

## The mode of action of aspirin-like drugs: Effect on inducible nitric oxide synthase

ASHOK R. AMIN\*†‡§, PRANAV VYAS\*, MUKUNDAN ATTUR\*, JOANNA LESZCZYNSKA-PIZIAK\*,  
INDRAVADAN R. PATEL¶, GERALD WEISSMANN‡, AND STEVEN B. ABRAMSON\*‡

\*Department of Rheumatology, Hospital for Joint Diseases, New York, NY 10003; Departments of †Pathology and ‡Medicine, New York University Medical Center, New York, NY 10016; and §Department of Biochemistry, Glaxo, Inc., Research Triangle Park, NC 27709

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**ABSTRACT** Nitric oxide synthesized by inducible nitric oxide synthase (iNOS) has been implicated as a mediator of inflammation in rheumatic and autoimmune diseases. We report that exposure of lipopolysaccharide-stimulated murine macrophages to therapeutic concentrations of aspirin ( $IC_{50} = 3$  mM) and hydrocortisone ( $IC_{50} = 5$   $\mu$ M) inhibited the expression of iNOS and production of nitrite. In contrast, sodium salicylate (1–3 mM), indomethacin (5–20  $\mu$ M), and acetaminophen (60–120  $\mu$ M) had no significant effect on the production of nitrite at pharmacological concentrations. At suprapharmacological concentrations, sodium salicylate ( $IC_{50} = 20$  mM) significantly inhibited nitrite production. Immunoblot analysis of iNOS expression in the presence of aspirin showed inhibition of iNOS expression ( $IC_{50} = 3$  mM). Sodium salicylate variably inhibited iNOS expression (0–35%), whereas indomethacin had no effect. Furthermore, there was no significant effect of these nonsteroidal anti-inflammatory drugs on iNOS mRNA expression at pharmacological concentrations. The effect of aspirin was not due to inhibition of cyclooxygenase 2 because both aspirin and indomethacin inhibited prostaglandin  $E_2$  synthesis by >75%. Aspirin and *N*-acetylimidazole (an effective acetylating agent), but not sodium salicylate or indomethacin, also directly interfered with the catalytic activity of iNOS in cell-free extracts. These studies indicate that the inhibition of iNOS expression and function represents another mechanism of action for aspirin, if not for all aspirin-like drugs. The effects are exerted at the level of translational/posttranslational modification and directly on the catalytic activity of iNOS.

Nitric oxide (NO), first identified as an endothelium-derived relaxation factor (1), is now recognized to be an intra- and extracellular mediator of cell function (2–5). NO produced by the constitutive isoform of nitric oxide synthase (NOS) is a key regulator of homeostasis, whereas the generation of NO by inducible NOS (iNOS) plays an important role in inflammation, host-defense responses, and tissue repair (2–4). NO formation is increased during inflammation (rheumatoid arthritis, and ulcerative colitis, Crohn disease), and several classic inflammatory symptoms (erythema and vascular leakiness) are reversed by NOS inhibitors (2–4). Vane and co-workers (6) have implicated NO as an important mediator of inflammation in animal models. Furthermore, because iNOS is up-regulated by endotoxin, interleukin 1, tumor necrosis factor, and interferon  $\gamma$ , the increased synthesis of NO has been implicated in autoimmune diseases, allograft rejection, graft-versus-host disease, and systemic response to sepsis. Recent studies by Salvemini *et al.* (7) have shown that NO modulates the activity of prostaglandin endoperoxide H synthase 2 [cyclooxygenase 2 (COX-2)] in a concentration-

dependent manner, through a mechanism independent of cGMP.

Although nonsteroidal antiinflammatory drugs (NSAIDs) clearly inhibit the synthesis and release of prostaglandins (8, 9), these actions are by no means sufficient to explain all the antiinflammatory effects of NSAIDs. NSAIDs also inhibit activation of neutrophils (10), which provoke inflammation by releasing products other than prostaglandins (11). In these studies we examined the effect of NSAIDs on NO production.

