Deficiency of Nrf2 Accelerates the Effector Phase of Arthritis and Aggravates Joint Disease

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Abstract

Aims: Although oxidative stress participates in the etiopathogenesis of rheumatoid arthritis, its importance in this inflammatory disease has not been fully elucidated. In this study, we analyzed the relevance of the transcription factor Nrf2, master regulator of redox homeostasis, in the effector phase of an animal model of rheumatoid arthritis, using the transfer of serum from K/BxN transgenic mice to Nrf2−/− mice. Results: Nrf2 deficiency accelerated the incidence of arthritis, and animals showed a widespread disease affecting both front and hind paws. Therefore, the inflammatory response was enhanced, with increased migration of leukocytes and joint destruction in front paws. We observed an increased production of tumor necrosis factor-α, interleukin-6, and CXCL-1 in the joint, with small changes in eicosanoid levels. Serum levels of CXCL-1 and receptor activator for nuclear factor κB ligand were enhanced and osteocalcin decreased in arthritic Nrf2−/− mice. The expression of cyclooxygenase-2, inducible nitric oxide synthase, and peroxynitrite in the joints was higher in Nrf2 deficiency, whereas heme oxygenase-1 was downregulated. Innovation: Nrf2 may be a therapeutic target for arthritis. Conclusion: Our results support a protective role of Nrf2 against joint inflammation and degeneration in arthritis.

Introduction

The transcription factor Nrf2 (NF-E2-related factor 2) controls the expression of cytoprotective genes involved in electrophile conjugation (31, 34). Although Nrf2 is dispensable for mouse development (4), this transcription factor regulates an antioxidant defense system and its deficiency leads to augmented oxidative stress and cytokine production (47). Nrf2 may protect against chemical carcinogenesis (2) and cancer metastasis by the regulation of the immunological response (41). On the contrary, the presence of high constitutive Nrf2 levels in some tumor cells increases resistance to cancer therapy (53). Other biological roles have been proposed for this transcription factor, such as the control of adipocyte differentiation (37), the modulation of hepatic lipid homeostasis (16), or the promotion of atherogenesis (44).

Several lines of evidence have demonstrated that a defective Nrf2 activity results in greater sensitivity to oxidative and inflammatory disorders such as asthma, pulmonary fibrosis, neurodegeneration, colitis, and sepsis (8, 18, 23, 38, 40, 45). In addition, Nrf2 deficiency results in a higher sensitivity to inflammatory responses induced by bacteria or lipopolysaccharide. Thus, macrophages from Nrf2−/− mice generate 2-fold higher reactive oxygen species levels after lipopolysaccharide stimulation compared with cells from wild-type animals (25).

Innovation

It has been accepted that activation of Nrf2 results in anti-inflammatory effects in different biological systems. In contrast, knowledge on the role of this transcription factor in rheumatoid arthritis is scarce. We have shown in the K/BxN serum transfer model that Nrf2 deficiency results in a widespread disease with higher production of relevant mediators such as reactive oxygen and nitrogen species, cytokines and chemokines, enhanced migration of inflammatory cells, cartilage degradation, and bone erosion. Therefore, Nrf2 may counteract important processes in the effector phase of arthritis to protect joint structures. Our findings thus support that Nrf2 may be a therapeutic target for arthritis.

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mice (25). Similarly, deficiency in Nrf2 increases the inflammation involved in secondary brain injury after traumatic lesions (21) and leads to induction of angiogenic factors and promotion of ischemia-induced neovascularization (17).

Despite the established antioxidant and anti-inflammatory role of Nrf2, little is known about the involvement of this transcription factor in arthritis. Previous studies have suggested that Nrf2 may participate in the regulation of the immune response (27) and chondrocyte integrity (13). Recently, it has been shown that Nrf2 expression is upregulated in rheumatoid arthritis synoviocytes compared with healthy donors, and the deficiency in this transcription factor leads to increased joint damage in anti-collagen II antibody-induced arthritis (50). It has also been demonstrated that activation of Nrf2 and induction of heme oxygenase-1 mediate the anti-inflammatory and protective effects of cilostazol (35) and antirheumatic gold compounds (22). These findings highlight Nrf2 as a novel molecular target for arthritic diseases (24). In direct contrast, it has been reported that increased expression of Nrf2 in a subset of rheumatoid arthritis patients could lead to a more severe disease state (46). In addition, Nrf2 may play complex roles in articular cells as this transcription factor mediates the expression of antioxidant genes but negatively regulates chondrocyte and osteoblast differentiation (14, 15). Therefore, the role of Nrf2 in arthritis remains unclear.

