JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650

Title page

The CO-releasing molecule CORM-2 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; University of Valencia, Faculty of Pharmacy, 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.); Department of Surgery, Faculty of Medicine, University of Valencia, Spain (F.G.)

JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

Running title page

Running title: Effects of CORM-2 on chondrocytes.

Address correspondence to: Prof. Maria Jose Alcaraz, Department of Pharmacology, University of Valencia, Faculty of Pharmacy, Av. Vicent Andres Estelles s/n, 46100 Burjasot, Valencia, Spain. Tel: 34-963544292; Fax: 34-963544499; e-mail: maria.j.alcaraz@uv.es

NUMBER OF TEXT PAGES: 25

NUMBER OF TABLES: 0

NUMBER OF FIGURES: 6

NUMBER OF REFERENCES: 38

NUMBER OF WORDS:

Abstract: 136 Introduction: 393 Discussion: 932

ABBREVIATIONS

ADAMTS, a disintegrin and metalloproteinase with thrombospondin domain; CORM-2, tricarbonyldichlororuthenium(II) dimer; CO-RMs, carbon monoxide-releasing molecules; CT, cycle threshold; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase; HO-1, heme oxygenase-1; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMPs, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OA, osteoarthritis.

RECOMMENDED SECTION: INFLAMMATION, IMMUNOPHARMACOLOGY AND ASTHMA.

JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

We have investigated the effects of a CO-releasing molecule, tricarbonyldichlororuthenium(II) dimer (CORM-2) on catabolic processes in human osteoarthritis (OA) cartilage and chondrocytes activated with interleukin-1β. In these cells, pro-inflammatory cytokines induce the synthesis of matrix metalloproteinases (MMPs) and aggrecanases including members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin domain) 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 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 the inhibition of catabolic activities and the enhancement of aggrecan synthesis.

Introduction

Osteoarthritis (OA) is characterized by a progressive erosion of articular cartilage. After an initial anabolic reaction attempting 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 could be dependent on an accelerated rate of aggrecanolysis exceding the ability of biosynthetic replacement. Aggrecan cleavage is caused mainly by MMPs and aggrecanases including members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin domain) 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 pro-inflammatory 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).

CO-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 (reviewed in (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). Interestingly, CO-RMs have shown anti-inflammatory effects in 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., 2008). 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.

JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

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 N-terminal kinase (JNK) and p38 were from R&D Systems (Minneapolis, MN). The peroxidase-conjugated IgGs were purchased from Dako (Copenhagen, Denmark). CORM-2, $RuCl_3$ 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, 8 males, aged 72±3 years, mean±S.E.M.) 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 cut into small pieces. Chondrocytes were isolated by sequential enzymatic digestion: 1 h with 0.1 mg/ml hyaluronidase (Sigma) followed by 12 h with 1 mg/ml collagenase (type IA) (Sigma) at 37° in DMEM/Ham's F-12 (Sigma) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37ºC in 5% CO_2 atmosphere. The digested tissue was filtered through a 70 μm nylon mesh, washed and centrifuged. Cell viability was greater than 95% according to the Trypan blue exclusion test. The isolated chondrocytes were seeded at 2.5 x 10^5 cells/well in six-well plates. Cells were cultured in DMEM/Ham's F-12 supplemented with 10% human serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 incubator at 37°C. Chondrocytes in primary culture were allowed to grow until nearly confluence and then incubated with CORM-2 at different

concentrations or vehicle for 1 h before stimulation with IL-1β (100 U/ml) for different times. CORM-2 was dissolved in dimethyl sulfoxide and then diluted in culture medium (0.1% v/v). Control cells were treated with the same vehicle. Possible cytotoxicity of treatments was assessed by the mitochondria1-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan. After appropriate stimulation, cells were incubated with MTT (200 μg/ml) for 2 h. The medium was then removed and the cells solubilized in dimethyl sulfoxide (100 μl) to quantitate formazan at 550 nm (Gross and Levi, 1992). For explant cultures, full-thickness pieces of cartilage were removed from the femoral condyles. Slices measuring ~ 2 mm wide x 2 mm long were dissected from the tissue. Explants were transferred to 24-well plates (10 explants/well) containing DMEM/F-12 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum and incubated in a humidified 5% CO_2 incubator at 37°C for 2 days before experiments to allow then to stabilize.

Glycosaminoglycan degradation. Cartilage explants in DMEM/F12 medium +10% fetal bovine serum were labeled with [^{35}S] sulfate (4 μCi/ml) for 6 days. The unincorporated radioactivity was removed by extensive washing for 2 days with DMEM/F12 medium + 10% fetal bovine serum. Explants were incubated in fresh medium with IL-1β (100 U/ml) or IL-1β+CORM-2 for 6 days with renewal of medium and treatment every other day. The media were collected and explants were digested with papain (2 mg/ml) in 1 mM EDTA, 0.25 mg/ml dithiothreitol, 20 mM sodium phosphate pH 6.8, at 56ºC for 16 h. Sephadex G25 (GE Healthcare Life Sciences, Barcelona, Spain) chromatography was used to remove unincorporated [^{35}S] sulfate from medium and tissue digests. Radioactivity was measured

by liquid scintillation counting. Degradation was expressed as percentage of released radioactivity with respect to total radioactivity.

