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Influence of Heme Oxygenase 1 Modulation on the Progression of Murine Collagen-Induced Arthritis

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Objective. **Heme oxygenase 1 (HO-1) can be induced by inflammatory mediators as an adaptive response. The objective of the present study was to determine the consequences of HO-1 modulation in the murine collagen-induced arthritis (CIA) model.**

Methods. **DBA/1J mice were treated with an inhibitor of HO-1, tin protoporphyrin IX (SnPP), or with an inducer of HO-1, cobalt protoporphyrin IX (CoPP), from day 22 to day 29 after CIA induction. The clinical evolution of disease was monitored visually. At the end of the experiment, joints were examined for histopathologic changes. Cytokine levels in paws were measured by enzyme-linked immunosorbent assay. Levels of HO-1, cyclooxygenase 2 (COX-2), and prostaglandin E_2 (PGE_2) were determined. Effects of treatments on the early phase of disease and after prophylactic administration were also assessed.**

Results. **CoPP strongly induced HO-1, resulting in the inhibition of cartilage erosion accompanied by extensive fibrosis in the joint. Levels of tumor necrosis factor α (TNFα), interleukin-2 (IL-2), and IL-10 were inhibited by CoPP, whereas levels of vascular endothelial growth factor were increased. Treatment with SnPP significantly reduced the severity of CIA, with inhibition of joint inflammation and cartilage destruction. The levels of PGE_2, IL-1β, and TNFα were also significantly reduced by SnPP treatment, which did not modify COX-2 protein expression. SnPP was more effective than CoPP in preventing the development of CIA (prophylactic administration).**

Conclusion. **HO-1 is induced during CIA. Although overexpression of this protein causes some beneficial effects, strategies aimed at HO-1 overexpression cannot slow the progression of the chronic inflammatory disease, whereas treatment with SnPP, which inhibits HO-1, exerts prophylactic and therapeutic effects.**

Rheumatoid arthritis is characterized by chronic articular inflammation and progressive destruction of the joints (1). Some experimental models of autoimmune disease can be induced in animals by immunization with type II collagen. Collagen-induced arthritis (CIA) is an example of an autoimmune disease with a predominant Th1 response at the time of arthritis onset (2). This model of rheumatoid arthritis exhibits clinical and histopathologic features similar to those of the human disease (3). Studies of CIA models have helped in our understanding of the molecular mechanisms involved in the pathogenesis of chronic inflammatory joint disease, and the CIA model has been widely used in the search for new antiarthritis treatments (4–6).

Heme oxygenase (HO) activity catabolizes heme to biliverdin, which is reduced to bilirubin by biliverdin reductase, carbon monoxide, and iron. HO-1 is induced by a variety of stimuli or agents that cause oxidative stress, such as cytokines, reactive oxygen species, nitric oxide, heat shock, ultraviolet radiation, hypoxia, and hyperoxia (7,8). In inflammatory and immune conditions, high levels of mediators with the potential to induce HO-1 are produced, and the expression of this protein could be part of an adaptive mechanism for limiting cytotoxicity via several means, including radical scavenging or inhibition of cell proliferation and apoptosis (9,10). The beneficial effects of HO-1 overexpression have been shown in a number of conditions, such as endotoxemia (11), lung hyperoxia (12), atherogenesis (13), myocardial injury after ischemia-reperfusion (14), and cardiac allograft survival (15). HO-1 induction has also been shown to down-regulate the inflammatory response in animal models of acute inflammation. For example, we have recently demonstrated the antiinflammatory effects of HO-1 in the zymosan-injected

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mouse air-pouch model (16). In addition, several studies have suggested that HO-1 expression in response to different inflammatory mediators may contribute to the resolution of inflammation (17).

Because of the postulated effects of HO-1 on inflammatory responses, this study was directed toward exploring the possible effects of HO-1 modulation on chronic inflammatory diseases. Thus, we examined the consequences of HO-1 inhibition and HO-1 induction on the onset and progression of CIA in a murine model.

MATERIALS AND METHODS

Animals. Arthritis was induced in DBA/1J mice (Charles River, Barcelona, Spain) between 10 and 12 weeks of age. Water and food were provided ad libitum. 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.

