Heme oxygenase-1 end-products carbon monoxide and biliverdin ameliorate murine collagen-induced arthritis

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Abstract

Objectives

Heme oxygenase-1 (HO-1) which degrades Heme to free iron, biliverdin and carbon monoxide (CO) plays an important role in inflammation. There are, however, conflicting data concerning the role of HO-1 in rheumatoid arthritis (RA) and the therapeutic potential of individual heme degradation products remains to be determined. We therefore investigated the effect of CO and biliverdin upon therapeutic administration in the murine collagen-induced arthritis (CIA) model of RA.

Methods

CIA was induced in DBA/1 mice. Anti-CII antibody levels were determined by ELISA. Mice were scored for paw swelling and grip strength. After the first clinical signs of arthritis one group of animals was treated with biliverdin, the second group was treated with CO. After 60 days all animals were sacrificed and analysed for histomorphological signs of arthritis.

Results

All animals immunised with CII developed serum anti-CII antibodies. Antibody levels were decreased in the CO-treated group. Both, Biliverdin and the CO-treated animals, showed an improvement in clinical disease activity. Histological analysis revealed significantly less inflammation, erosion and reduced numbers of osteoclasts in CO-treated animals only, whereas cartilage degradation was prevented in both biliverdin and CO-treated animals.

Conclusion

Our data demonstrate a beneficial effect of CO, in particular, and biliverdin, on inflammation and bone destruction in the CIA mouse model.

Key words

heme oxygenase, arthritis, inflammation, biliverdin, CO

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Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease, which is characterised by chronic articular inflammation and progressive destruction of the joints (1). RA still remains an incurable disease and even most advanced treatment strategies are able to induce stringent remission in on average only 20% of the patients in clinical practice (2). Therefore, new therapies for RA are still needed.

HO-1 deficiency in mice can lead to a chronic inflammatory state (3), whereas HO-1 overexpression downregulates the inflammatory response in various disease conditions, including murine models of RA (4-7). These observations have led to a number of efforts in order to use the anti-inflammatory effect of HO-1 inducers or of heme degradation products for the treatment of various inflammatory conditions (8, 9).

Partially conflicting results, however, have been reported in studies where HO-1 inducers or heme degradation products were used for the treatment of arthritis in animal models of RA (6, 7, 10-12). Therefore the aim of this study was to further elucidate the therapeutic potential of the two heme degradation products CO and biliverdin in the CIA model. We observed a beneficial effect in regard to clinical signs of inflammation as well as individual effects concerning histomorphological signs of bone and cartilage destruction.

Materials and methods

Animals

DBA/1 mice were obtained from Charles River Laboratories (Charles River Inc., Boston, MA) and were maintained in our animal facility and cared for in accordance with institutional guidelines for animal welfare. All animal experiments were approved by a local ethics committee.

Induction of arthritis in mice

Six eight-week-old DBA/1 mice were immunised intra-dermally at the tail base with 200 μg of chicken collagen type II (CII; Sigma, St. Louis, MO) in 0.05 M acetic acid, emulsified with an equal volume of complete Freund's adjuvant (CFA; Sigma). The mice received a boost immunisation two weeks later with CII emulsified in incomplete Freund's adjuvant (IFA; Sigma). Only animals that developed signs of arthritis (paw swelling of score 1) were included in further experiments.

Clinical assessment

Mice were assessed for clinical signs of arthritis like paw swelling and grip strength three times per week in a blinded manner. Briefly, paw swelling was assessed by using a semiquantitative score: 0=no erythema or swelling, 0.5=swelling of 1 or more digits, 1=erythema and mild swelling of the ankle joint, 2=decent erythema and mild swelling of the entire paw, 3=erythema and moderate swelling involving the entire paw, 4=erythema and severe swelling involving the entire paw. A mean value of front and hind paws was calculated.

The grip strength of each paw was analysed on a wire of 3 mm diameter and recorded using a semiquantitative score: 3=normal grip strength, 2=mildly reduced grip strength, 1=severely reduced grip strength, 0=no grip at all. A mean value of front and hind paws was calculated.