Proteoglycan synthesis. Proteoglycan synthesis was quantified by monitoring [^{35}S] sulfate incorporation (Moulharat et al., 2004). After chondrocyte stimulation with IL-1β (100 U/ml) or IL-1β+CORM-2 for 24 h, cells were labeled with [^{35}S] sulfate (2 μCi/ml) for 24 h. Cells were washed with Hank's balanced salt solution and then extracted with 4M guanidinium HCl/5 mM EDTA/5 mM Na acetate, pH 7.2 for 48 h at 4ºC. Proteoglycans absorbed on Whatmann filter paper were precipitated by cetylpyridinium chloride monohydrate and radioactivity was measured by liquid scintillation counting. Total radioactivity (medium+cell) was calculated for each well and normalized with respect to protein content.

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

Western blot analysis. After 15 min stimulation with IL-1β (100 U/ml) or IL-1β+CORM-2 (100 μM), chondrocytes in primary culture were lysed in 100 μl of buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl and 25 mM Tris, pH 7.4) and centrifuged at 4°C for 10 min at 10,000 x *g*. Proteins (25 μg) in supernatants of cell lysates were separated by 12.5% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene

difluoride membranes (GE Healthcare Life Sciences). Membranes were blocked with 3% bovine serum albumin and incubated with specific antibodies for 2 h at room temperature. Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG and the immunoreactive bands were visualized by enhanced chemiluminescence (GE Healthcare Life Sciences) using the AutoChemi image analyzer (UVP Inc., Upland, CA).

Real-time PCR. Chondrocytes in primary culture were stimulated with IL-1β (100 U/ml) or IL-1β+CORM-2 (100 μM) for 12 h. Total RNA was extracted using the TRIzol reagent (Life Technologies Inc. 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). PCR reactions were performed using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Madrid, Spain). Primers were purchased from Superarray (Frederick, MD). PCR assays were performed in duplicate on an iCycler Real-Time PCR Detection System (Bio-Rad Laboratories) running the cycling conditions: 95ºC for 10 min, 40 cycles of 95ºC for 15s, and 60ºC for 1 min. Reaction specificity was determined by melt curve analysis which was performed by heating the plate from 55 to 95ºC and measuring SYBR Green I dissociation from the amplicons. Cycle threshold (C_T) values for each gene were corrected using the mean C_T value for β-actin. Relative gene expression was calculated using the ΔC_T method and expressed as fold change ($2^{-\Delta\Delta C_T}$) relative to the expression values in nonstimulated cells.

Enzyme-linked immunosorbent assay. Chondrocytes in primary culture were stimulated with IL-1β (100 U/ml) or IL-1β+CORM-2 for 24 h. Supernatants were harvested,

centrifuged and frozen at -80ºC until analysis. Pro-MMP-1, total MMP-3, total MMP-10 and pro-MMP-13 protein was quantified in supernatants by using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems Inc. (Minneapolis, MN) 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, Zürich, Switzerland).

Data analysis. Results are presented as mean ± S.E.M. Statistical analyses were performed using one-way ANOVA 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, while the negative control $RuCl_3$ 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 significative (Fig. 3A and 3B). The observed effects were not due to cytotoxicity, since cell viability was not significantly modified by CORM-2 (50-150 μM) either in the absence or presence of IL-1β as determined by the MTT assay (data not shown). As expected, the negative control $RuCl_3$ (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. Fig. 4A shows that CORM-2 treatment (100 or 150 μM) overcame the effects of IL-1β on glycosaminoglycan release. We next wished to determine whether CORM-2 treatment results in an interaction with anabolic processes in chondrocytes. Synthesis of aggrecan was investigated using $Na_2{}^{35}SO_4$ 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. Fig. 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. Interestingly, CORM-2 (100 μM) treatment maintained the expression of this extracellular matrix component in cells in basal conditions and restored collagen II expression in chondrocytes stimulated with IL-1β.

Effects on mitogen-activated protein kinase activation. In human articular chondrocytes, the activation of mitogen-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, CORM-2 (100 μM) inhibited ERK1/2 and p38 phosphorylation in primary chondrocytes stimulated with IL-1β, whereas JNK activation was not affected by this compound.

Discussion

In OA, extracellular matrix depletion is the consequence of decreased synthesis and increased catabolic activity of proteolytic enzymes. Cartilage destruction is a complex process involving a wide range of proteinases. Loss of aggrecan precedes collagen degradation in OA (Nagase and Kashiwagi, 2003) and both processes are central pathophysiological features contributing to cartilage erosion during the progression of degenerative joint diseases (Caterson et al., 2000). The presence of pro-inflammatory cytokines such as IL-1β in OA joints would lead to the induction of a number of catabolic enzymes including collagenases and aggrecanases (Goldring and Goldring, 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). To this respect, it is interesting to note the role of MMP-3 (stromelysin 1) and MMP-10 (stromelysin 2) in activating the pro-forms 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 plays a crucial role in catalyzing proteoglycan degradation induced by IL-1 (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. In the present study, we have shown that CORM-2 inhibits the degradation of aggrecan but 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 downregulation 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., 2005). Nevertheless, whether this exogenous source of CO could be comparable to 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 likely that compounds such as CO-RMs, able to control both degradative pathways could provide a better protection. These observations support the interest of further studies on this class of agents.

