the transcription factor interferon- γ -responsive factor 1 (γ RF-1) in our studies because its activation in RAW 264.7 cells is specific for interferon- γ , is rapid and does not require protein synthesis [15,16]. γ RF-1 in unstimulated cells is present in a latent form; its activation requires tyrosine phosphorylation; it is inducible by interferon- γ in a wide variety of murine and human cell lines and primary cell types; and it binds to a DNA sequence (γ RE-1) that is not recognized by STAT1 [16]. Studies using purified γ RF-1 have demonstrated that it contains a tetrameric form of STAT1 that does not bind to a single STAT1 binding site but binds to the dimeric STAT1 binding site (γ RE-1) present in the promoter of the *mig* gene ([16] and T. M. Wright, unpublished results).

In this report we demonstrate that salicylate enhances the activation of STAT1 and γ RF-1 by interferon- γ , apparently by prolonging tyrosine phosphorylation of these transcription factors. Enhanced activation of STAT1 also resulted in increased induction of interferon regulatory factor-1 (IRF-1), encoded by a gene that is a target of activated STAT1.

MATERIALS AND METHODS

Reagents

Aspirin and sodium salicylate were obtained from Sigma Chemical Co. Recombinant murine interferon- γ and Dulbecco's modified Eagle's medium (DMEM) were from Life Technologies. ³²P-labelled nucleotides and Gene Screen membranes were purchased from NEN Life Science Products. The ECL[®] system for developing immunoblots was from Amersham Corporation.

Abbreviations used: iNOS, inducible (type II) nitric oxide synthase; JAK, Janus kinase; γ RF-1, interferon- γ -responsive factor 1; IRF-1, interferon regulatory factor-1; EMSA, electrophoretic mobility-shift assay; DMEM, Dulbecco's modified Eagle's medium; STAT, signal transducer and activator of transcription; NF- κ B, nuclear factor κ B.

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Cell culture

RAW 264.7 cells were maintained in DMEM containing 10% (v/v) fetal calf serum. At the beginning of each experiment, cells were washed with serum-free DMEM and then incubated in serum-free DMEM to avoid the potential for additive or synergistic responses to interferon- γ and factors present in the serum. Aspirin or sodium salicylate was immediately added at the concentrations indicated in the Figures, followed by the addition of interferon- γ (100 units/ml) 30 min later.

Nuclear extracts and electrophoretic mobility shift-assays (EMSA)

Preparation of nuclear extracts and analyses of STAT1 and γ RF-1 DNA-binding activity were exactly as described [15,16]. The sequence of the duplex oligonucleotide used for EMSA of STAT1 was (5'-GATCTTCAGTTTCATATTACTCTAAATC-CAGGATC-3') and for EMSA of γ RF-1 was (5'-GATCCT-TACTATAAACTCCCCGTTTATGTGAAATGGAGATC-3') [16]. Unless indicated otherwise, all buffers used in the preparation of nuclear extracts contained 0.1 mM Na₃VO₄ to inhibit phosphatases.

Immunoblotting

Protein concentrations of nuclear extracts were determined by a Coomassie Blue protein assay (Bio-Rad). Immunoblotting was as described [11], using antibodies specific for STAT1 or phospho(Tyr701)-STAT1 (New England Biolabs).

RNA analysis

Isolation of RNA and Northern blotting were as described [17]. Northern blots were hybridized to cDNA encoding murine IRF-1 [18]. After quantification using the Molecular Dynamics PhosphorImager, hybridized blots were stripped and rehybridized to a labelled DNA probe for 18 S ribosomal RNA [19] to correct for any minor variations in sample loading.

Figure 2 Salicylate-enhanced tyrosine phosphorylation of STAT1 in nuclei of interferon- γ -treated cells

Immunoblots of the nuclear extracts used in Figure 1 were probed with antibodies specific for STAT1 or phospho(Tyr701)-STAT1 (P-STAT1). Arrows indicate positions of phosphorylated (pp) and nonphosphorylated (p) 91- and 84-kDa isoforms of STAT1.

RESULTS

Effects of sodium salicylate on DNA-binding activities of STAT1 and γ RF-1 in interferon- γ -treated RAW 264.7 cells are shown in Figure 1. In the presence of interferon- γ alone, DNA-binding activity was strongly induced by 30 min and declined to low levels within 2 h, in accordance with previous results [15]. Sodium salicylate, which had no effect by itself, had little effect on cells treated with interferon- γ for 30 min but clearly enhanced interferon- γ -inducible DNA-binding activity by 2 h. Similar to studies that showed salicylate-dependent inhibition of NF- κ B activation [3,5,8], concentrations of sodium salicylate ≥ 3 mM were required for enhanced activities of STAT1 and γ RF-1, with the greatest effects occurring at 10 and 20 mM sodium salicylate.

