Effects of Aspirin-Like Drugs on Nitric Oxide Synthesis in Rat Vascular Smooth Muscle Cells

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Abstract—The purpose of this study was to investigate the effects of aspirin-like drugs on nitric oxide (NO) synthesis in rat vascular smooth muscle cells (VSMCs). We measured the accumulation of nitrite, a stable oxidation product of NO, and the expression of inducible NO synthase (iNOS) mRNA and protein in rat cultured VSMCs. Sodium salicylate, aspirin, and indomethacin dose-dependently enhanced nitrite production by interleukin (IL)-1β-stimulated VSMCs at therapeutic plasma concentration ranges. Increased nitrite production by aspirin-like drugs was accompanied by increased iNOS mRNA and protein accumulation in VSMCs. Addition of IL-1β activated nuclear factor κB (NF-κB) in VSMCs, but sodium salicylate did not affect IL-1β-induced NF-κB activation. The nonselective lipoxygenase (LO) inhibitor nordihydroguaiaretic acid inhibited sodium salicylate–induced nitrite production, whereas the selective 5-LO inhibitor caffeic acid did not influence production of nitrite. The 12-LO product 12-HETE dose-dependently enhanced nitrite production by IL-1β-stimulated VSMCs, whereas the 15-LO product 15-HETE did not. Our study demonstrates that aspirin and the aspirin-like drugs, sodium salicylate and indomethacin, increase NO synthesis in IL-1β-stimulated VSMCs by upregulation of iNOS transcription via a 12-LO pathway. These effects were independent of NF-κB activation. In addition to the direct inhibition of platelet function, aspirin-like drugs may contribute to the reduction of atherothrombotic risk in myocardial ischemia via enhancing NO production by VSMCs. (Hypertension. 2000;35:1085-1091.)

Key Words: nitric oxide ▪ aspirin ▪ atherosclerosis ▪ lipoxygenase ▪ cyclooxygenase

Aspirin-like drugs, so-called NSAIDs, are commonly used to treat various inflammatory diseases. Their mechanism of action is inhibition of cyclooxygenase (COX). In cardiovascular disease, aspirin therapy is one of the most popular pharmacological strategies for the prevention and treatment of ischemic heart disease. Low doses of aspirin decrease in vivo platelet aggregation by selectively inhibiting thromboxane A2 production by platelets while maintaining prostacyclin production by endothelium. This inhibition of platelet activation has been the focus of several pharmacological strategies for the prevention and treatment of myocardial ischemia; however, the antithrombotic effects of aspirin cannot be explained by its effect on thromboxane A2 production alone.

Nitric oxide (NO), an extensively characterized endothelium-derived relaxing factor, is a short-lived free radical. It is synthesized from the amino acid L-arginine in a reaction catalyzed by 2 enzymes, Ca2+- and calmodulin-dependent constitutive NO synthase (constitutive NOS) and Ca2+- and calmodulin-independent inducible NOS (iNOS).1,2 Two isoforms of constitutive NOS have been identified in endothelial cells (endothelial, or type III, NOS) and in brain (neuronal, or type I, NOS). iNOS has been identified in endotoxin- and cytokine-treated neutrophils, hepatocytes, endothelial cells, and myocardium.1,3 Its activity is also induced in aortic rings and cultured vascular smooth muscle cells (VSMCs) by cytokines and endotoxin.4 NO plays important roles in the regulation of blood flow and vascular homeostasis; it inhibits vascular contraction, platelet aggregation, proliferation of VSMCs,5 and leukocyte adhesion to endothelial cells.6 Therefore, iNOS induction in VSMCs is believed to suppress the vascular injury associated with atherosclerosis.

Previously, it has been reported that aspirin and aspirin-like drugs, such as sodium salicylate, inhibit NO synthesis in macrophages7,8 and cardiac fibroblasts.9 However, there have been no reports concerning the effects of these drugs on the synthesis of NO, an antithrombotic factor, in VSMCs. In the present study, we investigated the effects of aspirin, sodium salicylate, and indomethacin on NO synthesis in rat cultured VSMCs.

Materials

Acetylsalicylic acid and sodium salicylate were purchased from Wako Pure Chemical Industries Ltd. Indomethacin was purchased from Sigma Chemical Co. Human recombinant interleukin (IL)-1β...
(specific activity ~2×10^6 U/mg) was a gift from Otsuka Pharmaceutical Co, Ltd (Tokushima, Japan). Nordihydroguaiaretic acid (NDGA) and caffeic acid were purchased from Research Biochemicals Inc. 12-HETE and 15-HETE were obtained from Cayman Chemical Co. A monoclonal anti-mouse iNOS antibody, which cross-reacts against rat iNOS, was obtained from Transduction Laboratories. A goat polyclonal anti-nuclear factor-κB (NF-κB) p65 antibody was purchased from Santa Cruz Biotechnology Inc. All other chemicals used were of the highest grade commercially available.