Activation of MAPK signaling pathways appears 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, as well as 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.

It is known the ability of CO to bind metal centers in metalloproteins (Roberts et al., 2004), which may result in a 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 (Piantadosi, 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 impact the development of new therapies for the protection or repair of cartilage in degenerative joint diseases.

References

Bani-Hani MG, Greenstein D, Mann BE, Green CJ and Motterlini R (2006) Modulation of thrombin-induced neuroinflammation in BV-2 microglia by carbon monoxide-releasing molecule 3. *J Pharmacol Exp Ther* **318**:1315-1322.

Barksby HE, Milner JM, Patterson AM, Peake NJ, Hui W, Robson T, Lakey R, Middleton J, Cawston TE, Richards CD and Rowan AD (2006) Matrix metalloproteinase 10 promotion of collagenolysis via procollagenase activation: implications for cartilage degradation in arthritis. *Arthritis Rheum* **54**:3244-3253.

Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E and Aigner T (2002) Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. *Arthritis Rheum* **46**:2648-2657.

Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H and Poole AR (1997) Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* **99**:1534-1545.

Boczkowski J, Poderoso JJ and Motterlini R (2006) CO-metal interaction: Vital signaling from a lethal gas. *Trends Biochem Sci* **31**:614-621.

Burrage PS, Mix KS and Brinckerhoff CE (2006) Matrix metalloproteinases: role in arthritis. *Front Biosci* **11**:529-543.

Caterson B, Flannery CR, Hughes CE and Little CB (2000) Mechanisms involved in cartilage proteoglycan catabolism. *Matrix Biol* **19**:333-344.

Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R and Motterlini R (2003) Cardioprotective actions by a water-soluble carbon monoxide-

releasing molecule. *Circ Res* **93**:e2-e8.

Desmard M, Amara N, Lanone S, Motterlini R and Boczkowski J (2005) Carbon monoxide reduces the expression and activity of matrix metalloproteinases 1 and 2 in alveolar epithelial cells. *Cell Mol Biol (Noisy -le-grand)* **51**:403-408.

Elson CJ, Mortuza FY, Perry MJ, Warnock MG, Webb GR and Westacott CI (1998) Cytokines and focal loss of cartilage in osteoarthritis. *Br J Rheumatol* **37**:106-107.

Fan Z, Soder S, Oehler S, Fundel K and Aigner T (2007) Activation of Interleukin-1 Signaling Cascades in Normal and Osteoarthritic Articular Cartilage. *Am J Pathol* **171**:938-946.

Foresti R, Hammad J, Clark JE, Johnson TR, Mann BE, Friebe A, Green CJ and Motterlini R (2004) Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. *Br J Pharmacol* **142**:453-460.

Foresti R, Shurey C, Ansari T, Sibbons P, Mann BE, Johnson TR, Green CJ and Motterlini R (2005) Reviewing the use of carbon monoxide-releasing molecules (CO-RMs) in biology: implications in endotoxin-mediated vascular dysfunction. *Cell Mol Biol (Noisy -le-grand)* **51**:409-423.

Goldring MB (2000) The role of the chondrocyte in osteoarthritis. *Arthritis Rheum* **43**:1916-1926.

Goldring MB, Birkhead J, Sandell LJ, Kimura T and Krane SM (1988) Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. *J Clin Invest* **82**:2026-2037.

Goldring SR and Goldring MB (2004) The role of cytokines in cartilage matrix degeneration in osteoarthritis. *Clin Orthop Relat Res*S27-S36.

Gouze JN, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, Fournel-Gigleux S and Ouzzine M (2001) Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis: influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. *Arthritis Rheum* **44**:351-360.

Gross SS and Levi R (1992) Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J Biol Chem* **267**:25722-25729.

Guillen MI, Megias J, Gomar F and Alcaraz MJ (2008) Heme oxygenase-1 regulates anabolic and catabolic processes in osteoarthritic chondrocytes. *J Pathol* (in press).

Knauper V, Murphy G and Tschesche H (1996) Activation of human neutrophil procollagenase by stromelysin 2. *Eur J Biochem* **235**:187-191.

Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, Antoniou J, Feige U and Poole AR (2005) Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. *Arthritis Rheum* **52**:128-135.

Malfait AM, Liu RQ, Ijiri K, Komiya S and Tortorella MD (2002) Inhibition of ADAM-TS4 and ADAM-TS5 prevents aggrecan degradation in osteoarthritic cartilage. *J Biol Chem* **277**:22201-22208.

