

Ectopic Expression of Caveolin-1 Induces COX-2 Expression in Rabbit Articular Chondrocytes via MAP Kinase Pathway

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ABSTRACT

Background: Caveolin-1 is a principal component of caveolae membranes in vivo. Although expression of caveolae structure and expression of caveolin family, caveolin-1, -2 and -3, was known in chondrocytes, the functional role of caveolae and caveolins in chondrocytes remains unknown. In this study, we investigated the role of caveolin-1 in articular chondrocytes. **Methods:** Rabbit articular chondrocytes were prepared from cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion. Caveolin-1 cDNA was transfected to articular chondrocytes using LipofectaminePLUS. The cyclooxygenase-2 (COX-2) expression levels were determined by immunoblot analysis, immunostaining, immunohistochemistry, and prostaglandin E₂ (PGE₂) assay was used to measure the COX-2 activity. **Results:** Ectopic expression of caveolin-1 induced COX-2 expression and activity, as indicated by immunoblot analysis and PGE₂ assay. And also, overexpression of caveolin-1 stimulated activation of p38 kinase and ERK-1/-2. Inhibition of p38 kinase and ERK-1/-2 with SB203580 and PD98059, respectively, led to a dose-dependent decrease COX-2 expression and PGE₂ production in caveolin-1-transfected cells. **Conclusion:** Taken together, our data suggest that ectopic expression of caveolin-1 contributes to the expression and activity of COX-2 in articular chondrocytes through MAP kinase pathway. (**Immune Network 2006;6(3):123-127**)

Key Words: Caveolin-1, COX-2, prostaglandin E₂, p38 kinase

Introduction

Arthritic joints produce large amounts of prostaglandins (PGs) that are involved in cartilage inflammation (1). The rate-limiting steps in PG synthesis are hydrolysis of phospholipids to produce free arachidonic acid and conversion of arachidonic acid to prostaglandin E₂ (PGE₂), which is catalyzed by two isoforms of cyclooxygenase (COX) (2,3). COX-1 is constitutively expressed in many cell types and participates in physiological activities, whereas COX-2 expression is induced by diverse mitogenic and proinflammatory factors and plays an important role in inflammation and tumorigenesis (4). Regulation of COX-2 expression has been shown to occur at both transcriptional and post-transcriptional levels, although the molecular mechanisms of this up-regulation remain unclear. Several studies indicate that its expression

is regulated by mitogen-activated protein kinase (MAPK) subtypes including extracellular signal-regulated protein kinases 1 and 2 (ERK-1/-2), p38 kinase, and c-Jun N-terminal kinase (JNK), depending on the types of extracellular stimuli and cells (5-7). PGE₂ exerts diverse effects on the immune response and the biological outcome in a variety of inflammatory diseases including rheumatoid arthritis (RA).

Caveolae are 50~100 nm vesicular invaginations of the plasma membrane and participate in the variety of cellular processes including of vesicular trafficking events and signal transduction processes (8,9). Caveolin, a family of 21~24 kDa integral membrane proteins are a principal component of caveolae membranes (10). Although expression of caveolae structure and expression of caveolin family, caveolin-1, -2 and -3, was known in chondrocytes, the exact functional role of caveolae and caveolins in chondrocytes remain unclear (11).

The metabolic activities of COX-1 and COX-2 are regulated by multiple factors. Several lines of evidence suggest that their subcellular locations are an important determinant for their metabolic activities

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and cellular functions. It has been reported that COX-1 and COX-2 were similarly localized to endoplasmic reticulum (ER) and nuclear envelope (NE) (12). Liou (13) demonstrate that a fraction of COX-2 in human foreskin fibroblasts (HFFs) were colocalized with caveolin-1 to plasma membrane caveolae, and subcellular fractionation analysis confirmed the presence of COX-2 with caveolin-1 in the detergent-insoluble membrane fraction.

The current study investigated the role of caveolin-1 in the regulation of COX-2 expression and PGE₂ production. Our results suggest that the expression of caveolin-1 might be responsible for COX-2 expression and PGE₂ production via MAP kinase signaling dependent pathway.