Among the agents studied in an effort to elucidate the effect of NSAIDs on iNOS expression and function, we have selected three: an acetylated salicylate (aspirin, an effective inhibitor of COX); a nonacetylated salicylate (sodium salicylate, an ineffective inhibitor of COX); and a nonacetylated nonsteroidal compound (indomethacin, a potent inhibitor of COX). We tested the hypothesis that NSAIDs, which inhibit COX activity, might inhibit inflammation by modifying iNOS expression/activity. Aspirin, sodium salicylate, and indomethacin, which reach therapeutic concentrations in plasma of 1–3 mM, 1–3 mM, and 5–20  $\mu$ M, respectively (12), were tested for their capacities to inhibit iNOS expression/catalytic activity at the clinically relevant concentrations. In the present study we report that aspirin and, to a lesser extent, sodium salicylate (but not indomethacin) inhibit iNOS expression in murine macrophages activated with lipopolysaccharide (LPS). In addition, aspirin inhibits the catalytic activity of iNOS, an effect mimicked by *N*-acetylimidazole (NAI), another acetylating agent. We therefore conclude that the aspirin-like drugs differ in their mode of action and that acetylation may be a critical difference.

### MATERIALS AND METHODS

**Cell Lines and Reagents.** Murine macrophage cells (RAW 264.7) were obtained from the American Type Culture Collection. An antimurine iNOS antibody was obtained from Transduction Laboratories (Lexington, KY). Aspirin, sodium salicylate, indomethacin, acetaminophen, NAI, and imidazole were obtained from Sigma.

**Immunoblot Analysis.** Equal amounts of protein (50  $\mu$ g) estimated by bicinchoninic acid reagent (Pierce) were loaded onto SDS/PAGE gels and stained to verify the concentrations of various protein fractions by examining the intensities of the protein bands on the gels. Immunoblot analysis was done from the same cell extracts. The immunoblotted membrane was probed with a specific anti-iNOS monoclonal antibody, as specified by Transduction Laboratories. The blots were devel-

Abbreviations: COX, cyclooxygenase (prostaglandin endoperoxide H synthase 2); NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; NAI, *N*-acetylimidazole; NSAIDs, nonsteroidal antiinflammatory drugs; LPS, lipopolysaccharide(s).

§To whom reprint requests should be addressed at: Department of Rheumatology, Hospital for Joint Diseases, 301 East 17th Street, Room 1600, New York, NY 10003.

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oped by using the enhanced chemoluminescence immunoblot system (Amersham). Density of the bands was measured with a densitometer from Molecular Dynamics.

**Northern Blot Analysis.** Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati). Northern blot analysis was done as described by Church and Gilbert (13). Thirty micrograms of RNA was subjected to electrophoresis in 1% agarose/formaldehyde gel. The gel was then transferred via capillary action onto a nylon membrane (Zeta probe, Bio-Rad). The membrane was hybridized with [<sup>32</sup>P]dCTP-labeled iNOS cDNA (4-kb *Sma* I fragment), from James Cunningham (Harvard Medical School, Boston). After hybridization, the blot was exposed to Kodak x-ray film for 24–48 hr with intensifying screens at –70°C. The  $\beta$ -actin probe was purchased from Clontech and probed as described above. Measurement of the intensity of the iNOS/ $\beta$ -actin bands was done by using a PhosphorImager (Molecular Dynamics).