Materials. We obtained [5,6,8,11,12,14,15(*N*)-^{3}H]-prostaglandin E_2 (PGE_2) from Amersham Biosciences (Barcelona, Spain). Cyclooxygenase 2 (COX-2)–specific polyclonal antibody was purchased from Cayman Chemical (Ann Arbor, MI). Cobalt protoporphyrin IX (CoPP) and tin protoporphyrin IX (SnPP) were obtained from Frontier Scientific Europe (Carnforth, UK). Peroxidase-conjugated goat anti-rabbit IgG was purchased from Dako (Glostrup, Denmark). Kits for enzyme-linked immunosorbent assay (ELISA) of tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and vascular endothelial growth factor (VEGF) were purchased from R&D Systems (Minneapolis, MN). The detection limits of these kits were 33–2,700 pg/ml, 25–2,000 pg/ml, and 8–500 pg/ml, respectively. ELISA kits for interferon-γ (IFNγ), IL-2, and IL-10 were obtained from eBioscience (San Diego, CA), with detection limits of 15–2,000 pg/ml for IFNγ and IL-10, and 2–200 pg/ml for IL-2. The rest of the reagents were from Sigma (St. Louis, MO).

Induction of CIA. Bovine type II collagen (CII) was prepared as previously described (18) and then diluted in 0.05*M* acetic acid to a concentration of 2 mg/ml. This was emulsified in equal volumes of Freund's complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis* strain H37Ra; Difco, Detroit, MI). On day 0, DBA/1J mice were immunized at the base of the tail with 100 μg of bovine CII. On day 21, mice received an intraperitoneal booster injection of 100 μg of CII dissolved in phosphate buffered saline (PBS).

Mice were considered to have arthritis when significant changes of redness and/or swelling were noted in the digits or in other parts of the paws. Joint inflammation in each paw was scored visually, using a scale of 0–2, where 0 = uninflamed, 1 = mild inflammation, 1.5 = marked inflammation, and 2 = severe inflammation. Scoring was performed by independent observers (ID and MLF) without knowledge of the experimental groups.

Treatment groups. For therapeutic studies, animals with a minimum inflammation score of 1 on day 22 were randomized into treatment groups. CoPP (2.5 mg/kg of body weight, intraperitoneally twice a day) or SnPP (6 mg/kg of body weight, intraperitoneally twice a day) was administered from day 22 to day 29 after immunization (therapeutic day 10 group), and animals were killed on day 30 after CIA induction. These doses were selected in preliminary experiments.

In another series of experiments, CoPP and SnPP were administered as described above from day 22 to day 25 after immunization (therapeutic day 5 group), and the animals were killed on day 26 after CIA induction. For studies of prophylactic therapy, CoPP (2.5 mg/kg of body weight, intraperitoneally twice a day) or SnPP (6 mg/kg of body weight, intraperitoneally twice a day) was administered from day 20 (before clinical evidence of CIA) until day 34 after immunization (prophylactic group), and animals were killed by cervical dislocation on day 35.

Measurement of cytokine and PGE_2. Hind paws were amputated above the ankle and homogenized in 1 ml of 10 m*M* HEPES buffer, pH 7.4, containing 0.32*M* sucrose, 100 m*M* EDTA, 1 m*M* dithiothreitol, 2 m*M* phenylmethylsulfonyl fluoride, and 100 m*M* leupeptin. After centrifugation at 1,200*g* for 15 minutes at 4°C, supernatants were removed and used for determination of PGE_2 by radioimmunoassay (19) and for determination of cytokine levels by ELISA.

Western blot analysis. Supernatants from paw homogenates were centrifuged at 10,000*g* for 15 minutes at 4°C, and the new supernatants were removed and used to determine 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 12.5% sodium dodecyl sulfate–polyacrylamide gels and transferred onto polyvinylidene difluoride membranes for 90 minutes at 125 mA. Membranes were blocked with PBS–Tween 20 (0.02*M*, pH 7.0) containing 3% weight/volume nonfat milk and incubated with a specific polyclonal antibody against HO-1 (20) or COX-2 (1:1,000 dilution). Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:20,000 dilution). The immunoreactive bands were visualized using an enhanced chemiluminescence system.

Histologic analysis. Mice were killed, and whole knee joints were removed and fixed in 10% formalin. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding (21). Tissue sections (7 μm) were stained with hematoxylin and eosin or with Safranin O.

Histopathologic changes were scored on hematoxylin and eosin–stained sections using the following parameters. Cellular infiltration was scored on a scale of 0–3, according to the number of inflammatory cells in the synovial cavity (exudate) and synovial tissue (infiltrate), where 0 = none and 3 = the highest number of cells in an arthritic specimen. Proteoglycan depletion was determined on Safranin O–stained sections. Loss of proteoglycans was scored on a scale of 0–3, where 0 = fully stained cartilage and 3 = destained cartilage or complete loss of articular cartilage.

A characteristic parameter of CIA is the progressive loss of articular cartilage. This feature was graded separately for the appearance of dead chondrocytes (empty lacunae) and the loss of articular cartilage (cartilage surface erosion). The degree of chondrocyte death was scored on a scale of 0–3, where 0 = no 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 (18).

Histopathologic changes in the knee joints were scored

on 5 semiserial sections of the joint. Two observers who were unaware of the experimental group performed all scorings.