Materials and Methods

Monolayer culture of rabbit articular chondrocytes and experimental culture condition. Rabbit articular chondrocytes were released from cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion. To summarize, after aseptic dissection cartilage slices were aseptically dissected and then dissociated enzymatically for 6 h in 0.2% collagenase type II (381 U/mg solid, Sigma) in PBS, and individual cells were then obtained by collecting the supernatant after brief centrifugation. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine-calf serum, 50 µg/ml streptomycin, and 50 units/ml penicillin, after which and they were then plated on culture dishes at a density of 5×10^4 cells/cm². The medium was changed every 2 days after seeding, and cells reached confluence in approximately 5 days. In some experiment, chondrocytes at day 3 were transfected with wild-type caveolin-1 (Cav-1). The transfected cells, which were cultured in complete medium for 24 hours, were used for further analysis as indicated in each experiment.

Immunoblot analysis. Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecylsulfate, supplemented with protease inhibitors [10 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml aprotinin and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride] and phosphatase inhibitors (1 mM NaF and 1 mM Na₃VO₄). The proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was then blocked with 3% non-fat dry milk in Tris-buffered saline. COX-2 was detected using antibody purchased from Cayman Chemical (Ann Arbor, MI). Caveolin-1, pERK, ERK-2 and p38 were detected using antibodies purchased from Santa Cruz Biotech.

(Santa Cruz, CA). The bands were visualized using peroxidase-conjugated secondary antibodies and chemiluminescence.

p38 kinase assay. p38 kinase activity was determined by immune complex kinase assays as described previously (14,15). Briefly, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate and inhibitors of proteases and phosphatases, as described above. Total cell lysates were precipitated with polyclonal anti-p38 kinase antibody and the immune complexes collected using protein-A Sepharose beads. The beads were re-suspended in 20 µl kinase reaction buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.1 mM sodium orthovanadate, 10 mM MgCl₂, 5 µCi [γ -³²P] ATP and 1 µg activating transcription factor-2 (ATF-2) protein as a substrate (New England Biolabs, Beverly, MA). The kinase reaction was performed for 30 min at 30°C and phosphorylated ATF-2 was detected by autoradiography following gel electrophoresis.

PGE₂ Assay. PGE₂ production was determined by measuring the levels of cellular and secreted PGE₂ using an assay kit (Amersham Pharmacia Biotech, NJ, UK). Briefly, chondrocytes were seeded in standard 96-well microtiter plates at 2×10^4 cells/well. Following addition of the indicated pharmacological reagents, total cell lysate was used to quantify the amount of PGE₂, according to the manufacturer's protocol. PGE₂ levels were calculated against a standard curve of PGE₂ and normalized against the amount of genomic DNA.

Immunofluorescence microscopy. Expression and distribution of caveolin-1 and COX-2 in rabbit articular chondrocytes were determined by indirect immunofluorescence microscopy, as described previously (16). Briefly, chondrocytes were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized and blocked with 0.1% Triton X-100 and 5% fetal calf serum in PBS for 30 min. The fixed cells were washed and incubated for 1 h with antibody (10 µg/ml) against caveolin-1 and COX-2. The cells were washed, incubated with rhodamine- or fluorescein- conjugated secondary antibodies for 30 min, and observed under a fluorescence microscope.

Data analyses and statistics. The results are expressed as the means ± S.E. values calculated from the specified number of determinations. A Student's test was used to compare individual treatments with their respective control values. A probability of $p < 0.05$ was taken as denoting a significant difference.

Results

Ectopic expression of caveolin-1 induces COX-2 expression and PGE₂ production. To examine the relationship between caveolin-1 and COX-2 signaling, and the role of caveolin-1 in articular chondrocyte primary culture, the protein was overexpressed by cDNA transfection. Ectopic expression of caveolin-1 induced COX-2 expression (Fig. 1A) and subsequent PGE₂ production (Fig. 1B). Immunofluorescence double staining of caveolin-1 and COX-2 in chondrocytes transfected with caveolin-1 are also positive for COX-2 staining, whereas cells that do not express caveolin-1 (indicated by white arrow head) are negative for COX-2 staining (Fig. 2). Therefore, ectopic expression of

