Potential role of heme oxygenase-1 in the progression of rat adjuvant arthritis

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Rat adjuvant arthritis is an experimental model widely used to evaluate etiopathogenetic mechanisms in chronic inflammation. We have examined the participation of heme oxygenase-1 (HO-1) in this experimental arthritis. In this study, an increased nitric oxide (NO) production in the paw preceded the upregulation of HO-1, whereas selective inhibition of inducible NO synthase (iNOS) after the onset of arthritis decreased HO-1 expression, suggesting that the induction of this enzyme may depend on NO produced by iNOS. Therapeutic administration of the HO-1 inhibitor tin protoporphyrin IX was able to control the symptoms of arthritis. This agent significantly decreased leukocyte infiltration, hyperplastic synovitis, erosion of articular cartilage and osteolysis, as well as the production of inflammatory mediators. In this experimental model, HO-1 can be involved in vascular endothelial growth factor production and angiogenesis. These results support a role for HO-1 in mediating the progression of the disease in this model of chronic arthritis.

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Rheumatoid arthritis is a chronic relapsing inflammatory disease characterized by a proliferative synovitis that can lead to joint destruction. An autoimmune response would induce T-cell activation, synovial infiltration of leukocytes and induction of proinflammatory mediators in the synovium. The inflammatory cytokines can stimulate the proliferation of the mesenchymal and fibroblast-like synovial cells leading to the formation of pannus (reviewed in Lee and Weinblatt¹).

Nitric oxide (NO) is synthesized from L-arginine by three isoforms of NO synthase (NOS). The constitutive enzymes endothelial NOS (eNOS) and neuronal NOS (nNOS) generate low levels of NO under physiological conditions, whereas the expression of inducible NOS (iNOS) is upregulated by cytokines and bacterial products to generate large and sustained amounts of NO.² NO produced by constitutive isozymes may exert anti-inflammatory effects by preventing the adhesion and release of oxidants by activated leukocytes.³ In

contrast, NO and species derived from it such as peroxynitrite contribute to tissue injury in different rheumatic diseases including rheumatoid arthritis. Generation of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, hydroxyl radical and hypochlorous acid has also been involved in chronic inflammation.⁴ In particular, increased NO_x levels have been found in the serum, urine and synovial fluid of patients from rheumatoid arthritis^{5,6} accompanied by a high expression of iNOS in synoviocytes, chondrocytes, endothelial cells and infiltrating cells in the arthritic joints.⁷

Adjuvant arthritis is a rat model of autoimmune erosive arthritis widely used to evaluate etiopathogenetic mechanisms in rheumatoid arthritis as well as for testing anti-inflammatory drugs.⁸ Although animal models can only represent partial aspects of the complex pathology of human disease, they allow the development of therapeutic approaches for rheumatoid arthritis.⁹ The development of rat arthritis induced by adjuvant is accompanied by the induction of cyclooxygenase-2 (COX-2) and iNOS, and high levels of derived metabolites can be measured in the serum and synovium of arthritic rats.^{10,11}

CO may function as a signaling molecule in different physiological responses with a role similar

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to NO. The main endogenous source of CO is heme oxygenase (HO), which exists in inducible (HO-1) and constitutive isoforms (HO-2 and HO-3). This enzyme activity oxidizes heme to biliverdin, converted to bilirubin by biliverdin reductase, CO and free iron. HO-1 is rapidly induced in response to cytokines, NO and peroxynitrite, ROS, etc. Over-expression of HO-1 confers protection against oxidative injury or can lead to anti-inflammatory effects, which could be due to biliverdin and bilirubin production, CO or ferritin induction (reviewed in Alcaraz *et al*¹²). Protection by HO-1 can be of relevance during endotoxemia,¹³ liver failure¹⁴ or lung hyperoxia.¹⁵ HO-1 also prevents atherogenesis¹⁶ and myocardial injury after ischemia–reperfusion.¹⁷ Interestingly, metabolites derived from HO-1 activity suppress T-cell proliferation and improve cardiac allograft survival.¹⁸

