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Endoplasmic reticulum stress (ER-stress) by 2-deoxy-D-glucose (2DG) reduces cyclooxygenase-2 (COX-2) expression and N-glycosylation and induces a loss of COX-2 activity *via* a Src kinase-dependent pathway in rabbit articular chondrocytes

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Abbreviation: 2DG, 2-deoxy-D-glucose

Abstract

Endoplasmic reticulum (ER) stress regulates a wide range of cellular responses including apoptosis, proliferation, inflammation, and differentiation in mammalian cells. In this study, we observed the role of 2-deoxy-D-glucose (2DG) on inflammation of chondrocytes. 2DG is well known as an inducer of ER stress, *via* inhibition of glycolysis and glycosylation. Treatment of 2DG in chondrocytes considerably induced ER stress in a dose- and time-dependent manner, which was demonstrated by a reduction of glucose regulated protein of 94 kDa (grp94), an ER stress-inducible protein, as determined by a Western blot analysis. In addition, induction of ER stress by 2DG led to the expression of COX-2 protein with an apparent molecular mass of 66-70kDa as compared with the normally expressed 72-74 kDa protein. The suppression of ER stress with salubrinal (Salub), a selective inhibitor of eif2-alpha dephosphorylation, successfully prevented grp94 induction and efficiently recovered 2DG-modified COX-2 molecular mass and COX-2 activity might be associated with COX-2 N-glycosylation. Also, treatment of 2DG increased phosphorylation of Src in chondrocytes. The inhibition of the Src signaling pathway with PP2 (Src tyrosine kinase inhibitor) suppressed grp94 expression and restored COX-2 expression, N-glycosylation, and PGE2 production, as determined by a Western blot analysis and PGE2 assay. Taken together, our results indicate that the ER stress induced by 2DG results in a decrease of the transcription level, the molecular mass, and the activity of COX-2 in rabbit articular chondrocytes *via* a Src kinase-dependent pathway.

Keywords: cartilage, articular; chondrocytes; cyclooxygenase 2; deoxyglucose; proto-oncogene proteins pp60(c-src)

Introduction

Chondrocytes in joint cartilage are differentiated from mesenchymal cells during embryo development (Sandell and Adler, 1999; DeLise *et al*., 2000; Singh Khillan, 2007). They are comprised of a single-cell population responsible for the biosynthesis of matrix molecular components. Differentiated chondrocytes are characterized by the ability to synthesize cartilage-specific extracellular matrix molecules including type II collagen and sulfated proteoglycans (Archer *et al*., 1990; Hauselmann *et al*., 1994; Reginato *et al*., 1994; Martel-Pelletier *et al*., 2008). The integrity of the matrix is crucial for the unique biosynthetic properties of cartilage and depends on conservation of the quality of the matrix constituents (Chandrasekhar and Phadke, 1988; Jennings *et al*., 2001; L'Hermette *et al*., 2006).

Balance of anabolic and catabolic processes within the tissue is important for homeostasis maintenance of cartilage. This homeostasis is destroyed in degenerative diseases, such as rheumatoid arthritis. Arthritis is characterized by loss and degeneration of matrix cartilage (Brandt, 1998; Tatari, 2007; Hashimoto *et al*., 2008). Prostaglandins are synthesized from arachidonic acid (AA) by mediation of the cyclooxygenase (COX) enzymes, either constitutively or in response to cell specific stress, stimuli, or signaling molecules (Smith *et al*., 1996; Vane *et al*., 1998). COXs isoforms, cy-