This study was conducted to investigate the involvement of Nrf2 in the effector phase of arthritis. We sought to characterize the consequences of Nrf2 deficiency on key aspects of the inflammatory response and joint lesion to gain new insights into the underlying mechanisms of action. To address this question, we used an animal model that exhibits important features of human rheumatoid arthritis, the K/BxN serum transfer model (49).

**Results**

**Evolution of arthritis**

In our experimental conditions, administration of serum from K/BxN transgenic mice causes severe arthritis affecting mainly hind paw joints. We have followed the progression of arthritis in wild-type and Nrf2<sup>−/−</sup> mice. Figures 1A and 1B show the time-course of arthritis incidence (as percentage of arthritic animals) and the arthritic score, as well as representative pictures of paws. In wild-type mice, maximal incidence of disease was reached on day 7 (hind paws) and 10 (front paws). Interestingly, maximal incidence appeared earlier in Nrf2<sup>−/−</sup> mice (day 4 in hind paws and day 7 in front paws). A higher incidence of arthritis was observed in these animals from days 2 to 6 in hind paws and from days 4 to 8 in front paws, as compared with wild-type animals. As expected, arthritic score values were lower in front paws of wild-type mice compared with hind paws of the same group. In accordance with the high inflammatory response present in hind paws, Nrf2<sup>−/−</sup> and wild-type mice showed similar score values.
in both groups. In contrast, Nrf2 deficiency significantly enhanced the arthritic score of front paws from days 6 to 10.

**Histological analysis of joint sections**

In line with the results obtained for the macroscopic arthritic score, the histological analysis of ankles on day 10 (Figs. 2A and 2B) revealed important inflammatory and erosive changes with the presence of synovial infiltrate and exudate, depletion of proteoglycan and bone erosion in both Nrf2-/- and wild-type arthritic animals without significant statistical differences between both groups.

The histological analysis of front joint sections at the end of the experiment (day 10) (Figs. 3A, 3B, and 3C) indicated very little change in the arthritic wild-type group compared with its naive control. In contrast, the arthritic process was intense in Nrf2-/- mice and therefore we observed a marked infiltration of inflammatory cells into joint tissues as well as the presence of inflammatory exudate in the joint space. We also observed in these animals significant increases in proteoglycan depletion and bone erosion in comparison with arthritic wild-type mice.

**Levels of inflammatory mediators in serum**

As shown in Table 1, no significant differences were observed between naive Nrf2-/- mice and their wild-type counterparts in constitutive levels of inflammatory mediators on day 10. The arthritic process in wild-type animals was accompanied by significant increases in serum IL-6, PGD2, 6-ketoPGF1a, and LTB4, whereas in Nrf2-/- mice besides these mediators CXCL-1, RANKL, and PGE2 were significantly enhanced with respect to naive animals and osteocalcin decreased. In arthritic animals, Nrf2 deficiency increased CXCL-1 and reduced 6-ketoPGF1a, LTB4, and osteocalcin.