Complex interactions between NO and CO have been described. For instance, CO may elevate the steady-state level of NO leading to peroxynitrite production by endothelial cells and perivascular oxidative injury,¹⁹ whereas NO can bind to human HO-1 leading to reversible inhibition of this enzyme.²⁰ In fact, endogenous NO functions as an inhibitory regulator of CO production by HO-1 in rat kidney.²¹ In addition, a synergy between NO and CO to provide cytoprotection has been reported in tumor necrosis factor- α (TNF α)-induced hepatocyte cell death.¹⁴ HO-1 activity could reduce NO production through decrease in heme availability, release of iron²² or interaction of CO with the heme group of iNOS,²³ which results in decreased iNOS expression or activity. We have shown that iNOS participates in HO-1 modulation in RAW 264.7 macrophages²⁴ and in a model of acute inflammation.²⁵ However, the interaction between both pathways during chronic arthritis has not been examined previously.

The aim of this study was to characterize the role of HO-1 during chronic inflammation. For that purpose, we determined the time course of HO-1 induction in relation with inflammatory parameters and assessed the consequences of HO-1 inhibition. We also examined the relationships between HO-1 and iNOS during the chronic phase of the rat adjuvant arthritis, a model of rheumatoid arthritis.

Materials and methods

Adjuvant Arthritis

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 Committee. Adjuvant arthritis was elicited in male Lewis rats (175–200 g) by injection of 0.1 ml of *Mycobacterium butyricum* (Difco, Detroit, MI, USA; 10 mg/ml) in mineral oil into the base of the tail.²⁶

Paw volumes were measured at the beginning of the experiment by using a water displacement plethysmometer. Animals were housed in propylene cages with food and water *ad libitum*. The light cycle was automatically controlled (on 0700 hours; off 1900 hours) and the room temperature thermostatically regulated to $21 \pm 1^\circ\text{C}$.

In this experimental model, a high level of protein expression is present on day 14 for iNOS and HO-1 with a significant paw swelling that reaches maximal levels on day 17. The magnitude of the inflammatory response was thus evaluated by measuring the volume of both hind paws at day 17. Animals with edema values of 1.1 ml larger than normal paws were then randomized into treatment groups. The pharmacological treatment was started after the clinical symptoms had appeared, since this approach is more relevant in relation with a chronic inflammatory process. Thus, this therapeutic arthritis model was used instead of a prophylactic one. For this purpose, drugs were administered from day 17 and not before. Experience with this experimental model indicates that by day 24, antiarthritic effects are observed with anti-inflammatory drugs. Thus, day 25 was the first final time point and day 35 was chosen to assess if the effects were lasting. These last phases of the response are closer to a chronic inflammatory disease.

For pharmacologic treatment, N-iminoethyl-L-lysine (L-NIL, 10 mg/kg p.o.) or tin protoporphyrin IX (SnPP, 6 mg/kg i.p.) was administered twice-daily from days 17 to 24 and the volume of hind paws was measured by plethysmometry (Ugo Basile, Comerio, Italy). Radiographic analysis of hind paws was performed by X-ray (Uniret LX 160, Multimage, Italy) with a 40 kW exposition for 0.01 s. Blood was collected by retro-orbital puncture on the last day of the experiment (day 25 or 35), under light anesthesia. Serum was used for determination of prostaglandin E₂ (PGE₂) by radioimmunoassay²⁷ and TNF α and vascular endothelial growth factor (VEGF) levels by ELISA (R&D Systems Inc., Minneapolis, MN, USA). Serum was filtered by centrifugation for 30 min at 12 000 g using 10 000 NMWL filters. Nitrate was then reduced enzymatically to nitrite with nitrate reductase and the total amount of nitrite was measured by reaction with 2,3-diaminonaphthalene according to Misko *et al*.²⁸ Animals were killed by cervical dislocation and hind paws were amputated above the ankle and homogenized in 2.5 ml of 10 mM HEPES buffer, pH 7.4 containing 0.32 M sucrose, 100 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride and 100 mM leupeptin. After centrifugation at 1200 g for 15 min at 4°C, supernatants were used for the determination of PGE₂ by radioimmunoassay,²⁷ nitrite by fluorometry²⁸ and TNF α and VEGF levels by ELISA. For histological analysis, hindlimbs from three animals in each group were fixed in 10% neutral buffered formalin for 24–48 h, cut longitudinally and then

