Nonsteroidal Anti-inflammatory Drugs Suppress T-cell Activation by Inhibiting p38 MAPK Induction*

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In addition to antagonizing inflammation by inhibiting the activity of cyclooxygenases (COX), nonsteroidal anti-inflammatory drugs (NSAID) block T-cell activation. The immunosuppressant activity of NSAID correlates with their ability to block transcription factors required for the expression of inducible response genes triggered by T-cell antigen receptor (TCR) engagement. Whereas the inhibition of nuclear factor-κB by aspirin and sodium salicylate can be partly accounted for by their binding to IκB kinase-β, the broad range of transcriptional targets of NSAID suggests that the products of COX activity might affect one or more among the early steps in the TCR-signaling cascade. Here we show that the inhibition of NF-AT activation by NSAID correlates with a selective inhibition of p38 MAP kinase induction. The suppression of TCR-dependent p38 activation by NSAID can be fully overcome by prostaglandin E₂, underlining the requirement for COX activity in p38 activation. Furthermore, the inhibition of COX-1 results in defective induction of the COX-2 gene, which behaves as an early TCR responsive gene. The data identify COX-1 and COX-2 as integral and sequential components of TCR signaling to p38 and contribute to elucidate the molecular basis of immunosuppression by NSAID.

Nonsteroidal anti-inflammatory drugs (NSAID)¹ block the activity of cyclooxygenases (COX), which catalyze the first step in the biosynthesis of prostaglandins (PG) and related eicosanoids. Two COX isomers have been identified in eucaryotic cells. Although closely related by their enzymatic activity, COX-1 and COX-2 are implicated in different facets of the interaction of cells with their internal and external environments. COX-1 is constitutively expressed in most cells and is thought to play an important role in tissue homeostasis. In contrast, COX-2 is inducibly expressed in a more limited array of cell types in response to proinflammatory and mitogenic stimuli and is considered as the crucial mediator of inflammation (1). In agreement with the dominant role of COX-2 in inflammation, COX-2-selective NSAID have anti-inflammatory activity but limited unwanted side effects such as gastric damage and hemorrhage, typical of nonselective NSAID, which are likely to be accounted for by the inhibition of COX-1 (2). NSAID harbor additional immunomodulatory properties that have been correlated with their ability to block the activation of transcription factors implicated in the expression of proinflammatory cytokines and are likely to result, at least in part, from their well characterized activity on COX as exemplified by the inhibition of NF-κB by cyclopentenone PG through covalent modification of IκB kinase β (3). However, additional COX-independent targets of NSAID are emerging, including IκB kinase itself (4) and the nuclear receptor peroxisome proliferator-activated receptor δ (5).

Although the principal cellular targets of NSAID are inflammatory cells such as macrophages and neutrophils, NSAID suppress T-cell proliferation, expression of surface activation markers such as CD25 and CD71, and the production of cytokines such as interleukin-2, interferon-γ, and tumor necrosis factor-α (6–9). The immunosuppressant activity of NSAID correlates with their ability to block transcription factors required for the expression of inducible response genes triggered by the T-cell antigen receptor (TCR) following encounter with antigens, including NF-κB, NF-AT, and activated protein 1 (5, 6, 9, 10). The broad range of transcriptional targets of NSAID suggests that these drugs not only inhibit the distal components of individual transcription factor activation pathways but might also affect one or more steps in the early TCR-signaling cascade leading to transcription factor activation, potentially implicating COX in this process. In support for a potential role of COX in T-cell activation, COX-1 is constitutively expressed in T-cells, whereas the gene encoding COX-2 is inducibly activated as an early response gene in response to T-cell-activating stimuli (9).