**Cell Cultures**

Primary cultures of VSMCs were obtained from the thoracic aortas of Sprague-Dawley rats (200 to 250 g), as described previously.10 Cells (3×10^5/mL) passaged 5 to 10 times were plated in 24-well or 100-mm culture dishes in 10% FBS containing DMEM (GIBCO Laboratories) and allowed to grow to subconfluence for 24 to 48 hours, after which they were preincubated in DMEM containing 0.5% FBS for 24 hours and used for the experiments described below.

**Measurement of Nitrite**

NO production by the cultured cells was determined by measuring the nitrite content of the culture medium, as reported previously.11

**Assay for iNOS Protein**

The expression of iNOS protein was analyzed by immunoblotting with an anti-iNOS antibody, as previously described.3

**Assay for iNOS mRNA**

The expression of iNOS mRNA was analyzed by quantitative reverse transcription (RT)–polymerase chain reaction (PCR). Total RNA was extracted from VSMCs plated in 100-mm culture dishes by use of the guanidinium isothiocyanate–phenol–chloroform method. RT-PCR was performed under the conditions recommended in the RNA PCR kit (AMV, version 2.1, Takara Shuzo Co). The synthesis of first-strand cDNA was performed with the use of oligo(dT) primers and avian myeloblastosis virus reverse transcriptase. The PCR amplifications were performed with the use of rat iNOS–specific primers (sense 5′-TCGAGCCTGGAAGACCCATCTG-3′, antisense 5′-GGTTGTTCCTCTTCCAAGGTGTTTGCCTTAT-3′) and GAPDH primers (sense 5′-TATTGGGCCGCTGTTGACCA-3′, antisense 5′-CCACCTCTTGATGTCATCA-3′). GAPDH mRNA levels served as an internal standard for normalization of iNOS mRNA levels. RT-PCR conditions were optimized to ensure that the procedure was performed in the linear portion of the reaction. The PCR products (10 μL) were electrophoresed on ethidium bromide–stained 1.8% agarose gels and visualized and digitally photographed by use of a Luminescent Image Analyzer (LAS-1000, Fuji Photo Film Co, Ltd) and quantified by use of Image Gauge (version 3.0, Fuji Photo Film).

**Gel Retardation Assays**

The levels of NF-κB proteins in nuclear extracts from VSMCs were analyzed by gel retardation assays. Nuclear extract preparation and gel retardation experiments were performed as described previously.12 A chemically synthesized oligonucleotide that contained a recognition site for NF-κB (5′-TCAACAGAGGGGGACTTTCCAGGCGCA-3′) was annealed with its complementary sequence oligonucleotide.13 The resultant oligonucleotide was 5′-labeled with γ-[^32]P]ATP and polynucleotide kinase and used as a probe. For 15 μL DNA-binding reaction mixture, 5 to 8 fmol probe was incubated with nuclear extract (6 μg protein) and 1 μg poly[d(I-C)]·d[I-C)] at room temperature for 30 minutes in 60 mM/L KC, 20 mM/L HEPES (pH 8.4), and 4% Ficoll. The DNA-bound protein complexes in the reaction mixtures were resolved by electrophoresis on a 4% polyacrylamide gel in 0.25× Tris/borate/EDTA buffer. NF-κB consensus oligonucleotides (5′-AGTTAGGGAGGACTTTCCAGGCG-3′, Santa Cruz Biotechnology Inc) or mutated oligonucleotides (5′-AGTTGAGGGCAGTCTCCAGGCG-3′, Santa Cruz Biotechnology Inc) were used as competitors. An anti–NF-κB p65 antibody (goat polyclonal IgG, 200 μg/0.1 mL) was used for supershift assays.

**Statistical Analysis**

Data are expressed as mean±SEM of 4 samples, which represented at least 3 separate experiments. Differences were analyzed by 1-way ANOVA combined with the Scheffé test, and values of P<0.05 were considered to be statistically significant.