**Assays for iNOS in Cell-Free Extracts.** Specific activity of iNOS was determined in cell-free extracts by monitoring the conversion of L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H]citrulline, as described by Misko *et al.* (14) and modified by us (unpublished work). RAW 264.7 cells were induced with LPS in the presence and absence of NSAIDs for 16–18 hr. After induction, the cells were pelleted at 4°C and resuspended in Tris buffer (10 mM, pH 7.4) containing chymostatin, antipain, leupeptin, and pepstatin each at 10  $\mu$ g/ml, as well as dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Cells were lysed in a Polytron PT 1200 homogenizer (Kinematica, Lucerne, Switzerland) after three cycles of rapid freeze–thawing. The lysate was centrifuged at 30,000  $\times$  g for 60 min at 4°C, and the supernatants were used as cell-free extracts. The protein was measured by bicinchoninic acid assay reagent using bovine serum albumin as standard (16). The reaction mixture for iNOS assay consists of 50  $\mu$ M Tris (pH 7.8); bovine serum albumin at 1 mg/ml; 1 mM dithiothreitol; 2 mM CaCl<sub>2</sub>; 10  $\mu$ M FAD; 10  $\mu$ M tetrahydrobiopterin; 30  $\mu$ M L-arginine; 1 mM NADPH (11). The reaction mixture was treated with 1  $\mu$ l (250 nM) of L-[<sup>3</sup>H]arginine (DuPont/NEN) (1 mCi/ml = 37.0 MBq/ml). After 20 min the assays were terminated by heating the reaction mixture at 90°C for 5 min. The precipitates were removed by centrifuging at 27,000  $\times$  g for 20 min. Ten microliters ( $\approx$ 50,000 cpm) of the supernatant was spotted on activated Avicel TLC plates (Analtech). The TLC plates were developed in a solvent system of ethanol/water/ammonia, 80:16:4. The spot for L-[<sup>3</sup>H]citrulline was quantitated by using a Bioscan (Washington, DC) system 200 imaging scanner. Briefly, total cpm per lane were averaged, and the cpm of each lane was then normalized to the mean. The quantity of [<sup>3</sup>H]arginine converted to [<sup>3</sup>H]citrulline was calculated from the specific activity of [<sup>3</sup>H]arginine added to the assay mixture (2 cpm = 1 pmol of [<sup>3</sup>H]arginine or [<sup>3</sup>H]citrulline).

**Assay for COX-2 in Whole Cells.** Cells were incubated with LPS (1  $\mu$ g/ml) for 16 hr to induce COX-2, exposed to NSAIDs for 1 hr, and subsequently harvested. The harvested cells were then incubated with radiolabeled arachidonic acid (100,000 cpm, 57 mCi/mM) in 1 ml of Tris-HCl (together with 3  $\mu$ M of cold arachidonic acid) for 10 min. Specific enzyme activity (whole-cell assays) was measured by the conversion of [<sup>14</sup>C]arachidonic acid to prostaglandin E<sub>2</sub> after separation by TLC (6, 17). Authentic prostaglandin and monohydroxy standards were run in parallel. The transformed products were quantitated by a Bioscan system 200 imaging scanner, as described above.

## RESULTS AND DISCUSSION

**Effects of NSAIDs on Nitrite Accumulation.** Murine macrophage cells (RAW 264.7) were selected because the iNOS regulation in these cells has been well-characterized, both at the biochemical and molecular levels (2, 18, 19). RAW 264.7 cells were activated with LPS at 100 ng/ml to induce iNOS (18) with and without aspirin (1–3 mM), sodium salicylate (1–3 mM), and indomethacin (5–20  $\mu$ M). Expression and activity of iNOS were monitored by estimating the stable end-product nitrite, as described for these cells by other investigators (7, 18, 19). Table 1 shows a concentration-dependent inhibition of nitrite accumulation in cells stimulated with LPS in the presence of 1–3 mM aspirin. Only 2 and 3 mM concentration showed a significant effect on nitrite accumulation. Suprapharmacological concentrations of aspirin (5 and 10 mM) further inhibited nitrite accumulation [by 50%  $\pm$  6 and 80%  $\pm$  5 ( $p$  < 0.005), respectively] above that seen at 3 mM (data not shown). Sodium salicylate (3 mM) and indomethacin (5  $\mu$ M) did not significantly inhibit nitrite production ( $\approx$ 7%). Suprapharmacological concentrations of sodium salicylate (5 mM) inhibited nitrite accumulation by 15% under identical conditions. However, the IC<sub>50</sub> of sodium salicylate with respect to nitrite accumulation was 20 mM, whereas its ability to inhibit fMet-Leu-Phe-induced neutrophil aggregation was 3 mM (data not shown) (11). Although indomethacin is effective therapeutically at 20  $\mu$ M, the extent to which it inhibited nitrite accumulation (10  $\pm$  9.6%) was only marginally greater than that seen with 5  $\mu$ M and was not statistically significant. Our results on the effect of indomethacin on nitrite accumulation in RAW 264.7 cells were identical to those seen by Salvemini *et al.* (7). Acetaminophen (60–120  $\mu$ M), an analgesic agent closely related to salicylates, failed to block nitrite production (1  $\pm$  1%) in LPS-stimulated macrophages at therapeutic concentrations. As previously shown by Moncada and coworkers (21) in murine macrophages (J774 cells) and as seen here (Table 2), hydrocortisone (5  $\mu$ M) inhibited endotoxin-induced NO production by >60%.