**Levels of inflammatory mediators in paw homogenates**

In knee homogenates of day 10 (Fig. 4), we observed increases in IL-1β, CXCL-1, PGE2, PGD2, and TXB2 after the induction of arthritis in wild-type mice. Interestingly, the arthritic process in
the presence of Nrf2 deficiency led to the upregulation of all the inflammatory mediators assayed. In addition, the comparison of this group with arthritic wild-type animals showed significant increases in TNF-α, IL-6, and CXCL-1. The levels of myeloperoxidase activity, a marker of neutrophils, were also determined in knee homogenates. No significant differences were observed between naïve wild-type animals and naïve Nrf2-/- mice (6982.3–2400.7 and 5711.9–890.9 Units/g joint, respectively, FIG. 3. Histological analysis of frontal sections of front joints on day 10. (A) Hematoxylin and eosin-stained sections. A, E, I, front joint of naïve wild-type mouse; B, F, J, front joint of naïve Nrf2-/- mouse; C, G, K, front joint of arthritic wild-type mouse; D, H, L, front joint of arthritic Nrf2-/- mouse. C, cartilage; JS, joint space; R, radius; S, synovium; U, ulna. The solid arrows indicate areas of cell infiltration. Broken arrows (…) indicate exudate. Original magnification X100 (I–L). (B) Safranin O and fast green-stained sections. A, E, I, front joint of naïve wild-type mouse; B, F, J, front joint of naïve Nrf2-/- mouse; C, G, K, front joint of arthritic wild-type mouse; D, H, L, front joint of arthritic Nrf2-/- mouse. C, cartilage; JS, joint space. Broken arrow indicates proteoglycan depletion from cartilage matrix (—). Original magnification X100 (A–D). Original magnification X200 (E–H). (C) Histological score. Data represent mean ± S.E.M, n = 4; *p < 0.05 each arthritic group compared with its respective naïve group, #p < 0.05 arthritic Nrf2-/- group with respect to arthritic wild-type group. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

<table>
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<th>Table 1. Levels of Inflammatory Mediators in Serum on Day 10</th>
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<td><strong>TNF-α (pg/ml)</strong></td>
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<td>3.58 ± 0.10</td>
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<td>326.5 ± 26.88</td>
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<td>0.44 ± 0.08</td>
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WT (wild-type mice). Values are mean ± S.E.M (n = 4–8); **p < 0.01, *p < 0.05 each arthritic group compared with its respective naïve group, #p < 0.01, #p < 0.05 arthritic Nrf2-/- mice with respect to arthritic wild-type animals. Mann-Whitney U-test (two-tailed).
The induction of arthritis resulted in a significant increase ($p < 0.01$) in myeloperoxidase activity with respect to the corresponding naïve animals (23229.3 ± 2048.0 and 22582.7 ± 2237.0 Units/g joint, $n = 8$, in arthritic wild-type and arthritic Nrf2-deficient mice, respectively).

Figure 5 shows that the arthritic process in front paws increased IL-1β, LTB₄, PGE₂, PGD₂, and TXB₂ in wild-type animals on day 10, whereas in Nrf2-/− mice, the levels of IL-1β, TNF-α, CXCL-1, PGE₂, PGD₂, and TXB₂ were significantly enhanced. The induction of arthritis in Nrf2-/− mice resulted in higher TNF-α and lower LTB₄ levels compared with arthritic wild-type animals.

In front paws, increased myeloperoxidase activity was observed in naïve Nrf2-deficient mice with respect to naïve wild-type animals, although they did not reach statistical significance (41582.0 ± 3209.0 and 19203.7 ± 3898.1 Units/g joint, $n = 4$, respectively). The arthritic process slightly increased myeloperoxidase activity in wild-type animals (33781.3 ± 6371.5 Units/g joint, $n = 8$, with respect to naïve wild-type mice). Interestingly, arthritic Nrf2-deficient mice showed a dramatic increase with values of 88690.2 ± 4838.6 Units/g joint, $n = 8$, $p < 0.001$ with respect to naïve Nrf2-deficient mice and also to arthritic wild-type animals.

**HO-1 and COX-2 protein expression in paw homogenates**

We studied the expression of HO-1, a target of Nrf2, in paw homogenates of day 10. As shown in Figures 6A and B, there is a low level of HO-1 in naïve animals and Nrf2 deficiency does not modify the constitutive expression of this protein. In arthritic animals, HO-1 protein was induced in both front paws and knees and this induction was decreased in Nrf2-/− mice. We also determined the expression of COX-2 in paw homogenates. Although no significant differences were observed between both groups of naïve animals, the induction of arthritis enhanced COX-2 expression in front paws and knees, and this expression was increased in Nrf2-/− mice compared with wild-type animals, with statistical significance in front paws.

