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Aspirin and Salicylates Inhibit the IL-4- and IL-13-Induced Activation of STAT6

Moises Perez-G.,* Marco Melo, † Achsah D. Keegan, † and Jose Zamorano2*†

Allergic diseases, including asthma, represent a major threat to human health. Over the three last decades, their incidence has risen in western countries. Aspirin treatment has been shown to improve allergic diseases, especially asthma, and the decreased use of aspirin has been hypothesized to contribute to the increase in childhood asthma. Because salicylate compounds suppress a number of enzymatic activities, and signaling through IL-4R participates in the development of allergic responses, we tested the effect of salicylates on IL-4 signal transduction. We found that treatment of cell lines and primary cells with aspirin and salicylates, but not acetaminophen, inhibited the activation of STAT6 by IL-4 and IL-13. This effect correlated with the inhibition of IL-4-induced CD23 expression. Although salicylates inhibited the in vivo activation of Janus kinases, their kinase activity was not affected in vitro by salicylates, suggesting that other kinases were involved in IL-4-induced STAT6 activation. Furthermore, we found that an Src kinase was involved in STAT6 activation because 1) Src kinase activity was induced by IL-4, 2) Src kinase activity, but not Janus kinase, was inhibited by salicylates in vitro, 3) cells expressing viral Src had constitutive STAT6 phosphorylation, and 4) cells lacking Src showed low STAT6 phosphorylation in response to IL-4. Because STAT6 activation by IL-4 and IL-13 participates in the development of allergic diseases, our results provide a mechanism to explain the beneficial effects of aspirin and salicylate treatment of these diseases. The Journal of Immunology, 2002, 168: 1428–1434.

Human allergic diseases represent a serious threat to the health and well being of individuals (1, 2). Over the three last decades, their incidence has risen at an alarming rate in western countries; currently, ~30% of western populations suffer from allergies, including asthma, rhinitis, and atopic eczema (1, 2). A simple explanation for this phenomenon has not been found, and multiple potential causative factors have been proposed (1–9). They include exposure to indoor and outdoor air pollution, extensive vaccination programs, reduction in exposure to childhood infections, responses to certain early childhood viral infections, and responses to medications (1–9). Among medications, it has been hypothesized that the decreased use of aspirin to treat febrile respiratory infections may be a contributing factor to the increase in childhood asthma observed in the United States (5).

Aspirin (acetyl salicylic acid (ASA)) has been one of the most widely used drugs in history. Since 1899, it has been used as an analgesic, an antipyretic, and an anti-inflammatory agent (10). Over the years, aspirin has been substituted by other agents due to its side effects, especially in children (5, 10, 11). However, there are studies that support a beneficial effect of aspirin in asthma patients. Several groups showed that salicylates could protect against allergic responses. Aspirin (12), lysine acetylsalicylate (13, 14), and sodium salicylate (NaSal; Ref. 14) were shown to protect the early and late asthmatic response to allergens. Salicylate pretreatment also attenuates the intensity of bronchial and nasal symptoms in aspirin-induced asthma (AIA; Ref. 15), and treatment of aspirin-sensitive patients with aspirin after desensitization results in clinical improvement of inflammatory respiratory disease (16).

The many effects of aspirin are believed to be mediated by the inhibition of cyclooxygenase (COX)-1 and COX-2 enzymes, thereby blocking the production of PG (10, 17). However, the concentrations of aspirin required to obtain a beneficial effect in asthma are higher than those required to inhibit COX (16). Furthermore, NaSal, shown to have a protective effect in asthma, has little effect on COX activity. These findings raise the possibility that the beneficial effect of salicylates on allergic processes is independent of COX inhibition. Recent observations indicated that salicylates can target a wider variety of enzymes. They can regulate the activation and inhibition of several kinases such as p38 mitogen-activated protein kinase (18) and IkB kinase complex (19), therefore inhibiting the activation of NF-κB (20). Recently, it was shown that aspirin can inhibit IL-4 gene transcription in CD4+ human T cells by an unknown mechanism (21).

