The Carbon Monoxide-Releasing Molecule Tricarbonyldichlororuthenium(II) Dimer Protects Human Osteoarthritic Chondrocytes and Cartilage from the Catabolic Actions of Interleukin-1β

Javier Megías, María Isabel Guillén, Antonio Bru, Francisco Gomar, and María José Alcaraz

Department of Pharmacology, Faculty of Pharmacy, University of Valencia, Burjasot, Valencia, Spain (J.M., M.I.G., M.J.A.); Department of Chemistry, Biochemistry, and Molecular Biology, Cardenal Herrera-CEU University, Moncada, Valencia, Spain (M.I.G.); Department of Orthopaedic Surgery and Traumatology, General Hospital, Valencia, Spain (A.B.); and Department of Surgery, Faculty of Medicine, University of Valencia, Spain (F.G.)

Received November 23, 2007; accepted January 11, 2008

ABSTRACT

We have investigated the effects of a carbon monoxide-releasing molecule, tricarbonyldichlororuthenium(II) dimer (CORM-2), on catabolic processes in human osteoarthritis (OA) cartilage and chondrocytes activated with interleukin-1β. In these cells, proinflammatory cytokines induce the synthesis of matrix metalloproteinases (MMPs) and aggrecanases, including members of a disintegrin and metalloproteinase with thrombospondin domain (ADAMTS) family, which may contribute to cartilage loss. CORM-2 down-regulated MMP-1, MMP-3, MMP-10, MMP-13, and ADAMTS-5 in OA chondrocytes, and it inhibited cartilage degradation. These effects were accompanied by increased aggrecan synthesis and collagen II expression in chondrocytes. Our results also indicate that the inhibition of extracellular signal-regulated kinase 1/2 and p38 activation by CORM-2 may contribute to the maintenance of extracellular matrix homeostasis. These observations suggest that CORM-2 could exert chondroprotective effects due to the inhibition of catabolic activities and the enhancement of aggrecan synthesis.

Osteoarthritis (OA) is characterized by a progressive erosion of articular cartilage. After an initial anabolic reaction to attempt repair, the process of matrix degradation is carried out by matrix metalloproteinases (MMPs) and aggrecanases (Goldring, 2000). OA depletion of the most abundant cartilage proteoglycan, aggrecan, is thought to initiate the events leading to cartilage degradation, and it could be dependent on an accelerated rate of aggrecanolysis, exceeding the ability of biosynthetic replacement. Aggrecan cleavage is caused mainly by MMPs and aggrecanases, including members of a disintegrin and metalloproteinase with thrombospondin domain (ADAMTS) family. Recent studies support a key role for ADAMTS-mediated aggrecanolysis in cartilage destruction (Malfait et al., 2002; Sandy, 2006).

Interleukin (IL)-1β and other proinflammatory cytokines have been detected in OA synovial fluid and chondrocytes. These mediators have been shown to induce MMP and aggrecanase synthesis in chondrocytes in an autocrine/paracrine manner, which may contribute to cartilage loss in OA (Elson et al., 1998; Tetlow et al., 2001). IL-1β is involved in collagenase-mediated cleavage of collagen II, degradation of aggrecan, and the inhibition of gene expression of matrix molecules (Kobayashi et al., 2005). It has also been demonstrated that this cytokine reduces the production of cartilage matrix components such as aggrecan (Gouze et al., 2001) and type II collagen (Goldring et al., 1988).