**Results**

**Effects of Aspirin-Like Drugs on Nitrite Production by VSMCs**

We initially investigated the effects of IL-1β on nitrite production by VSMCs. Unstimulated VSMCs produced only low levels of nitrite, but the addition of IL-1β (10 ng/mL) stimulated nitrite production by VSMCs in a time-dependent manner, as reported previously.11 We then investigated the concentration-response effect of aspirin-like drugs on nitrite production by VSMCs stimulated with IL-1β. As shown in Figure 1A, the addition of sodium salicylate enhanced nitrite production by IL-1β-stimulated VSMCs in a concentration-dependent manner, whereas sodium salicylate itself did not affect the basal levels of nitrite production. Aspirin and indomethacin also dose-dependently increased nitrite production by IL-1β-stimulated VSMCs (Figure 1B and 1C).

**Effects of Aspirin-Like Drugs on iNOS Protein and mRNA Expression**

We then investigated whether aspirin-like drugs enhanced IL-1β–induced nitrite production at the iNOS protein level. Figure 2 shows the result of immunoblot analysis. Unstimulated VSMCs showed no detectable iNOS protein expression. After 24 hours of incubation with IL-1β, the expression of iNOS protein was upregulated, and its expression was further enhanced in the presence of sodium salicylate in a concentration-dependent manner.

We further examined whether aspirin-like drugs enhanced IL-1β–induced nitrite production at a transcriptional level. Unstimulated VSMCs did not show any detectable iNOS mRNA expression. Incubation with IL-1β for 24 hours induced the expression of iNOS mRNA, and its expression was further enhanced in the presence of sodium salicylate, aspirin, and indomethacin (Figure 3A). To determine iNOS mRNA turnover without grossly affecting posttranscriptional steps leading to mRNA degradation, we performed an RNA polymerase inhibitor actinomycin D chase.14 As shown in Figure 3B, the half-life of iNOS mRNA induced by IL-1β was ~6 hours. Sodium salicylate did not affect the stability of iNOS mRNA.

**Involvement of NF-κB Activation**

It was reported that the transcription factor NF-κB is critical for the transcriptional regulation of iNOS.15 Recently, salicylate and aspirin have been shown to regulate the activation of NF-κB in several cell types.16 Thus, we examined whether aspirin-like drugs activate NF-κB in VSMCs. Figure 4A shows the results of a gel retardation assay. Addition of IL-1β induced specific retardation complexes (lane 2 compared with lane 1). These complexes competed with nonradioabeled NF-κB consensus oligonucleotides but not with mu-
tated NF-κB oligonucleotides (lanes 4 and 5). Supershift experiments with anti–NF-κB p65 antibody confirmed that these complexes contained NF-κB (lane 7). Sodium salicylate did not affect IL-1β–induced activation of NF-κB (lane 3).

Figure 4B shows the time course of NF-κB activation. Maximal NF-κB activation occurred within 30 minutes and was then reduced, but it persisted >24 hours. Addition of sodium salicylate did not affect NF-κB activity at each time point.

Involvement of Lipoxygenase Pathway

It has been reported that aspirin-like drugs activate the lipoxygenase (LO) pathway, another pathway of arachidonic acid metabolism, in a number of cell types.17 We next examined whether the LO pathway is involved in nitrite production by aspirin-like drugs (Figure 5). The nonselective LO inhibitor NDGA inhibited sodium salicylate–induced nitrite production by VSMCs, whereas the selective 5-LO inhibitor caffeic acid did not affect nitrite production, suggesting that the 12-LO and/or the 15-LO pathway, but not the 5-LO pathway, may be involved.

We then further examined the effect of 12- and 15-LO products. The 12-LO product 12-HETE dose-dependently enhanced nitrite production by IL-1β–stimulated VSMCs, whereas the 15-LO product 15-HETE did not influence nitrite production (Figure 6).

Discussion

The present study was designed to examine whether aspirin-like drugs affected NO synthesis in VSMCs. We have selected 3 kinds of drugs: an acetylated salicylate (aspirin), a nonacetylated salicylate (sodium salicylate), and a nonacetylated nonsteroidal compound (indomethacin). Although these aspirin-like drugs by themselves had no effect on nitrite accumulation, all of them significantly enhanced IL-1β–induced NO production at therapeutic plasma concentrations (1 to 3 mmol/L aspirin, 1 to 3 mmol/L sodium salicylate, and 5 to 20 μmol/L indomethacin). These effects occur after a lag period of several hours (data not shown), suggesting that the upregulation of NO production is due to enhanced induction of iNOS. Indeed, these drugs increased IL-1β–induced iNOS mRNA at a transcriptional level.