Megias J, Busserolles J and Alcaraz MJ (2007) The carbon monoxide-releasing molecule CORM-2 inhibits the inflammatory response induced by cytokines in Caco-2 cells. *Br J Pharmacol* **150**:977-986.

Milner JM, Elliott SF and Cawston TE (2001) Activation of procollagenases is a key control point in cartilage collagen degradation: interaction of serine and

metalloproteinase pathways. *Arthritis Rheum* **44**:2084-2096.

Moulharat N, Lesur C, Thomas M, Rolland-Valognes G, Pastoureau P, Anract P, De Ceuninck F and Sabatini M (2004) Effects of transforming growth factor-beta on aggrecanase production and proteoglycan degradation by human chondrocytes in vitro. *Osteoarthritis Cartilage* **12**:296-305.

Murphy G, Cockett MI, Stephens PE, Smith BJ and Docherty AJ (1987) Stromelysin is an activator of procollagenase. A study with natural and recombinant enzymes. *Biochem J* **248**:265-268.

Murphy G and Lee MH (2005) What are the roles of metalloproteinases in cartilage and bone damage? *Ann Rheum Dis* **64**:iv44-iv47.

Nagase H and Kashiwagi M (2003) Aggrecanases and cartilage matrix degradation. *Arthritis Res Ther* **5**:94-103.

Piantadosi CA (2002) Biological chemistry of carbon monoxide. *Antioxid Redox Signal* **4**:259-270.

Roberts GP, Youn H and Kerby RL (2004) CO-sensing mechanisms. *Microbiol Mol Biol Rev* **68**:453-473.

Sandouka A, Fuller BJ, Mann BE, Green CJ, Foresti R and Motterlini R (2006) Treatment with CO-RMs during cold storage improves renal function at reperfusion. *Kidney Int* **69**:239-247.

Sandy JD (2006) A contentious issue finds some clarity: on the independent and complementary roles of aggrecanase activity and MMP activity in human joint aggrecanolysis. *Osteoarthritis Cartilage* **14**:95-100.

Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ and Motterlini R (2005) Carbon

monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br J Pharmacol* **145**:800-810.

Stanton H, Rogerson FM, East CJ, Golub SB, Lawlor KE, Meeker CT, Little CB, Last K, Farmer PJ, Campbell IK, Fourie AM and Fosang AJ (2005) ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. *Nature* **434**:648-652.

Tetlow LC, Adlam DJ and Woolley DE (2001) Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. *Arthritis Rheum* **44**:585-594.

Urquhart P, Rosignoli G, Cooper D, Motterlini R and Perretti M (2007) Carbon monoxide-releasing molecules modulate leukocyte-endothelial interactions under flow. *J Pharmacol Exp Ther* **321**:656-662.

Wu W, Billinghurst RC, Pidoux I, Antoniou J, Zukor D, Tanzer M and Poole AR (2002) Sites of collagenase cleavage and denaturation of type II collagen in aging and osteoarthritic articular cartilage and their relationship to the distribution of matrix metalloproteinase 1 and matrix metalloproteinase 13. *Arthritis Rheum* **46**:2087-2094.

Yoon YM, Kim SJ, Oh CD, Ju JW, Song WK, Yoo YJ, Huh TL and Chun JS (2002) Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J Biol Chem* **277**:8412-8420.

Footnotes

This work was supported by grants SAF2004-03835 (Ministerio de Educación y Ciencia-FEDER) and ACOMP2007/066 (Generalitat Valenciana). J. Megías thanks Spanish Ministerio de Educación y Ciencia for a fellowship.

JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Fig. 1. Effect of CORM-2 on MMPs protein levels released by human OA chondrocytes. Cells in primary culture were stimulated with IL-1β (100 U/ml) for 24 h in the presence or absence of CORM-2 or the negative control $RuCl_3$ and the levels of pro-MMP-1, total MMP-3, total MMP-10 and pro-MMP-13 protein were measured in supernatants by ELISA and expressed as ng MMP protein/mg cell protein. Data are mean±S.E.M. of independent cultures with cells from seven different donors. *p<0.05, **p<0.01 with respect to IL-1β; ## p<0.01 with respect to nonstimulated cells.

Fig. 2. Effect of CORM-2 on aggrecanase activity released by human OA chondrocytes. Cells in primary culture were stimulated with IL-1β (100 U/ml) for 24 h in the presence or absence of CORM-2 or the negative control $RuCl_3$ and aggrecanase activity was measured in supernatants by ELISA. Data are expressed as mean±S.E.M. of independent cultures with cells from seven different donors. **p<0.01 with respect to IL-1β. ; ## p<0.01 with respect to nonstimulated cells.

Fig. 3. Effect of CORM-2 on mRNA expression of MMPs and aggrecanases. Cells in primary culture were stimulated with IL-1β (100 U/ml) for 12 h in the presence or absence of CORM-2 (100 μM). mRNA expression was determined by real-time PCR. Data are expressed as mean±S.E.M. of independent cultures with cells from three different donors. *p<0.05, with respect to IL-1β.

