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The role of peroxynitrite in cyclooxygenase-2 expression of rheumatoid synovium

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ABSTRACT

Objective. *Reactive oxygen intermediates play an important role in the inflammatory processes of rheumatoid arthritis. Cyclooxygenase-2 is an inducible form of an enzyme involved in prostanoid biosynthesis. This study linked peroxynitrite ($ONOO^-$) to the signaling pathways that induce COX-2.*

Results. *Exposure of rheumatoid synovial cells to peroxynitrite resulted in COX-2 protein expression in a dose-dependent manner. RT-PCR analysis also demonstrated that COX-2 mRNA was induced in peroxynitrite-treated rheumatoid synovial cells. Dexamethasone markedly inhibited this peroxynitrite-mediated COX-2 expression at therapeutic concentrations.*

Conclusion. *This study demonstrates that oxidant stress is an important inducer of COX-2 in rheumatoid synovium. This induction may contribute to the amplification of prostanoids in the rheumatoid inflammatory process.*

Introduction

Rheumatoid arthritis (RA) is a chronic multisystem disease characterized by the overgrowth of synovium and subsequent cartilage destruction. Many years ago it was suggested that reactive oxygen species might be involved in the tissue damage observed in rheumatoid joints (1). The overproduction of oxygen-derived species such as the superoxide radical O_2^- was demonstrated in RA synovial fluids (2). There is also considerable evidence that the production of nitric oxide (NO) is increased in synovial fluids from RA patients (3). Nitric oxide reacts with O_2^- to lead to peroxynitrite ($ONOO^-$) which has been implicated in a variety of human inflammatory diseases (4). A "footprint"molecule of $ONOO^-$, 3-nitrotyrosine, has been detected in the synovial fluid as well as synovium of RA patients (5, 6).

In addition prostaglandin E2, an important inflammatory mediator, is produced in rheumatoid synovium (7). The inducible isoform of cyclooxygenase (COX-2), which plays a major role in the production of inflammatory prostaglandins (PGs), was found to be expressed in rheumatoid synovium (8). Peroxynitrite has been shown to be implicated in the enzymatic activation of COX through interaction with its catalytic site (9). Based on these observations, we examined the functional interaction of peroxynitrite and COX-2 which is responsible for the synthesis of pro-inflammatory prostanoids in rheumatoid synovium.

Materials and methods

Antibodies and reagents

Rabbit anti-human COX-2 polyclonal antibody was purchased from Immuno-Biological Laboratories (IBL, Fujioka, Japan). SIN-1 (N-Morpholino syndnonimine hydrochloride) and peroxynitrite were purchased from Dojindo Laboratories (Kumamoto, Japan). All other reagents were purchased from Sigma Chemical CO. (St. Louis, MO).

Synovial cell culture

The experimental protocol was approved by the local ethics committee and a signed consent form was obtained from each patient. Synovial tissue samples were obtained from 8 patients with rheumatoid arthritis (RA) during synovectomy. The synovial membranes were minced aseptically, then dissociated enzymatically with collagenase (4.0 mg/ml,Sigma) in RPMI 1640 for 4 hrs at 37°C. The cells obtained were plated on culture dishes and allowed to adhere. To eliminate non-adherent cells from the synovial cell preparations, the plated cells were cultured for 18 hrs with RPMI 1640 supplemented with 10% FCS at 37°C in humidified 5% CO_2 in air. The cells were then washed thoroughly with phosphate-buffered saline (PBS) solution. Adherent synovial cells were removed by adding trypsin-EDTA, followed by washing with PBS containing 2% FCS. The collected synovial cells were used at the third or fourth passages for subsequent experiments. Synovial cell preparations were less than 1% reactive with monoclonal antibodies CD3, CD20, CD68 (Coulter Immunology, FL), and anti-human von Willebrand factor (Immunotech, Marseille, France), indicating that these preparations were almost free of mature T lymphocytes, B lymphocytes, monocytes/macrophages, and vas-

cular endothelial cells.
These synovia cells were cultured with SIN-1 or peroxynitrite. No endotoxin was detected (<0.5 U/ml) under the present experimental culture conditions, as confirmed by an endotoxin-specific detection kit (Toxicolor system, Seikagaku corporation, Tokyo, Japan).