The transcription of NF-κB is critical for the transcriptional regulation of iNOS. It has been shown that iNOS
induction depends on the unique NF-kB sequence containing nucleotides 285 to 276 of the murine iNOS promoter and the binding to this region of a cycloheximide-sensitive complex containing both p50/c-Rel and p50/RelA heterodimers of NF-kB, in partnership with additional unidentified nuclear protein(s). Additionally, 2 NF-kB consensus sequences have been demonstrated in the murine iNOS promoter. The cytokines IL-1 and tumor necrosis factor have signal transduction pathways that culminate in the activation of NF-kB. Recently, salicylate and aspirin have been shown to modulate the activation of NF-kB in several cell types. This suggests that the induction of iNOS by aspirin-like drugs may be mediated via NF-kB. Thus, we investigated whether aspirin-like drugs regulate NF-kB and found that IL-1β markedly stimulated NF-kB activity, whereas sodium salicylate did not modulate NF-kB activity in IL-1β–stimulated VSMCs. Similarly, Farivar and Brecher reported that salicylate modulates iNOS mRNA expression in cytokine-stimulated cardiac fibroblasts; however, its effect was independent of NF-kB activation. To date, several different transcription factors other than NF-kB have been implicated in the regulation of iNOS by inflammatory mediators, including signal transducer(s) and activator(s) of transcription-1, interferon regulatory factor-1, CAAT box/enhancer binding protein, and cAMP-responsive element binding protein. We have not determined any transcription factor other than NF-kB in the present study, and further studies are needed to isolate the target molecule involved in their action of aspirin-like drugs.

VSMC activation occurs early in the development of lesions in arteriosclerosis and hypertension or after balloon angioplasty. IL-1 is 1 of the cytokines that mediate the response to injury. In addition to the induction of iNOS, IL-1 stimulates the expression of a number of other gene products, including the inducible COX isoform COX-2. This isoform is considered to be involved in the overproduction of prostanoids under pathological conditions. In VSMCs, it has been shown that prostaglandins E2 and I2 enhance iNOS expression and NO production, whereas prostaglandin D2 inhibits iNOS expression. The effects of prostanoids on iNOS expression are variable, and the involvement of prostaglandins in the effects of aspirin-like drugs has not been fully determined. IL-1 also stimulates the expression of the type II secretory form of phospholipase A2. Stimulation of this form of phospholipase A2 activity and/or expression can result in enhanced generation of arachidonic acid, which is an important substrate for COX isoforms LO (which is involved in the production of HETEs and leukotrienes) and p450 monooxygenases. LaPointe and Sitkins demonstrated that arachidonate metabolites, possibly LO products, were involved in IL-1–mediated expression of iNOS in cardiac myocytes. Furthermore, it has been reported that aspirin-like drugs enhance leukotriene synthesis in a number of inflammatory cell types, including neutrophils and macrophages. There-

Figure 3. Effects of aspirin-like drugs on iNOS mRNA accumulation. A, Quantitative RT-PCR for iNOS was performed. Cells were incubated for 24 hours with IL-1β (10 ng/mL) in the presence or absence of sodium salicylate (3 mmol/L), aspirin (3 mmol/L), and indomethacin (30 μmol/L). PCR products (10 μL) were run on 1.8% agarose gels stained with ethidium bromide. The sizes of PCR products for GAPDH and iNOS were 747 and 277 bp, respectively. Relative iNOS mRNA levels are represented as a ratio of iNOS mRNA levels after normalization with GAPDH mRNA levels. Values are mean±SEM for 3 different experiments. B, For actinomycin D chase, cells were treated with IL-1β and sodium salicylate for 24 hours and then incubated further with actinomycin D (5 μg/mL) for the indicated times. Graph shows the stability of iNOS mRNA. The intensities of iNOS mRNA bands corrected with GAPDH mRNA bands were plotted as a percentage of the zero-hour value against time. Solid line represents IL-1β; broken line, IL-1β and sodium salicylate. Values are mean±SEM for 3 different experiments.
fore, we hypothesize that an activated LO pathway, either directly or via COX inhibition, is involved in increased NO production caused by aspirin-like drugs. It has been reported that rat aortic smooth muscle cells have 5-, 12-, and 15-LO. In the present study, the nonselective LO inhibitor NDGA at 10 μmol/L (EC50 was 0.2 μmol/L for 5-LO and 30 μmol/L for 12- and 15-LO) inhibited sodium salicylate–induced NO production. However, the selective 5-LO inhibitor caffeic acid at 10 μmol/L (EC50 4 μmol/L) did not affect nitrite production by VSMCs. Furthermore, 12-HETE enhanced nitrite production by IL-1β-stimulated VSMCs, whereas 15-HETE did not influence the nitrite production. These results suggest that the LO pathway, possibly 12-LO, is involved in increased NO production caused by aspirin-like drugs, although the precise mechanism is unknown. On the other hand, the cytochrome P-450 inhibitor SKF-525A did not affect sodium salicylate– and IL-1β–induced NO production (data not shown).