Table 1. Effect of NSAIDs on nitrite accumulation and specific activity of iNOS in murine macrophages induced with LPS

Modulating agent	Nitrite released			Specific activity		
	Nitrite, $\mu$ M	Inhibition, %	<i>P</i> value	pmol/min per mg protein	Inhibition, %	<i>P</i> value
Control (uninduced)	0.5 $\pm$ 0.5			11.7 $\pm$ 1.5		
LPS-induced	29.2 $\pm$ 6.8			310.0 $\pm$ 54.6		
Aspirin (1 mM)	26.6 $\pm$ 4.3	10	<0.267	271.7 $\pm$ 17.2	12	<0.155
Aspirin (2 mM)	22.9 $\pm$ 5.3	22	<0.071	231.3 $\pm$ 29.8	25	<0.046
Aspirin (3 mM)	20.3 $\pm$ 3.9	32	<0.025	162.3 $\pm$ 25.9	48	<0.006
Sodium salicylate (3 mM)	27.1 $\pm$ 8.8	7	<0.345	304.0 $\pm$ 48.1	2	<0.446
Indomethacin (5 $\mu$ M)	27.5 $\pm$ 7.7	7	<0.365	304.0 $\pm$ 39.4	2	<0.441

Murine macrophage cells (RAW 264.7) were incubated with NSAIDs for 2 hr followed by addition of LPS at 100 ng/ml. After 16–18 hr of incubation, the medium was used to estimate nitrite accumulation by the modified Greiss method (20). Specific activity of iNOS was determined in cell-free extracts at a given time, as described. Nitrite and specific activity data are representative of means  $\pm$  SD values, as determined by Student's *t* test, for four independent experiments. The *P* values indicate comparisons with LPS-stimulated cells.

Table 2. Action of NSAIDs on expression of iNOS and COX-2

Modulating agent	Nitrite release	Inhibition of iNOS at 16 hr, %			Specific activity in <i>in vitro</i> assay	Inhibition of COX-2, %
		Specific activity in cell-free extracts	Protein expression	mRNA		
Aspirin (3 mM)	32.0*	47†	≈53	NS	≈45 (1mM)	>75†
Sodium salicylate (3 mM)	7.0‡	2§	≈15	NS	≈1 (1mM)	NS
Indomethacin (5 μM)	7.0‡	2§	0	NS	0 (5 μM)	>75†
Hydrocortisone (5 μM)	63.0¶	ND	ND	ND	ND	ND
NAI (1 mM)	ND	ND	ND	ND	≈74 (1mM)	ND

The data (expressed as percentage inhibition) are compiled from this study. RAW 264.7 cells were induced with LPS at 100 ng/ml to stimulate iNOS and COX-2 activity. After 16–18 hr of incubation, COX-1/COX-2 activity was assayed, as described by Mitchell *et al.* (17). COX-1 activity was not detected in these cells, as described (7). *In vitro* assay indicates the effect of NSAIDs (at concentrations in parentheses) on catalytic activity of iNOS in cell-free extracts. Protein expression data are represented as approximate percentage inhibition based on the densitometry data from one of the two representative experiments. ND, not done; NS, not significant. Note *P* values: §, *P* < 0.45, ‡, *P* < 0.36, \*, *P* < 0.05, ¶, *P* < 0.01, †, *P* < 0.006 (see text and Table 1 for absolute values).

