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SB 203580 Inhibits p38 Mitogen-Activated Protein Kinase, Nitric Oxide Production, and Inducible Nitric Oxide Synthase in Bovine Cartilage-Derived Chondrocytes

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Nitric oxide (NO) is implicated in a number of inflammatory processes and is an important mediator in animal models of rheumatoid arthritis and in vitro models of cartilage degradation. The pyridinyl imidazole SB 203580 inhibits p38 mitogen-activated protein (MAP) kinase in vitro, blocks proinflammatory cytokine production in vitro and in vivo, and is effective in animal models of arthritis. The purpose of this study was to determine whether SB 203580 could inhibit p38 MAP kinase activity, NO production, and inducible NO synthase (iNOS) in IL-1 stimulated bovine articular cartilage/chondrocyte cultures. The results indicated that SB 203580 inhibited both IL-1 stimulated p38 MAP kinase activity in isolated chondrocytes and NO production in bovine chondrocytes and cartilage explants with an IC$_{50}$ value of approximately 1 µM. To inhibit NO production, SB 203580 had to be present in cartilage explant cultures during the first 8 h of IL-1 stimulation, and activity was lost when it was added 24 h following IL-1. SB 203580 did not inhibit iNOS activity, as measured by the conversion of arginine to citrulline, when added directly to cultures where the enzyme had already been induced, but had to be present during the induction period. Using a 372-bp probe for bovine iNOS we demonstrated inhibition of IL-1-induced mRNA by SB 203580 at both 4 and 24 h following IL-1 treatment. The iNOS mRNA levels were consistent with NO levels in 24-h cell culture supernatants of the IL-1-stimulated bovine chondrocytes used to obtain the RNA.


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2 Abbreviations used in this paper: RA, rheumatoid arthritis; OA, osteoarthritis; NO, nitric oxide; iNOS, inducible nitric oxide synthase; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; CSBP, CSAID binding protein; RK, reactivating kinase; GST, glutathione-S-transferase; ATF2, activating transcription factor 2; hsp, heat shock protein.

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at SmithKline Beecham. DMEM and Ham’s F-12 medium were obtained from Life Technologies (Grand Island, NY). FBS was obtained from HyClone (Logan, UT). The media were supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L-glutamine (Life Technologies). Dulbecco’s PBS was obtained from Life Technologies and contained 2% antibiotics. 1-ascorbic acid, N6-mono-methyl-L-arginine (L-NMA), pronase E from Streptomyces griseus, and hyaluronidase type V were obtained from Sigma (St. Louis, MO); collagene D from Clostridium histolyticum was purchased from Boehringer Mannheim (Indianapolis, IN). [3H]Arginine was obtained from Amersham (Arlington Heights, IL). SeaPlaque agarose was obtained from FMC Bio-products (Rockland, ME). Other standard buffer components and chemicals, unless specifically indicated otherwise, were obtained from Sigma.

**Cartilage and chondrocyte cultures**

Bovine chondrocytes were established in 10-cm tissue culture petri dishes (Costar, Cambridge, MA) at a density of 2 x 10⁶ cells/ml in Ham’s F-12 containing 10% FCS, 50 μg/ml ascorbic acid, and antibiotic/antimycotic. Cells were allowed to adhere and were washed twice in DMEM with 10% FCS. This medium was changed 48 to 72 h later to DMEM with 0.5% FCS (10 ml, 1 x 10⁶ cells/ml). Cells were washed twice in medium and then incubated in fresh medium for a further 20 min. During this period a second set of wells that had been treated with IL-1 alone was treated with similar doses of SB 203580 to determine the compound’s effect on the already induced enzyme. [3H]Arginine (3 μCi/ml) was added to the cultures for 20 min, and incorporation of labeled arginine was terminated by rapid aspiration of extracellular medium and washing twice with ice-cold PBS. Cells were collected and the following steps were performed as described previously (28, 29). The columns were washed three times with 1 ml of water, and the radioactive material in the flow-through fractions (i.e., that containing almost exclusively [3H]citrulline) was quantified by scintillation spectroscopy. All measurements were made in duplicate, and the data are presented as the recovery of radioactivity compared to untreated controls.