clooxygenase-1 (COX-1), and cyclooxygenase-2 (COX-2), encoded by distinct genes, have been identified in eukaryotic cells. COX-1 is constitutively expressed in most cell types and acts to maintain physiological functions. In contrast, COX-2 is largely an inducible enzyme, by pro-inflammatory cytokines, tumor promoters, oncogenes, and growth factors, and is involved mainly in the regulation of inflammation responses (Smith *et al*., 1994; Langenbach *et al*., 1999; O'Banion, 1999; Crofford, 2000; Crofford *et al*., 2000) in numerous types of cells such as monocytes, fibroblasts, and endothelial cells (Rodriguez *et al*., 1993; Perkins and Kniss, 1997; Rich *et al*., 1998; Tanabe and Tohnai, 2002; Zaric and Ruegg, 2005; Mbonye *et al*., 2006). Previous evidence suggested that inflammatory mediators such as cyclooxygenases (COXs) and prostaglandins (PGs) impact the matrix homeostasis of articular chondrocytes by altering their metabolism (Lee *et al*., 2008). Also, inflammatory response mediators are involved with osteoarthritis (OA) (Hardy *et al*., 2002; Yoon *et al*., 2002; Gosset *et al*., 2006, 2008).

Cox-2 expression is regulated at transcription, post-transcription, and translation. COX-2 transcription is induced by various exogenous stimuli that regulate the intracellular signaling pathway, which in turn modulates the activity of transcription factors (Herschman *et al*., 1997). Stabilization and nuclear export of COX-2 mRNA at post-transcriptional levels are also necessary for maximal COX-2 induction (Ristimaki *et al*., 1994; Srivastava *et al*., 1994; Jang *et al*., 2003). In addition, activities of MAPKs, including ERKs, p38MAPK, and JNKs, were reported to be important for COX-2 expression (Chen *et al*., 2001; Hunot *et al*., 2004). COX-2 is an N-glycoprotein with four glycosylation sites (Hla and Neilson, 1992; Nemeth *et al*., 2001). It has been previously shown that inhibition of COX-2 N-glycosylation by site-directed mutagenesis or tunicamycin (TN), a protein N-glycosylation inhibitor, results in expression of COX-2 with reduced molecular mass and activity (Otto *et al*., 1993), indicating the importance of this co-translational modification in COX-2 enzyme catalysis.

The lumen of the endoplasmic reticulum (ER) is a specialized organelle for the production of secretory and membrane proteins, and provides an environment for this synthesis. In addition, the ER provides a cellular site to assist protein folding and to assure that the accurately folded protein is delivered to the correct secretory pathway (Chevet *et al*., 2001; Kaufman, 2002; Ron, 2002). If confusion such as redox status and perturbation of calcium homeostasis occurs in the endoplasmic reticulum, misfolded and/or unfolded proteins will be accumulated in the endoplasmic reticulum (Zhang and Kaufman, 2006a, 2006b; Malhotra and Kaufman, 2007). This perturbation in the endoplasmic reticulum results in destruction of the homeostasis for principle cellular responses, including production of properly folded proteins by reducing endoplasmic reticulum efficiency, resulting in activation of the unfolded protein response (UPR) pathway. To maintain cellular homeostasis against any ER dysfunction, the UPR pathway increases the expression of molecular chaperones including glucose regulated protein (grp) 78 and grp94, a molecular chaperone that acts within the ER, to accumulate the protein folding activity and thus prevent the aggregation of unfolded proteins in the ER (Kozutsumi *et al*., 1988).

The damage of these ER functions has been implicated in several human pathologies including viral infection, ischemic injury, neurodegenerative disorders, and metabolic diseases. In addition, inhibition of N-glycosylation has been reported to be associated with inflammation induced by endoplasmic reticulum stress (Aridor and Balch, 1999; Hung *et al*., 2004; Schroder and Kaufman, 2005). 2-deoxy-D-glucose (2DG) is a synthetic glucose wherein the hydroxyl group at the second position of the glucose molecule is replaced with a hydrogen group (Sols and Crane, 1954; Tower, 1958). 2DG is known as a potent inducer of endoplasmic reticulum stress (ER stress) by inhibiting glycolysis and N-glycosylation of proteins (Harding *et al*., 2002; Zhang and Kaufman, 2004; Schroder and Kaufman, 2005). Treatment of 2DG in cells induces ER stress and activates the UPR in the endoplasmic reticulum in rabbit articular chondrocytes. In a recent study we reported that the Src kinase pathway is a key modulator in the regulation of cellular responses such as inflammation and differentiation in chondrocytes (Yu *et al*., 2006). Here, we show that the treatment of chondrocytes