Immunohistochemistry for IL-1β. Tissue sections were prepared as described above. After treatment with 3% H_2O_2 for 10 minutes at room temperature followed by 10 m*M* citrate (pH 6.0) for 2 hours, sections were incubated for 1 hour with rabbit anti-mouse IL-1β antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit Ig antibody (Dako, Carpinteria, CA) was used as control. After rinsing, sections were incubated with biotinylated horseradish peroxidase–conjugated goat anti-rabbit IgG (Dako). Peroxidase staining was developed with diaminobenzidine (Sigma). Counterstaining was performed with hematoxylin. IL-1β–positive cells were counted in 5 random high-power fields by 2 independent observers who were unaware of the experimental group.

Statistical analysis. Results are presented as the mean ± SEM. The level of statistical significance was determined by analysis of variance, followed by Dunnett's *t*-test for multiple comparisons.

Table 1. Effects of treatment with SnPP and CoPP on the pathologic condition of knee joints from mice with collagen-induced arthritis*

Treatment	Synovial infiltrate	Synovial exudate	Cartilage erosion	Chondrocyte death	Proteoglycan depletion
Control	2.0 ± 0.1	0.8 ± 0.1	2.3 ± 0.2	1.8 ± 0.2	2.0 ± 0.1
SnPP	1.2 ± 0.3†	0.2 ± 0.1†	0.6 ± 0.2‡	0.5 ± 0.2‡	1.1 ± 0.3‡
CoPP	2.0 ± 0.1	ND	1.4 ± 0.3†	0.9 ± 0.2†	1.9 ± 0.2

* Joints were examined for histopathologic changes after treatment with tin protoporphyrin IX (SnPP; 6 mg/kg of body weight, intraperitoneally twice a day), an inhibitor of heme oxygenase 1 (HO-1), or with cobalt protoporphyrin IX (CoPP; 2.5 mg/kg of body weight, intraperitoneally twice a day), an inducer of HO-1, from day 22 to day 29 after immunization with bovine type II collagen. On day 30, synovial infiltrates, synovial exudates, cartilage erosion, chondrocyte death, and proteoglycan depletion were scored on a scale of 0–3. Values are the mean ± SEM (n = 10–12 mice per group). ND = not determined (because of the presence of fibrosis).
† $P < 0.05$ versus control arthritic mice.
‡ $P < 0.01$ versus control arthritic mice.

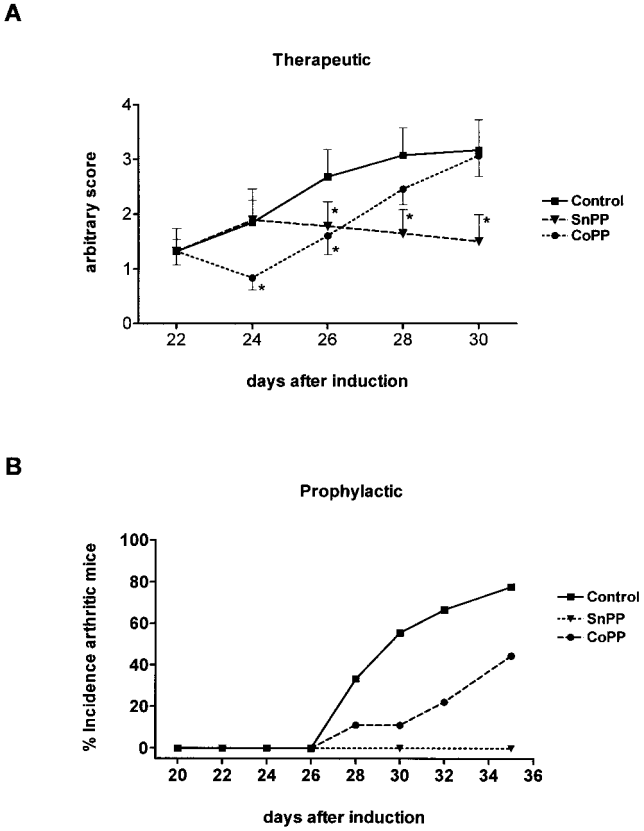

Figure 1. Effect of treatment with tin protoporphyrin IX (SnPP; 6 mg/kg of body weight, intraperitoneally twice a day) and cobalt protoporphyrin IX (CoPP; 2.5 mg/kg of body weight, intraperitoneally twice a day) on the clinical symptoms of collagen-induced arthritis. **A,** Clinical scores in mice with CIA after therapeutic administration (therapeutic day 10) of SnPP and CoPP. Values are the mean ± SEM of 10–12 mice per group. * = $P < 0.05$ versus arthritic mice (controls). **B,** Percentages of mice with arthritis at the different time points assessed after prophylactic administration of SnPP and CoPP (n = 10 mice per group).