decalcified in 5% nitric acid for 24 h. The limbs were washed with water for 1 h, dehydrated through graded alcohols and embedded in paraffin. Sections of 5 μ m were made and stained with hematoxylin–eosin. In another series of animals, bone of the rat hind paw was removed with a scalpel and soft tissue was fixed in formalin and embedded in paraffin. Sections of 5 μ m were used for TUNEL with an 'In situ cell death detection kit, POD' (Roche Applied Science, Barcelona, Spain), and immunohistochemistry with a monoclonal mouse anti-human CD31, endothelial cell marker (Dako, Glostrup, Denmark) following the manufacturer's instructions.

Western Blot Analysis

Supernatants from paw homogenates were centrifuged at 100 000 g for 100 min at 4°C. Cytosolic fractions were used for iNOS and microsomal fractions for COX-2 and HO-1 protein expression. Protein was measured by the Bradford method using bovine serum albumin as standard. Equal amounts of protein were loaded on 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate-buffered saline–Tween-20 (0.02 M, pH 7.0) containing 3% w/v unfatted milk and incubated with specific polyclonal antibody against HO-1, iNOS, COX-2 or actin (1/1000). Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/20 000). The immunoreactive bands were visualized using an enhanced chemiluminescence system.

Materials

[5,6,8,11,12,14,15(*n*-³H)]PGE₂ was from Amersham Biosciences (Barcelona, Spain). L-NIL and iNOS and COX-2-specific polyclonal antisera were purchased from Cayman Chemical (Ann Arbor, MN, USA). Anti-HO-1 antibody was from StressGen Biotech, (Victoria, BC, Canada) and SnPP from Frontier Scientific Europe Ltd. (Carnforth, UK). The peroxidase-conjugated goat anti-rabbit IgG and the monoclonal mouse anti-human CD31, endothelial cell were purchased from Dako (Glostrup, Denmark). The rest of reagents were from Sigma (St Louis, MO, USA).

Statistical Analysis

The results are presented as mean $\pm$ s.e.m.; *n* represents the number of experiments or animals. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

Results

Clinical and Histological Evaluation of Adjuvant Arthritis Rats

In rats injected with *M. butyricum*, a significant swelling was observed by day 14 in hind paws, which reached maximal levels on day 17 (Figure 1a). Weight loss occurred from day 17 until the end of the experiment (day 35) (Figure 1b). Treatment of arthritic animals with the HO inhibitor SnPP from days 17 to 24 after administration of adjuvant, caused a progressive reduction of paw edema, which reached values near to those of naïve rats at day 35. This therapeutic effect was accompanied by improving the weight loss during the progression of chronic inflammation. In contrast, the administration of the selective iNOS inhibitor L-NIL was ineffective on edema progression and weight loss. Arthritic changes were evident in radiographs of hind paws and were confirmed by histological analysis (Figure 2). Evidence of chronic inflammation was apparent in joints from arthritic rats on

Figure 1 Effect of SnPP (12 mg/kg; i.p.) and L-NIL (20 mg/kg p.o.) on the development of adjuvant-induced arthritis. Arthritis was induced by injection of *M. butyricum* on day 0. Compounds were administered twice-daily from day 17 to 24 after the arthritis induction. End of treatment ($\downarrow$). (a) Hind paw edema and (b) body weight were measured to assess the magnitude of the inflammatory response. AR is the arthritic control group of animals. Data represent mean $\pm$ s.e.m. (*n* = 6–12). **P* < 0.05; ***P* < 0.01 compared with AR.