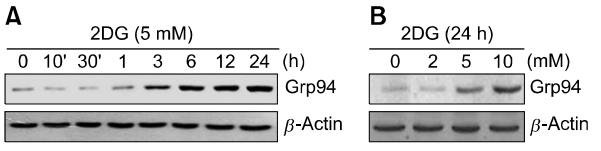

Figure 1. Treatment of 2DG led to ER stress in primary rabbit chondrocytes. (A) Chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods in rabbit articular chondrocytes. (B) Chondrocytes were untreated or treated with specific concentrations of 2DG for 24 h in rabbit articular chondrocytes. (A, B) Expressions of glucose-regulated protein of 94 kDa (grp94) were detected by a Western blot analysis. Expressions of β-actin were used as a loading control. The data represent a typical experiment, and similar results were obtained from four independent experiments.

with 2DG induced ER stress and promoted expression of inflammation molecules such as COX-2 and PGE_2. Furthermore, the COX-2 expression and PGE_2 production induced by 2DG occurred through a Src kinase pathway.

Results

Treatment of 2DG induced ER stress and reduced COX-2 expression/N-glycosylation in chondrocytes

2-deoxy-D-glucose (2DG) is known as an ER stress inducer, acting *via* prevention of N-glycosylation of proteins, and this compound may regulate cellular responses. To confirm the effects of 2DG, chondrocytes were treated with 5 mM 2DG for the indicated time periods or treated with the specific concentrations of 2DG for 24 h (Figure 1). We determined the expression level of glucose-regulated protein of 94 kDa (Grp94), an indicator for ER stress, in 2DG treated condrocytes (Figure 1). As shown in Figure 1, the expression of grp94 was dramatically enhanced in response to 2DG in a dose- and time-dependent manner, as determined by a Western blot analysis, respectively. The increase in grp94 was apparent ~3 h after treatment of 2DG (Figure 1A). These data indicated that 2DG induces ER stress in rabbit articular chondrocytes (Figure 1). Actin was not