Carbon monoxide-releasing molecules (CO-RMs) are a new group of drugs able to reproduce the biological actions of CO...
derived from heme oxygenase-1 (HO-1) activity (for review, see Foresti et al., 2005). Therefore, the vasoactive (Foresti et al., 2004) and cardioprotective (Clark et al., 2003) effects of CO-RMs have been demonstrated. These agents are also able to deliver CO and protect isolated kidneys against cold preservation and ischemia-reperfusion (Sandouka et al., 2006). It is interesting to note that CO-RMs have shown anti-inflammatory effects in some cell lines, including RAW 264.7 macrophages (Sawle et al., 2005), microglia (Bani-Hani et al., 2006), and Caco-2 (Megias et al., 2007). In addition, these compounds can modulate leukocyte-endothelial cell interactions (Urquhart et al., 2007). However, studies on the regulation of chondrocyte or cartilage metabolism by CO-RMs have not yet been reported. Previous observations from our laboratory suggest a beneficial role for HO-1 in OA chondrocytes (Guillen et al., 2007). We postulate that CO-RMs may exert protective effects on these cells. To test this hypothesis, we have investigated whether the CO-RM tricarbonyldichlororuthenium(II) dimer (CORM-2) may modulate catabolic processes in human OA cartilage and chondrocytes activated with IL-1β.

Materials and Methods

Reagents. IL-1β was from PeproTech EC Ltd. (London, UK). Antibodies against phosphorylated or total extracellular signal-regulated kinase (ERK)1/2, c-Jun NH₂-terminal kinase (JNK), and p38 were from R&D Systems (Minneapolis, MN). The peroxidase-conjugated IgGs were purchased from Dako Denmark A/S (Copenhagen, Denmark). CORM-2, RuCl₃, and other reagents were from Sigma-Aldrich (St. Louis, MO).

Chondrocyte and Explant Culture. Cartilage specimens were obtained from patients with the diagnosis of advanced OA [16 females and 8 males, 72 ± 3 (mean ± S.E.M.) years old] undergoing total knee joint replacement. Diagnosis was based on clinical, laboratory, and radiological evaluation. Samples were obtained under the Institutional Ethical Committee-approved protocol. Cartilage slices were removed from the femoral condyles and tibial plateaus, and they were cut into small pieces. Chondrocytes were isolated by sequential enzymatic digestion: 1 h with 0.1 mg/ml hyaluronidase (Sigma-Aldrich) followed by 12 h with 1 mg/ml collagenase (type IA) (Sigma-Aldrich) at 37°C in DMEM/Ham’s F-12 medium (Sigma-Aldrich) containing 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂ atmosphere. The digested tissue was filtered through a 70-µm nylon mesh, and then it was washed and centrifuged. Cell viability was greater than 95% according to the trypan blue exclusion test. The isolated chondrocytes were seeded at 2.5 × 10⁶ cells/well in six-well plates. Cells were cultured in DMEM/Ham’s F-12 medium supplemented with 10% human serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified 5% CO₂ incubator at 37°C. Chondrocytes in primary culture were allowed to grow until near confluence, and then they were incubated with CORM-2 at 37°C. Chondrocytes in primary culture were allowed to grow until near confluence, and then they were incubated with CORM-2 for 24 h, cells were labeled with 2 µCi/ml [³⁵S]sulfate for 24 h. Cells were washed with Hanks’ balanced salt solution, and then they were extracted with 4 M guanidinium HCl, 5 mM EDTA, and 5 mM Na acetate, pH 7.2, for 48 h at 4°C. Proteoglycans absorbed on Whatman filter paper (Whatman, Maidstone, UK) were precipitated by ethylenediamine chloride monohydrate, and radioactivity was measured by liquid scintillation counting. Total radioactivity (medium + cell) was calculated for each well, and the value was normalized with respect to protein content.

Immunocytochemistry. Chondrocytes in primary culture were allowed to grow until near confluence, and then they were incubated with 100 µM CORM-2 in the presence or absence of 100 U/ml IL-1β for 15 days, with renewal of medium and treatment every 4 days. Cells were fixed with 4% formaldehyde in phosphate-buffered saline for 30 min at 4°C, and collagen II was detected using the type II collagen staining kit (MD Biosciences, Zurich, Switzerland), following the manufacturer’s instructions.