Figure 2 X-ray images and histological correlations at day 25 or 35 after arthritis induction. Hind paw tissues were prepared as indicated in Materials and methods. Sections of 5 μ m were stained with hematoxylin–eosin.

days 25 and 35. The arthritic control group showed a characteristic periarticular inflammation with edema and infiltration of polymorphonuclear neutrophils and lymphocytic cells, synovitis and synovial hyperplasia as well as a severe loss of articular cartilage. Periostitis and bone resorption were associated with a marked osteoclastic activity, new bone formation and ankylosis. Histological analysis of joint sections indicated that administration of SnPP inhibited leukocyte infiltration, inflammation, hyperplastic synovitis, erosion of articular cartilage and osteolysis. Stabilized lesions and new bone formation were observed on day 35. Joints of animals treated with L-NIL showed on day 25 erosion of articular cartilage with pannus accompanied by a regenerative process including fibrosis and new bone formation. By day 35, there was a general increase in inflammatory parameters in the joint, with leukocyte infiltration, erosion of cartilage with pannus as well as osteolysis and a marked new bone formation.

Time Course of iNOS, COX-2 and HO-1 Protein Expression: Levels of Inflammatory Mediators

COX-2 expression in paws was already elevated on day 7, whereas iNOS or HO-1 protein levels were too low to be detected (Figure 3a). The expression of the first protein peaked on day 14 and then decreased until day 35. A high level of iNOS and HO-1 expression was evident on day 14 and decreased during the later phase of this experimental response (days 28–35). NO production, as indicated by $\text{NO}_2^- + \text{NO}_3^-$ levels was already elevated in paws on day 7 and peaked on day 14 (Figure 3b), whereas

increased serum levels appeared later, with a maximum on day 21 (Figure 4). PGE_2 production followed a similar pattern, with lower changes in serum (Figure 4) with respect to paw levels (Figure 3b). As shown in Figure 3c, a highly significant increase in $\text{TNF}\alpha$ was noted in paws on day 14, which was not accompanied by increases in serum (data not shown). It is known that systemic $\text{TNF}\alpha$ increases rapidly after adjuvant injection (12 h) and then returns to near-control concentrations.²⁹ Thus, at the time points selected in the present experiments serum $\text{TNF}\alpha$ levels were under the limit of detection of the assay system (5 pg/ml).

We also found that paw levels of VEGF progressively increased from day 21 until the end of the experiment (Figure 3c), whereas a significant elevation in systemic VEGF was noted on day 14 and reached maximal levels from days 21 to 35 (Figure 4).

Administration of SnPP resulted in a slight reduction in iNOS and HO-1 expression at day 25, whereas COX-2 was not affected (Figure 5). At the same time, SnPP also inhibited the levels of $\text{NO}_2^- + \text{NO}_3^-$ in paw and serum at day 25 (Figure 6a), whereas PGE_2 was significantly reduced at days 25 and 35 only in paws (Figure 6b). SnPP also inhibited $\text{TNF}\alpha$ and VEGF (Figure 7) measured in paws homogenates at days 25 and 35 after adjuvant injection as well as VEGF present in serum at the first time.

Reduction of iNOS activity by the selective inhibitor L-NIL decreased HO-1 protein expression at days 25 and 35 (Figure 5). As expected, L-NIL administration resulted in a lower NO production, as measured by $\text{NO}_2^- + \text{NO}_3^-$ levels in paw homogenates and serum (Figure 6a). This compound also slightly reduced PGE_2 levels at day 25 (Figure 6b) in

Figure 3 (a) Time course of iNOS, COX-2 and HO-1 expression in paw homogenates. This figure is representative of three similar experiments. Levels of (b) PGE₂ and NO (measured as NO₂⁻ + NO₃⁻) and (c) VEGF and TNFα in paw homogenates. Data represent mean ± s.e.m. (*n* = 6 animals per group). **P* < 0.05; ***P* < 0.01 compared with naïve rat group.

paw homogenates, without modifying TNFα or VEGF (Figure 7).