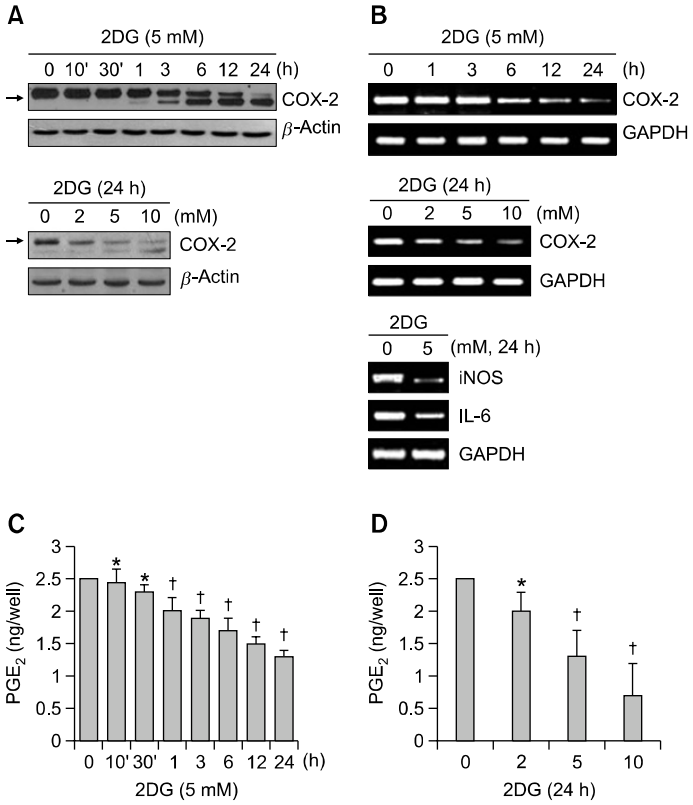

Figure 2. Treatment of 2DG decreased the expression and activity of COX-2 in chondrocytes. (A) Primary chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods (upper panel) or with the specified concentrations of 2DG for 24 h (lower panel). Expression of COX-2 was detected by a Western blot analysis and expression of β-actin was used as a loading control. (B) Articular chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods (upper panel) or with the specificied concentrations of 2DG for 24 h (middle and lower pannels). Expressions of COX-2, iNOS and IL-6 were detected by RT-PCR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (C, D) Chondrocytes were untreated or treated with 5 mM 2DG for the indicated time periods (C) or with the specificied concentrations of 2DG for 24 h (D). Production of PGE_2 was performed by a PGE_2 assay kit. Data are presented as results of a typical experiment (A-B) and as mean values with standard deviation (C, D) (n = 4). *, $P < 0.05$, †, $P < 0.01$ compared with untreated cells. The *arrow* indicates expression of the reduced molecular mass of COX-2 (~66 kDa) following 2DG treatment.

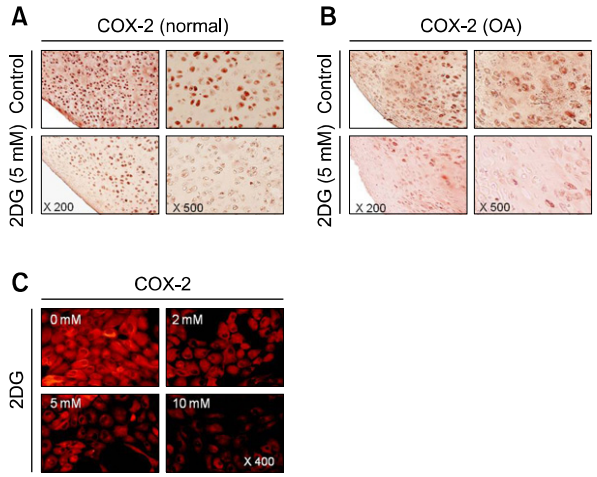

Figure 3. Treatment of 2DG caused reduction of COX-2 in both cartilage explants and chondrocytes. (A) Cartilage explants from undamaged part of rabbit cartilage (normal) were untreated or treated with 5 mM 2DG for 48 h. Expressions of COX-2 were detected by immunohistochemical staining. (B) Cartilage explants from osteoarthritis-affected human joint cartilage (OA) were untreated or treated with 5 mM 2DG for 48 h. Expressions of COX-2 were detected by immunohistochemical staining. (C) Primary rabbit articular chondrocytes were untreated or treated with 5 mM 2DG for 24 h. Expressions of COX-2 were detected by immunofluorescence staining. The data are typical results from four independent experiments with similar results.

affected by 2DG-induced ER stress in chondrocytes. Accordingly, we used actin as a loading control (Figure 1).

To determine the role of ER stress on COX-2 expression of chondrocytes, we investigated COX-2 expression in 2DG treated cells. Treatment of cells with 2DG led to a dramatic reduction of COX-2 expression and a shift in the molecular mass of COX-2 from 72-74 to 66-70 kDa (marked with *arrow*) in a time- and dose-dependent manner, determined *via* a Western blot analysis (Figure 2A). Remarkably, the 2DG-mediated decrease in COX-2 molecular mass is associated with inhibition of COX-2 N-glycosylation during translation. The reduction in COX-2 protein levels appears to be due to a time- and dose-dependent decrease in transcription upon 2DG treatment (Figure 2B). 2DG did not affect COX-1 protein expression (data not shown). Next, we tested whether there are other inflammatory-related genes such as inducible nitric oxide synthase (iNOS) and interleukin (IL)-6 influenced by 2DG in condrocytes. As expected, 2DG led to decrease in iNOS and IL-6, as shown by RT-PCR. These results provided that 2DG regulates not only COX-2 expression but also other inflammatory-related genes in rabbit articular chondrocytes (Figure 2B, lower pannel). We subsequently evaluated whether 2DG-modified COX-2 is associated with (non) proteolytic mechanisms. However, inhibition of COX-2 glycosylation by 2DG at 10 mM did not interfere with MG132 (data not shown). To assess whether 2DG-modified COX-2 is functional, the effect of 2DG on the production of PGE_2, a major and stable COX-2 metabolite, in articular chondrocytes was next investigated. As shown in Figures 2C and 2D, production of PGE_2 was significantly decreased in cells treated with 2DG, which was considerably detectable ~1 h after 2DG treatment. A maximum of ~40% inhibition of PGE_2 production by 2DG treatment during 24 h was observed. These results suggest that 2DG inhibits COX-2 activity by preventing COX-2 N-glycosylation. Taken together, these data indicate that 2DG-modified COX-2 molecular mass and activity might be associated with COX-2 N-glycosylation.