COX-2 expression analysis by Western blot
The expression of COX-2 was analyzed by the Western blot method as described previously by Crofford *et al.* (8). For this purpose, the cells were washed with cold PBS and lysed by the addition of a lysis buffer containing 1% nonidet P-40, 50 mM Tris, (pH 7.5), 100 mM NaCl, 5 mM EDTA, 10 µg/ml aprotinin, and 10 µg/ml leupeptin for 20 min at 4°C. Insoluble material was removed by centrifugation at 15,000 xg for 15 min at 4°C. The supernatant was saved and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An identical amount of protein (50 µg) from each lysate was subjected to 10% SDS-PAGE. The fractionated proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL) and the membranes were blocked for 16 hrs in a solution containing 5% BSA, 10 mM Tri-HCl (pH 8.2), and 140 mM NaCl. The blots were incubated with an anti-COX-2-specific antibody (IBL Co., Fujioka, Japan) for 2 hrs at room temperature. The membranes were further incubated with peroxidase-conjugated anti-rabbit IgG antibodies (Amersham, Arlington Heights, IL) for 60 min and developed using enhanced chemiluminescence (ECL) system (Amersham).

Prostaglandin E2 measurements
Rheumatoid synovial cell-conditioned culture media were collected and PGE2 was measured uusing specific enzyme-linked immunosorbent assay (ELISA) according to the manufacturesís instructions (Amersham).

RNA preparation and RT-PCR assay
Total cellular RNA was extracted from synovial fibroblasts using guanidium thiocyanate and phenol (RNAzol B, Cinna/Bioek Labs Intl. Inc., Friendswood, TX). First-strand cDNA was synthesized by reverse transcription at 42°C for 45 min in a 50 µl reaction mixture containing 1 µg of total RNA and MuLV reverse transcriptase (Gibco BRL, Gaithersburg, ND). After heating at 99°C for 5 min to denature, followed by cooling at 5°C, the cDNA was used for amplification. For the PCR reactions, 2 µl of denatured cDNA was amplified in a 20 µl final volume with 1 U Taq DNA polymerase (Gibco BRL), 1 mM of both primers and Taq polymerase buffer contained 1.5 mM $MgCl_2$ with 1.5 mM of each dNTP. PCR was performed in a thermal cycler (Perkin Elmer Cetus, Foster City, CA) using a program of 25 cycles at 94°C for 1 min, 60°C for 1 min 72°C for 1 min and followed by a 10 min extension at 72°C. The amplified products were subjected to electrophoresis on 1.5% agarose gel.
The specific primers used included COX-2 (10), 5'-TTCAAATGAGATT GTGGGAAAATTGCT-3'(forward primer), and 5'-AGATCATCTCTGCCT-GAGTATCTT-3' (reverse primer).
The predicted size of the fragment was 301 bp.
-actin, 5'-GACGAGGCCCAGAGC AAGAGAG-3' (forward primer), 5'-ACGTACATGGCTGGGGTGTTG-3' (reverse primer).
The predicted size of the fragment was 284 bp.

Results
We stimulated rheumatoid synovial cells with SIN-1 (3-morpholino syononimine hydrochloride), which mimics the production of $ONOO^-$ from simultaneously generated O_2^- and NO (11), for 24 hr. After stimulation, lysates of synovial cells were analyzed by anti-COX-2 immunoblot. Treatment of the synovial cells with SIN-1 resulted in COX-2 protein expression in a dose-dependent manner (Fig. 1). To determine the direct effects of peroxynitrite, synovial cells were treated with low concentrations (up to 50 M) of peroxynitrite, at which concentrations peroxynitrite is not cytotoxic for synovial cells. As shown in Figure 2, peroxynitrite induced COX-2 expression on synovial cells in a dose-dependent manner. The concentration of prostaglandin E2 was increased in peroxynitrite-stimulated synovial cells compared to those of the control (without stimulation: 1202 ± 263 pg/ml, peroxynitrite-stimulated 1754 ± 342 pg/ml).
To investigate the molecular mechanism of peroxynitrite-mediated COX-2 expression in synovial cells, we examined the COX-2 mRNA expression in