The efficacy of aspirin-like drugs on NO production has been reported in several cell types. Amin et al7 showed that aspirin but not sodium salicylate or indomethacin inhibited iNOS expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Kepka-Lenhart et al8 reported that aspirin and sodium salicylate inhibited iNOS mRNA induction in LPS-stimulated RAW264.7 cells but enhanced iNOS mRNA induction in interferon-γ–stimulated RAW264.7 cells. Farivar et al9 revealed that sodium salicylate and aspirin inhibited the induction of iNOS in rat cardiac fibroblasts stimulated with interferon-γ and IL-1β. In the present study, we examined the effects of aspirin-like drugs in VSMCs and in RAW264.7 cells. Aspirin, sodium salicylate, and indomethacin all enhanced NO production by IL-1β–stimulated VSMCs. On the other hand, these drugs significantly inhibited NO production in LPS-stimulated RAW264.7 cells (data not shown). We also observed that these drugs enhanced NO production by LPS-stimulated VSMCs (data not shown).

Several lines of evidence from in vitro and in vivo studies have suggested the role of NO as an antithrombotic factor. NO inhibits aggregation of platelets and restores blood flow in the balloon-injured artery.26 NO also inhibits the proliferation of VSMCs,5 the production of cytokines by endothelial cells,26 and leukocyte adhesion to endothelial cells.6 Joly et al27 demonstrated that in vivo balloon injury induced NOS activity in rat carotid arteries, even in the absence of endothelium. Hansson et al28 reported that arterial smooth muscle cells in the neointima that formed after a deendothelializing balloon injury to the rat carotid

Figure 4. Analysis of the NF-κB activation by gel retardation assays. VSMCs were pretreated with sodium salicylate for 1 hour and treated with IL-1β for 24 hours. A, 32P-labeled NF-κB consensus oligonucleotides were incubated with nuclear extract from nonstimulated (lane 1), IL-1β (10 ng/mL)–stimulated (lanes 2 and 4 to 7), and IL-1β and sodium salicylate (3 mmol/L)–costimulated VSMCs (lane 3). An anti–NF-κB antibody was used for supershift (lane 7). Gel retardation complexes of NF-κB are indicated by solid arrows. A supershift complex is indicated by open arrow. Competitors are as follows: K, NF-κB consensus oligonucleotides; M, mutated NF-κB oligonucleotides. B. Time course of NF-κB activation in the presence (+) or absence (−) of sodium salicylate is shown. VSMCs were pretreated with sodium salicylate for 1 hour and further treated with IL-1β for 0.5 to 24 hours. Two independent experiments yielded identical results.

Figure 5. Concentration dependence of the effects of LO inhibitors on nitrite production by VSMCs. VSMCs stimulated with IL-1β (10 ng/mL) were incubated for 24 hours with or without sodium salicylate (3 mmol/L) in the presence of various concentrations of NDGA (○) or caffeic acid (●). Nitrite accumulation in culture medium was measured. Values represent the percent inhibition of sodium salicylate–mediated effects on nitrite production by LO inhibitors. Values are mean±SEM (n=4).
artery expressed iNOS. Recently, Buttery et al.\(^\text{29}\) also reported that iNOS mRNA and protein were present within human arteriosclerotic lesions. NO production by VSMCs may in part compensate for the absence of endothelial NO synthesis by inhibiting VSMC proliferation and by limiting thrombus formation by preventing platelet adhesion and aggregation.\(^\text{26}\) This hypothesis is supported by the observation that in animals l-arginine attenuates neointimal formation after balloon injury\(^\text{30}\) and that iNOS suppresses the development of allograft arteriosclerosis.\(^\text{31}\)

In the present study, we demonstrated that aspirin and the aspirin-like drugs sodium salicylate and indomethacin enhance NO production by IL-1β-stimulated VSMCs. The enhancement of iNOS transcription was independent of NF-κB activation. This effect was possibly via activation of the 12-LO pathway. In addition to the direct inhibition of platelet function, aspirin and aspirin-like drugs possibly contribute to the reduction of atherothrombotic risk in myocardial ischemia via enhanced NO production by VSMCs.

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