**Immunohistochemical analysis of iNOS expression in joint sections**

No significant iNOS positive cells were observed in naïve animals in joint sections of day 10. In contrast, iNOS was induced in the joint during the inflammatory process mainly in chondrocytes of ankles (Fig. 7A). In addition, iNOS induction...
was significantly higher in front paws of arthritic Nrf2−/− mice with respect to arthritic wild-type animals (Fig. 7B).

**Immunohistochemical analysis of nitrotyrosine expression in joint sections**

Constitutive nitrotyrosine immunostaining was detected mainly in Nrf2-deficient mice in both ankles and front paw joints on day 10 (Figs. 8A and B), whereas the induction of arthritis increased nitrotyrosine expression that was apparent in chondrocytes, synovium, and infiltrate. We observed in arthritic animals that nitrotyrosine expression was enhanced in Nrf2−/− mice compared to their wild-type counterparts with statistical significance in front paws.

**Discussion**

K/BxN serum transfer to healthy mice leads to an autoimmune and inflammatory response mediated by IgG1 autoantibodies (28, 30). This animal model of arthritis exhibits clinical similarities to human rheumatoid arthritis, with cell infiltration, synovitis, pannus formation, and erosion of cartilage and bone (49). The K/BxN serum transfer model emulates rheumatoid arthritis specificity as there is a joint-specific inflammation that may be dependent on autoantigen binding to cartilage surface (43). In this study, the induction of arthritis in naïve mice slightly increased neutrophil migration, an essential process to initiate and maintain arthritis (49), in front paws, but its effect in hind paws was strong. Of note, Nrf2 deficiency dramatically upregulated neutrophil migration in front paws. Therefore, maximal severity of arthritis was observed in hind paws of wild-type animals, limiting a further increase in inflammation in the presence of Nrf2 deficiency. In contrast, front paws exhibited a low-grade inflammation after induction of arthritis in wild-type animals that was significantly enhanced in Nrf2−/− mice. Interestingly, our data have revealed that Nrf2 deficiency accelerates the incidence of arthritis in this experimental model and leads to a widespread disease, with important inflammation and lesions in both hind and front joints.

Induction of HO-1 during inflammatory conditions could be part of an adaptive mechanism to limit cytotoxicity via scavenging of reactive oxygen or nitrogen species, regulation of cell proliferation, and prevention of apoptosis (reviewed in (1)). We have shown previously that induction of HO-1 can exert partial anti-arthritic effects (9). In the present study, Nrf2 deficiency during K/BxN serum transfer arthritis led to increased iNOS expression and production of reactive oxygen and nitrogen species, whereas the expression of HO-1 was downregulated. Our results thus suggest that the increased production of oxidative stress due to Nrf2 deficiency may...
mediate an enhanced inflammatory response and joint lesion in the K/BxN serum transfer model of arthritis which is in line with data from anti-collagen II antibody-induced arthritis (50).

Increased eicosanoid production has been shown to contribute to the development of arthritis in human patients and animal models (5). There are differences in the eicosanoid profile according to the experimental model, as it has been reported a more relevant role for COX-1 and PGI2 compared to COX-2 and PGE2 in the K/BxN serum transfer arthritis (5). In addition, our previous research suggests the participation of another prostanoid pathway, PGD2, in the effector phase of this arthritis model (29). Joint inflammation is dependent on neutrophil recruitment via LTB4 (6) which also stimulates synovial fibroblasts migration and invasion (7). In this study, the PGI2 metabolite 6-ketoPGF1α, PGD2, and LTB4 were significantly increased in the serum of arthritic wild-type mice on day 10, compared with naive animals, whereas the small increase in PGE2 was not significant. The levels of these eicosanoids and TXB2 were also enhanced in the joints at that time point. In Nrf2 deficiency, the arthritic process significantly induced the production of eicosanoids, although the effect on LTB4 was weak and the production of serum 6-ketoPGF1α, and LTB4 in Nrf2-deficient mice was lower than in wild-type animals. These observations suggest the participation of Nrf2 in the modulation of eicosanoid synthesis and/or metabolism during inflammation. Little is known about the possible modification by this transcription factor of the expression or activity of enzymes involved in eicosanoid pathways. In our study, Nrf2 deficiency resulted in increased COX-2 expression in paws, which is in line with reports in ischemia-induced neovascularization (17). The regulation of enzymes involved in eicosanoid pathways is very complex in vivo. Although the Nrf2 enhancer element is present in thromboxane synthetase, different trans-activating factors may confer cell-type preferential expression (52). On the other hand, in situations of oxidative stress, PGD2 and cyclopentenone isoprostanes can be generated in a nonenzymatic manner (11) and therefore, this mechanism may contribute to the increased serum levels of this prostaglandin in arthritic Nrf2−/− animals which have a reduced antioxidant defense system. Interestingly, our data indicate that Nrf2 deficiency results in a higher migration of
pro-inflammatory cells into the joints during the development of arthritis, which may be dependent on several inflammatory mediators.