The salicylate-dependent increases in DNA-binding activity could reflect either increased cellular concentration of STAT1 and γ RF-1 or increased activation of these transcription factors. Both STAT1 and γ RF-1 require tyrosine phosphorylation for activation [16,20]. We therefore analysed STAT1 phosphorylation by Western blotting. We used an antibody specific for STAT1 phosphorylated at Tyr701 [20] to evaluate the phos-

Figure 1 Effect of sodium salicylate on interferon- γ -induced DNA-binding activities of γ RF-1 (left panel) and STAT1 (right panel) in RAW 264.7 cells

EMSA were performed with nuclear extracts of cells treated with interferon- γ (IFN γ) in the presence or absence of the indicated concentrations of sodium salicylate (Sal) for 30 min or 2 h.

Figure 3 Comparison of Na₃VO₄ and sodium salicylate in preserving γ RF-1 and STAT1 activity in nuclear extracts

For these experiments Na₃VO₄ was omitted from the buffers used to isolate nuclei and prepare nuclear extracts. Different aliquots of a nuclear extract from cells treated with interferon- γ for 30 min were incubated at 25 °C for 18 h in the presence or absence of the indicated concentrations of Na₃VO₄ or sodium salicylate; a control aliquot (C) was not subjected to incubation at 25 °C. The nuclear extracts were subsequently analysed for DNA-binding activity of γ RF-1 (A) and STAT1 (B), and for phosphorylation of STAT1 (C). Other abbreviations are as in Figures 1 and 2.

phorylation state of STAT1 in nuclear extracts, as well as a different anti-STAT1 antibody to monitor total STAT1 levels. The phosphorylation profile of STAT1, including the dose response for sodium salicylate, paralleled the DNA-binding activity profile in Figure 1; i.e., sodium salicylate prolonged STAT1 phosphorylation in interferon- γ -treated RAW 264.7 cells, but sodium salicylate alone had no effect (Figure 2). The predominant phosphorylated species was the 91-kDa isoform of STAT1, which is the isoform responsible for transcriptional responses to interferon- γ [20,21]. Overall levels of STAT1 in the nuclear extracts of interferon- γ -treated cells were not increased by sodium salicylate (Figure 2), indicating that sodium salicylate was affecting the fraction of nuclear STAT1 that was phosphorylated. In the absence of interferon- γ , however, nuclear levels of p91 STAT1 increased during the interval from 30 min to 2 h, apparently as a consequence of changing to fresh serum-free medium. Presumably the absence of serum factors or a change in concentration of metabolites or autocrine factors led to this temporal increase in nuclear levels of p91 STAT1.

The preceding results indicated that salicylate-enhanced activation of γ RF-1 and STAT1 could reflect prolonged activation of a kinase, inhibition of a protein-tyrosine phosphatase, or a combination of the two. As previous experiments have shown that γ RF-1 activity in nuclear extracts can be preserved by Na₃VO₄ but not by inhibitors of serine/threonine phosphatases or by protease inhibitors [16], we asked whether sodium salicylate could be a protein-tyrosine phosphatase inhibitor by testing whether it would block inactivation of γ RF-1 and STAT1 in nuclear extracts. Sodium salicylate (10 or 20 mM), which potently enhanced γ RF-1 activity in RAW 264.7 cells, failed completely to block inactivation of γ RF-1 or STAT1 DNA-binding activity *in vitro*, whereas the phosphatase inhibitor Na₃VO₄ preserved these activities quite effectively (Figure 3A). Similarly, Na₃VO₄ preserved STAT1 phosphorylation but sodium salicylate did not (Figure 3B). The Western blots also ruled out the possibility that loss of DNA-binding activity was due simply to loss of STAT1 by proteolysis. Although this result demonstrated that sodium salicylate does not directly inhibit nuclear protein-tyrosine phos-

Figure 4 Effect of aspirin and sodium salicylate on induction of IRF-1 mRNA in interferon- γ -treated cells

Northern blotting was performed on total RNA (10 μ g each lane) isolated from RAW 264.7 cells that had been treated for 6 h with interferon- γ (100 units/ml) in the presence or absence of aspirin (Asp) or sodium salicylate as indicated. The graph presents averages of results from two independent experiments. Levels of IRF-1 mRNA are expressed relative to the IRF-1 mRNA level (arbitrarily assigned a value of 100) in cells treated with interferon- γ alone.

phatase activity, it does not rule out the possibility that sodium salicylate acts indirectly to inhibit nuclear protein-tyrosine phosphatases in the intact cell.