**Isolation of chondrocyte RNA and Northern blot analysis**

Total RNA was isolated from chondrocytes treated with IL-1 and SB 203580 by a modified guanidine isothiocyanate extraction using Tri-Reagent (MRC, Cincinnati, OH) according to the manufacturer’s instructions. RNA concentration and purity were determined spectrophotometrically. All RNA samples had an A260/A280 ratio > 1.8. Approximately 10 μg of the RNA samples were electrophoresed in 1% agarose gels containing 2.5% formaldehyde, 20 mM 3-[(N-morpholino)propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0. After electrophoresis, the RNA was transferred to positively charged nylon membranes (Bio-Rad, Hercules, CA). Membranes were prehybridized for 1 h at 68°C with

**FIGURE 1.** SB 203580: 4-(4-fluorophenyl)-2-(4-methylsulfanyl-phenyl)-5-(4-pyridyl)imidazole.
A, IL-1 activation of CSBP/p38 and MAPKAP kinase-2 by IL-1. Chondrocytes (1 × 10^7 cells in 10 ml of medium) were plated in 100-mm plates and allowed to adhere for 24 h. Cells were treated with 20 ng/ml of IL-1 for different time periods as indicated. At the end of the incubation, cells were harvested, p38 or MAPKAP kinase-2 was immuno-precipitated, and the immune complex kinase assay was performed as described in Materials and Methods. The upper and lower panels show the time course of activation of p38 and MAPKAP kinase-2, respectively. Lane 1, Control untreated; lanes 2 through 5, IL-1 treated at 5, 15, 30, and 60 min. B, SB 203580 inhibits CSBP/p38 and MAPKAP kinase-2 activity. Upper panel, Chondrocytes were treated with IL-1 for 5 min and harvested. CSBP was immunoprecipitated from the cell lysates, and a kinase assay was performed in the absence (lane 1) or the presence (lanes 2–5) of SB 203580. Lower panel, Chondrocytes were pretreated with the indicated concentrations of SB 203580 and then exposed to IL-1 for 5 min. Cells were harvested, and MAPKAP kinase-2 was assayed with hsp27 as a substrate as described in Materials and Methods.

ExpressHyb (Clontech, Palo Alto, CA). Hybridization was performed under identical conditions as prehybridization with addition of 32P-labeled (Amersham) specific probe for bovine iNOS. The 372-bp probe was provided by Dr. T. Jungi (35) (University of Berne, Berne, Switzerland) and corresponds to nucleotides 682 to 1053 of the human iNOS cDNA provided by Dr. T. Jungi (35) (University of Berne, Berne, Switzerland). Radiolabel on the blots was analyzed and integrated using phosphorimaging.

Statistical analysis

Comparisons between treated and control groups were determined by Student’s t test, with p < 0.05 considered significant. All experiments were performed at least three times, and the results shown are those from representative experiments.

Results

Activation of p38 MAP kinase activity by IL-1 and inhibition by SB 203580

IL-1 induced a rapid activation of p38 MAP kinase in the chondrocyte cultures (Fig. 2A). A fourfold activation of p38 was achieved by a 5-min treatment with IL-1. At 60 min postactivation, p38 activity was still increased threefold over the basal level. We also analyzed the activation of MAPKAP kinase-2, a physiologic substrate of p38, by IL-1 in the same cell lysate. MAPKAP kinase-2 was activated fivefold within 5 min after treatment with IL-1, but unlike P38, its activity level fell rapidly. At 60 min, MAPKAP kinase-2 activity was only twofold over the basal level (Fig. 2A). Immunoblots verified that the same amount of kinase was immunoprecipitated from different samples (data not shown).

To determine the inhibition of p38 MAP kinase activity by SB 203580, different concentrations of the compound were added directly to the p38 immune complex purified from IL-1-activated chondrocytes and were assayed for activity using GST-ATF2 as substrate. As shown in Figure 2B, SB 203580 inhibited the activity of p38 in a dose-dependent manner, with an IC50 value of about 0.1 μM. Complete inhibition occurred at 1 and 10 μM. While SB 203580 inhibits the activity of p38 MAP kinase, it has no effect on the activation of p38 by upstream kinase in response to IL-1 or any other stimuli (31). It has been shown that MAPKAP kinase-2 is a physiologic substrate of p38 in vivo (32). The activity of MAPKAP kinase-2 itself is not inhibited by SB 203580, but activation of the enzyme by p38 MAP kinase in cells is inhibited by SB 203580 (31). Therefore, to show that SB 203580 inhibits p38 MAP kinase in bovine chondrocytes, we pretreated the chondrocytes with SB 203580 at different concentrations before activation with IL-1 and then assayed for MAPKAP kinase-2 activity using hsp27 as substrate. As shown in Figure 2B, SB 203580 inhibited the activation of MAPKAP kinase-2 in a dose-dependent manner with an IC50 value of about 0.4 μM, suggesting that SB 203580 treatment results in the inhibition of p38 MAP kinase activity within the cell. Almost complete inhibition was achieved at 1 and 10 μM. Similar IC50 values have been reported in several earlier reports for other cell types (31–33).