Pro-inflammatory cytokines and chemokines play an important role in the effector phase of experimental arthritis. The arthritic process is associated with the increase of cytokines such as IL-1β, TNF-α, and IL-6, which are highly expressed in the joints of rheumatoid arthritis patients (10). In particular, experimental data support the view that TNF-α plays an important role in rheumatoid arthritis (48). Our results have shown an upregulation of cytokine expression during arthritis in Nrf2-deficient animals. Therefore, we observed significant differences in TNF-α and IL-6 levels with respect to wild-type animals. This is in line with recent reports indicating a higher infiltration of inflammatory cells into skeletal muscle and higher levels of TNF-α mRNA in Nrf2-deficient animals following ischemia (17). Similarly, pulmonary inflammation induced by lipopolysaccharide was enhanced in Nrf2−/− mice with increased mRNA expression of IL-6 in the lungs (25).

Pro-inflammatory cytokines inhibit bone formation and stimulate osteoblasts to produce RANKL leading to osteoclast activation and bone resorption (reviewed in (3)). In this study, the arthritic process resulted in enhanced serum levels of RANKL in the presence of Nrf2 deficiency whereas osteocalcin levels were significantly decreased. Therefore our data suggest that increased bone resorption and reduced bone formation contribute to arthritic changes in these animals and Nrf2 may be a protective factor for bone metabolism in the presence of inflammation.

It is known that oxidative stress can induce chemokine expression during inflammatory responses and thus activation of Nrf2 and induction of target genes such as HO-1 would inhibit chemokine production, as reported for IL-8 in HT-29 cells (26). Nevertheless, the role of Nrf2 may be more complex and in
some cell types the activation of this transcription factor has been related to IL-8 mRNA stabilization (54). Chemokines are also upregulated in inflamed joints during K/BxN serum transfer arthritis and could mediate neutrophil recruitment to the joints. Gene expression of CXCL1 is upregulated in ankles and synovial fluid in parallel with the progression of arthritis in this experimental model (20). This chemokine is a ligand of CXCR2 mediating the migration of different cell types during inflammatory conditions (39, 42). Recent studies have demonstrated that CXCR2 signaling is critical for K/BxN serum transfer arthritis (20). It is interesting to note that CXCL-1 induction during the arthritic process is augmented by Nrf2 deficiency, which may play an important role in the potentiation of the inflammatory response observed in these animals.

MCP-1 is another inflammatory mediator upregulated during arthritis (20, 33). This chemokine enhances migration and proliferation of synovial fibroblasts as well as matrix metalloproteinase expression (12). Nevertheless, the influence of MCP-1 upregulation on arthritis progression is not clear, as recent studies using CCL2-deficient mice have shown no differences in disease severity with respect to wild-type animals in the K/BxN serum transfer model (20). Our data indicate that Nrf2 deficiency in arthritic animals results in the upregulation of MCP-1 in hind paws which is consistent with results in muscle ischemia and pulmonary inflammation (17, 25). It is known that MCP-1 promoter activation depends on nuclear factor κB activity that responds to reactive oxygen species (51) and therefore increased oxidative stress in Nrf2 deficiency would facilitate MCP-1 synthesis.