Here we have assessed the effect of NSAID on the TCR-signaling cascade. Using as a read-out NF-AT activation, we show that NSAID affect the MAP kinase cascade and specifically the activation of p38 MAP kinase, an effect that can be reversed by PGE₂ and is therefore dependent on COX activity. Furthermore, the inhibition of COX-1 results in defective induction of COX-2 expression in response to TCR engagement.
highlighting a sequential role of COX-1 and COX-2 in TCR signaling to p38.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Cell lines included a stably transfected Jurkat T-cell line expressing luciferase under the control of a trimer of the distal NF-AT binding site on the human interleukin-2 gene promoter (11), the monocyte line U937, and the Raji B-leukemia cell line. A stably transfected Jurkat T-cell line expressing luciferase under the control of a trimer of a NF-κB binding site was generated as described previously (11). Peripheral blood mononuclear cells were isolated from whole blood by density centrifugation on Ficoll-Paque (Amersham Biosciences, Inc.) and subsequently depleted of macrophages by adherence. NSAID, SB203580, PD098059, and PGE2 were purchased from Calbiochem and Sigma. Phosphospecific antibodies recognizing the phosphorylated active forms of p38 and Erk/Erk2 were from Cell Signaling Technology (Beverly, MA). Anti-p38 and anti-Erk2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-actin mAb was from Amersham Pharmacia, and anti-COX-2 mAb was from Transduction Laboratories (Lexington, UK). IgG antibodies from OKT3 hybridoma supernatants were purified on Mabtrap (Amersham Biosciences, Inc.) and titrated by flow cytometry. Anti-IgM antibodies were purchased from Cappel (Durham, NC). All NSAID at the highest concentration used were tested for lack of toxicity by trypan blue exclusion. RAV-2 reverse transcriptases and Taq polymerase were purchased from Takara Shuzo Co. (Shiga, Japan), and Roche Diagnostics SpA (Milan, Italy).

Transfections and Confocal Microscopy—Confocal microscopy was carried out on Jurkat cells transiently transfected by the DEAE/dextran procedure as described previously (12) with the plasmid pEGFP/NF-AT1-D (13), either as such or following treatment for 20 min with 500 ng/ml A23187 in the presence or absence of either 500 ng/ml cyclooxygenase A inhibitor (Sandoz, East Hanover, NJ) or 800 μM ibuprofen, using a Leica Microsystems confocal microscope (Heidelberg, Germany).

Sequence Analysis—Scanning of the human COX-2 gene promoter (GenBank accession number AF276953) for transcription factor binding sites was carried out using the matrix search program TRANSFAC MatInspector (14).

RESULTS AND DISCUSSION

To assess their impact on the TCR-signaling cascade, we assayed the capacity of NSAID to inhibit the activation of the transcription factor NF-AT using as a read-out a reporter Jurkat T-cell line stably transfected with a plasmid encoding luciferase under the control of NF-AT (11). The nonselective COX inhibitors, aspirin and ibuprofen, the COX-1-selective inhibitor resveratrol (15), and the COX-2-selective inhibitor NS-398 (16) all inhibited in a dose-dependent fashion the activation of NF-AT induced either by triggering the TCR/CD3 complex with agonistic mAb (Fig. 1) or pharmacologically using a combination of phorbol ester and a calcium ionophore (data not shown). A similar profile of inhibition was also observed in a Jurkat line stably transfected with an NF-κB/luciferase reporter and activated using the same pharmacological agonists (data not shown). Hence, both COX-1 and COX-2 activities are required for TCR signaling in T-cells. Although COX-2-specific mRNA was detectable as early as 2 h after stimulation (Fig. 2A), NS-398 only partially blocked NF-AT (Fig. 1) and NF-κB (data not shown) activation, suggesting a dominant role for the costitutively expressed COX-1 in this process.

TCR triggering initiates a tyrosine kinase-based signaling cascade leading within minutes to the activation of two main pathways, the Ras/MAPK and the Ca2+/calcineurin pathways, which synergistically activate NF-AT (17). We assessed the effect of NSAID on both pathways. The inhibitors of both Erk and p38 MAPKs reduced NF-AT activation in response to TCR engagement (Fig. 1). However TCR-induced p38 activity but not Erk/Erk2 activity was dramatically inhibited both by the nonselective and by the COX-1-selective NSAID as assessed by immunoblot analysis of Jurkat T-cell lysates with phosphospecific antibodies (Fig. 3A). Consistent with the lack of COX-2 expression before cell stimulation (Fig. 2A and Ref. 9), no effect on early p38 activation was observed in the presence of the COX-2 inhibitor NS-398. Similar results were obtained on purified peripheral blood lymphocytes (data not shown). Hence, whereas Erk and p38 MAPKs are both required for TCR signaling to NF-AT, NSAID selectively inhibited p38 activation.