Measurement of serum anti-CII antibody levels

On day 30 after the first immunisation, approximately 50 μl of blood was collected of each animal by bleeding animals from the tail vein. Serum samples were prepared and anti-CII antibody levels were determined by ELISA. Briefly, ELISA plates (Nunc, Rochester, NY) were coated overnight at 4°C with 0.5 μg/ml chicken CII in PBS. After washing with PBS containing 0.05% Tween-20 (Pierce, Rockford, IL), non-specific binding was blocked with PBS 3% Gelatine for 1 h at room temperature. After washing three times, serum samples diluted 1/10000 were added and incubated for 1 h at room temperature. After four washes, horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotech, Birmingham, AL) were added and incubated at room temperature for 1 h, followed by five washes. Plates were developed using 3,3',5,5' -tetram-

ethybezidine (TMB) (Biomedica, Vienna, Austria) as substrate. The OD was measured at 405 nm using a microplate reader (Titertek, Huntsville, AL). The quantity of specific antibody was measured for each animal and data are expressed as mean relative units of activity based on a standard anti-CII serum that was generated from pooled sera of arthritic mice. Antibody values >0.15 units/ml were considered as positive.

Treatment

After the development of first clinical sings of arthritis, animals were randomly divided into four groups. The first group of animals with CIA received PBS i.p. and was allowed to breathe fresh air. The next two groups with CIA were treated with CO or biliverdin, respectively. One group of animals without CIA was left untreated. All animals were sacrificed on day 60 by cervical dislocation.

CO inhalation treatment

Animals that developed clinical signs of arthritis (n=6) were exposed to CO at a concentration of 250 ppm in a closed chamber as described previously (13). Briefly, CO at a concentration of 1% (10,000 p.p.m.) in compressed air was mixed with compressed air in a stainless steel mixing cylinder before being delivered into the exposure chamber. Flow into the 3.70-ft2 plexiglass animal chamber was maintained at rate of 12 l/min. A CO analyser (Interscan, Chatsworth, CA) was used to measure CO levels continuously in the chambers. Gas samples were introduced to the analyser through a port in the top of the chambers at a rate of 1 l/min and were analysed by electrochemical detection, with a sensitivity of 10–600 p.p.m.. Concentration levels were measured hourly and there were no fluctuations in the CO concentrations after the chamber had equilibrated (approximately 5 min). Animals were exposed to CO for one hour per day on 14 consecutive days.

Biliverdin treatment

Animals that developed clinical signs of arthritis (n=6) were treated with 35 mg/kg biliverdin (Frontier Scientific, Logan, UT) for 14 days twice per day by intraperitoneal injection of biliverdin. In order to determine whether biliverdin is reduced to its active metabolite bilirubin, blood was drawn 15 minutes after biliverdin injection. Serum levels of bilirubin were measured by a diazo method using a Hitachi 747 analyser (Roche Diagnostics, Basel, Switzerland) with a detection limit for bilirubin of 0.0039 mg/dl.

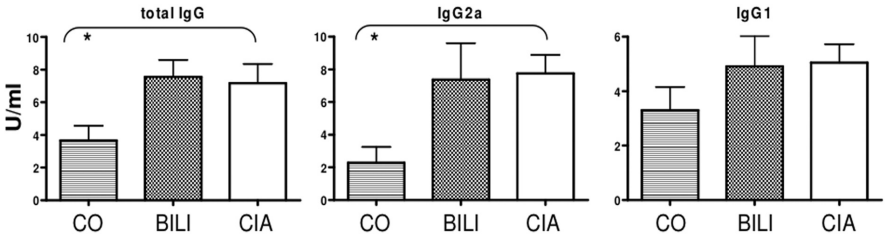

Fig. 1. Analysis of anti collagen II antibody in CO- and bilverdin treated animals.
On day 30 after the first immunisation, serum samples from each animal were analysed for anti-chicken collagen II antibodies. Immunised animals (n=6) generated IgG (7.2±1.2 U/ml), IgG2a (7.8±0.1 U/ml) and IgG1 (5.1±0.7 U/ml) antibody levels. CO-treated animals (CO, n=6) show a significant reduction in antibody levels for whole IgG (3.7±0.9 U/ml; p=0.046) and IgG2a (2.3±0.9 U/ml; p=0.01) as compared to mock treated animals (CIA). No significant reduction in antibody levels was observed in biliverdin treated animals (BILI, n=6) as compared to mock treated animals.