Effect of SnPP on TUNEL Positivity and CD31 Expression

As SnPP showed therapeutic effects in this experimental model, we next examined its influence on some parameters relevant to the maintenance of arthritis. Apoptosis was determined by TUNEL positivity. Figure 8 shows a high number of positive cells in paw sections from arthritic animals at day 25. In contrast, there was a marked decrease in

Figure 4 Time course of PGE₂, VEGF, and NO (measured as NO₂⁻ + NO₃⁻) serum levels. Data represent mean ± s.e.m. (*n* = 6 animals per group). **P* < 0.05; ***P* < 0.01 compared with naïve rat group.

the number of TUNEL-positive cells in animals treated with SnPP. As neovascularization plays an important role in the pathophysiology of synovitis, the presence of microvessels was assessed by immunohistochemistry. For this purpose, endothelial cells were revealed using a specific antibody against the endothelial marker CD31 (Figure 9). Interestingly, paw sections from SnPP-treated rats showed a lower number of CD31-positive endothelial cells and blood vessels, in comparison with arthritic controls.

Discussion

In adjuvant arthritis, chronic inflammation is characterized by prominent paw edema, monocytic and neutrophilic infiltration of the synovium, hyperplasia, pannus invasion and erosion of cartilage and bone. In this experimental model, a similarity has been reported between the development of arthritis and iNOS expression and activity.¹¹ Augmented NO production after iNOS induction may be involved in the pathogenesis of adjuvant arthritis by causing defects in lymphocyte function.³⁰ We have examined the temporal pattern of HO-1 expression in rat adjuvant arthritis and found that an increased NO production in the paw preceded the upregulation of HO-1. In this study, therapeutic L-NIL administration significantly inhibited iNOS activity, as reflected by the reduction in NO₂⁻ + NO₃⁻ levels, which was ineffective in controlling arthritis, in agreement with previous studies.¹¹ It has been found that iNOS activity upregulates HO-1 in inflammatory models such as rat nephritis³¹ or mouse air pouch.²⁵ In the chronic phase of rat adjuvant arthritis, we have shown that selective iNOS inhibition results in decreased HO-1 expression, suggesting that endogenously NO generated by this enzyme participates

Figure 5 Effect of SnPP (12 mg/kg; i.p.) and L-NIL (20 mg/kg; p.o.) on iNOS, COX-2 and HO-1 expression in paw homogenates from adjuvant-induced arthritic rats. Compounds were administered twice-daily from day 17 to day 25 and paw tissues were recovered on day 25 or 35 after arthritis induction for analysis. The figure is representative of three experiments ($n=6$ animal per group).

Figure 6 Effect of SnPP (12 mg/kg; i.p.) and L-NIL (20 mg/kg; p.o.) on (a) NO (measured as NO₂⁻ + NO₃⁻), and (b) PGE₂ levels in paw homogenates and serum from adjuvant-induced arthritic rats. Compounds were administered twice-daily from day 17 to 24 after arthritis induction. Paw tissues and serum were recovered on day 25 or 35 after arthritis induction for analysis. AR, arthritic control group. Data represent mean $\pm$ s.e.m. ($n=6$ animals per group). * $P<0.05$; ** $P<0.01$ compared with AR on day 25 after arthritis induction. # $P<0.05$; ## $P<0.01$ compared with AR on day 35 after arthritis induction.

Figure 7 Effect of SnPP (12 mg/kg; i.p.) and L-NIL (20 mg/kg; p.o.) on (a) VEGF and (b) TNF α levels in paw homogenates and serum from adjuvant-induced arthritic rats. Compounds were administered twice-daily from day 17 to 24 after arthritis induction. Paw tissues and serum were recovered on day 25 or 35 after arthritis induction for analysis. AR, arthritic control group. Data represent mean $\pm$ s.e.m. ($n=6$ animals per group). * $P<0.05$; ** $P<0.01$ compared with AR on day 25 after arthritis induction. # $P<0.05$; ### $P<0.01$ compared with AR on day 35 after arthritis induction.