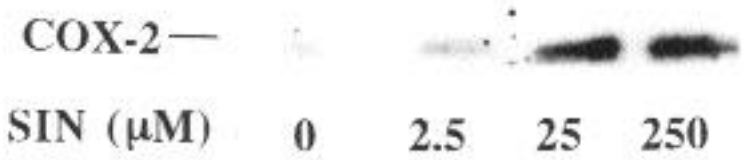

Fig. 1. Effects of SIN-1 on COX-2 protein expression of synovial cells. Synovial cells were cultured with various concentrations of SIN-1 for 24 hrs. The cells were lysed, and the lysates were analyzed by anti-COX-2 Western blot. Data are representative of at least three experiments.

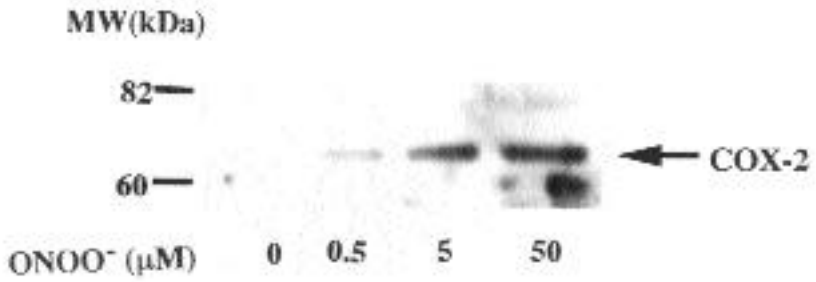

Fig. 2. Peroxynitrite-induced COX-2 protein expression synovial cells. Synovial cells were cultured with various concentrations of peroxynitrite ($ONOO^-$) for 24 hrs. The cells were lysed, and the lysates were analyzed by anti-COX-2 Western blot. Data are representative of at least 4 experiments.

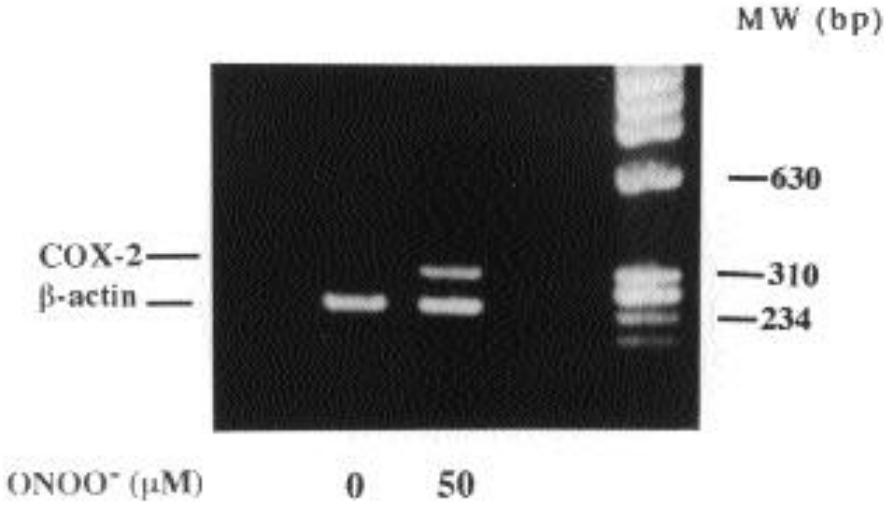

Fig. 3. RT-PCR analysis of mRNA for COX-2 in synovial cells treated with peroxynitrite. Synovial cells were treated with or without peroxynitrite ($ONOO^-$, 50 M) for 6 hrs as described in the Methods section. Total RNA was reverse transcribed following PCR amplification with primers for COX-2 and -actin. The PCR products were stained with ethidium bromide after 25 cycles of amplification and separated in a 1.5% agarose gel. Data are representative of at least 3 experiments.