Histological assessment of arthritis

Joint histology and assessment were performed as described previously (14). Hind paws were fixed in 4.0% formalin and then decalcified in 14% EDTA (Sigma, St. Louis, MO). Serial paraffin sections (2 μm) of hind paws were stained with haematoxylin (HE) and eosin for assessment of synovial inflammation and bone erosions, with toluidine blue (TB) for cartilage degradation and with tartrate-resistant acid phosphatase (TRAP) for detection of osteoclasts. TRAP staining was performed using a leukocyte acid phosphatase staining kit (Sigma). Synovial inflammation, bone erosions, osteoclast numbers, and cartilage destruction were quantified with the use of an Axioskop 2 microscope (Zeiss, Marburg, Germany) equipped with a digital camera and an image analysis system (Osteomeasure; Osteometrics, Decatur, GA), as described previously (14).

Statistical analysis

All results are expressed as means ±SEM unless otherwise stated. For comparison of differences, the Student's t-test was used. If adequate, one- or two-way ANOVA were performed. Differences were considered to be significant if the p-value was less than 0.05. All analyses were performed using the GraphPad Prism IV program (GraphPad Software Inc., San Diego, CA).

Results

Biliverdin is metabolised to bilirubin in vivo

Animals were treated with 35 mg/kg biliverdin. This dosage has previously been shown to be effective in inducing tolerance to cardiac allografts (15), in protecting against endotoxin-induced acute lung injury (16), lethal endotoxemia and acute hepatitis (17). In all biliverdin treated animals, significant serum concentrations of bilirubin (0.27±0.15 mg/dl) were detected in contrast to untreated animals in which serum bilirubin levels were below the detection limit.

CO treatment decreases serum anti-collagen II antibody levels

After the induction of CIA, serum samples were analysed for anti-C II antibodies using isotype-specific ELISAs on day 30. As shown in Figure 1, immunised animals generated IgG (7.2±1.2 U/ml), IgG2a (7.8±1.1 U/ml) and IgG1 (5.1±0.7 U/ml) antibodies. No anti-C II antibodies were detected in animals that were injected with CFA alone (data not shown). A significant reduction in antibody levels was observed in CO-treated animals for whole IgG (3.7±0.9 U/ml; p=0.05) and IgG2a (2.3±0.9 U/ml; p=0.01) as compared to mock treated animals. In contrast, biliverdin treatment did not cause a significant reduction in anti-C II antibody levels.