We next compared the capacities of selected drugs to inhibit the specific activity of COX-2 in RAW 264.7 cells exposed to LPS, as shown in Table 2. Aspirin (3 mM) and indomethacin (20 μM) inhibited the specific activity of COX-2 by  $79 \pm 6.7\%$  (*P* < 0.001) and  $84 \pm 4.0\%$  (*P* < 0.002), respectively, whereas sodium salicylate (3 mM) had no effect [ $16 \pm 11\%$  (*P* < 0.28)]. These data indicate that aspirin does not inhibit nitrite production by inhibiting COX because aspirin shares this latter effect with indomethacin.

We further examined the mechanism of action of aspirin by determining its effects on (i) the specific activity of the enzyme, (ii) the synthesis of iNOS at the protein level, (iii) the synthesis of mRNA, and (iv) the catalytic activity of iNOS in cell-free extracts and also by comparing it with sodium salicylate and indomethacin.

**Effect of NSAIDs on Expression and Catalytic Activity of iNOS.** Because nitrite accumulation, which represents the cumulative effect of iNOS expression from induction of the enzyme, does not directly assess the effects of pharmacologic agents (i.e., NSAIDs) on specific enzyme activity, we analyzed these two parameters in tandem.

The specific enzyme activity of iNOS from cells exposed to aspirin in cell-free extracts showed a significant inhibition in activity in a dose-dependent fashion ( $IC_{50} = 3$  mM). Sodium salicylate and indomethacin did not inhibit the specific activity of iNOS (Table 1). These observations raised the following hypotheses. Aspirin may (i) decrease the expression of iNOS protein and therefore decrease the specific activity of the enzyme and subsequently the production of nitrite; (ii) decrease only the catalytic activity of iNOS without influencing the expression of iNOS protein; or (iii) decrease both the catalytic activity of iNOS and the expression of iNOS protein, which in turn cumulatively leads to decrease in the accumulation of nitrite in the medium.

We therefore analyzed (by immunoblot) iNOS protein in cells treated with LPS with and without NSAIDs for 16–18 hr. Fig. 1A shows a significant decrease in iNOS expression in cells treated with aspirin, thus accounting, in part, for the decrease in the specific activity of iNOS and thus eliminating hypothesis ii described above. Aspirin at 10 mM further decreased iNOS expression by ≈70%, as determined by immunoblot analysis (Fig. 1B). Therapeutic concentration of sodium salicylate (2 mM) caused ≈15% inhibition of iNOS expression, whereas 5 μM indomethacin had no effect, as assessed by immunoblot analysis. Sodium salicylate (2–3 mM) caused a variable (0–35%) inhibition of iNOS expression at therapeutic concentrations in four independent experiments, one of which is presented in Fig. 1B. However, increased sodium salicylate concentration (5 and 20 mM) did not increase inhibition of iNOS expression, unlike the increasing effects seen with 10 mM aspirin (≈70%). These results are not easily interpreted, but we assume that sodium salicylate at lower concentrations interferes with enzyme synthesis, whereas at higher concen-

trations this salicylate inhibits the catalytic activity of iNOS. This biphasic effect would account for a decrease in nitrite production without apparent decrements of protein synthesis, as assessed by immunoblot analysis.

Previous studies have shown that induction of iNOS and COX-2 are both achieved by LPS in RAW 264.7 cells after 12–16 hr (7). Indomethacin (5 μM) inhibited COX-2 activity by >75% but had no effect on iNOS expression in immunoblot analysis (Fig. 1A). Furthermore, because indomethacin had minimal effects on iNOS activity at therapeutic concentrations, COX-2 or its products are unlikely to be regulators of iNOS activity *per se*, at least in murine macrophages.