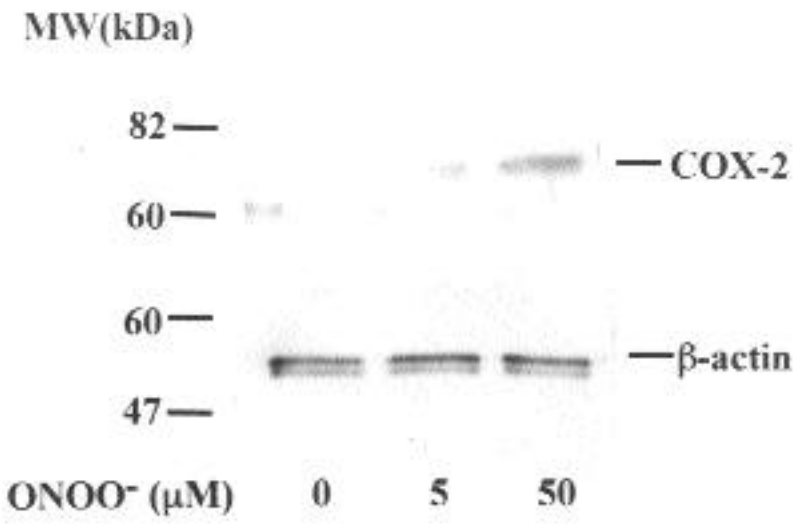

Fig. 4. Peroxynitrite induced COX-2 protein expression without affecting -actin protein expression in synovial cells. Synovial cells were cultured with or without peroxynitrite (50 M) for 24 hr. The cells were lysed, and the lysates were analyzed by anti-COX-2 and anti- -actin Western blot.

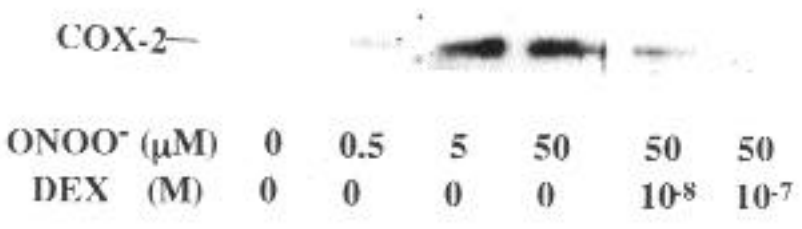

Fig. 5. Effects of dexamethason on COX-2 protein expression in peroxynitrite-treated synovial cells. Synovial cells were cultured with peroxynitrite (50 M) in the presence or absence of dexamethasone for 24 hrs. The cells were lysed, and the lysates were analyzed by anti-COX-2 Western blot. Data are representative of at least 3 experiments.

peroxynitrite-treated synovial cells. COX-2 and -actin cDNA were amplified by PCR. There was a linear correlation between the number of cycles (n = 19-31) and the yield of PCR products for both -actin and COX-2 mRNA (data not shown). Although COX-2 mRNA was not detected in synovial cell, stimulation of these cells with peroxynitrite for 6 hr induced COX-2 mRNA expression. No change was noted in the amplified fragments of housekeeping gene -actin (Fig. 3). Similarly, peroxynitrite induced COX-2 protein expression without affecting -actin protein expression (Fig. 4).

It has been suggested that glucocorticoid down-regulates COX-2 expression by inhibiting transcription factor, such as nuclear factor-kappa B (12). Therefore, we investigated the effects of dexamethasone (DEX) on peroxynitrite-induced COX-2 expression in synovial cells. As shown in Figure 5, DEX completely inhibited peroxynitrite-induced COX-2 expression in synovial cells at therapeutic concentrations (10^{-7} ~ 10^{-8} M).