RESULTS

Effects of therapeutic administration of CoPP and SnPP in the therapeutic day 10 group. To determine whether the induction or the inhibition of HO-1 is capable of modulating the severity of CIA, mice were treated with CoPP or SnPP from day 22 to day 29 after CIA induction and were killed on day 30. Figure 1A shows the changes in the macroscopic arthritis scores after drug treatment compared with the control arthritic group. CoPP administration resulted in an initial improvement in clinical disease activity, since it significantly reduced joint swelling and redness on days 24–26, but was followed by a loss of efficacy. In contrast, administration of SnPP ameliorated the clinical expression of CIA from day 26 until the end of the experiment (day 30).

Histologic analysis indicated the presence of numerous granulocytes and mononuclear cells in the joints of control arthritic mice on day 30 (Table 1 and Figure 2). At this time, CoPP treatment failed to modify cell influx into the synovium and induced a high degree of fibrosis. In contrast, SnPP reduced cell infiltration into synovial tissues as well as the number of cells present in the joint cavity (exudate). Control animals also showed cartilage erosion, chondrocyte death, and proteoglycan depletion. All these parameters were significantly reduced by SnPP treatment, whereas CoPP treatment inhibited cartilage erosion and chondrocyte

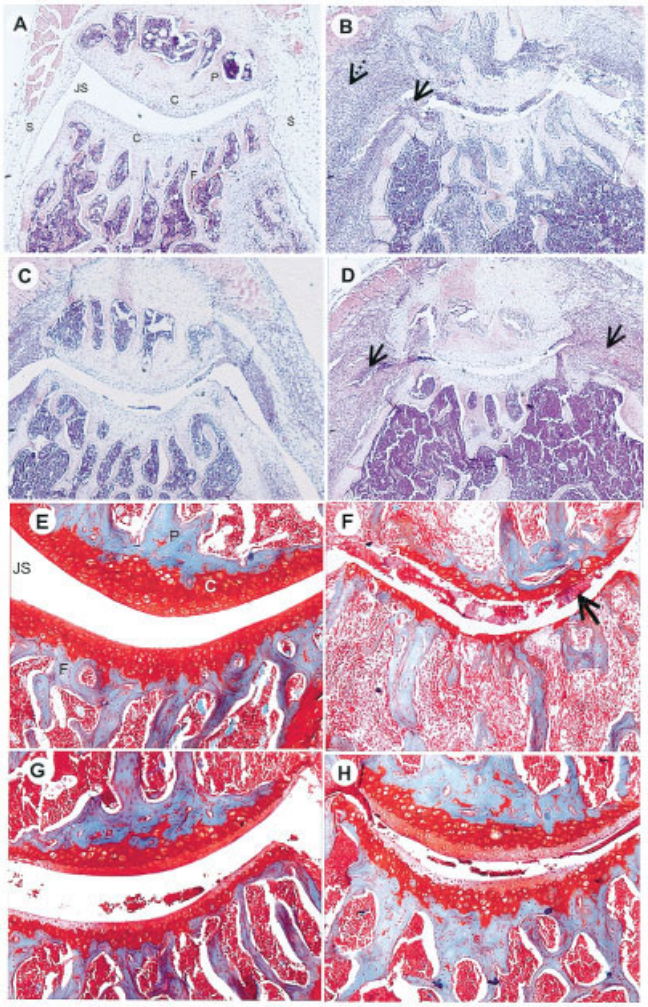

Figure 2. Histologic analysis of knee joints from mice treated with tin protoporphyrin IX (SnPP) and cobalt protoporphyrin IX (CoPP) (therapeutic day 10). Knee joints were harvested on day 30 of collagen-induced arthritis. **A–D,** Frontal sections of the knee joint stained with hematoxylin and eosin. **A,** Section from a naive mouse, showing normal joint architecture. **P** = patella; **C** = cartilage; **JS** = joint space; **S** = synovium; **F** = femur. **B,** Section from an arthritic control mouse, showing marked synovial infiltrate **(broken arrow)** and exudate **(arrow)**. **C,** Section from a mouse treated with SnPP (6 mg/kg of body weight, intraperitoneally twice a day). **D,** Section from a mouse treated with CoPP (2.5 mg/kg of body weight, intraperitoneally twice a day), showing marked fibrosis **(arrows)**. **E–H,** Frontal sections of the knee joint stained with Safranin O. **E,** Section from a naive mouse, showing normal joint architecture. **F,** Section from an arthritic control mouse, showing marked cartilage destruction and chondrocyte death **(arrow)**. **G,** Section from a mouse treated with SnPP. **H,** Section from a mouse treated with CoPP. (Original magnification × 40 in **A–D**; × 100 in **E–H**.)

death, but to a lesser extent and without modification of proteoglycan depletion.