**Effect of NSAIDs on Expression of iNOS mRNA.** Aspirin may suppress iNOS expression early in the course of enzyme induction, leading to inhibition or delay in nitrite accumulation. This assumption is based on the observation that, in macrophages, transforming growth factor β1 suppresses iNOS expression by decreasing mRNA stability and translation and

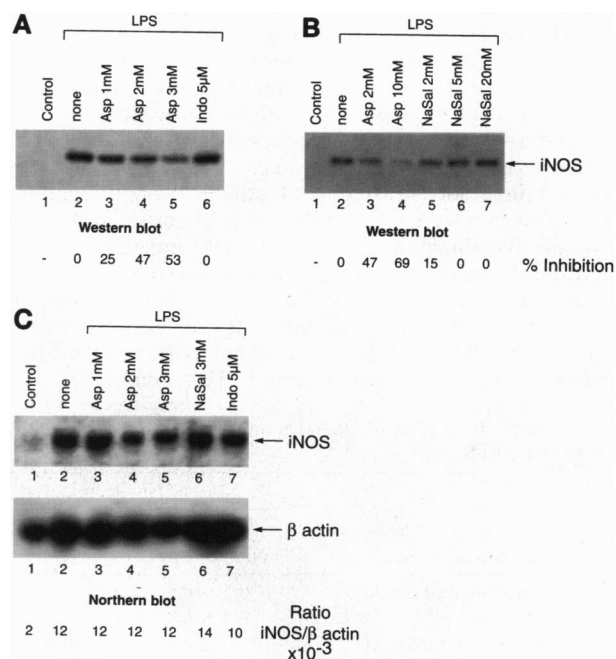


FIG. 1. Immuno- and Northern blot analyses of iNOS from RAW 264.7 cells activated with LPS with and without NSAIDs. (A and B) Western (immunoblot) analysis of cells treated with LPS at 100 μg/ml with or without NSAIDs for 16 hr. (C) RNA blot analysis of iNOS and β-actin from RAW 264.7 cells activated with LPS and treated with NSAIDs for 16 hr. The iNOS/β-actin ratio was determined by using a PhosphorImager. Data represent one of the four representative experiments. Asp, aspirin; Indo, indomethacin; NaSal, sodium salicylate.

increasing the degradation of iNOS protein in macrophages (22). There was no significant difference in the expression of iNOS mRNA (at 16 hr) in cells treated with LPS with or without NSAIDs because the iNOS mRNA/ $\beta$ -actin mRNA ratios were either identical or not significantly different from cells stimulated with LPS alone (Fig. 1). Recent studies by Tetsuka *et al.* (23) have demonstrated that indomethacin addition enhanced interleukin 1 $\beta$ -induced steady-state level of iNOS mRNA and nitrite production in rat mesangial cells. Hence, our studies indicate that the effect of indomethacin may differ in different cell types. Kopp and Ghosh (24) showed that aspirin (3 mM) or sodium salicylate (5 mM) inhibit NF- $\kappa$ B-dependent transcription, using sensitive assays based on plasmids containing two IgK- $\kappa$ B sites driving a luciferase reporter gene. However, in the same studies, the same concentrations of aspirin and sodium salicylate had no significant effect on NF- $\kappa$ B activation, judged by gel-shift assays. Nathan and coworkers (19) have shown that NF- $\kappa$ B expression is one of the integral components of iNOS transcription/expression, which can be inhibited by an NF- $\kappa$ B inhibitor, pyrrolidine dithiocarbamate, at 30  $\mu$ M. Our studies indicate that 3 mM aspirin is probably not sufficient to block the transcription of the iNOS gene, as seen with 30  $\mu$ M of pyrrolidine dithiocarbamate, which blocked >90% of nitrite accumulation in our studies (data not shown). Furthermore, the lack of significant effects of aspirin and sodium salicylate on iNOS mRNA expression and the differential effect of aspirin and sodium salicylate on iNOS expression support the above notion that aspirin and sodium salicylate have no significant effect on iNOS expression at the gene level, at least in murine macrophages activated with LPS *in vitro*. These experiments further reinforce the notion that the mechanism of action of aspirin (at pharmacological concentrations) in inhibiting iNOS expression is due to its interference in translational/posttranslational modification of the enzyme and/or inhibiting the catalytic activity of iNOS. However, direct experiments with an iNOS promoter and a reporter gene are needed to confirm this observation.

**Effect of NSAIDs on Catalytic Activity of iNOS in Cell-Free Extracts.** The potency of aspirin in inhibiting iNOS activity compared to the other NSAIDs may be attributable to the acetylation by aspirin of proteins such as COX-1 and COX-2 (25, 26). We therefore examined the effects of aspirin, sodium salicylate, and indomethacin in *in vitro* iNOS enzyme assays.