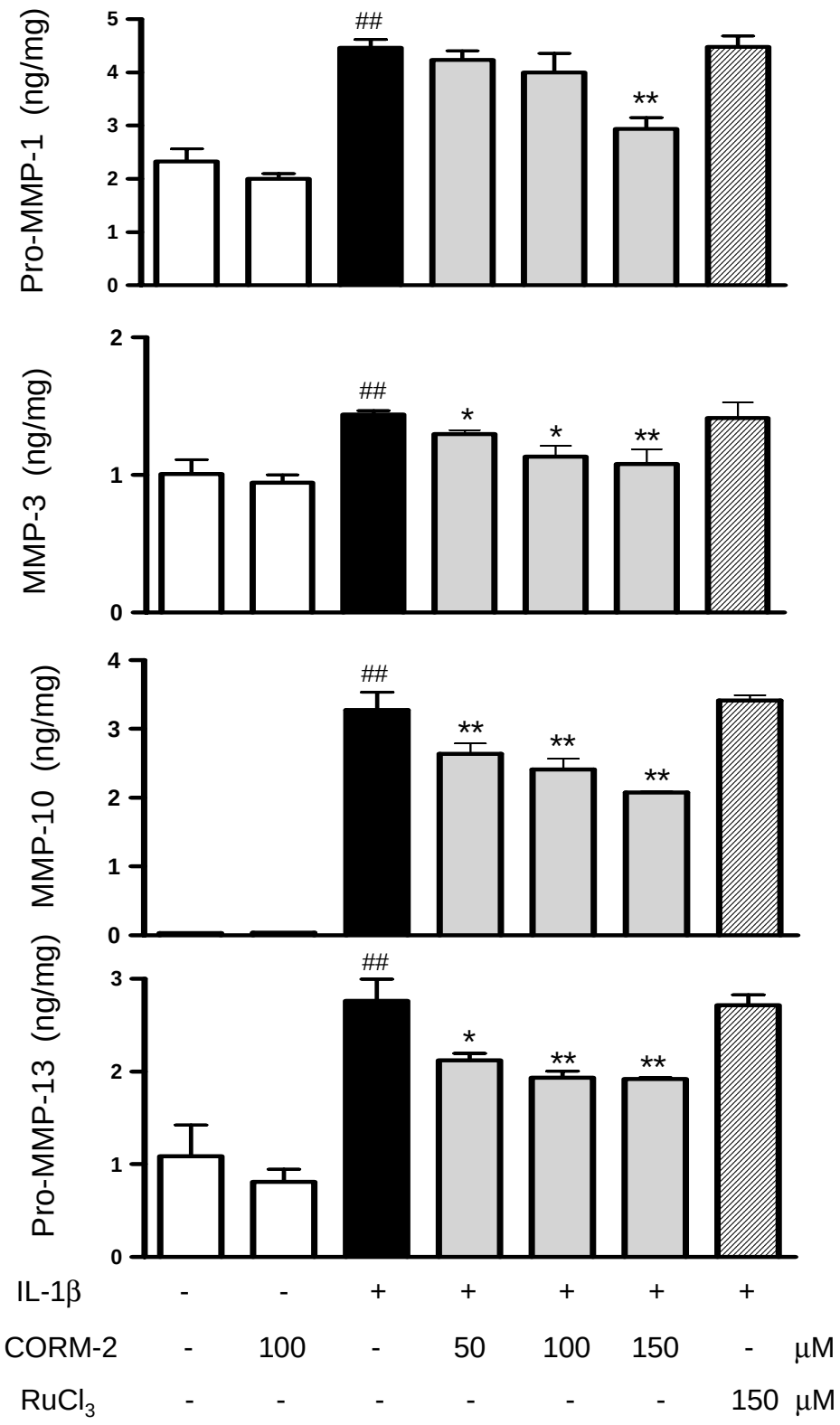
Fig. 4. Effect of CORM-2 on glycosaminoglycan degradation in OA explants (A) or glycosaminoglycan synthesis in OA chondrocytes in primary culture (B). Explants or cells were stimulated with IL-1β (100 U/ml) in the presence or absence of CORM-2 or the negative control $RuCl_3$ and glycosaminoglycan degradation or synthesis was measured by radiometric procedures, as indicated in Materials and methods. Data are expressed as mean±S.E.M. of independent cultures with explants or cells from three different donors. ** $p<0.01$ with respect to IL-1β. ## $p<0.01$ with respect to nonstimulated cells.

Fig. 5. Effect of CORM-2 (100 μM) on collagen II expression in OA chondrocytes. Cells were incubated with CORM-2 in the presence or absence of IL-1β (100 U/ml) for 15 days. Chondrocytes were fixed and stained with a monoclonal antibody against human collagen II, as indicated in Materials and methods. Original magnification: 200-fold.