Discussion

Nitrogen species play an important role in the inflammatory process of RA (13). Increased levels of the marker for peroxynitrite, nitrotyrosine, were found in serum and synovial fluids from RA patients (5,6). Similarly, increased expression of COX-2, which accounts for the release of pro-inflammatory prostaglandins at the site of inflammation, has been demonstrated in rheumatoid synovium (8). COX, which converts arachidonic acid to PGs, is a critical enzyme in many inflammatory diseases (8). In inflammatory processes the inducible isoform, COX-2, is expressed in many cells including fibroblasts and macrophages and accounts for the release of large quantities of pro-inflammatory PGs at the site of inflammation (14).

These inflammatory mediators may play a profound role in the pathological process of RA. Recent studies have suggested that the interaction between COX and nitrogen species exists at multiple levels (15,16). In RA there is known to be overproduction of oxygen

derived species such as superoxide radicals O_2^- (1). There is also considerable evidence that the production of nitric oxide (NO) is increased in synovial fluids from patients with RA (15). Peroxinitrite ($ONOO^-$) is a harmful oxidant that is formed when NO reacts with superoxide anion (4). Peroxynitrite generation *in vivo* has been implicated in inflammatory diseases (5,6).

Cyclooxygenase activity is known to be sensitive to free radicals. Furthermore, peroxynitrite has been implicated in the enzymatic activation of COX-2 to produce prostaglandins (9). Thus the cross-talk between COX-2 and peroxynitrite plays an important role during the pathological process of RA. Our data clearly indicated that peroxynitrite serves as an efficient inducer of COX-2 in rheumatoid synovium. RT-PCR analysis also demonstrated COX-2 mRNA induction in peroxynitrite-treated synovial cells. These findings strongly suggest that a harmful oxidant, peroxynitrite, that is formed from NO and superoxide anion, plays a critical role in the COX-2 expression in rheumatoid synovium.

Marnett *et al*. demonstrated that PGE_2 formation was abolished in iNOS-deficient mice (17). These studies support the hypothesis that NO-derived species such as peroxynitrite can modulate cyclooxygenase-2 activity. However, COX-2 protein expression of IFN- and LPS-stimulated macrophages was not affected in iNOS-deficient animals. The contribution of NO-derived species may be minimal and other molecules are involved in cytokine-induced COX-2 induction. Furthermore, it was demonstrated that peroxynitrite activates human recombinant COX-2 enzymatic activity at 140 M (18). This concentration is higher than those of our experimental conditions to induce COX-2 mRNA and protein induction (50 M). We could not examine the effects of peroxynitrite at higher concentrations because peroxynitrite is toxic to rheumatoid synovial cells. The appropriate concentrations of peroxynitrite for COX-2 protein induction may be different from those for COX-2 enzymatic activation.

The expression of COX-2 is regulated at a transcriptional level. The promoter region of the COX-2 gene contains putative transcriptional elements such as NF-IL-6 and NF-kB (12). NF-kB is a redox-sensitive transcription factor. Recent data indicate that redox regulation occurs in the degradative process of I-kB which binds to NF-kB (19). Our data indicated that dexamethasone dramatically inhibited the COX-2 expression of peroxynitrite-treated synovial cells. Ligand-binding glucocorticoid receptor rapidly translocates to the nucleus and interact with transcription factors such as NF- B and functions as a negative regulator (20). More recently, it was shown that glucocorticoid prevents NF- B translocation by inducing I -B (21). Therefore, it is suggested that one mechanism by which dexamethasone inhibited peroxynitrite-mediated COX-2 expression may be due to the inhibition of NF- B nuclear translocation.

In summary, the pathophysiological significance of our findings is that peroxynitrite generation in synovium may exacerabate the iflammatory process through the additional pro-inflammatory mediator, COX-2. Furthermore, these pleotropic mediators are targets for pharmacological intervention.

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