Western Blot Analysis. After 15-min stimulation with 100 U/ml IL-1β or IL-1β + CORM-2 for 24 h, cells were labeled with 2 µCi/ml [³⁵S]sulfate for 24 h. Cells were washed with Hanks’ balanced salt solution, and then they were extracted with 4 M guanidinium HCl, 5 mM EDTA, and 5 mM Na acetate, pH 7.2, for 48 h at 4°C. Proteoglycans absorbed on Whatman filter paper (Whatman, Maidstone, UK) were precipitated by ethylenediamine chloride monohydrate, and radioactivity was measured by liquid scintillation counting. Total radioactivity (medium + cell) was calculated for each well, and the value was normalized with respect to protein content.

Real-Time PCR. Chondrocytes in primary culture were stimulated with 100 U/ml IL-1β or IL-1β + 100 µM CORM-2 for 12 h. Total RNA was extracted using the TRIzol reagent (Invitrogen, Barcelona, Spain) according to the manufacturer’s instructions. Reverse transcription was accomplished on 1 µg of total RNA using random primers (TaqMan reverse transcription reagents; Applied Biosystems Spain, Madrid, Spain). PCR reactions were performed using SYBR Green PCR Master Mix (Bio-Rad, Madrid, Spain). Primers were purchased from Superarray Bioscience Corporation (Frederick, MD). PCR assays were performed in duplicate on an iCycler Real-Time PCR Detection System (Bio-Rad) running the following cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Reaction specificity was determined by melt curve analysis that was performed by heating the plate from 55 to 95°C and measuring SYBR Green 1 dissociation from the amplicons. Cycle thresh-
old (C<sub>T</sub>) values for each gene were corrected using the mean C<sub>T</sub> value for β-actin. Relative gene expression was calculated using the ΔΔC<sub>T</sub> method, and values are expressed as fold change (2^-ΔΔC<sub>T</sub>) relative to the expression values in nonstimulated cells.

**Enzyme-Linked Immunosorbent Assay.** Chondrocytes in primary culture were stimulated with 100 U/ml IL-1β or IL-1β + CORM-2 for 24 h. Supernatants were harvested, and then they were centrifuged and frozen at −80°C until analysis. Pro-MMP-1, total MMP-3, total MMP-10, and pro-MMP-13 protein were quantified in supernatants by using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems, with sensitivity of 21.0, 9.0, 4.1, and 7.7 pg/ml, respectively. Aggrecanase activity was measured in supernatants by a sensitive ELISA kit detecting ADAMTS-1, ADAMTS-4, and ADAMTS-5, with sensitivity of 2 pM (MD Biosciences).

**Data Analysis.** Results are presented as mean ± S.E.M. Statistical analyses were performed using one-way analysis of variance followed by Dunnett’s t test for multiple comparisons and unpaired Student’s t test for dual comparisons.

## Results

**Effects on MMPs and Aggrecanases.** We determined the effects of CORM-2 on IL-1β-mediated induction of several enzymes relevant to cartilage degradation. After stimulation of OA chondrocytes with IL-1β for 24 h, enhanced levels of MMP-1, MMP-3, MMP-10, and MMP-13 were detected in the medium by ELISA (Fig. 1). CORM-2 significantly reduced the protein levels of these enzymes, whereas the negative control RuCl<sub>3</sub> was ineffective. CORM-2 treatment resulted in a concentration-dependent decrease in MMP-3, MMP-10, and MMP-13 levels, whereas MMP-1 was inhibited at the highest concentration (150 μM). In addition, aggrecanase activity was measured in chondrocyte supernatants as the release of specific aggrecan neoepitopes, detected by ELISA, which was significantly decreased by CORM-2 in a concentration-dependent manner (Fig. 2). We then examined the effects of CORM-2 on mRNA expression of these enzymes. Analysis of mRNA levels by real-time PCR showed that CORM-2 treatment reduced the expression of MMP-10, MMP-13, and ADAMTS-5 mRNA, whereas the reductions in MMP-1, MMP-3, and ADAMTS-4 were not significant (Fig. 3, A and B).