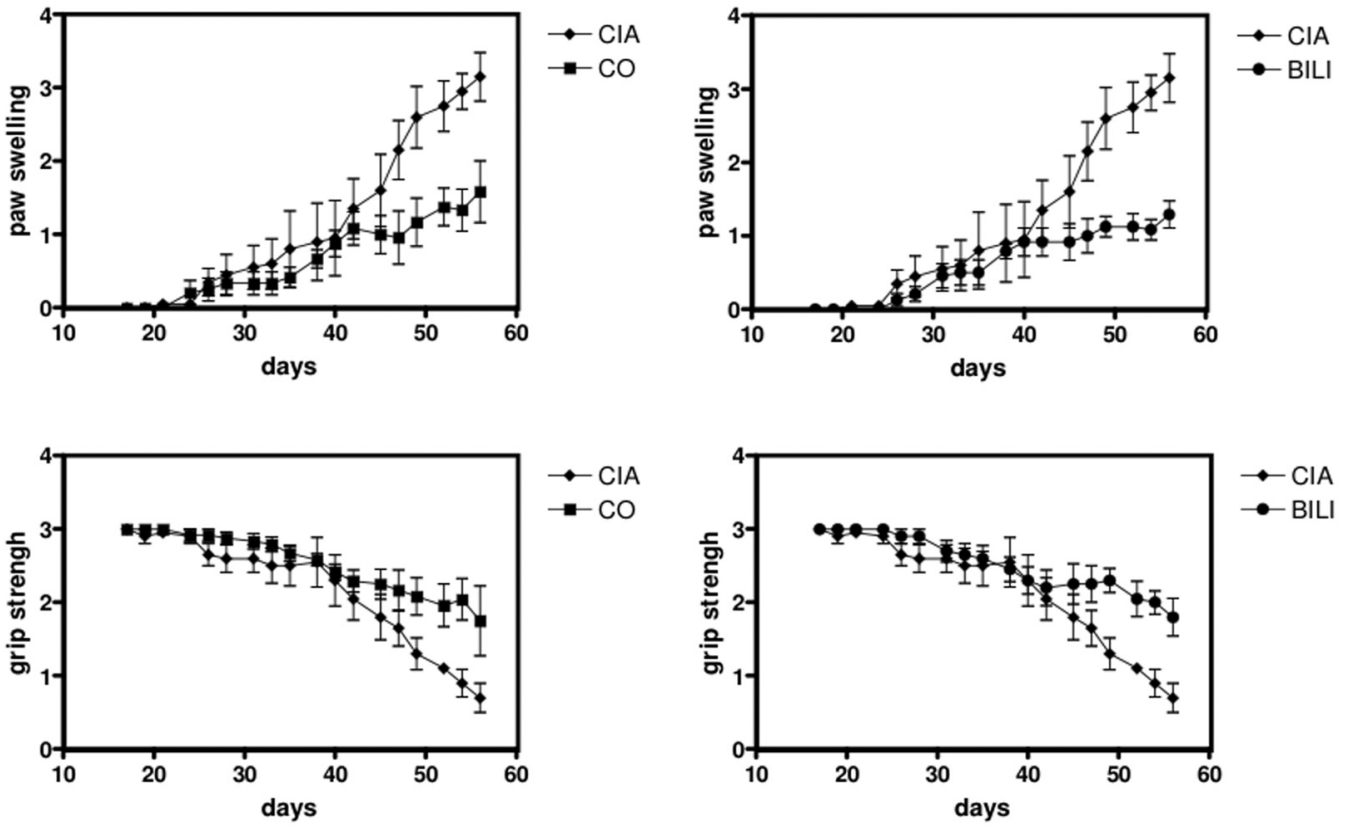

Fig. 2. Analysis of clinical signs of arthritis in CO- and biliverdin treated groups as compared to mock treated animals.
All animals were analysed 3 times per week for the extent of paw swelling and the loss of grip strength. CO (CO, n=6, $p<0.05$ from day 47 onwards) and biliverdin (BILI, n=6, $p<0.05$ from day 47 onwards) treatment reduced the extent of paw swelling and both CO (CO, $p<0.05$ from day 49 onwards) and biliverdin (BILI, $p<0.05$ from day 49 onwards) treated animals showed a preserved grip strength as compared to mock treated (CIA, n=6) animals. Data represent mean values ±SEM.