RAW 264.7 cells were incubated overnight with LPS to induce iNOS. Cell-free extracts were prepared from these cells and used as a source of iNOS. These enzyme extracts were preincubated with NSAIDs for 20 min before initiating the enzyme reactions as described. Aspirin at 0.1 and 1 mM inhibited conversion of L-[ $^3$ H]arginine to L-[ $^3$ H]citrulline in cell-free extracts by 10–12% and 45–68%, respectively (Figs. 2 and 3), whereas no significant differences (7%) were observed in extracts treated with 1 mM of sodium salicylate. Similarly, 5  $\mu$ M indomethacin or an equivalent volume of absolute alcohol had no effect (Fig. 2). These studies demonstrated that aspirin, but not sodium salicylate or indomethacin, directly interfered with the catalytic activity of iNOS, possibly by acetylating an important functional component of the enzyme or its cofactors. However, because sodium salicylate at therapeutic (2 mM) and suprapharmacological (5 and 20 mM) concentrations inhibits accumulation of nitrite (by 6%, 15%, and 50%, respectively) and has only minimal and insignificant effect on the expression of the iNOS protein, the possibility of sodium salicylate interfering with iNOS catalytic activity at suprapharmacological concentrations cannot be ruled out.

**Effect of NAI on Catalytic Activity of iNOS in Cell-Free Extracts.** Unlike aspirin, which acetylates Ser-530 of COX and inactivates the COX and not the peroxidase activity (26), NAI acetylates and inhibits both the COX and the peroxidase activity of COX (27). We therefore tested the effect of NAI on

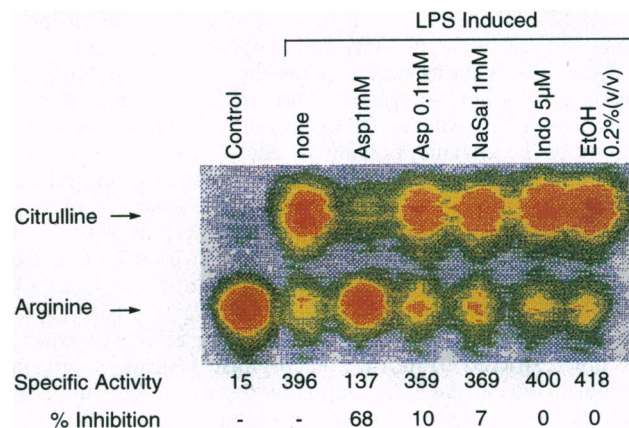


FIG. 2. Effect of NSAIDs on iNOS activity in cell-free extracts. RAW 264.7 cells were induced with LPS for 16 hr, and enzyme extracts were prepared to examine iNOS activity in cell-free extracts by monitoring conversion of L-[ $^3$ H]arginine to L-[ $^3$ H]citrulline by TLC, as described. NSAIDs were preincubated with enzyme extracts 20 min before starting the reaction, and formation of L-[ $^3$ H]citrulline was monitored for 30 min. An equivalent volume of alcohol used as a solvent for NSAIDs was also added as an additional control. Data represent the TLC analysis of one of two similar experiments. Percentage inhibition is determined after removing the control value (=15), which represents specific activity of iNOS in uninduced cells. The specific activity of LPS-treated cells after 16–18 hr was 396 pmol of [ $^3$ H]citrulline released/min per mg of protein at 37°C. Asp, aspirin; Indo, indomethacin; NaSal, sodium salicylate.

the catalytic activity of iNOS in cell-free extracts and compared it with equivalent amounts of aspirin in the same experiment. Fig. 3 shows the dose-dependent inhibition of iNOS by NAI and aspirin. In contrast to aspirin, which does not seem to inhibit the iNOS activity significantly (10–12%) at 0.1 mM (Figs. 2 and 3), NAI at similar concentrations inhibited  $\approx$ 45% of iNOS activity. However, at 1 mM, aspirin and NAI inhibited the catalytic activity of iNOS by 45% and 74%, respectively, whereas imidazole at similar concentrations had no significant effect (<5% inhibition). These experiments further indicate that acetylation of iNOS [and/or essential cofactor(s)] inactivates its catalytic activity and that the potency of NAI is relatively greater than that of aspirin. NAI, which is commonly used for acetylation of tyrosine hydroxyl