The observed effects were not due to cytotoxicity, because cell viability was not significantly modified by 50 to 150 μM CORM-2 either in the absence or presence of IL-1β as determined by the MTT assay (data not shown). As expected, the negative control RuCl<sub>3</sub> at 150 μM used in some assays also showed no cytotoxicity. Taken together, these results suggest that CORM-2 blocks the effects of IL-1β on primary chondrocytes by down-regulation of several matrix-degrading enzymes, thereby preventing cartilage damage.

**Effects on the Degradation of Cartilage Matrix and the Synthesis of Glycosaminoglycan.** We next examined whether CORM-2 activity on degrading enzymes translates into protective effects on cartilage. IL-1β treatment of cartilage explants induced the degradation of matrix, which was measured as the release of glycosaminoglycan. Figure 4A shows that CORM-2 treatment at 100 or 150 μM overcame the effects of IL-1β on glycosaminoglycan release. We next wanted to determine whether CORM-2 treatment results in an interaction with anabolic processes in chondrocytes. Synthesis of aggrecan was investigated using Na<sub>2</sub>SO<sub>4</sub> incorporation into chondrocytes. Basal aggrecan synthesis in OA chondrocytes was significantly decreased by IL-1β (Fig. 4B). CORM-2 at the concentrations of 50 and 100 μM significantly counteracted the down-regulation of glycosaminoglycan synthesis by this cytokine.

**Effects on Collagen II Expression.** To evaluate the influence of CORM-2 on collagen II, we carried out immunocytochemical analyses. Figure 5 shows that OA chondrocytes in culture exhibit a high level of collagen II expression. In contrast, IL-1β stimulation results in a dramatic reduction of this protein with respect to basal incubations. It is interesting to note that 100 μM CORM-2 treatment maintained the expression of this extracellular matrix component in cells in basal conditions, and it restored collagen II expression in chondrocytes stimulated with IL-1β.

**Effects on Mitogen-Activated Protein Kinase Activation.** In human articular chondrocytes, the activation of mi-
togen-activated protein kinase (MAPK) signaling by IL-1β plays an important role in anabolic and catabolic processes (Fan et al., 2007). Stimulation of OA chondrocytes with IL-1β led to a rapid induction of ERK1/2, JNK, and p38 phosphorylation. As shown in Fig. 6, 100 \( \mu \)M CORM-2 inhibited ERK1/2 and p38 phosphorylation in primary chondrocytes stimulated with IL-1β, whereas JNK activation was not affected by this compound.
enzymes, including collagenases and aggrecanases (Goldring et al., 2004). There is clear evidence that MMP-1 (collagenase-1) and MMP-13 (collagenase-3) play an important role in the degradation of collagen II in the extracellular matrix (Billinghurst et al., 1997; Wu et al., 2002). It has also been reported that MMP-13 is the major collagenase in OA cartilage (Bau et al., 2002; Burrage et al., 2006). The present findings demonstrate the ability of CORM-2 to inhibit the production of both enzymes, with a higher effect on MMP-13.

The activation of collagenolytic MMPs depends on proteolysis by serine proteinases and other MMPs, which may be a rate-limiting step in cartilage collagenolysis (Milner et al., 2001). In this respect, it is interesting to note the role of MMP-3 (stromelysin 1) and MMP-10 (stromelysin 2) in activating the proforms of collagenases (Murphy et al., 1987; Knauper et al., 1996), leading to a significant increase in cartilage collagenolysis (Barksby et al., 2006). Our studies have shown the inhibitory effects of CORM-2 on both enzymes, indicating that this agent may act at different levels in the cascade of reactions leading to collagen degradation.