IL-4 is a cytokine that participates in the immune response against parasitic infections (22) and in the development of allergic diseases (23–26). Its effects are mediated by a cell surface receptor expressed in most cell types. This receptor consists of two subunits, the IL-4R α-chain (IL-4Rα) and the common γ-chain (γc) (27). IL-4Rα binds IL-4 with high affinity and specifies the signals transmitted to the interior of the cell. The γc is shared by several cytokine receptors, including the IL-2R. In some cases, the γc can be substituted by the IL-13Rα1 that, along with the IL-4Rα, forms the IL-4R type II (28). Interestingly, the IL-4Rα is also a member of the IL-13R complex (28), which explains why these two cytokines share many biological functions. At the intracellular level, signaling by IL-4 and IL-13 induces the activation of the transcription factor STAT6 via the Janus kinase (JAK) kinases (28–30). It has clearly been shown that the evolution of symptoms in the
DNA was analyzed by EMSA. Then, IL-4 was added, and STAT6 binding to indicated concentrations of acetaminophenol. Furthermore, STAT6 activation is critical for efficient Th2 development in response to protein Ags and contributes to the asthma responses (33).

Because salicylates can regulate a number of enzymatic activities, we investigated the effect of salicylates on IL-4 and IL-13 signaling. We found that aspirin and NaSal inhibited the activation of STAT6 by a mechanism that likely involves the tyrosine kinase Src. The inhibition of STAT6 activation by salicylates may explain their beneficial effect on the treatment of allergic diseases.

Materials and Methods

Cells and reagents

NIH3T3-expressing viral Src (v-Src) were obtained from Dr. J. Moscat (Centro de Biología Molecular, Cantoblanco, Spain). Wild-type and Src knockout embryonic fibroblasts were obtained from Dr. X. Zhan (Holland Laboratory, American Red Cross, Rockville, MD) and have been previously described (34). Aspirin, NaSal, acetaminophenol, cycloheximide, and Na3VO4 were purchased from Sigma-Aldrich (St. Louis, MO), and MGI32, lactacystin, and herbimycin A were obtained from Biomol (Plymouth Meeting, PA). Anti-JAK1 and -JAK3 were purchased from Upstate Biotechnology (Lake Placid, NY). RC20 anti-phosphotyrosine Ab was obtained from BD Transduction Laboratories (Lexington, Kentucky), and anti-STAT6, anti-phosphorylated STAT6, and src2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD23 Ab was a kind gift from Dr. J. M. Bergua (Hematology Department, Hospital San Pedro de Alcantara, Caceres, Spain). All cytokines were from R&D Systems (Minneapolis, MN).

EMSA

After culture, cells were washed and resuspended in lysis buffer (50 mM Tris, pH 8.0, 0.5% Nonidet P-40, 10% glycerol, 200 mM NaCl, 1 mM DTT, 5 mM NaF, 0.1 mM EDTA, 1 mM PMSF, and protease inhibitor mixture) for 5 min on ice. Cellular extracts were measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), and 1 μg of protein was incubated with 1 ng of 32P-labeled oligonucleotide in reaction buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTT, 6% glycerol, and 0.1 mg/ml poly(dIdC)) for 20 min at room temperature. To determine STAT6 DNA-binding activity, we used the IFN-γ promoter (5'-CAGCCCAAGAAGCAGA-3'). Polyacrylamide gels (4.5%) containing 0.22% Tris borate-EDTA were pre-run for 1 h at 200 V. After loading the samples, gels were run at 200 V for ~3 h. Afterward, gels were dried and exposed to film.

Immunoprecipitation and immunoblotting

After stimulation, cell pellets were lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 10 mM pyrophosphate, 1 mM PMSF, and protease inhibitor mixture) and clarified. To perform precipitations, the soluble fraction was immunoprecipitated with the indicated Ab followed by incubation with protein G-agarose. The washed precipitates were separated on 7.5% SDS-polyacrylamide gel before transfer to a polyvinylidene difluoride membrane. Membranes were then probed with the indicated Ab. The bound Ab was detected using ECL (Amersham, Arlington Heights, IL).