NO production by bovine articular cartilage and chondrocytes

Bovine articular cartilage produces large amounts of NO following stimulation with IL-1 (34). In these studies cartilage explants were cultured for 48 h in DMEM with 10% FCS and then transferred to medium containing 0.5% FCS. Twenty-four hours later the cultures were stimulated with 20 ng/ml of IL-1, and SB 203580 was added at doses ranging from 0.15 to 10 μM. The explants were incubated for an additional 72 h, and secreted NO was determined in the supernatants. In the presence of IL-1 there was a two- to threefold increase in NO production compared with that in the untreated control cultures. This increase was inhibited by SB 203580 with an IC50 value that ranged from 0.6 to 1 μM, and maximal inhibition was observed at 5 to 10 μM. The pooled results of two experiments are shown in Figure 3. For comparison, in this culture system, IL-1-induced NO production was inhibited by L-NMMA at 500 μM (L-NMMA is a specific competitive inhibitor of all isoforms of NOS) and by SB 203580 (5 μM), but not by the metalloprotease inhibitor BB94 (Fig. 4). For SB 203580 to significantly inhibit IL-1 induced NO production in cartilage explants,
the compound had to be present early during the incubation period. Maximum inhibition was obtained when the compound was added during the first 4 h of culture with IL-1. The effect became weaker over the next 4 h and was lost by 24 h (Fig. 5).

To demonstrate that SB 203580 was equally active on isolated chondrocytes as in the cartilage explants, bovine chondrocytes were isolated from the cartilage by enzymatic digestion and established either as monolayer cultures or in agarose. In agarose the chondrocytes maintain their chondrocyte phenotype for several weeks. The effects of SB 203580 were examined on chondrocytes that had been incubated in agarose culture for 2 wk. At this time the chondrocytes have formed large aggregates that can be stimulated with IL-1 (20 ng/ml) to produce NO, which accumulates in the supernatant. IL-1 (20 ng/ml) and varying doses of SB 203580 were added on day 14 of culture, and the NO accumulated in the supernatant was determined 72 h later. There was a dose-related inhibitory effect with SB 203580, with an IC50 value of approximately 1 μM (Fig. 6).

Effect of SB 203580 on iNOS activity of chondrocytes

To show that the activity of SB 203580 was due to its ability to inhibit the synthesis of iNOS and not to a direct effect on NOS activity, we examined the ability of the compound to inhibit IL-1-induced conversion of radiolabeled arginine to citrulline during or after the iNOS synthetic phase. As shown in Figure 7, SB 203580 inhibited nitrite production when the compound was added to the cell cultures in combination with IL-1 (Fig. 7A), but not when it was added at the time of iNOS assay (Fig. 7B). No significant effects of SB 203580 on arginine uptake into cells were observed (data not shown). This would indicate that the compound has no direct effect on NOS enzyme activity directly, but has a dose-related effect on its induction.

Expression of iNOS RNA

To determine the level at which SB 203580 regulated iNOS, the effect on iNOS mRNA was examined using Northern analysis. Chondrocytes were established in monolayers in six-well tissue culture plates (72 h), treated with SB 203580, and stimulated with IL-1 (100 ng/ml) for 4 or 24 h, and then RNA was isolated as described in Materials and Methods. Following electrophoresis of the RNA and blotting onto nylon membranes, the blots were hybridized with the labeled iNOS probe and analyzed using the PhosphorImager. Figure 8A shows that there was a large increase in expression of iNOS mRNA following treatment with IL-1 and that this was inhibited in a dose-related manner by SB 203580. Similar results were obtained at both 4 and 24 h, with slightly more inhibition being obtained with SB 203580 at the 4 h point as measured by the PhosphorImager counts (Fig. 8B). Normalization of the PhosphorImager counts based on the 28S ribosomal RNA were within 10% of the data shown in Figure 8B. The inhibition of induced message was in agreement with the NO levels in the 24-h cell supernatants of the IL-1-stimulated bovine chondrocytes used to obtain the RNA (Fig. 8C).
Discussion