We next examined whether ER stress by 2DG causes reduction of COX-2 in cartilage explants and chondrocytes. Cartilage explants were treated with 5 mM 2DG for 24 h (Figure 3A). 2DG caused a reduction of COX-2, as demonstrated by immunocytochemical staining in cartilage (Figure 3A). We also investigated the expression of COX-2 by 2DG in human osteoarthritic cartilage obtained from patients. The decrease in COX-2 levels by 2DG was also detected in human osteoarthritic cartilage (Figure 3B). Similar to the effects on cartilage, immunofluorescence staining also revealed that 2DG caused a reduction of COX-2 in chondrocytes (Figure 3C). The above results clearly indicate that 2DG regulated reduction of COX-2 expression in both cartilage explants and chondrocytes (Figure 3).

ER stress effect by 2DG modulated COX-2 expression and activity in primary chondrocytes

The above results show that 2DG regulated reduction of COX-2 by the ER stress pathway in rabbit articular chondrocytes. We further investigated the relationship between ER stress and COX-2 expression by the treatment of 2DG in rabbit articular chondrocytes. We examined the effects of salubrinal (inhibitor of ER stress, Salub) on the reduction of COX-2 expression by 2DG-induced ER stress in chondrocytes (Figure 4). As expected, treatment of cells with 2DG in the presence of salubrinal abrogated the 2DG-induced increase in grp94 protein level (Figure 4A). Furthermore, the expression level and N-glycosylation of COX-2 were recovered by inhibition of ER stress with salubrinal in cells (Figure 4A). Consistent with the effect of COX-2 expression, the production of PGE_2 was rescued by salubrinal, detected by a PGE_2 assay (Figure 4B). These

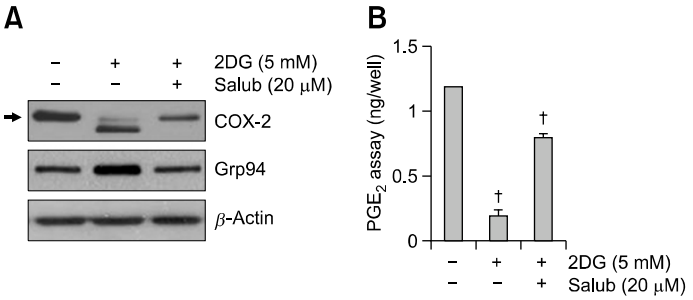

Figure 4. ER stress by treatment of 2DG reduced COX-2 expression and PGE_2 production in rabbit articular chondrocytes. (A, B) Chondrocytes were treated with 5 mM 2DG for 24 h in the presence of 20 uM salubrinal (salub). (A) Expression levels of COX-2 and grp94 were detected by a Western blot analysis. (B) Production levels of PGE_2 were determined using a PGE_2 assay kit. These data are results of a typical experiment (A) and given as mean values with standard deviation (B) (n = 4). *, $P < 0.05$, †, $P < 0.01$ compared with untreated cells. The *arrow* indicates expression of the reduced molecular mass of COX-2 (~66 kDa) following 2DG treatment.

findings suggest that the reduction of COX-2 by 2DG is caused via an ER stress related pathway (Figure 4).