Kinase assays

JAK1, JAK3, and Src kinases were precipitated as described above. Precipitates were washed in kinase buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 50 μM Na3VO4, and protease inhibitors) and incubated in the presence of mentioned inhibitors. The enzymatic reaction was initiated with the addition of [γ-32P]ATP (Amersham). Samples were separated on polyacrylamide gels, dried, and exposed to film.

Analysis of CD23 expression

CD23 expression on monocytic cells was analyzed as previously described with little modification (35). PBMC were cultured for 30 h in the presence of indicated compounds. The cells were then stained with FITC-conjugated anti-human CD23 Ab (Immunotech, Marseille, France) and analyzed by flow cytometer (FACScan, BD Biosciences, Mountain View, CA). Viable monocytic cells were selected using forward-scatter and side-scatter parameters.

Results

Salicylates inhibit STAT6 activation

In experimental asthma, IL-4 and IL-13 have been shown to regulate a number of asthma symptoms (23–26, 31–33), likely mediated through STAT6 (33). Because salicylates also ameliorate asthma symptoms (12–16), we set out to test whether salicylates had an effect on IL-4- or IL-13-activated signal transduction. We first analyzed the effect of NaSal and ASA on the activation of STAT6 (Fig. 1). Pretreatment of the murine B cell lymphoma M12 with either NaSal or ASA blocked the ability of IL-4 to induce the DNA-binding activity of STAT6 in a concentration-dependent manner (Fig. 1A). A concentration of 20 mM completely blocked the activation of STAT6, whereas 5 mM inhibited STAT6 activation by >60%. Because ASA suppresses COX-1 and -2 activity and NaSal does not (16), these results suggest that inhibition of STAT6 activation by salicylates is not via suppression of COX activity. This inhibition was observed in all cell lines tested, including 32D, FDCP-1, M12, A1.1, U937, CH-31, and Wehi-231 (Figs. 1–3 and J. Zamorano, unpublished observations). The effect of salicylates in IL-4 signaling was not due to inhibition of IL-4 binding to its receptor because incubation of 32D cells with 20 mM NaSal did not affect the binding of IL-4 to its receptor (J. Zamorano, unpublished observations). We found similar results for IL-13 signaling; treatment of cells with NaSal and ASA also

FIGURE 1. Salicylates, but not acetaminophenol, inhibit STAT6 activation. A, M12 cells were cultured with the indicated concentrations of NaSal and ASA for 1 h before stimulation with IL-4 (10 ng/ml) for 30 min. STAT6 DNA-binding activity in cell extracts was analyzed by EMSA using the IFN-γ activation site sequence contained in the Ce promoter. B, U937 cells were cultured with NaSal or ASA for 1 h, and then stimulated for 30 min with IL-13 (400 ng/ml). STAT6 activation was then analyzed by EMSA. C, M12 cells were cultured with nothing, NaSal (20 mM), or the indicated concentrations of acetaminophenol. Then, IL-4 was added, and STAT6 binding to DNA was analyzed by EMSA.
STAT6 DNA-binding activity was analyzed by EMSA. MG132 (30 μM/H9262) inhibited DNA-binding activity of STAT6 (Fig. 1B). Thus, a concentration of 20 mM salicylates completely blocked STAT6 activation, and a significant inhibition, greater than 50%, was still observed at 5 mM. In contrast to salicylates, treatment of cells with acetylsalicylic acid (ASA) at 10 mM did not block STAT6 activation by IL-4 (Fig. 1C). High concentrations of acetylsalicylic acid (20 mM) did not abrogate STAT6 activation as compared with NaSal, and lower concentrations (10 and 5 mM) resulted in no inhibition. Cell viability was not affected under the experimental conditions used in these experiments, suggesting a specific effect of salicylates in inhibiting STAT6 activation.