In conclusion, our study provides novel insights into the role of Nrf2 in the inflammatory response in vivo. Taken together, our data suggest that Nrf2 acts upon the production of inflammatory mediators to modulate the systemic and local processes driving erosive arthritis.

![Image](image-url)
Materials and Methods

Induction of arthritis

K/BxN mice were generated by crossing KRN−TCR−transgenic mice (B10.BR genetic background) with NOD mice. The C57BL/6J Nrf2 knockout mice were kindly provided by Dr. Masayuki Yamamoto (University of Tsukuba, Japan) (19). These mice exhibit a normal phenotype and lifespan, but are hypersensitive to treatments that lead to oxidative or inflammatory stress. For instance, these animals exhibit exacerbated neuron death, gliosis, and neuroinflammation after treatment with the parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (40) or lipopolysaccharide (18).

Genotyping of wild-type (Nrf2+/+) C57BL/6J mice and Nrf2-deficient (Nrf2−/−) littermates was done as reported previously (19). Arthritis was induced in 16−24-week-old mice by intraperitoneal injection of 150 μl serum from arthritic K/BxN mice on day 0 and 2. All mice were maintained in cages with a 12-hour light/dark cycle and free access to standard diet and water. Mice were housed and cared for by the veterinary staff in accredited facilities and were routinely screened for health status. Mice were assigned to their experimental group (n = 8 in arthritic wild-type and arthritic Nrf2−/− groups, n = 4 in naïve wild-type and naïve Nrf2−/− groups). Blood was collected from the retro-orbital venous plexus on day 10, and animals were then sacrificed by cervical dislocation. Hind and front paws from randomly selected animals in each group were isolated for histological and immunohistochemical analyses (8 ankles and 4 front paws in arthritic groups, 4 ankles and 4 front paws in naïve groups) and the rest of them were used for measurement of inflammatory markers. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols were approved by the institutional Animal Care and Use Committees (University of Valencia and Autonomous University of Madrid, Spain).

Arthritis score

Joint inflammation was scored visually in each paw. The clinical severity of arthritis was graded using a scale of 0−2 for each paw where 0 = uninfamed, 1 = mild, 1.5 = marked, and 2 = severe inflammation. Scoring was performed by two independent observers without knowledge of the experimental groups.

Measurement of inflammatory mediators in serum

To determine the levels of prostaglandin(PG)D2 and 6-ketoPGF1α in serum samples, ELISA kits were used (Cayman Chemical, Ann Arbor, MI) with a range of detection of 78−10000 and 1.6−1000 pg/ml, respectively. PGE2 and leukotriene B4 (LTB4) levels were measured by radioimmunoassay (32). CXCL-1 was determined by ELISA (Promokine, PromoCell GmbH, Heidelberg, Germany, 8−1000 pg/ml). The levels of tumor necrosis factor α (TNF-α), interleukin(IL)-6, osteocalcin, and receptor activator for nuclear factor κB ligand (RANKL) in serum were determined by the LINCOplex™ system (Millipore Iberica, Madrid, Spain), with sensitivity of 0.8, 0.6, 7.0, and 3.3 pg/ml, respectively.

Measurement of inflammatory mediators in paw homogenate

Hind paws (knees) and front paws were amputated, homogenized in liquid N2 with 1 ml of A buffer pH 7.46 (10 mM HEPES, pH 8, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 5 mM NaF, 1 mM NaN3, 1 μg/ml leupeptin, 0.1 μg/ml aprotinine, and 0.5 μM phenylmethyl sulfonyl fluoride). The tissue homogenates obtained were sonicated (10 sec three times at 20% with a 10-sec incubation on ice between bursts) in an ultrasonic processor (VC130PB, Sonics & Materials Inc., Newtown, CT) and centrifuged at 1200 g, 10 min at 4°C. Supernatants were removed and used for determination of eicosanoid, cytokine, and chemokine levels. TNF-α and IL-1β were determined by ELISA (R&D Systems Inc., Minneapolis, MN, with a range of detection of 32−2700 and 25−2000 pg/ml, respectively). IL-6 and monocyte chemoattractant protein-1 (MCP-1) were measured using the ELISA kits from eBioscience Inc. (San Diego, CA), with sensitivity of 2 and 7 pg/ml, respectively. CXCL-1 and PGD2 were determined by ELISA, as indicated above, and PGE2, thromboxane B2 (TXB2) and LTB4 were quantified by radioimmunoassay (32). Myeloperoxidase activity was assayed as described previously (36).