COX-2 expression by 2DG-induced ER stress was regulated through the SRC pathway

Next, we investigated the molecular mechanisms of COX-2 modulation by regulating ER stress via 2DG. We examined the possible signaling pathways, that is, ERK-1/-2, p38 kinase, and the SRC pathway, because these pathways, identified in previous reports, are related with COX-2 expression of chondrocytes (Yu *et al*., 2006). Among these signaling pathways, phosphorylation of SRC was dominantly increased by 2DG treatment. Activity of ERK-1/-2 and p38 kinase, respectively, was also increased by 2DG treatment alone (data not shown). However, inhibition of ERK-1/-2 and p38 kinase with PD98059 or SB203580 slightly inhibited grp94 expression and COX-2 expression was vaguely recovered by 2DG (data not shown).

To examine the effects of the SRC pathway on 2DG modulated COX-2 expression, cells were precisely examined with various concentrations of PP2 (a potent inhibitor of Src kinase) for 1 h before 2DG treatment (Figure 5). The inhibition of Src with PP2 significantly blocked grp94 expression in a

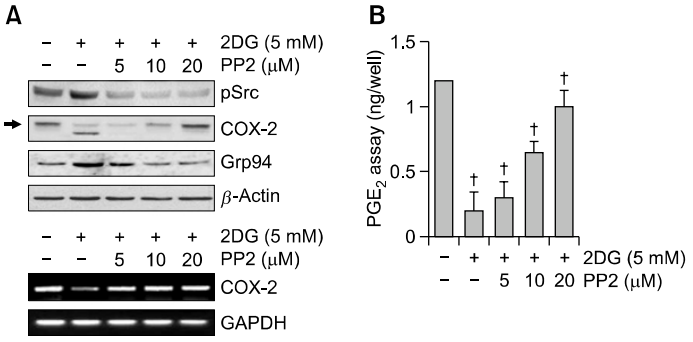

Figure 5. COX-2 expression by 2DG-induced ER stress was regulated *via* the Src pathway in primary chondrocytes. (A, B) Articular chondrocytes were treated with 2DG for 24 h in the presence of PP2 (a potent inhibitor of Src kinase). (A) Expression levels of pSrc, COX-2, and grp94 were detected by a Western blot analysis (A, upper panel). Transcriptional level of COX-2 was determined by RT-PCR (A, lower panel). GAPDH was used as a loading control (A, lower panel). (B) Production of PGE_2 was determined using a PGE_2 assay kit. The data are typical results from four independent experiments with similar results and are given as mean values with standard deviation (B) (n = 4). *, $P < 0.05$, †, $P < 0.01$ compared with untreated cells. The *arrow* indicates expression of the reduced molecular mass of COX-2 (~66 kDa) following 2DG treatment.

dose-dependent manner. Also, 2DG treatment dose-dependently inhibited COX-2 glycosylation, resulting in expression of the low molecular mass of COX-2 (mostly ~66 kDa), as demonstrated by a Western blot analysis (Figure 5A, upper panel). As expected, PP2 did not affect 2DG-mediated expression of steady-state COX-2 mRNA 2 (Figure 5B, lower panel). Also, production of PGE_2 was recovered via inhibition of pSrc with PP2 treatment, detected by a PGE_2 assay (Figure 5B). These results collectively demonstrate that the Src pathway is necessary for 2DG induced reduction of COX-2 expression/N-glycosylation and a loss of COX-2 activity in rabbit articular chondrocytes (Figure 5).