To test whether increases in STAT1 DNA-binding activity and phosphorylation state correlate with STAT1 transcriptional activity, we measured levels of mRNA encoding IRF-1. Transcription of the *IRF-1* gene is induced by interferon- γ via activation of the 91-kDa isoform of STAT1 [21–23]. Sodium salicylate and aspirin enhanced the induction of IRF-1 mRNA by interferon- γ in a dose-dependent fashion (Figure 4), indicating that transcriptional activity of STAT1 correlated with the increased phosphorylation and DNA-binding shown in Figures 1 and 2. Although our data do not rule out the alternative interpretation that the enhanced level of IRF-1 mRNA is due to an aspirin/salicylate-dependent increase in IRF-1 mRNA half-life, we consider this possibility to be unlikely. There was no induction of IRF-1 mRNA in cells treated with sodium salicylate or aspirin alone, consistent with the absence of STAT1 activation (Figures 1 and 2). There was no significant difference in the effects of aspirin or sodium salicylate, indicating that acetylation of cellular components is not required for the responses seen here.

DISCUSSION

Our results demonstrate that sodium salicylate can enhance specific transcriptional responses to interferon- γ mediated via the JAK–STAT signalling pathway, exemplified by enhanced

activation of STAT1 and γ RF-1 and by enhanced induction of IRF-1 and iNOS mRNAs. The effects of sodium salicylate on STAT1 activation contrast markedly with those on activation of NF- κ B. In the latter case, salicylate can prevent the phosphorylation and subsequent degradation of inhibitory κ B protein [8,24] by inhibiting the activity of inhibitory κ B protein kinase- β [25] or by activating p38 mitogen-activated protein kinase [24]. Prolonged activation of STAT1 and γ RF-1 could reflect inhibition of a protein-tyrosine phosphatase, prolonged activation of JAK1 or JAK2 kinases, or a combination of these two processes. It is important to note that the effects of sodium salicylate on γ RF-1 activation differ from those of pervanadate, a potent inhibitor of protein-tyrosine phosphatases. Pervanadate enhanced γ RF-1 activation at both early and late times of interferon- γ treatment [16], whereas sodium salicylate enhanced activation only at later times of interferon- γ treatment, indicating that the modes of action of these agents are not identical. Although our experiments show that sodium salicylate does not directly inhibit nuclear protein-tyrosine phosphatase activity, we cannot exclude the possibility that salicylate indirectly inhibits nuclear protein-tyrosine phosphatase activity in intact cells. For example, by virtue of its ability to scavenge hydroxyl radicals, salicylate may alter the cellular redox state and thereby reduce the activity of redox-sensitive phosphatases. Alternatively, the same mechanism could stimulate the activity of redox-sensitive kinases.

The effects of sodium salicylate reported here also differ from its effects on heat-shock transcription factor 1 in mammalian cells. Although sodium salicylate alone induced DNA-binding activity of pre-existing heat-shock transcription factor 1, this induced form of heat-shock transcription factor 1 is transcriptionally inert [26–28]. In our studies sodium salicylate alone was incapable of inducing DNA-binding activity of STAT1 or γ RF-1.

Though slight, effects on interferon- γ -induced responses were detectable at 3 mM sodium salicylate, which is at the upper range of plasma salicylate levels in individuals routinely taking high doses of aspirin for chronic pain [29,30]. This raises the possibility that the effects seen here may be relevant to the clinical effects of these drugs. The salicylate concentrations required to maximally enhance the responses to interferon- γ exceed those sufficient to inhibit activities of cyclo-oxygenase-1 or -2 in intact cells [31], consistent with our previous findings [11] that the inhibition of prostaglandin synthesis is not the mechanism underlying the effects of sodium salicylate reported here.

The results presented here expand our view of the immunomodulatory actions of sodium salicylate by demonstrating that at high dosage levels it can have pro-inflammatory as well as anti-inflammatory effects. More importantly, elucidation of the mechanism(s) whereby salicylate elicits its effects should provide new insights into regulation of the JAK–STAT signalling pathway and thus may identify new targets for development of immunomodulatory drugs.

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