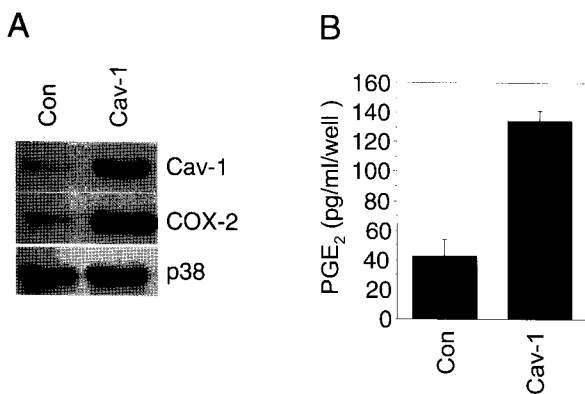


Figure 1. Ectopic expression of caveolin-1 induces COX-2 expression and PGE₂ production. Chondrocytes were transfected with empty vector as a control (Con) or caveolin-1 (Cav-1). Following incubation in complete medium for 24 h, expression of COX-2, Cav-1 (caveolin-1) and p38 (A) and production of PGE₂ (B) were determined by immunoblot analysis and using a PGE₂ assay kit, respectively. The data in A represent the results of a typical experiment conducted four times, and B signifies the average values with standard deviation (n=4).

caveolin-1 appears to be sufficient to induce COX-2 expression in articular chondrocytes. Also, these data indicate that expression of caveolin-1 regulates inflammatory responses in articular chondrocytes (i.e., COX-2 expression and PGE₂ production).

Caveolin-1 regulates COX-2 expression and activity in chondrocytes through MAP kinase pathway. Ectopic expression of caveolin-1 in chondrocytes was accompanied by increased phosphorylation of ERK-1/-2 and p38 kinase (Fig. 3A). Caveolin-1 transfected chondrocyte cells were treated with PD98059, an inhibitor of ERK-1/-2 and SB203580, an inhibitor of p38 kinase, respectively. Blocking ERK-1/-2 and p38 kinase activity inhibit caveolin-1-induced COX-2 expression in a dose-dependent manner (Fig. 3B). Similar to the effects on COX-2 expression, caveolin-1-induced PGE₂ production was completely blocked by direct inhibition of two MAP kinase inhibitor (Fig. 4). Taken together, these results indicate that caveolin-1 induced COX-2 expression and PGE₂ production is regulated by ERK-1/-2 and p38 kinase activity. Levels of caveolin-1 and COX-2 protein were increased in rheumatoid arthritic cartilage, suggesting that caveolin-1 may play a role in the inflammatory responses of arthritic cartilage (data not shown).

Discussion

Caveolin-1 was not only the first protein to be localized to caveolae but due to its apparent involvement in the structural integrity of caveolae was also the first caveolar "marker protein" (10). The issue that required clarification, however, was whether caveolae could also serve as platforms for the aggregation and/or concentration of other proteins. Clearly, evidence for the presence of other caveolar resident proteins would be important in the understanding of caveolar function. In this regard, Lisanti and coworkers were the first investigators to broadly address

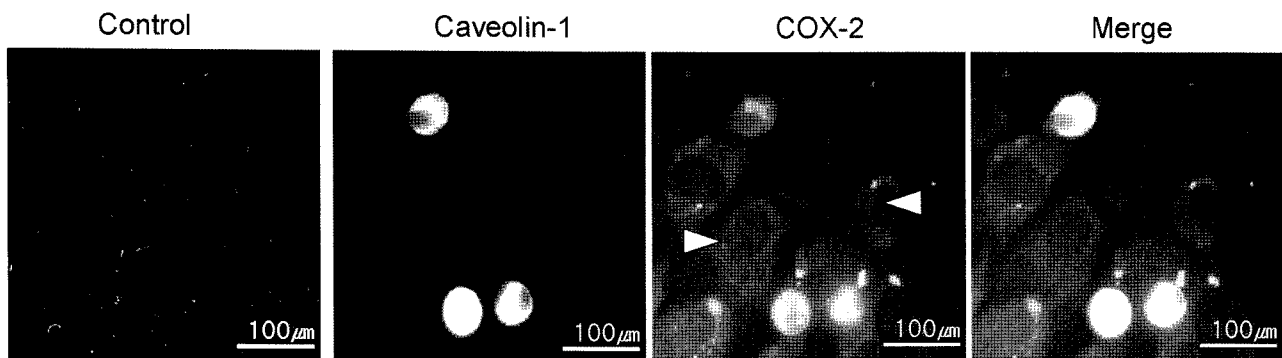


Figure 2. Overexpression of caveolin-1 causes COX-2 expression. Chondrocytes were transfected with empty vector as a control (rhodamine) or wild type caveolin-1, cultured for 24 h, double stained for caveolin-1 (fluorescein) and COX-2 (rhodamine), and analyzed by immunofluorescence microscopy. The data represent the results of a typical experiment conducted at least three times with similar results.