Discussion

COX-2 has four glycosylation sites and N-glycosylation of COX-2 is prevented by glycosylation inhibitors such as 2-deoxy-D-glucose and tunicamycin (Hla and Neilson, 1992; Otto *et al*., 1993; Nemeth *et al*., 2001). Interestingly, inhibition of N-glycosylation leads to the expression of COX-2 with declined molecular mass and activity, which is important for COX-2 enzyme catalysis. Endoplasmic reticulum (ER) stress can regulate several cellular responses including apoptosis, proliferation, inflammation, and differentiation of numerous cells (Hotamisligil, 2005; Pallet *et al*., 2008; Ulianich *et al*., 2008; Hsu *et al*., 2009). Glucosamine (GS) has been well established as a substitutable medical drug for rheumatoid arthritis or osteoarthritis (Reginster *et al*., 2001; Hua *et al*., 2005). Previous studies have demonstrated that GS regulates expression of COX-2 and production of PGE_2 *via* IL-1β treatment in A549 human lung epithelial cells (Jang *et al*., 2007). Also, GS reduced IL-1β induced COX-2 expression at both transcriptional and translational levels in chondrocytes and synoviocytes (Nakamura *et al*., 2004). GS also has been implicated in ER stress in skeletal muscle cells, carydiomyocytes, and astrogilal cells (Matthews *et al*., 2007; Ngoh *et al*., 2009; Raciti *et al*., 2010). ER-stress can be induced by N-glycosylation inhibition of COX-2 *via* treatment of 2DG. The mechanism by which ER stress regulates COX-2 expression in articular chondrocytes is presently unknown. The present findings obtained from GS treated cells closely coincide with the observed effect on COX-2 expression by 2DG. Based on this finding, therefore, we demonstrated that the induction of ER stress by 2DG reduced COX-2 expression/N-glycosylation and activity, and this effect was regulated by the Src signaling pathway in rabbit articular chondrocytes. As shown in Figure 1, treatment of 2DG induced GRP94 expression in a time- and dose-dependent manner, as determined by a Western blot analysis, in rabbit articular chondrocytes. We also confirmed that this ER stress by 2DG regulated inflammation of chondrocytes. COX-2 is mainly an inducible enzyme, by stimulating cellular stresses and inflammatory cytokines, and is involved in the modulation of inflammation (Smith *et al*., 1996; Vane *et al*., 1998). As shown in Figure 2, similar to the expression levels of GRP protein, the expression of COX-2 by 2DG mediated ER stress was increased in a dose- and time-dependent manner in chondrocytes. It is of particular note that the expression pattern of COX-2 by 2DG treatment is different compared with that of normal COX-2. There are two apparent patterns of COX-2, ~66-70 kDa protein and ~72-74 kDa protein, in 2DG treated cells. The lower molecule protein of ~66-70 KDa is an unglycosylated COX-2 due to the effects of 2DG and the higher molecule protein of ~72-74 KDa is a normally glycosylated COX-2 in chondrocytes. In chondrocytes treated with 2DG, the unglycosylated COX-2 protein increased in quantity whereas the glycosylated COX-2 protein decreased (Figure 2A). Consistent with the COX-2 expression level, a PGE_2 assay also revealed that 2DG reduced production of PGE_2 in a dose- and time-dependent manner in chondrocytes (Figure 2C, D). The production of PGE_2 decreased within 1 h after 2DG treatment (Figure 2C). Furthermore, immunohistochemical staining and immunofluorescence staining reflected a reduction of COX-2 expression in articular cartilage and chondrocytes (Figure 3). Treatment of 2DG in the presence of salubrinal reduced GRP94 expression while COX-2 expression/N-glycosylation and PGE_2 production were recovered by salubrinal in rabbit articular chondrocytes (Figure 4). These effects accurately showed that 2DG decreased expression and the molecular mass of COX-2, and the loss of COX-2 activity was regulated by ER stress.

To elucidate the mechanism regulating the 2DG reduced expression of COX-2, we evaluated several signaling pathways under consideration of the findings of previous reports. Since MAP kinase and SRC pathways are the major signaling pathways involved in COX-2 expression of chondrocytes, we tested whether the above results were attributable to regulation of these pathways. Interestingly, MAP kinase, ERK-1/2, and p38 kinase were activated, at least in part, by treatment of 2DG; however, the inhibition of ERK-1/2 and p38 kinase with PD98059 or SB203580 failed to completely recover COX-2 expression (data not shown).