Histological analysis

For standard histological assessment, isolated ankles and front paws were kept in 10% formalin for 4 days, decalcified in 5% formic acid, and subsequently dehydrated and embedded in paraffin. Standard frontal sections (7 μm) of the joint tissue were mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany). Hematoxylin and eosin staining was performed to study joint inflammation. The severity of inflammation in the joints was scored on a scale of 0−3 (0 = no cells, 1 = mild cellularity, 2 = moderate cellularity, and 3 = maximal cellularity). To study proteoglycan depletion from the cartilage matrix, sections were stained with safranin O, followed by counterstaining with fast green. Depletion of proteoglycan was determined using an arbitrary scale of 0−3, ranging from normal, fully stained cartilage to destained cartilage that was fully depleted of proteoglycan. Bone destruction was graded on a scale of 0−3, ranging from normal cellularity, with empty lacunae and 3 = complete loss of chondrocytes from the cartilage layer. Cartilage surface erosion was scored on a scale of 0−3, where 0 = no cartilage loss and 3 = complete loss of articular cartilage. Histopathologic changes were scored on three semiserial sections of the joint, with sections spaced 70 μm apart. Scoring was performed in a blind manner by two independent observers.

Immunohistochemistry

For immunohistochemical analyses, isolated ankles and front paws were fixed for 4 days in 10% formalin, decalcified in EDTA (10%), and subsequently dehydrated and embedded in paraffin. Tissue sections (7 μm) were deparaffinized, rehydrated, and treated with 2% H2O2 for 10 min at room temperature. Sections were incubated for 15 min with goat antiserum (1:10), and thereafter incubated for 2 h with rabbit anti-mouse antibodies against inducible nitric oxide synthase.
Western blot analysis

 Supernatants from paw homogenates (knees and front paws) were sonicated (10 sec twice at 20% with a 10-sec incubation on ice between bursts) in an ultrasonic processor (VC130PB, Sonics & Materials Inc.) and centrifuged at 10,000 g for 15 min at 4°C. The new supernatants were used to assess heme oxygenase-1 (HO-1) and cyclooxygenase (COX)-2 protein expression. Equal amounts of protein were loaded on 10% SDS-PAGE and transferred onto polyvinylidene difluoride Hybond-P membranes (GE Healthcare, Barcelona, Spain) for 120 min at 125 mA. Membranes were blocked in phosphate-buffered saline (0.02 M, pH 7.0)-Tween 20 (0.1%) containing 4% w/v unfatted milk, and incubated with a specific polyclonal antibody against HO-1 (1/1000) (SPA-896, Stressgen, Victoria, British Columbia, Canada), COX-2 (1/1000) (160116, Cayman Chemical) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1/10000) (G9545, Sigma-Aldrich). Finally, blots were washed and membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/5000, Dako). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL™ Western Blotting Detection reagents, GE Healthcare). Images were captured with the AutoChemi image analyzer (UVP Inc., Upland, CA) and protein band densitometry was performed.

Statistical analysis

 Differences between experimental groups were tested using the two-tailed Mann-Whitney U test. P values less than 0.05 were considered significant.

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Author Disclosure Statement

 The authors declare no competing financial interests.

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### Abbreviations Used

- **COX** = cyclooxygenase
- **GAPDH** = glyceraldehyde 3-phosphate dehydrogenase
- **HO-1** = heme oxygenase-1
- **IL** = interleukin
- **iNOS** = inducible nitric oxide synthase
- **LTB4** = leukotriene B4
- **MCP-1** = monocyte chemoattractant protein-1
- **Nrf2** = NF-E2-related factor 2
- **PG** = prostaglandin
- **RANKL** = receptor activator of nuclear factor κB ligand
- **TNFα** = tumor necrosis factor-α
- **TXB2** = thromboxane B2