**Salicylates inhibit tyrosine phosphorylation by a JAK-independent pathway**

Protein kinases, phosphatases, and proteasome are involved in the regulation of STAT6 (36). To address the molecular mechanisms by which salicylates affected STAT6 activation, we analyzed the effect of proteasome and phosphatase inhibitors in this process (Fig. 2). We found that addition of salicylate after IL-4 stimulation also resulted in the inhibition of STAT6. This inhibition was partially reverted by the phosphatase inhibitor Na3VO4, but not by cycloheximide or proteasome inhibitors. Because Na3VO4 enhances the phosphorylation of STAT6 by IL-4 (37), likely through the inhibition of a constitutive phosphatase, our data suggests that kinases or phosphatases could be the target for NaSal because vanadate partially reverted its inhibitory effect.

Previous studies have established an effect of salicylates on kinase activation (18, 19). We focused on tyrosine kinases because serine phosphorylation of STAT6 is not required to bind DNA (38). Treatment of cells with salicylates resulted in the inhibition of tyrosine phosphorylation of STAT6 in the unrelated cell lines 32D, CH31, and A1.1 to the same extent they inhibited the ability of STAT6 to bind DNA (Fig. 3). In this case, NaSal and ASA completely abrogated the ability of IL-4 to promote STAT6 tyrosine phosphorylation at a concentration of 20 mM, and a substantial inhibition (~60%) was also present at 5 mM (Fig. 3, A and B). A significant inhibition was also observed at lower concentrations (~<4 mM; Fig. 3C). Incubation of A1.1 cells with low doses of NaSal for 1, 4, and 12 h resulted in a reproducible inhibition of STAT6 phosphorylation. Similar results were obtained with 32D cells (data not shown). In these cases, densitometric scanning indicated that the levels of STAT6 phosphorylation are ~50% reduced in the presence of 4 mM NaSal and ~30% in the presence of 1 mM NaSal. Cell viability was not affected under these conditions of culture. Concentrations of salicylates in the millimolar range can be reached in plasma during treatment of rheumatic diseases and during analgesic and anti-pyretic regimens (10), suggesting that STAT6 activation may be inhibited during therapeutic treatment with salicylates.

We have shown that salicylates inhibit the activation of STAT6 in multiple cell lines. We next investigated the effect of salicylates on primary cells. Pretreatment of human PBMC with NaSal also inhibited the activation of STAT6 by IL-4 (Fig. 4A). The pattern of inhibition was similar to cell lines. Thus, 20 mM of NaSal completely block STAT6 activation and there was still a significant inhibition at low concentrations of 1–2 mM. We next investigated the physiological effects of STAT6 inhibition by salicylates. To this end, we analyzed the effect of salicylate treatment in the IL-4-induced CD23 expression on monocyctic primary cells (Fig. 4B). We found that treatment of human PBMC with NaSal inhibited the induction of CD23 by IL-4 in a dose-dependent manner. Thus, 10 mM NaSal almost completely abrogated the induction of CD23,
Salicylates inhibit an Src kinase involved in STAT6 activation

In addition to JAK kinases, several laboratories have demonstrated that the Src family of kinases can participate to varying degrees in the activation of STATs (40, 41). Although little is known about the role of Src kinases in IL-4 signaling, it has been shown that IL-4 stimulation can result in their activation (42). Interestingly, it has been reported that salicylates can inhibit the activation of Src kinases (43, 44). Therefore, we examined the IL-4-induced activation of Src family kinases and the effects of salicylates on this activation (Fig. 6). We found that IL-4 treatment of M12 cells activated an Src kinase that was precipitated by the anti-Src Ab, src2. We observed an increase in autophosphorylation activity in precipitates obtained from cells that had been stimulated with IL-4, and this increase was abrogated when cells were cultured in the presence of NaSal before IL-4 stimulation (Fig. 6A). Furthermore, the incubation of src2 immunoprecipitates with NaSal inhibited in vitro kinase activity (Fig. 6B). These results demonstrate that salicylates not only suppress the IL-4-induced activation of a Src kinase in cells, but also suppress the in vitro activity of this kinase.