Furthermore, the phosphorylation of SRC occurred by the addition of 2DG in chondrocytes. The inhibition of phosphorylated SRC with PP2 treatment also recovered COX-2 expression/N-glycosylation and PGE_2 production while GRP94 was decreased *via* the addition of PP2 in chondrocytes. RT-PCR revealed the same results as the above results in chondrocytes. In conclusion, 2DG-induced ER stress reduced COX-2 expression/N-glycosylation, and PGE2 production was associated with the SRC pathway in rabbit articular chondrocytes.

Methods

Culture of primary chondrocytes and experimental conditions

Rabbit articular chondrocytes from joint cartilage slices of 2-week-old New Zealand White rabbits were isolated with 0.2% collagenase type II (381 units/ml of solid, Sigma, MO), as described previously (Ryu *et al*., 2002). To summarize, cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 units/ml of solid, Sigma, St.Louis, MO) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA). Individual cells were obtained by brief centrifugation. The cells were suspended in DMEM supplemented with 10% (v/v) bovine calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin, and were then plated on culture dishes at a density of 5×10^4 cells/cm^2. The medium was replaced every 2 days after seeding. The 3 day cell cultures were treated with various reagents with a glucose-free medium before treatment with the reagent.

Western blot analysis

Whole cell lysates were prepared as described previously. Proteins from chondrocytes were extracted using a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors and phosphatase inhibitors. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and the nitrocellulose membrane was blocked with 5% non-fat dry milk in Tris-buffered saline. The following antibodies were employed to detect proteins: anti-COX-2 (Cayman Chemical, Ann Arbor, MI), anti-grp94 (Santa Cruz, CA), anti-phospho-Src (Santa Cruz, CA), and anti-β-actin (Santacruz, CA). The blots were developed using a peroxidase-conjugated secondary antibody and an ECL system.

PGE_2 assay

PGE_2 production was determined by measuring the levels of cellular and secreted PGE_2 using a PGE_2 linked immunosorbent assay kit purchased from Amersham Biosciences. Chondrocytes were seeded in standard 96-well microtiter plates at 2×10^4 cells/well. After treatment with reagents, total cell lysates were used to quantify the amount of PGE_2 according to the manufacturer's protocol. PGE_2 levels were calculated against a standard curve of PGE_2.

Immunocytochemical staining and immunofluorescence staining

Rabbit joint cartilage explants or human osteoarthritic cartilage were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, washed with PBS, dehydrated with graded ethanol, embedded in paraffin, and sectioned at 4 μM thickness. The sections were stained by standard procedures using antibodies against COX-2 (Cayman Chemical, Ann Arbor, MI) and visualized by developing with a kit purchased from DAKO (Carpinteria, CA), following the procedure recommended by the manufacturer. Expression of COX-2 in rabbit articular chondrocytes was determined by indirect immunofluorescence microscopy, as described previously (Yoon *et al*., 2002). Briefly, chondrocytes were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized and blocked in PBS containing 0.1% Triton X-100 and 5% fetal calf serum for 30 min. The fixed chondrocytes were washed with PBS and incubated for 1 h with the antibody (10 μg/ml) against COX-2. The cells were washed for 30 min, and observed under a fluorescence microscope.

RNA extraction and reverse transcriptation-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIZOL (Life Technology. MD) according to the manufacturer's protocol. Total RNA was reverse-transcribed using a Maxime RT Premix kit (Intron Biotechnology, Seoul, Korea) in accordance with the manufacturer's instructions with a PCR thermal cycler. RNA PCR conditions were 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s for a total of 25 cycles. The PCR primers used were COX-2 (298-bp product) sense, 5-TCAGCCACGCAGCAAATCCT-3, and antisense, 5-GTGATCTGGATGTCACG-3, and glyceraldehyde-3-phosphate dehydrogenase (363-bp product) sense, 5-CATCATCCCTGCCTCTACTGG-3, and antisense, 5-TCCACCACCCTGTTGCTGTA-3. The amplified products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Data analysis and statistics

The results are expressed as mean values with standard deviation. Values calculated from the specified number of determinations. An Anova test was used to compare individual treatments with their respective control values. Significance was defined at the $P < 0.05$ or 0.01 level.

Acknowledgements

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