in HO-1 induction. In a number of *in vitro* and *in vivo* systems, there is a negative feedback interaction between iNOS and HO-1 and thus overexpression of HO-1 results in inhibition of iNOS expression or activity, which can be protective against NO-mediated toxicity.^{25,31,32} In this study, the HO-1 inhibitor SnPP partially inhibited iNOS expression and NO production in the rat adjuvant arthritis. It should be noted that although protoporphyrins are widely used as pharmacological tools to inhibit HO-1 activity, some of these agents can exert direct inhibitory effects on iNOS. Nevertheless, this seems unlikely in the present experiments as inhibition of either NOS or soluble guanylate cyclase activities by SnPP have been observed at high concentrations only³³ and even stimulatory effects have been reported in some *in vitro* systems.³⁴ In addition, the lack of beneficial effects of the selective iNOS inhibitor in the present experimental model supports the view that the anti-inflammatory activity of SnPP would not depend on inhibition of iNOS.

Cytokines and chemokines are important mediators of the adjuvant arthritis process.³⁵ In particular, TNF α production may play an important role in leukocyte recruitment to the joints in this experimental model,³⁶ and iNOS and COX-2 expression in

the synovial tissue and cartilage of arthritic joints is regulated by inflammatory cytokines such as TNF α and IL-1.³⁷ It is worth noting that SnPP inhibited experimental polyarthritis when treatment was initiated after clinical symptoms had appeared. Our data have shown that TNF α levels in paws coincided with the onset of arthritis and that treatment of arthritic animals with SnPP significantly reduced the levels of this cytokine, which could be relevant for the antiarthritic effects of this agent. The ability to decrease in part the cellular infiltrate in arthritic joints could contribute to the observed reduction in the levels of iNOS and HO-1 in paws.

In the rat adjuvant arthritis, upregulation of COX-2 on the first days of the response results in a high PGE₂ production associated with the development of paw swelling. It has been reported that this eicosanoid enhances local COX-2 expression and thus either selective or nonselective COX-2 inhibition decreases COX-2 expression in paws.³⁸ In this experimental model, SnPP administration after the onset of the arthritis did not modify COX-2 expression and reduced PGE₂ levels in paws, suggesting a partial inhibition of COX-2 activity by this treatment. Inhibitory effects on NO production could

Figure 8 Representative hind paw sections after TUNEL analysis of (a) arthritic control group and (b) SnPP-treated arthritic rats; original magnification $\times 200$. TUNEL analysis demonstrates decreased levels of programmed cell death in the treated rat compared to the control arthritic rats.

Figure 9 Immunohistochemical analysis of CD31 expression in hind paw sections of (a) arthritic control group and (b) SnPP-treated arthritic rats. Representative photomicrographs of hind paw sections immunostained using a monoclonal anti-CD31 antibody and counterstained with hematoxylin; original magnification $\times 200$.

contribute to lower PGE_2 synthesis,³⁹ as observed in the group treated with L-NIL. In addition, our results suggest that products derived from HO-1 activity may be involved in COX activation. This is in line with the reported increase in PGE_2 release induced by bilirubin in rat astrocytes.⁴⁰ Nevertheless, in systems where high levels of HO-1 expression can be induced, this enzyme activity would be able to deplete cellular heme leading to decreased COX activity and PG production, as reported in microvessel endothelial cells transfected with the HO-1 gene or treated with CoCl_2 .⁴¹

Joint tissues are subject to a complex network of regulatory mediators. ROS production in rheumatoid arthritis affects signaling pathways and may cause damage to matrix components through reduction of synthesis, induction of apoptosis or activation of metalloproteinases.⁴² Production of NO in arthritis is also associated with matrix degradation and cellular apoptosis.⁴³ Inadequate apoptosis is believed to contribute to synovial hyperplasia and progression of chronic arthritis. Similarly, in adjuvant arthritis there is a lack of apoptosis in synovial tissues during the initial phases accompanied by expression of antiapoptotic proteins such as Bcl-2,⁴⁴ whereas overexpression of p53 is

greater during the latter stages of this model.⁴⁵ Thus, apoptotic cells are present when the chronic arthritis develops although apoptosis may be impaired.⁴⁵ Our data suggest that SnPP anti-inflammatory effects on chronic arthritis are accompanied by a reduction in the number of apoptotic cells in inflamed tissues.