The inhibition of p38 by both the nonselective and the COX-1-selective NSAID was also observed in Raji B-cells stimulated with agonistic mAb antibodies (data not shown). Although both mitogenic stimuli and oxidative stress can induce p38 activation (18), no effect of NSAID on p38 activation was observed either in cyclooxygenase and/or COX-2 selective inhibitor-resistant for further incubation of primary human peripheral blood mononuclear cells in media alone at 37 °C, washed in phosphate-buffered saline/1% serum, and analyzed by flow cytometry in a Becton Dickinson FACScan (San Jose, CA).

Reverse Transcription-PCR—Total RNA was extracted from Jurkat cells and either not activated or activated for 2, 4, 6, 8, and 16 h by TCR/CD3 cross-linking as described above using the RNA WIZ reagent (Ambion Inc, Austin, Texas). Reverse transcription-PCR was carried out using the oligo(dT), which hybridizes to polyadenylated RNA of primary human cells. PCR products were separated by agarose gel electrophoresis, and the intensity of the ethidium bromide-stained bands was quantitated by laser densitometry. The identity of the COX-2-specific reverse transcription-PCR product was confirmed by automatic sequencing.
NSAID did not affect the Ca\textsuperscript{2+}/calcineurin pathway. As shown in Fig. 3D, NF-AT localization was completely cytosolic in Jurkat cells transiently transfected with a construct encoding green fluorescent protein-tagged NF-AT1 in the absence of stimulation, whereas a massive NF-AT translocation to the nucleus fully repressible by cyclosporin A was observed following treatment with a calcium ionophore. Ibuprofen did not affect the inducible nuclear translocation of NF-AT (Fig. 3D).

Hence, the suppression of NF-AT activation by NSAID is achieved by inhibition of the MAPK pathway and specifically of p38 activation but not of the Ca\textsuperscript{2+}/calcineurin pathway. Of note, although in heterologous cells, active p38 has been shown to dampen NF-AT activity by promoting its phosphorylation-dependent export from the nucleus (21). In Jurkat T-cells, both NSAID and the p38 inhibitor SB203580 blocked NF-AT activation (Fig. 1) in agreement with the requirement for p38 in interleukin-2 production (22), suggesting that p38 might play opposing roles in the early activation and subsequent deactivation of gene expression.

The block by NSAID of TCR-dependent p38 activation suggests that the products of COX activity are required for TCR signaling. PGE\textsubscript{2} indeed increased both the low basal level of NF-AT activity and its response to TCR triggering (Fig. 1). Furthermore, the treatment of Jurkat cells with PGE\textsubscript{2} resulted in the up-regulation of p38 activity, but not Erk1/Erk2, in the absence of stimulation (Fig. 3C). Of note, PGE\textsubscript{2} could fully overcome the inhibition of TCR-dependent p38 activation by both ibuprofen (data not shown) and the COX-1-selective inhibitor resveratrol (Fig. 3C). Hence, the reduction in PGE\textsubscript{2} synthesis by COX can account for the inhibitory effect of NSAID on TCR-dependent p38 and NF-AT activation. In agreement with a role for COX upstream of p38 activation, PGE\textsubscript{2} failed to overcome the inhibition of NF-AT activation by the p38 inhibitor SB203580 (data not shown). p38 can be activated by distinct pathways triggered by mitogenic stimuli and cellular stress (18). The selective effect of NSAID on the activation of p38 triggered by the TCR and B-cell antigen receptor as opposed to LPS or H\textsubscript{2}O\textsubscript{2} (Fig. 3, A and B) suggests that PGE\textsubscript{2}, which is produced in response to all these stimuli, does not directly activate p38 but a component upstream of p38 in the antigen receptor-signaling cascade. Specifically, because tyrosine kinase-dependent pathways leading to Erk1/Erk2 activation are believed to diverge at the level of the small GTPases Ras and Rac, respectively (23), our data indicate a requirement...
for COX in the serine-threonine kinase cascade initiated by Rac.