It is becoming apparent that aggrecan breakdown would be dependent mainly on the activity of aggrecanases such as ADAMTS-4 and ADAMTS-5 (Malfait et al., 2002), although some MMPs such as MMP-13 are also able to degrade aggrecan (Burrage et al., 2006). ADAMTS-5 is the most strongly expressed aggrecanase in OA cartilage (Bau et al., 2002), and it plays a crucial role in catalyzing proteoglycan degradation induced by IL-1β (Stanton et al., 2005). Aggrecanase-mediated aggrecan degradation is an early feature of OA that may provide the basis to cartilage protection as the aggrecan macromolecule protects the collagen fibrillar structure from the proteolytic attack by collagenases (Malfait et al., 2002). Our data indicate that CORM-2 treatment reduces aggrecanase activity and ADAMTS-5 gene expression in human OA chondrocytes.

Herein, we have described for the first time the protective effects of a member of the CO-RM family on human OA cartilage. We have shown that CORM-2 inhibits the degradation of aggrecan but that it increases its synthesis and collagen II expression. As a result, the maintenance of aggrecan and collagen II content by CORM-2 would result in cartilage protection, avoiding the loss of tissue function. In addition, our results suggest that these actions may be mediated through the down-regulation of enzymes that target both collagen II and aggrecan, major components of extracellular matrix.

Our results indicate that CO released by CORM-2 can reproduce the protective effects of HO-1 induction in OA chondrocytes (Guillen et al., 2007). Nevertheless, whether this exogenous source of CO could be comparable with endogenous CO levels derived from HO-1 activity is not known. These limitations have been discussed in a recent study using CORM-3 (Urquhart et al., 2007). Concerning CORM-2 effects, it is known that this agent can induce HO-1 (Sawle et al., 2005; Megias et al., 2007). Nevertheless, HO-1 up-regulation by CORM-2 in chondrocytes stimulated with IL-1β is weak (Guillen et al., 2005), suggesting that this mechanism does not make a significant contribution to the observed protective effects.

To date, new strategies in cartilage protection focused on MMP inhibitors has not resulted in clinical benefit (Murphy and Lee, 2005). Therefore, the inhibition of ADAMTS-4/ADAMTS-5 has been proposed as a new pharmacological target
to develop cartilage-protecting agents (Malfait et al., 2002). It is possible that compounds such as CO-RMs, which are able to control both degradative pathways, could provide better protection. These observations support the interest of further studies on this class of agents.

Activation of MAPK signaling pathways seems to mediate IL-1β-dependent regulation of extracellular matrix components and MMPs expression in human articular chondrocytes (Fan et al., 2007). In particular, ERK1/2 is a negative regulator of chondrogenesis and chondrocyte differentiation (Yoon et al., 2002). Our results show that CORM-2 inhibits ERK1/2 and p38 phosphorylation, which plays an important role in MMPs induction, and in the down-regulation of aggrecan and collagen II expression. Interestingly, this study using primary human chondrocytes from OA patients and not cell lines suggests that the inhibition of ERK1/2 and p38 activation by CORM-2 may contribute to the maintenance of the chondrocytic phenotype and extracellular matrix homeostasis.

The ability of CO to bind metal centers in metalloproteins is known (Roberts et al., 2004), which may result in direct regulation of MAPK phosphorylation-dephosphorylation processes through possible interactions with protein phosphatase 2C (Boczkowski et al., 2006). In addition, CO could inhibit NADPH oxidase (Boczkowski et al., 2006) and increase mitochondrial reactive oxygen species production (Plantadosi, 2002), leading to the indirect modulation of MAPK signaling. Further studies would be necessary to determine the mechanisms responsible for MAPK inhibition by CORM-2 in chondrocytes. The ability of CO to react with transition metals could also result in the inhibition of metalloproteins relevant to cartilage degradation such as MMPs (Desmard et al., 2005).

In summary, CORM-2 exhibits protecting effects on cartilage metabolism through the depression of catabolic activities and the stimulation of glycosaminoglycan synthesis. These findings could affect the development of new therapies for the repair or protection of cartilage in degenerative joint diseases.

References