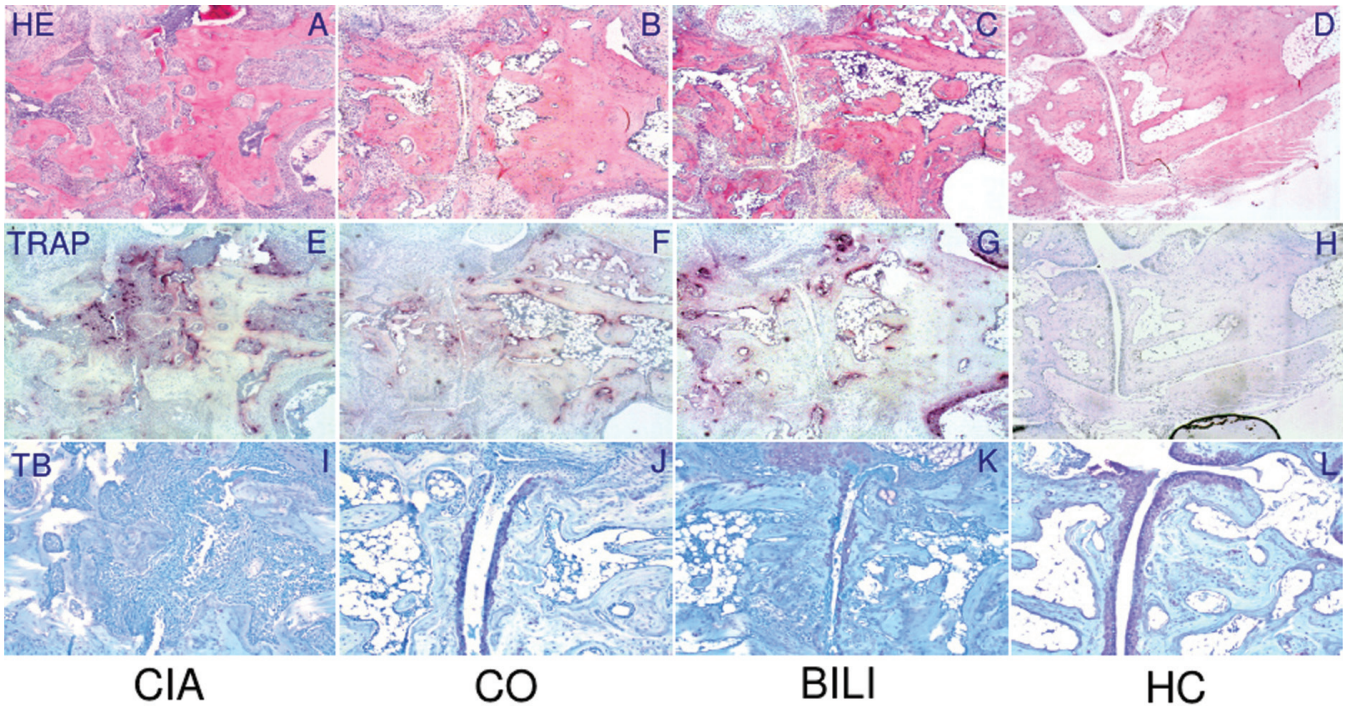

Fig. 3. Histomorphological analysis of paraffin section from hind paws.
Representative examples of paraffin sections from hind paws of mock treated (CIA, n=6), CO-treated (CO, n=6), biliverdin treated (BILI, n=6) or healthy control (HC, n=6) animals stained for HE, TRAP and TB are shown.

CO or biliverdin treatment reduces clinical signs of arthritis

As depicted in Figure 2, a rapid increase in joint swelling and a loss of grip strength was observed in mock treated animals: both CO and biliverdin treatment led to a significant reduction in the extent of paw swelling from day 47 onwards and both CO- and biliverdin treated animals showed a preserved grip strength as compared to mock treated animals. This difference was statistically significant from day 49 onwards for both CO- and biliverdin treated animals. No significant difference was observed between CO- and biliverdin treated animals in respect to the amelioration of paw swelling or the preservation of grip strength.

CO and biliverdin treatment reduces histomorphological signs of arthritis inflammation

As shown in Figure 3A, mock treated animals with CIA showed large areas of inflammation affecting the entire tarsus, covering a mean area of 0.3±0.1 mm^2. Treatment with either CO or biliverdin (Fig. 3B, C) led to a reduction of the synovial inflammation by reducing the area covered by inflamed synovial tissue to 0.04±0.03 mm^2 in CO-treated animals and to 0.2±0.1 mm^2 in biliverdin treated animals. This difference was found to be significant for CO-treated (p=0.02) but not for biliverdin treated (p=0.49) animals.