At the end of the experimental period (day 30), the levels of IL-1β, TNFα, VEGF, IFNγ, IL-2, and IL-10 were determined in hind paw homogenates. As shown in Table 2, animals treated with CoPP showed no significant changes in IL-1β levels, but a significant inhibition of TNFα levels and an increase in VEGF levels. SnPP treatment significantly inhibited IL-1β and TNFα levels, but not VEGF levels. None of the treatments affected IFNγ. In contrast, IL-2 and IL-10 levels were reduced in the animals treated with CoPP, whereas SnPP did not appreciably alter the levels of these cytokines.

Effects of treatment on levels of IL-1β were confirmed by immunohistochemistry of knee joint sections (Figure 3). For the arthritic control group, a significant increase ($P < 0.05$) in cells positive for IL-1β was observed (108.3 $\pm$ 19.0) with respect to naive animals (53.7 $\pm$ 20.1). In the group treated with SnPP, the immunoreactivity for this cytokine was significantly reduced (56.1 $\pm$ 13.4; $P < 0.05$ versus the arthritic control group), whereas CoPP did not affect IL-1β expression (94.9 $\pm$ 20.5).

Effects of therapeutic administration of CoPP and SnPP in the therapeutic day 5 group. To confirm the observed effects on the early phase of arthritis and to determine the levels of inflammatory mediators, another series of experiments in mice with established CIA was performed. In this therapeutic day 5 group, CoPP and SnPP were administered from day 22 to day 25, and animals were killed on day 26. At this time point, both treatment groups showed a partial reduction in the clinical score (0.8 $\pm$ 0.1 in CoPP mice and 0.8 $\pm$ 0.4 in SnPP mice; $P < 0.05$) with respect to the arthritic control group (1.9 $\pm$ 0.3; n = 9 mice per group). Histologic analysis did not show significant differences in any of the treatment groups as compared with the arthritic control group (data not shown).

As shown in Table 2, the effects on inflammatory mediators measured in paw tissues were similar to those seen on day 30. CoPP significantly reduced only the TNFα levels, whereas SnPP significantly reduced the levels of TNFα, IL-1β, VEGF, and PGE_2.

Effects of prophylactic treatment with CoPP and SnPP. To determine whether the induction or the inhibition of HO-1 is able to prevent the development of arthritis, the HO-1 inducer CoPP and the HO inhibitor SnPP were administered to collagen-immunized mice before the onset of disease (from day 20 until day 34), and animals were killed on day 35. CoPP treatment resulted in a lower incidence of CIA (Figure 1B) and a significantly reduced mean clinical score on day 35 (0.6 $\pm$ 0.3; $P < 0.01$) compared with that in the arthritic control mice (2.1 $\pm$ 0.4). Interestingly, clinical signs of

Table 2. Effect of treatment with SnPP and CoPP on cytokine and PGE_2 levels in paw homogenates from mice with collagen-induced arthritis*

Group	TNFα, pg/ml	IL-1β, pg/ml	IFNγ, pg/ml	IL-10, pg/ml	IL-2, pg/ml	VEGF, pg/ml	PGE_2, ng/ml
Therapeutic day 10							
Control	2,867.0 ± 257.0	1,803.0 ± 237.0	32.7 ± 2.7	442.0 ± 34.6	9.4 ± 0.5	58.2 ± 4.7	213.7 ± 13.1
SnPP	2,047.0 ± 156.0†	864.0 ± 28.0‡	32.4 ± 2.8	453.7 ± 27.4	8.4 ± 0.7	66.7 ± 6.9	114.3 ± 9.6‡
CoPP	1,801.0 ± 134.0‡	1,686.0 ± 171.0	31.2 ± 2.1	349.9 ± 33.3†	6.4 ± 0.5‡	82.0 ± 5.7‡	201.9 ± 17.6
Therapeutic day 5							
Control	161.4 ± 7.9	1,073.0 ± 189.1	62.3 ± 6.8	695.7 ± 29.6	18.3 ± 4.3	152.1 ± 9.2	141.2 ± 16.3
SnPP	116.7 ± 6.4†	317.1 ± 33.3‡	52.2 ± 6.8	593.3 ± 67.1	16.3 ± 4.9	95.7 ± 5.9†	95.3 ± 9.9†
CoPP	104.6 ± 19.8†	751.3 ± 169.4	64.6 ± 9.2	583.1 ± 76.7	26.7 ± 7.3	160.6 ± 14.7	103.4 ± 8.8
Prophylactic therapy							
Control	217.5 ± 11.0	629.0 ± 107.0	316.2 ± 80.7	588.0 ± 37.8	15.1 ± 2.0	61.2 ± 2.1	65.0 ± 5.2
SnPP	142.8 ± 18.5†	485.7 ± 48.8†	200.0 ± 24.1	472.1 ± 37.0	15.7 ± 2.1	49.6 ± 1.3†	44.9 ± 4.4‡
CoPP	269.7 ± 16.6	696.2 ± 73.7	369.7 ± 15.8	763.6 ± 88.8	21.2 ± 3.2	74.3 ± 5.1†	66.2 ± 3.4