Pyridinyl imidazoles have been shown to inhibit the p38 kinase-mediated synthesis of cytokines such as IL-1 and TNF-α (IC_{50} = 0.1–1 μM) (17, 18). SB 203580 as well as other members in this series of compounds have shown efficacy in several animal models of inflammation where cytokines play a definitive role. Examples are collagen-induced arthritis in the DBA/LacJ mouse (20, 36); adjuvant-induced arthritis in the Lewis rat, in which protective effects on both bone and cartilage have been observed (20); and mouse models of endotoxin shock (37, 38). In addition, these compounds are active in the fetal rat long bone resorption assay, thus demonstrating a direct therapeutic effect on bone integrity (39).

The role of cytokines in these models is well documented, and inhibition of TNF-α and IL-1 by the CSAID compounds is well established. SB 203580 inhibits TNF-α synthesis at the translational rather than the transcriptional level (40, 41), and the target of this and other CSAID compounds is known as CSBP/p38 kinase (17). SB 203580 is a highly selective and potent inhibitor of p38 MAP kinase (32). One of the physiologic substrates of CSBP/p38 is MAPKAP kinase-2, which, in turn, phosphorylates hsp27. SB 203580 inhibits the activation of MAPKAP kinase-2 with an IC_{50} of about 0.4 μM, and subsequently the phosphorylation of hsp27 in stress-stimulated cells is inhibited by this compound (32). In this study we report for the first time that there is clearly an elevation in specific p38 kinase activity in bovine chondrocytes treated with IL-1 and that the CSAID inhibitor SB 203580 inhibits this activity and blocks the production of the proinflammatory enzyme iNOS.

One of the documented proinflammatory actions of IL-1 and TNF-α is the induction of NO from a variety of tissues and cell types, including bone, cartilage, and cartilage-derived chondrocytes (12, 34, 42). In fact, the induction of iNOS by IL-1 results in copious amounts of NO that can contribute to tissue regulation and damage, and the spontaneous production of NO has been observed in OA-affected chondrocytes (43). NO has also been shown to mediate the IL-1-induced inhibition of proteoglycan synthesis in rat articular cartilage in vitro (44). In recent studies, the knockout of iNOS has been shown to block both the IL-1-induced inhibition
of proteoglycan biosynthesis and the release of cartilage proteoglycan in the zymosan-induced arthritis model (W. van den Berg, unpublished observations). The role of NO in vivo is inflammatory disease has also been well documented, and inhibitors of iNOS have been shown to protect against both inflammation and cartilage matrix loss in a number of experimental models, including inflammatory arthritides (45–48). Together, these data suggest that direct inhibition of iNOS or inhibition of iNOS production could be therapeutically beneficial in diseases such as OA and RA, in which there is both significant cartilage proteoglycan loss as well as inflammatory components.

In addition to inducing iNOS and the production of NO in cartilage/chondrocytes, IL-1 has been shown to induce the activation of p38 kinase in both human (23) and rabbit (49) articular cartilage. In the in vivo studies described here we have investigated the effect of IL-1 on the p38 kinase in bovine articular chondrocytes and also its effects on NO production and iNOS expression. IL-1 activated CSBP/p38 kinase within 5 min of treatment, and the level of activation was maintained over a 60-min period. In contrast, activation of MAPKAP kinase-2 peaked 5 min following the addition of IL-1 and then rapidly decreased. SB 203580 inhibited the activity of p38 MAP kinase in vitro when added directly to the p38 kinase assay (Fig. 2B, upper panel). It also inhibited the activity of p38 in cells; this was shown by the inhibition of activation of MAPKAP kinase-2 by p38 when SB 203580 was added to the cell cultures (Fig. 2B, lower panel). The IC50 value of inhibition of p38 MAP kinase when the compound was either added directly to the kinase assay or to the cells was about 0.1 to 0.4 μM, similar to that described in previous reports (31–33).