Fig. 6. Effect of CORM-2 on MAPK phosphorylation in human OA chondrocytes stimulated with IL-1β (100 U/ml) for 15 min in the absence or presence of CORM-2 (100 μM). The expression of phosphorylated and total ERK1/2, JNK and p38 in the cellular fraction was analyzed by Western blotting. Relative expression of phosphorylated and total protein bands was calculated after densitometric analysis. The immunoblot is representative of three independent experiments. Data are expressed as mean±S.E.M. * $p<0.05$; ** $p<0.01$ with respect to IL-1β. ## $p<0.01$ with respect to nonstimulated cells.

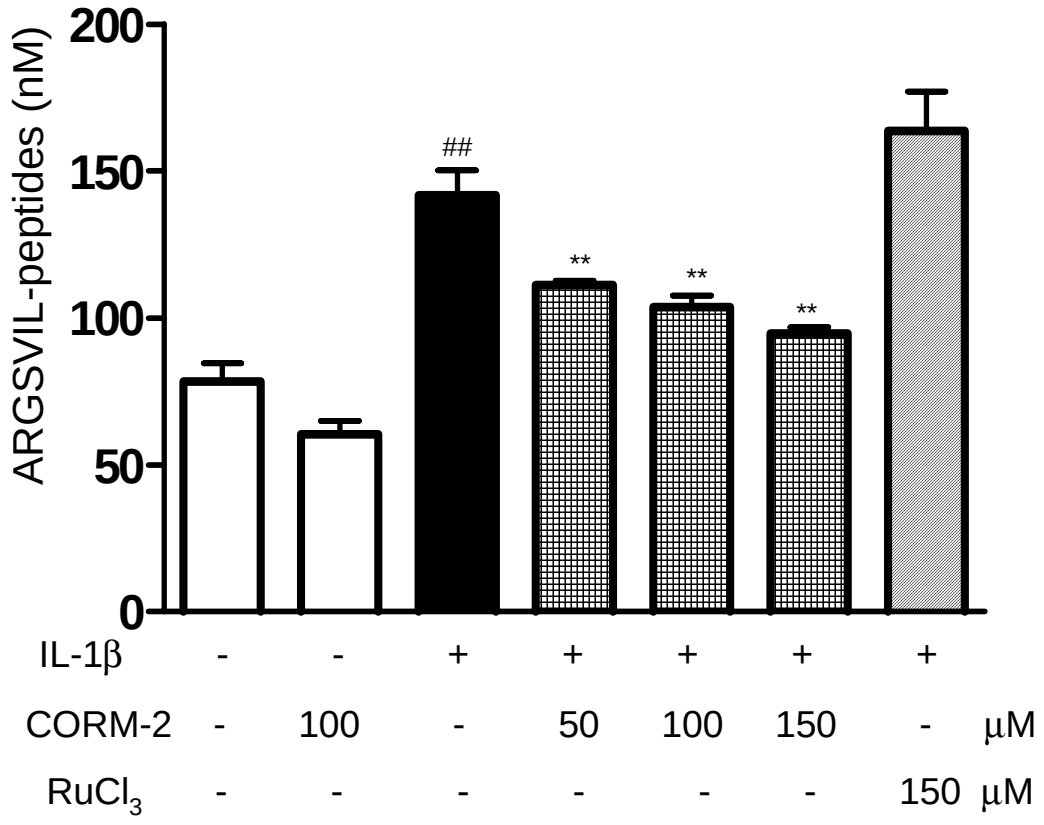
JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