* Values are the mean ± SEM (n = 9–12 mice per group). See Materials and Methods for a description of the experimental groups. SnPP = tin protoporphyrin IX; CoPP = cobalt protoporphyrin IX; PGE_2 = prostaglandin E_2; TNFα = tumor necrosis factor α; IL-1β = interleukin-1β; IFNγ = interferon-γ; VEGF = vascular endothelial growth factor.
† $P < 0.05$ versus control arthritic mice.
‡ $P < 0.01$ versus control arthritic mice.

arthritis did not develop in mice treated with SnPP. After prophylactic administration of CoPP, VEGF levels in paw tissues were increased (Table 2). In contrast, administration of SnPP resulted in significantly decreased paw tissue levels of TNFα, IL-1β, and VEGF, as well as significantly reduced PGE_2 levels.

Effects of CoPP and SnPP treatment on HO-1 and COX-2 protein expression. Western blot analysis was performed on hind paw homogenates from animals of the therapeutic day 10, therapeutic day 5, and prophylactic groups (Figure 4). In the control animals with CIA, HO-1 protein was present in the therapeutic day 10 and prophylactic groups. CoPP administration strongly augmented HO-1 protein expression after prophylactic or therapeutic treatments, whereas the HO-1 inhibitor SnPP caused only a partial reduction in the expression of this protein in the therapeutic day 10 group compared with the arthritic control group. COX-2 was always detected in paw homogenates from control mice, and its expression was not modified by CoPP or SnPP treatment.

DISCUSSION

Our data indicate that HO-1 is induced in inflamed tissues in mice with CIA. The induction of HO-1 could be dependent on the generation of a number of stimuli, such as cytokines or reactive oxygen and nitrogen species. Cytokines play a key role in chronic inflammatory diseases. Increased production of the inflammatory cytokines IL-1 and TNFα by synovial cells can be seen in rheumatoid arthritis (22). Although blocking of IL-1 or TNFα results in clinical improvement (23,24), other mediators are also involved in the pathogenesis of rheumatoid arthritis. Recently, a role in arthritis has been demonstrated for the T cell cytokines IL-17 (6) and IL-18 (25), which drive the production of IL-1 and TNFα and other mediators of inflammation.

TNFα is an important mediator of the inflammatory process (26). It is able to regulate chemokine levels and leukocyte migration into the joints of patients with rheumatoid arthritis (27). In experimental arthritis, TNFα drives early joint inflammation and the acute cellular infiltrate in the synovial tissue (28). Since TNFα plays a role in the onset of clinical symptoms and inflammation (29), the blockade of this cytokine would result in an antiinflammatory effect. We have shown that prophylactic administration of the HO-1 inducer CoPP partially reduced the incidence of CIA. In addition, after therapeutic administration, this compound caused an initial improvement in the arthritis, but failed to slow the progression of the disease. This could be because HO-1 induction by CoPP can partly modify the inflammatory component of this arthritis model by inhibition of TNFα.

The pathogenesis of articular erosion may differ from that of synovial inflammation. It has been reported that inflammation is not necessarily linked to the progression of joint destruction in rheumatoid arthritis, and thus, articular erosion continues despite clinical improvement and is accelerated in the presence of continuing synovitis (30). IL-1 mediates sustained cellular infiltration and erosive cartilage damage (28), and thus, cartilage destruction would be mainly dependent on this cytokine (26). It was recently demonstrated that IL-1 synergizes with mediators such as oncostatin M in