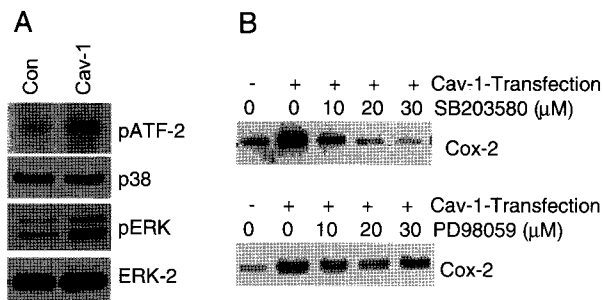


Figure 3. Activation of ERK-1/-2 and p38 kinase in caveolin-1-transfected chondrocytes. Chondrocytes were transfected with empty vector as a control (Con) or caveolin-1 (Cav-1). Following incubation in complete medium for 24 h, expression levels of phosphorylated ERK (pERK) and ERK-2 was determined by immunoblot analysis. p38 kinase activity was determined by immunocomplex kinase assay using ATF-2 as a substrate (A). Chondrocytes were pretreated with the indicated concentrations of SB203580 (B, upper panel) or PD98059 (B, lower panel) for 30 min, respectively, and cells were transfected with empty vector as a control (Con) or caveolin-1 (Cav-1) for 24 h (B). Expression of COX-2 was determined by immunoblot analysis. The data represent the results of a typical experiment conducted at least three times.

this issue (17,18). Using the insolubility of caveolae in mild detergents and their buoyancy in sucrose gradients, they were able to biochemically separate caveolae membranes and, in turn, determine the identity of cosegregated proteins. Of the numerous proteins identified in this manner, it was surprising to find that a large majority were signal transduction molecules, some at concentrations many fold higher than the bulk plasma membrane (17,18). This observation led Lisanti and colleagues (8) to put forth the "caveolae/raft signaling hypothesis": the compartmentalization of such molecules has distinct advantages as it provides a mechanism for the regulation of subsequent signaling events and explains cross-talk between different signaling pathways (18).

The exact functional role of caveolae and caveolins, and regulatory mechanism by which caveolin-1 induced expression of COX-2 in articular chondrocytes is currently unknown. Arthritic joints produce large amounts of prostaglandin via COX-2 (1,19,20). This study demonstrates for the first time that caveolin-1 caused inflammatory responses such as COX-2 expression and PGE₂ production. We also demonstrated that caveolin-1-induced COX-2 expression and PGE₂ production is regulated ERK-1/-2 and p38 kinase signaling. In addition, increasing number of evidence several studies linked COX-2 expression with MAP kinase subtypes, including ERK-1/-2, p38 kinase and c-Jun N-terminal kinase (JNK) (21-23). Therefore, our observation that caveolin-1 induced expression and activity of COX-2 is regulated by

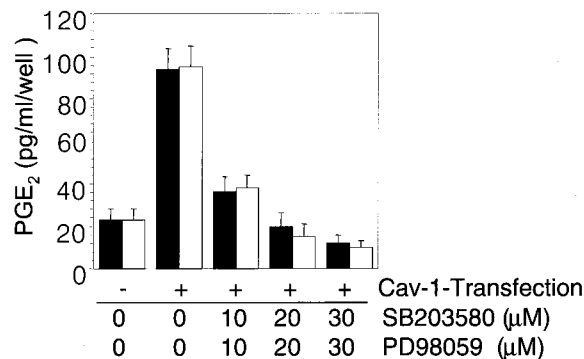


Figure 4. ERK-1/-2 and p38 kinase regulate caveolin-1-induced PGE₂ production. Chondrocytes were pretreated with the indicated concentrations of SB203580 or PD98059 for 30 min, respectively, and cells were transfected with empty vector as a control (Con) or caveolin-1 (Cav-1) for 24 h. Production of PGE₂ was determined by using a PGE₂ assay kit. The data represent the average values with standard deviation (n=4).

ERK-1/-2 and p38 kinase signaling is consistent with observations of others. We also investigated that expression levels of caveolin-1 and COX-2 were increased in experimental rheumatoid arthritic cartilage (data not shown). These results suggest that overexpression of caveolin-1 may contribute to the inflammatory responses of cartilage by inducing COX-2 expression in chondrocytes of arthritis-affected cartilage.

We considered the possibility that caveolin-1 induced COX-2 expression regulates dedifferentiation, because COX-2-mediated PGE₂ production is known to regulate differentiation (13,24,25) in chondrocytes. Therefore, further study is necessary to investigate whether or not caveolin-1 regulate dedifferentiation of chondrocytes.

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