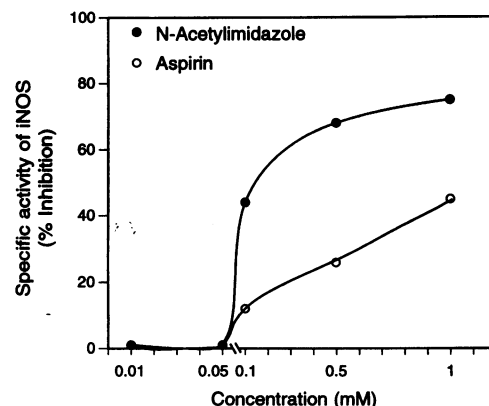


FIG. 3. Effect of acetylating agents aspirin and NAI on catalytic activity of iNOS in cell-free extracts. The enzyme reactions were set up as described for Fig. 3. Acetylating compounds were incubated with the enzyme extracts 20 min before starting the reaction and assayed as described. The specific activity of LPS-treated cells after 16–18 hr was 178 pmol of [ $^3$ H]citrulline released/min per mg of protein at 37°C. An equivalent volume of absolute alcohol, which was used as a carrier for the drugs, inhibited iNOS activity by  $\approx$ 10%.

groups (28–30), acetylates protein residues at rates proportional to their nucleophilicity and accessibility (27, 28).

These observations may explain the different potency of aspirin and sodium salicylate. Aspirin inhibits iNOS by effects on both synthesis of the iNOS protein and on the catalytic activity of the enzyme, possibly by acetylation of the enzyme and/or an essential cofactor, whereas indomethacin and sodium salicylate (which weakly inhibits iNOS protein expression) have no significant effect on the catalytic activity of iNOS. However, the possibility that aspirin interferes in the biosynthesis of other crucial cofactors cannot be excluded (3, 4).

In separate studies, we observed that the effects of aspirin are not restricted to murine macrophages. Slices of human osteoarthritic cartilage, in contrast with normal human cartilage, showed up-regulated NOS and accumulated  $>70 \mu\text{M}$  of nitrite in the medium, enough to damage tissue (31). Addition of 2 mM aspirin suppressed nitrite accumulation by at least 50% in *ex vivo* experiments (unpublished data), thus indicating that human chondrocyte NOS (which appears to be distinct from murine iNOS) is sensitive to aspirin and may also be sensitive to other NSAIDs.

It is clear from these experiments that aspirin does not inhibit iNOS expression completely at the therapeutic concentrations selected. However, partial suppression of iNOS may be sufficient to inhibit an inflammatory response. This assumption is supported by studies in animal models, where partial inhibition of NOS by NOS inhibitors in rats with induced adjuvant arthritis was sufficient to reduce paw swelling (indicating reduction in inflammation) without significantly affecting the elevated excretion of nitrite in the urine (32). Thus, small reductions in NO levels may profoundly affect the process of inflammation, and aspirin-like agents that incompletely inhibit iNOS expression at therapeutic concentrations may still be good candidates for pharmaceutical intervention to modulate iNOS. These data also show that at equivalent therapeutic levels, salicylates and indomethacin have divergent effects on iNOS. Such observations are consistent with previous studies that have shown important differences among NSAIDs with regard to their capacities to inhibit neutrophil function (11), COX-2 activity (17), NF- $\kappa$ B activation (24), and neurogenic inflammation (15).

In summary, we conclude that the inhibition of iNOS expression/function represents another mechanism of action for aspirin-like drugs and may explain individual differences in response to NSAIDs in patients with inflammatory diseases. In addition, a search for agents that can acetylate iNOS or its cofactors may be an important pharmacological strategy for developing newer aspirin-like drugs.

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