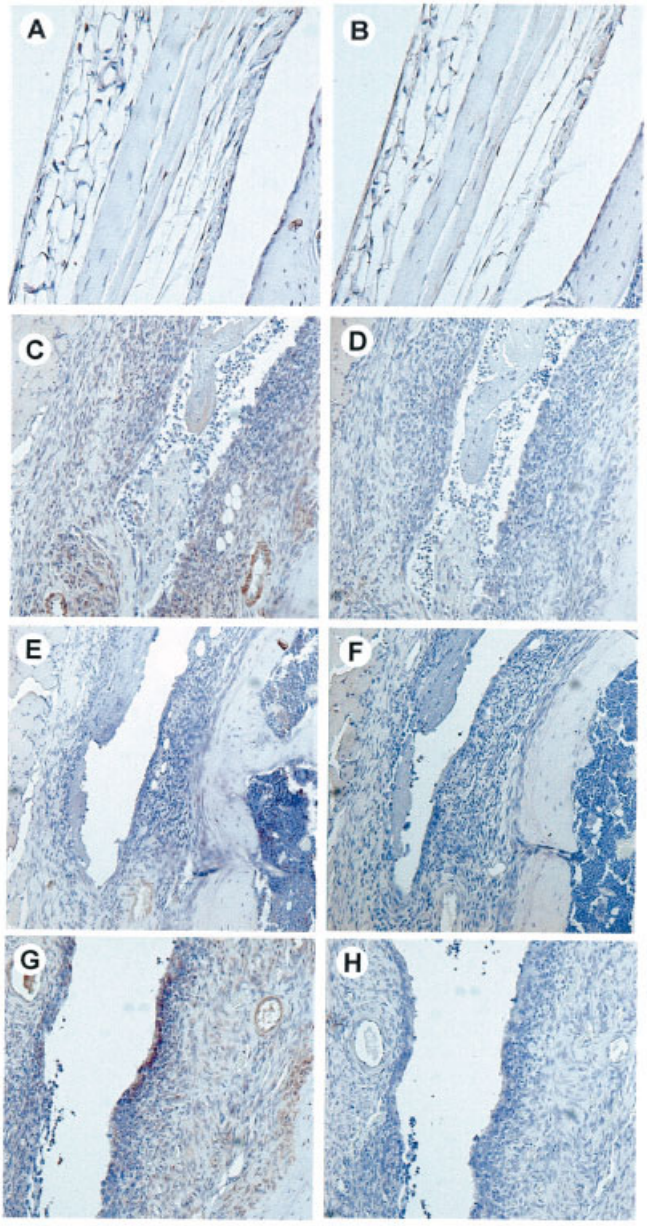

Figure 3. Effect of treatment with tin protoporphyrin IX (SnPP; 6 mg/kg of body weight, intraperitoneally twice a day) and cobalt protoporphyrin IX (CoPP; 2.5 mg/kg of body weight, intraperitoneally twice a day) (therapeutic day 10) on the expression of interleukin-1β (IL-1β) in the synovium. Knee joints were harvested on day 30 of collagen-induced arthritis, and sections were treated with **A, C, E,** and **G,** a specific anti–interleukin-1β antibody and **B, D, F,** and **H,** rabbit IgG control antibody. **A** and **B,** Sections from a naive mouse. **C** and **D,** Sections from an arthritic control mouse. **E** and **F,** Sections from a mouse treated with SnPP. **G** and **H,** Sections from a mouse treated with CoPP. Sections are representative of at least 3 experiments. (Original magnification $\times$ 200.)

rheumatoid joints to induce aggrecan-degrading and collagen-degrading enzymes (31). In murine CIA, we observed both prophylactic and therapeutic effects of SnPP administration, which significantly inhibited levels of IL-1β and TNFα in inflamed tissues.

Several lines of evidence indicate that IL-1β is the pivotal cytokine in CIA, with a main role in cartilage degradation and bone erosion (26). In this experimental model, anti-TNFα treatment does not inhibit the disease if given after the onset of arthritis. In contrast, anti–IL-1α/β treatment is effective in both early and late phases of CIA (29). Nevertheless, CoPP was not able to control the progression of murine CIA, and its protective effects on cartilage were lower than those of SnPP. It is