Cartilage damage

Mock treated mice with CIA showed a significant loss of cartilage (Figs. 3I, 4B) (mean cartilage area: 0.07±0.01 mm^2) as compared to healthy control animals (Fig. 3L) (mean cartilage area: 0.34±0.01 mm^2). CO and biliverdin treatment led to a significant reduction in articular cartilage damage as compared to mock treated animals with CIA (Figs. 3J, 4B) (mean cartilage area in CO-treated animals: 0.35±0.03 mm^2; p=0.001; in biliverdin treated animals 0.23±0.05 mm^2; p=0.001) (Figs. 3K, 4B).

Bone erosions

Mock treated CIA mice showed extensive bone erosions (Figs. 3E, 4C) (mean area of erosion: 0.3±0.1 mm^2).

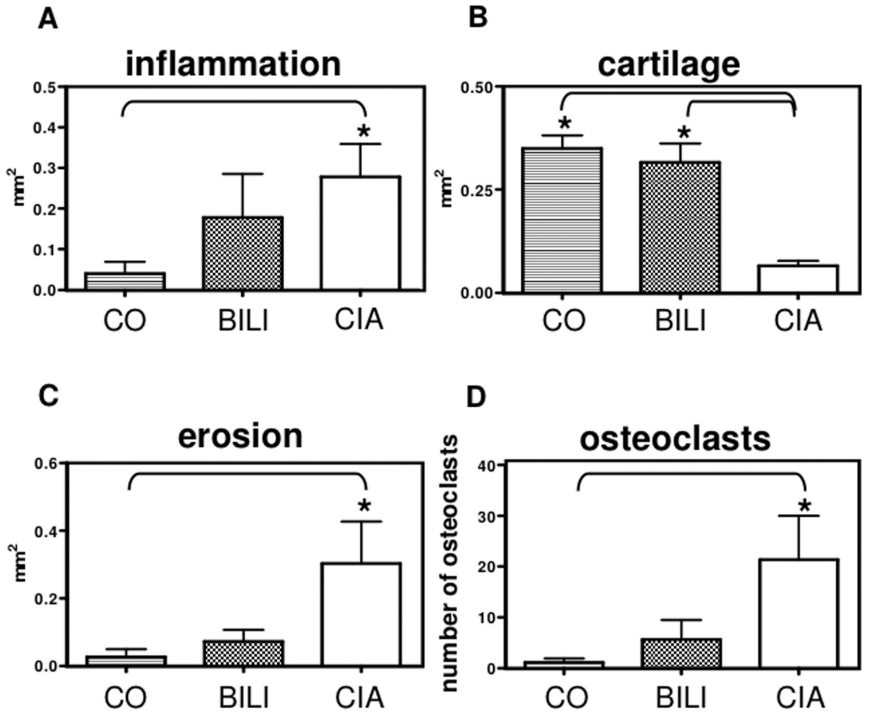

Fig. 4. Quantification of histomorphological analysis.
CO- (CO, n=6), biliverdin (BILI, n=6) and mock treated animals (CIA, n=6) were analysed for histological signs of arthritis. CO-treated animals showed a significantly reduced area of inflammation (p=0.02), erosion (p=0.04), as well as reduced numbers of osteoclasts (p=0.03). CO- and biliverdin treated animals displayed a significant reduction in loss of cartilage as compared to the mock treated group (p=0.001). Bars represent mean values ±SEM.

Treatment with either CO or biliverdin reduced the occurrence of bone erosions. This difference was found to be significant for CO-treated (Figs. 3F, 4C) (mean area of erosion: 0.03±0.02 mm^2; p=0.04) but had only a tendency for biliverdin treated (Figs. 3G, 4C) (mean area of erosion: 0.07±0.03 mm^2; p=0.08) animals.