To further confirm a role for Src in STAT6 activation, we analyzed the ability of IL-4 to signal STAT6 activation in cells expressing v-Src, a viral oncogenic form of Src (Fig. 7). NIH3T3 cells expressing v-Src showed a high basal tyrosine phosphorylation of STAT6 as compared with unstimulated wild-type cells (Fig. 7A). Stimulation of these cells with IL-4 also resulted in higher STAT6 phosphorylation than in control cells. As expected, treatment of parental NIH3T3 with NaSal inhibited the IL-4-induced phosphorylation of STAT6 (Fig. 7B). Salicylates also completely blocked the basal phosphorylation observed on v-Src-transformed cells. However, NIH3T3 cells expressing v-Src became more resistant to salicylate inhibition of IL-4-induced STAT6 phosphorylation. In this case, we observed a partial inhibition of phosphorylation on cells treated with 20 mM NaSal, but lower concentrations had no effect. This data suggests that the overexpression of src protects against salicylate inhibition, likely by augmenting the enzyme/drug ratio under these experimental conditions.
Salicylates inhibit an Src kinase involved in STAT6 activation. The role of Src in the IL-4-induced activation of STAT6 was also demonstrated in embryonic fibroblasts derived from Src-deficient mice (Ref. 34; Fig. 8). Treatment of the src<sup>−/−</sup> cells with IL-4 stimulated a weak tyrosine phosphorylation of STAT6, whereas in wild-type cells, IL-4 treatment induced robust phosphorylation (Fig. 8A). In this case, NaSal treatment had similar inhibitory effect on wild-type and src<sup>−/−</sup> cells (Fig. 8B). These data suggest that Src contributes to the IL-4-induced activation of STAT6, but other salicylate-sensitive kinases, probably members of the Src family, may substitute for this kinase.

**Discussion**

We have found in this study that salicylates, aspirin, and NaSal can inhibit STAT6 activation induced by IL-4 and IL-13, most likely by targeting a Src kinase. In contrast, acetaminophen (paracetamol) does not inhibit STAT6 activation. The effect of salicylates on STAT6 activation correlates with the inhibition of CD23 expression, supporting a physiological role of salicylates in inhibiting IL-4 signaling.

A number of observations indicate that the effects of salicylates in IL-4 signaling are specific. First, cell viability was not affected under the experimental conditions used. Second, concentrations of salicylates lower than 5 mM, which can be achieved therapeutically and are not toxic (10), exert a significant inhibition of STAT6. Third, other authors have found, under similar experimental conditions, the opposite effect of salicylate on IFN-γ signaling. They have found that salicylates enhanced STAT1 activation (45).

We have found that an Src kinase is a target for salicylates in IL-4 signaling. COX enzymes have been thought to be the main target for aspirin and other nonsteroidal anti-inflammatory drugs. However, over the last years, a number of new targets of salicylates have been described, including p38 mitogen-activated protein kinase (18) and IκB kinase complex (19). Our findings confirm previous reports showing the ability of salicylates to inhibit Src in vivo (43) and in vitro (44). Given the multiple functions associated with Src, it is reasonable that the effect of salicylates on Src could have important clinical applications.

Our study corroborates the evidence for a role of Src kinases in STAT activation (40, 41). Src kinases have been involved in the activation of several STATs by JAK-dependent (40) and -independent pathways (41). We have clearly shown that Src plays a critical role in the activation of STAT6. Furthermore, the fact that the absence or inhibition of Src abrogates JAK1, JAK3, and STAT6 activation suggests that Src activation is a very early event in IL-4 signaling. This effect on STAT6 is in contrast to a report showing the enhancement of STAT1 activation by aspirin (45), suggesting a divergence in the regulation of STAT1 and STAT6 by IFN-γ and IL-4, respectively. It is possible that this divergent effect could promote Th1-type responses while suppressing the Th2 type, with the result of inhibiting allergic disease.