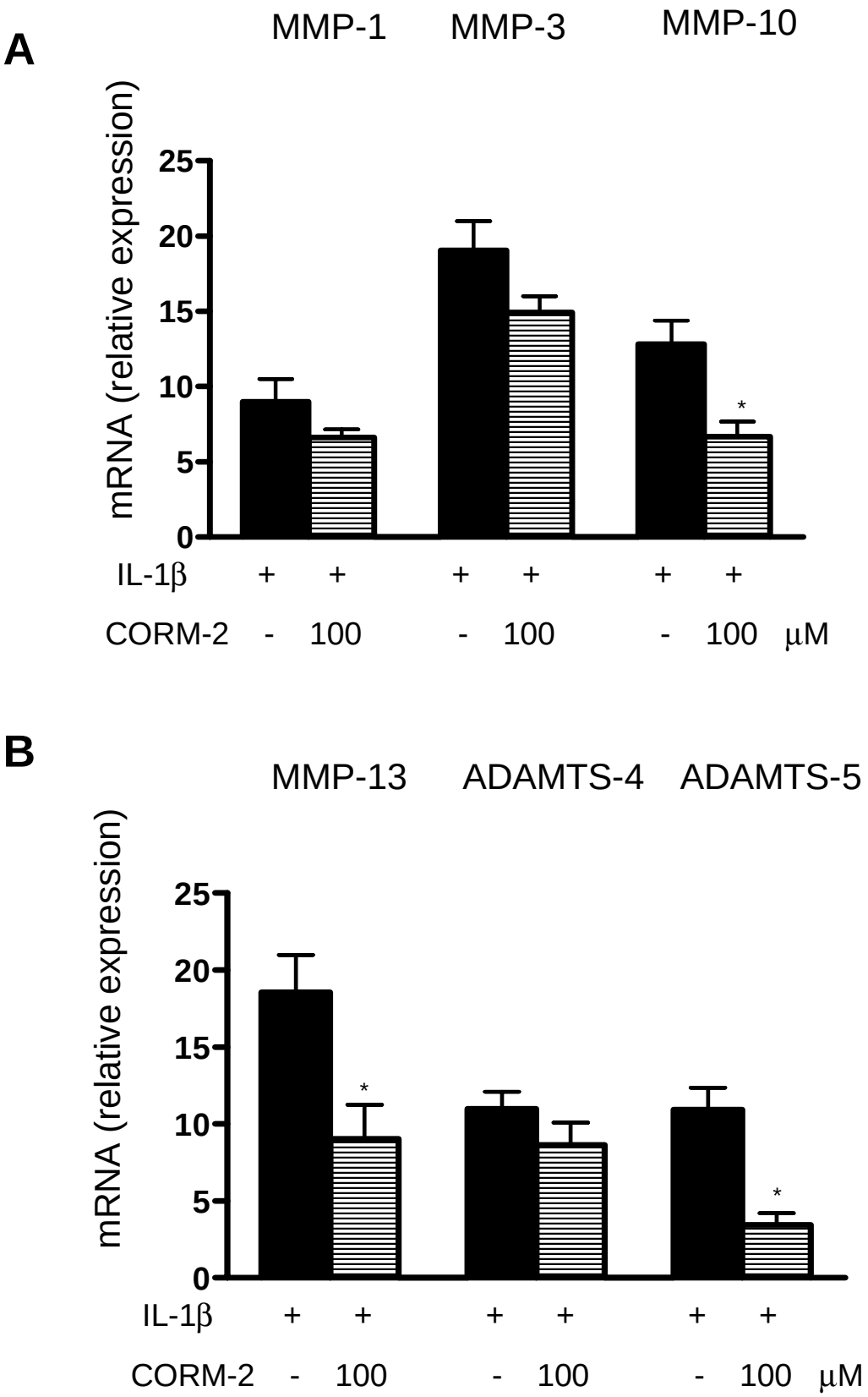
JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