The HO-1 and NOS pathways appear to participate in the maintenance of tissue oxygenation. It is known that HO-1 is upregulated by hypoxia in a number of cell types. In endothelial cells, redox reactions involving NO and S-nitrosothiols may regulate HO-1 gene expression by reduced oxygen levels.⁴⁶ Joint inflammation is associated with increased cellular hypoxia and overexpression of hypoxia-inducible factor-1, mediating the expression of hypoxic-sensitive genes been demonstrated in rheumatoid synovial tissue⁴⁷ and adjuvant arthritis.⁴⁸ Hypoxia is an inducer of cytokines, matrix-degrading enzymes and angiogenic factors such as VEGF. Hypoxia-driven angiogenesis is critical for extracellular matrix formation and remodeling in soft-tissue repair.⁴⁹

The inflamed synovium of rheumatoid arthritis shows a high level of angiogenesis¹ and increased

levels of VEGF in synovial fluid and serum of patients with rheumatoid arthritis correlate with disease severity.⁵⁰ VEGF is the main mediator of angiogenesis and also a potent vascular permeability agent and a chemoattractant for leukocytes.⁵¹ It is believed that VEGF plays a central role in pannus development and thus administration of specific inhibitors of the VEGF-RI pathway suppresses arthritis and prevents bone destruction in experimental arthritis.⁵² It has been demonstrated that HO-1 activity can be related to angiogenesis. Thus, this enzyme has been shown to induce VEGF in cultured endothelial cells by an increased transcription likely dependent on CO generated by HO-1 activity.⁵³ In adjuvant arthritis, local induction of TNF α , COX-2 and PGE $_2$, iNOS and NO as well as HO-1 preceded the elevation in VEGF. All these mediators may cooperate to induce VEGF during the established phase of this model. We have also shown that HO-1 inhibition results in reduction of VEGF production. This finding confirms the *in vitro* effects of SnPP in endothelial cells,⁵⁴ rat vascular smooth muscle cells and RAW 264.7 murine macrophages in basal conditions or after stimulation with IL-1 β or lipopolysaccharide, respectively.³⁴ The attenuation of VEGF after HO-1 inhibition could be responsible for the decreased formation of microvessels detected by immunohistochemistry in inflamed tissues from animals treated with SnPP. Our results thus suggest that HO-1 is involved in the angiogenic process in this experimental model. Since the full expression of experimental arthritis depends on the generation of new blood vessels,⁵⁵ the inhibition of angiogenesis may play a role in the therapeutic effects of SnPP in adjuvant arthritis.

Overexpression of HO-1 can be protective against cellular stress (reviewed in Alcaraz *et al*¹²). On the contrary, some studies indicate that HO-1 enzymatic actions are not always associated with beneficial effects. Thus, iron release by HO activity contributes to lipid peroxidation⁵⁶ and tissue damage,⁵⁷ whereas HO inhibition exerts antioxidant effects protecting against myoglobin cytotoxicity,⁵⁸ sepsis-induced liver dysfunction⁵⁹ or experimental allergic encephalomyelitis.⁵⁷ It has also been reported that hypoxia/reoxygenation activates the HO-1/CO pathway leading to cGMP production, which is a major stimulus for macrophage activation and synthesis of TNF α .⁶⁰

Our data show that HO-1 is upregulated during the maintenance of chronic inflammation in the rat adjuvant arthritis and may participate in the inflammatory features of this model. In particular, HO-1 activity is involved in VEGF production and angiogenesis during chronic inflammation. Although it has not been determined whether SnPP also acts on other factors that are related to the pathogenesis of adjuvant arthritis besides inhibition of HO activity, these findings suggest that HO-1 plays a role in this animal model of chronic arthritis.

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