To profile the effect of SB 203580 further in the bovine cartilage/chondrocyte assays, we examined its effects on NO production. The compound effectively inhibited IL-1-stimulated NO release into the culture medium with an IC50 value of approximately 0.6 μM (Fig. 3). SB 203580 appeared to inhibit only the inducible levels of NO (Figs. 3–5) (A. Badger, unpublished observations). To effectively inhibit NO production, SB 203580 needed to be added to the cultures within 4 h of IL-1. After this time the compound began to lose its inhibitory activity, indicating that its site of action is probably during the enzyme induction period (Fig. 5). The fact that IL-1 effects are still inhibited 2 h following induction may well be due to transport and availability in explant cultures.

However, in separate experiments, a significant amount of p38 remained active for up to 4 to 6 h after IL-1 treatment (data not shown). A similar profile of drug action was obtained in the case of HIV-1 LTR transcriptional activation, where the key role of p38 activity occurred some 2 to 4 h after cytokine and UV stimulation (33). Measurement of p38 kinase activity and its inhibition by SB 203580 could not be performed in cartilage explants due to the technical difficulty in quickly isolating the active p38 enzyme from this tissue. However, as chondrocytes are the only cell type residing in cartilage, we used cartilage-derived chondrocytes for these experiments. Modulation of p38 MAP kinase (Fig. 2) and the inhibitory effects of SB 203580 on IL-1-induced NO production were clearly demonstrated in cartilage-derived chondrocytes grown in agarose (Fig. 6) and monolayer culture (Fig. 8C). Experiments to determine whether SB 203580 affected iNOS activity in chondrocytes, measured by the conversion of arginine to citruline, resulted in the finding that inhibition occurred with an IC50 between 0.1 and 1 μM, and that the compound was inactive when added to the assay mixture, indicating inhibition of enzyme induction but not its activity (Fig. 7). We extended this observation to show that IL-1-induced expression of iNOS mRNA was inhibited in a dose-dependent manner by SB 203580 at both 4 and 24 h following activation with IL-1 (Fig. 8). Inhibition of iNOS mRNA at the 24 h point paralleled the inhibition of NO production in the supernatant of the cells used to prepare the RNA (Fig. 8). Since both p38 activity and iNOS expression are inhibited with comparable IC50 values (~0.5 μM), we hypothesize that p38 MAP kinase resides in the signal transduction pathway of IL-1-mediated NO inhibition. Activation of p38 MAP kinase appears to be an early event, whereas NO induction is a downstream late event mediated by IL-1.

Our results are consistent with those of a similar report in which the spontaneous activation of iNOS in chondrocytes was inhibited by SB 203580 (A. Amin, unpublished observations). In contrast to our findings described here, Guan et al. (50) found that an inhibitor of p38 MAP kinase, SC68376, enhanced NO biosynthesis in rat primary mesangial cells by increasing iNOS mRNA expression, protein expression, and nitrite production. A difference in the cell system (rat mesangial cells vs bovine articular chondrocytes) may explain the discrepancy between the two results. In addition, SC68376 is a relatively less well-characterized p38 inhibitor, with an IC50 value of 2 to 5 μM, compared with SB 203580, which has been extensively characterized and exhibits an IC50 value of at least 10-fold lower. It is possible that SC 68376 has other inhibitory activity and thus complicates the interpretation of the results.

Inhibition of the transcriptional regulation of iNOS and the subsequent production of NO by cartilage-derived chondrocytes by SB 203580 indicate that the CSAID compounds may be useful for therapy of disease states in which NO has been shown to play a proinflammatory role. In addition to the experimental models already discussed, additional evidence for the role of NO is provided by studies showing spontaneous production of this mediator by primary synovial cultures from both RA and OA patients, which may contribute to the pathology of these diseases (51). In addition, an increased expression of blood mononuclear cell iNOS has been described in RA patients (52). Thus, the CSAID compounds could be efficacious therapeutic agents by way of their ability to inhibit cytokines such as TNF and IL-1 and by inhibiting the downstream signal transduction pathways initiated by IL-1 and or TNF, including their ability to modulate iNOS expression. Given the central role that IL-1 plays in the pathology of RA and OA, an inhibitor of both IL-1 synthesis as well as IL-1 signal transduction and action would be of immense utility in the management of these debilitating diseases by controlling the inflammatory contribution as well as the effects on cartilage matrix biosynthesis and degradation.

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