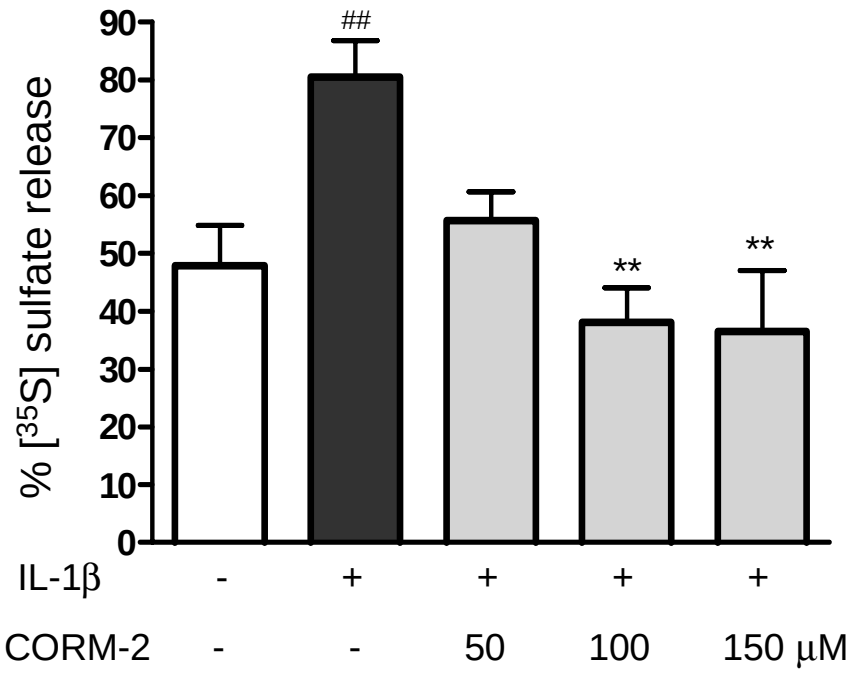
Figure 3



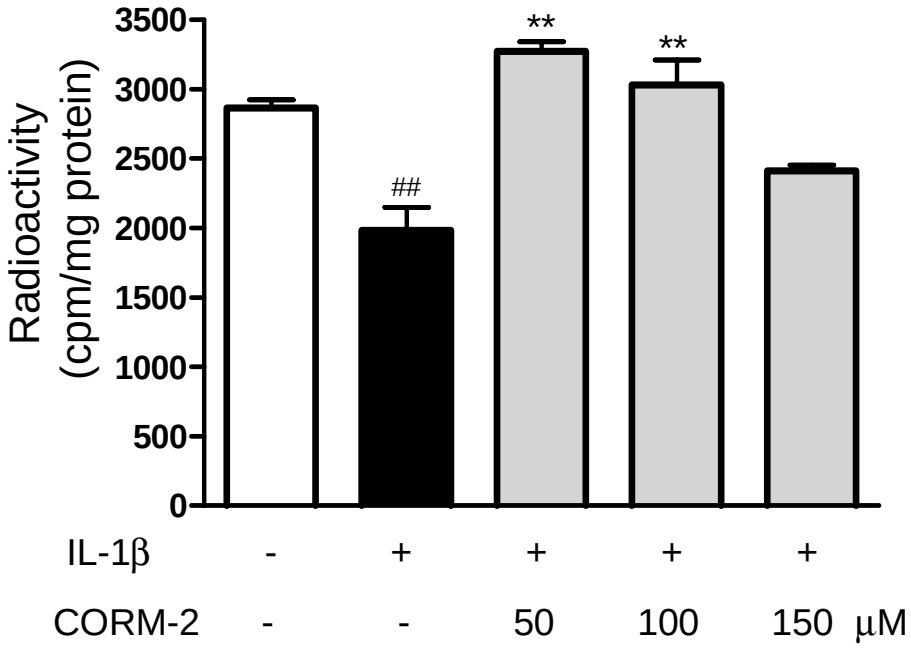
JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4

A

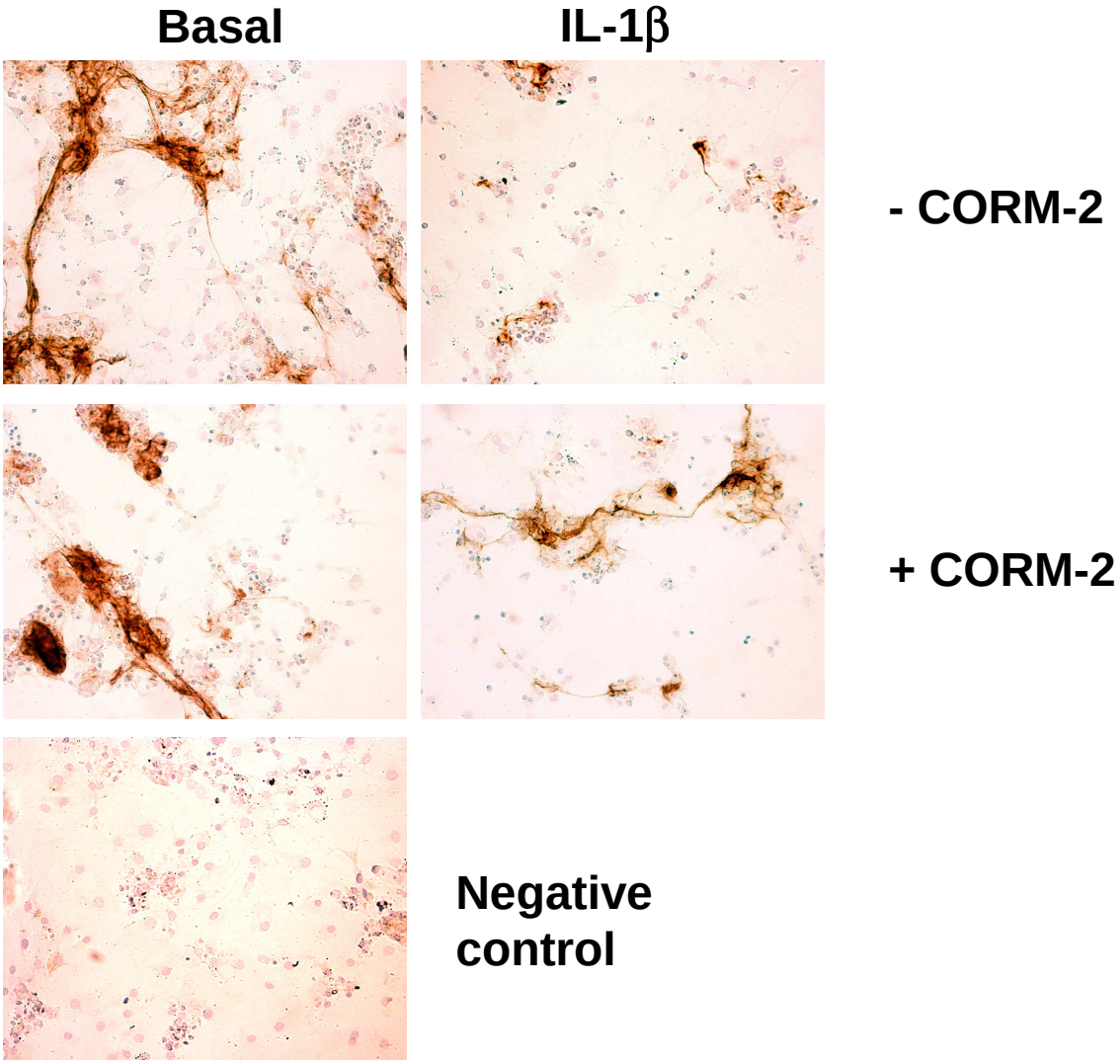


B



JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5



JPET Fast Forward. Published on January 14, 2008 as DOI: 10.1124/jpet.107.134650
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6