Clinical studies have found a beneficial effect of salicylates and aspirin in allergic diseases, especially asthma (12–16). It has also been proposed that the substitution of aspirin by acetaminophen could have contributed to the increased incidence of asthma in children (5). This is in apparent contradiction with the fact that aspirin can actually precipitate asthma in a subset of asthmatic patients.

**FIGURE 6.** Salicylates inhibit an Src kinase involved in STAT6 activation. A, M12 cells were treated or not with 20 mM of NaSal before stimulation with IL-4. Cell extracts were incubated with src2, an anti-Src Ab, and were precipitated with protein G-agarose. The kinase activity of the precipitates was analyzed as in Fig. 5. B, Src2 precipitates were prepared from M12 cells and were incubated with nothing, NaSal (20 mM), or herbinycin A (10 μM). Kinase activity was analyzed as above.

**FIGURE 7.** V-Src expression promotes STAT6 activation. A, STAT6 phosphorylation was analyzed in cell extracts obtained from wild-type or v-Src-expressing NIH3T3 cells. B, STAT6 phosphorylation was analyzed in wild-type NIH3T3 cells pretreated with NaSal before IL-4 stimulation (left), V-Src NIH3T3 cells cultured with NaSal without cytokine treatment (center), and V-Src NIH3T3 pretreated with NaSal and then stimulated with IL-4 (right). Culture conditions are the same as in Fig. 3A. The time of exposure in B was not the same in each sample. Upper panels, antiphosphotyrosine blots (Py). Lower panels, same membrane blotted with anti-STAT6 Ab (Stat6).

**FIGURE 8.** IL-4 induced low levels of STAT6 phosphorylation in cells lacking Src. A, STAT6 phosphorylation was analyzed as in Fig. 3 in embryonic fibroblasts derived from wild-type mice (src<sup>+/+</sup>) or src knockout mice (src<sup>−/−</sup>). B, STAT6 phosphorylation was analyzed in src<sup>+/+</sup> (left) and src<sup>−/−</sup> (right) cell lines pretreated with NaSal for 1 h with the indicated amount of NaSal before IL-4 stimulation for 30 min. Upper panels, antiphosphotyrosine blots (Py). Lower panels, same membrane blotted with anti-STAT6 Ab (Stat6).
patients (AIA) (17, 46). However, the effect of aspirin in these patients seems to be mediated by its direct effect on COX enzymes and its promotion of production of leukotriene C4, not by an immunological response against aspirin (17, 47). Perhaps, paradoxically, salicylates have been used successfully to treat AIA patients. Treatment of patients with high doses of salicylates has been shown to offer a moderate protection against AIA in predisposed individuals (15, 16). These beneficial effects of salicylates in asthma treatment cannot be explained by their ability to block COX enzymes. In these studies, the doses used are higher than those required to block COX. In addition, NaSal that does not inhibit COX also has a beneficial effect on asthma. Therefore, the molecular mechanisms involved in these processes likely do not require COX regulation.

Given the importance of STAT6 and IL-4 in the induction of asthma (23–26, 31–33, 48), our data suggest that the beneficial effect reported for aspirin and salicylates in asthma may be mediated by the inhibition of STAT6 activation and thereby by a Th2-type immune response. Concentrations of salicylate lower than 5 mM that can be achieved during analgesic, antipyretic, and anti-inflammatory treatments are able to partially inhibit the activation of STAT6. This effect correlates with the ameliorating effects of salicylates on asthma. In addition, salicylic compounds such as gentisic acid, gallic acid, and 2,3-dihydroxybenzoic acid, which are products of aspirin degradation (10), can also inhibit Src kinase activity (44). Therefore, they may also potentially contribute to the inhibition of IL-4-induced STAT6 activation under physiological conditions.

The importance of IL-4 and IL-13 signaling in human asthma (48) and animal models of asthma (23–26, 31–33) has been established. Therefore, their signaling pathways may be good targets for therapeutic intervention of allergic diseases. The finding that salicylates are able to inhibit the signaling of these cytokines may lead to the design of novel treatments for these diseases.

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References