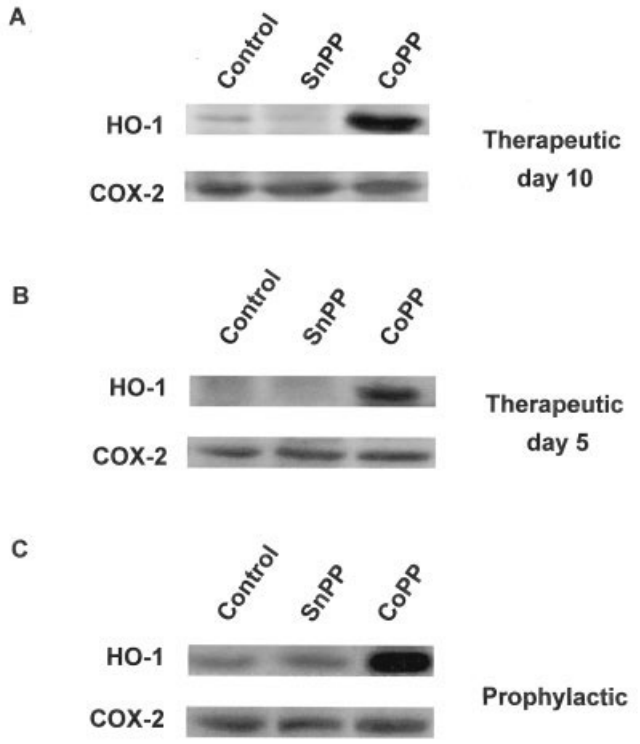

Figure 4. Effect of treatment with tin protoporphyrin IX (SnPP; 6 mg/kg of body weight, intraperitoneally twice a day) and cobalt protoporphyrin IX (CoPP; 2.5 mg/kg of body weight, intraperitoneally twice a day) on the expression of cyclooxygenase 2 (COX-2) and heme oxygenase 1 (HO-1) protein in paw homogenates from mice with collagen-induced arthritis. SnPP and CoPP were administered **A,** from day 22 to day 29 after immunization (therapeutic day 10 group), **B,** from day 22 to day 25 after immunization (therapeutic day 5 group), and **C,** from day 20 (before clinical evidence of arthritis) until day 34 after immunization (prophylactic therapy group). Results are representative of 3 experiments.

interesting to note that CoPP treatment inhibited TNFα and IL-2 levels but failed to modify IL-1β.

Complex interactions between regulatory cytokines occur in autoimmune arthritis. A growing body of evidence suggests that Th1 responses are predominant in the development of murine CIA (2). It has been reported that IL-2 can play an antiinflammatory role during early phases of CIA induction, but is proinflammatory in established disease (32). Previous studies have demonstrated that carbon monoxide inhibits T cell proliferation and IL-2 secretion (33), and biliverdin has been shown to inhibit IL-2 transcription and T cell proliferation in a murine model of heart transplantation (15). Our results in established CIA support the concept that HO-1 activity could exert immunosuppressive effects.

IL-10 inhibits the production of proinflammatory cytokines and has multiple immunomodulatory functions (34). This Th2 cytokine exhibits a wide range of interactions with HO-1. For example, carbon monoxide has been shown to mediate the antiinflammatory effects of IL-10 in a mouse model of septic shock (35). In addition, exogenous administration of HO-1 increases IL-10 production in macrophages, which leads to the resolution of neutrophilic migration in the lung after lipopolysaccharide administration (36). In contrast, HO-1 does not seem to play a significant role in the antiinflammatory activity of IL-10 in human monocytes or macrophages (37,38), and an autoregulatory feedback loop is present in lipopolysaccharide-stimulated human monocytes, involving proinflammatory cytokines and IL-10 (39), with a likely predominant role of TNFα (40). Further studies would be necessary to establish whether the inhibitory effects of CoPP on IL-10 may be the result of interference with feedback mechanisms driven by TNFα or the consequence of immunosuppressive effects on Th2 cells. Since IL-10 may participate in the suppression of arthritis during murine CIA (18), reductions in the levels of IL-10 by CoPP treatment would have detrimental consequences for the progression of disease.

Some studies have indicated the involvement of HO-1 in the resolution of the inflammatory response (41). The possibility exists that stimulation of repair mechanisms would lead to the appearance of fibrosis, as observed in the group treated with CoPP. It has been proposed that during chronic inflammation, HO-1 would exert an antiinflammatory action by inhibiting leukocyte infiltration as well as by facilitating tissue repair through VEGF-driven angiogenesis (42). Production of VEGF after HO-1 overexpression in murine CIA would promote angiogenesis. Nevertheless, VEGF expression correlates with disease severity (43), and it is likely that increased VEGF levels in animals treated with CoPP contribute to the progression of arthritis. This is different from the situation observed in the SnPP-treated mice, where VEGF levels were reduced after prophylactic administration or during the early phase of disease.

Our studies showed that prophylactic administration of the HO-1 inhibitor SnPP prevented the onset of CIA and that treatment after the onset of arthritis inhibited the clinical features of CIA during the late phase of disease. These therapeutic effects of SnPP may be dependent on the treatment-produced reduction of inflammatory cytokines. Interestingly, SnPP inhibited TNFα and IL-1β and reduced cell infiltration into the synovial tissues, which was accompanied by a protective effect on cartilage. SnPP also inhibited PGE_2 levels in inflamed tissues without modifying the expression of COX-2. These results suggest an inhibitory effect of SnPP on COX-2 activity, which is consistent with our findings in the rat adjuvant arthritis model (44).

HO-1 overexpression has shown antiinflammatory or immunomodulatory effects in models of acute disease (10,16,36,45–47). Less is known about the possible role of this enzyme in chronic inflammatory diseases, which involve complex interactions between different cell populations and a wide range of mediators. It has been reported that HO-1 deficiency in mice results in a chronic inflammatory state (48). It is interesting to note that we have recently observed a therapeutic effect of HO-1 inhibition in rat adjuvant arthritis (44). Our data suggest that although HO-1 overexpression exerts some antiinflammatory effects in this murine model of chronic inflammation, it is unable to effectively control the progression of disease and prevent cartilage damage.

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