p38 activity is required both for COX-2 gene expression and for post-transcriptional stabilization of COX-2 mRNA (24, 25). The block in p38 activation by nonselective and COX-1-selective NSAID (Fig. 3A) suggests that COX-1, which is constitutively expressed in T-cells (9), is implicated in the initial activation of p38 and might therefore be required for the induction of COX-2 expression. To assess this possibility, we analyzed COX-2 expression in normal PBL. TCR/CD3 stimulation as well as a combination of phospholipase ester and calcium ionophore induced the expression of COX-2, albeit at levels significantly lower than in macrophages stimulated with LPS or pharmacological agonists (Fig. 2B). Of note, the monocline line U937 constitutively expressed high levels of COX-2 (Fig. 2B), which were paralleled by a high constitutive p38 activity (Fig. 3B) in support of a key role for p38 in COX-2 expression. A pretreatment of PBL with the COX-1 inhibitor resveratrol completely blocked the TCR-dependent expression of COX-2 (Fig. 2C). Hence, COX-1 participates in the TCR-dependent induction of the gene encoding COX-2 probably by contributing to p38 activation. In agreement with the lack of effect of NSAID on LPS-dependent p38 activation in macrophages (Fig. 3B), the induction of COX-2 expression by LPS is not impaired in COX-1−/− macrophages (26). Interestingly, p38 is required for NF-AT activation (Fig. 1) and interleukin-2 expression (22). Furthermore, cyclosporin A, which potently blocks NF-AT activation, also inhibits the induction of COX-2 gene expression, suggesting a role for NF-AT in this process. In support of this possibility, scanning of the −500-bp region upstream of the COX-2 gene containing the essential elements for its transcriptional regulation (27) revealed four potential NF-AT binding sites at positions −309/−297, −292/−281, −109/−98 and −80/−69.

The immunosuppressant activity of NSAID on T-cells underlies a role for COX activity not only in the induction of proinflammatory cytokine gene expression during inflammation but also in the normal process of lymphocyte activation. COX-2 has indeed been identified as an early response gene in peripheral T-cells (9). Furthermore, genetic and pharmacological evidence supports a key role for COX-1, specifically mediated by PGE2, in the CD4−/CD8+ to the CD4+CD8+ transition during thymocyte development (28). COX-2-mediated PGE2 synthesis, by thymic stromal cells is also required for the maturation of CD4+ thymocytes (28). Our data show that COX-1 is a strategic component of the TCR-signaling cascade in that it promotes the activation of p38 and of transcription factors regulated by this MAPK. Among the transcriptional targets of these factors is COX-2. In this scenario, the initial burst of ROS production following TCR engagement, as well as the rapid early increase in the concentration of arachidonic acid due to the sequential activation of phospholipase Cγ and cytosolic phospholipase A2 (cPLA2), would result in the up-regulation of COX-1 activity and increased synthesis of PG, thereby promoting p38 activation. This would in turn lead to COX-2 expression and a delayed phase of PG synthesis. In this context, the partial inhibition of NF-AT activation by the COX-2 inhibitor NS-398 (Fig. 1 and Ref. 9) suggests that COX-2-dependent PG production might contribute to sustaining NF-AT activity at later stages of T-cell activation. Interestingly, mast cells from cPLA2−/− mice fail to express COX-2 in response to cytokines, however arachidonic acid restores COX-2 expression (29), supporting our proposed role for COX-1-dependent PG production in the regulation of COX-2 transcription. Collectively, our data not only provide novel insight into the mechanisms underlying the immunosuppressant activity of NSAID in T-cells but suggest that selective p38 inhibitors might be a valid alternative to classical immunosuppressants as a tool to control T-cell activation in disease.

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