Osteoclast numbers

Consistent with this finding as shown in Figures 3E and 4D, mock treated CIA mice showed an abundant accumulation of synovial osteoclasts (21.4±8.6 osteoclasts/section). CO treatment significantly reduced synovial osteoclast numbers (1.2±0.8, osteoclasts/section; p=0.03) (Figs. 3F, 4D), whereas biliverdin treatment did not lead to a significant reduction of osteoclast numbers (5.7±3.8 osteoclasts/section; p=0.1) (Figs. 3G, 4D).

Discussion

In this study, we showed that the products of heme degredation, CO and biliverdin, are able to ameliorate clinical signs of arthritis in the murine collagen-induced arthritis (CIA) model of human RA. CO treatment furthermore led to a reduction in anti-collagen II antibody levels and histomorphological signs of bone destruction whereas both, CO and biliverdin, diminished cartilage degradation.

Heme oxygenase is a rate-limiting enzyme in the oxidative degradation of heme into CO, biliverdin and Fe^{++} (18). HO-1 deficiency leads to an increased inflammatory state in mice and humans (3, 19), whereas HO-1 induction downregulates the inflammatory response in several conditions of acute and chronic inflammation. These include, but are not restricted to sepsis, malaria, transplant rejection, autoimmune neuroinflammation, myocardial infarction and diabetes (20). Concerning the role of HO-1 in RA, however, partially conflicting data have been published so far.

In principle, HO-1 can be induced with synthetic molecules such as heme arginate or synthetic protoporphyrins and treatment of human TNF-alpha transgenic arthritis mice with the HO-1 in-

ducer cobalt protoporphyrin IX (CoPP) inhibited osteoclastogenesis and reduced the area of local bone erosions *in vivo* (7). Likewise, CoPP treatment was found to diminish clinical signs, cartilage destruction and proinflammatory cytokine production in the K/BxN mouse arthritis model (21). Somewhat surprising, however, was the finding that the HO-1 inhibitor tin protoporhyrin IX (SnPP) turned out to be even more effective than CoPP in preventing the development of CIA (6).

Alternatively CO-releasing molecules (CORM) can be used to deliver small amounts of CO in biological systems, which has been shown to suppress clinical and histological signs of CIA (12). Finally, the direct administration of heme degradation products like CO or biliverdin can serve as an alternative treatment strategy. This might allow to circumvent difficulties that are caused by individual differences in the response to HO-1 activating agents. This appears even more important, since it has not been determined so far if these molecules can be induced at sufficient levels *in vivo* (20). One treatment study using CO showed an amelioration of clinical signs of arthritis in the anti-collagen II antibody induced arthritis model (22). Clinical scoring of arthritis, however, was only performed over a short period of time and the experimental set up was not designed to allow the distinction between a prophylactic and a therapeutic effect of CO administration.

In this study we therefore investigated the therapeutic potential of two distinct heme degradation products, CO and biliverdin, in the CIA mouse model of RA to evaluate efficacy and long term effects of therapeutic CO and biliverdin treatment.

Treatment of CIA animals with both CO and biliverdin not only led to a comparable reduction in paw swelling but also preserved grip strength. In contrast, however, only CO treatment significantly reduced anti-collagen antibody levels that have been suggested to play a pathogenic role in the development of CIA (23) (24). Treatment with CO furthermore induced a significant reduction in the extent of infiltration with inflammatory cells, the extent of bone erosions and numbers of osteoclasts. Both CO and biliverdin on the other hand had strong protective impacts on cartilage tissues as demonstrated by the prevention of cartilage degradation. Previous reports describing a suppressive effect of HO-1 induction on osteoclastogenesis *in vitro* and the development of bone erosions *in vivo* support our findings (7). In addition our observation of diminished cartilage destruction upon CO and biliverdin treatment is in line with a protective role of HO-1 for cartilage tissues (25).

Conclusion

In summary, HO-1 end-products were found to exert broad anti-inflammatory effects in the CIA model with a reduction of clinical and histological signs of arthritis. Certain discrepancies between the effect of CO and biliverdin on histomorphological signs of arthritis, however, were observed. Therefore future experiments will have to determine how individual HO-1 end-products interfere with pathogenetic mechanisms of arthritis.

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