Amino Acids (2008) 35: 397–402
DOI 10.1007/s00726-007-0605-1
Printed in The Netherlands

Amino Acids

Heme oxygenase-1 participates in the anti-inflammatory activity of taurine chloramine

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Received July 27, 2007
Accepted September 10, 2007
Published online January 25, 2008;

Summary. Interleukin-6 (IL-6) and interleukin-8 (IL-8) are implicated in the pathogenesis of rheumatic diseases. In affected joints fibroblast-like synoviocytes (FLS) are the major source of these pro-inflammatory cytokines. We have previously found that production of both cytokines is inhibited in vitro by taurine chloramine (Tau-Cl). Heme oxygenase (HO-1) activity was also reported to restrict synthesis of various inflammatory mediators, including IL-6 and IL-8. The aim of present study was to investigate whether this enzyme activity is implicated in the mechanism of Tau-Cl suppressive effect. We have shown that in rheumatoid FLS both hemin (known HO-1 inducer) and Tau-Cl significantly up-regulate HO-1 expression at the mRNA and protein levels and simultaneously inhibit IL-1β-triggered production of pro-inflammatory cytokines. However, the inhibitory potency of these compounds differs, because hemin is more potent inhibitor of IL-8 than IL-6 production, while Tau-Cl exerts opposite effect. Importantly, pretreatment of the cells with HO-1 inhibitor completely reverses the inhibitory effect of hemin on both cytokines production. However, in Tau-Cl treated cells this inhibitor fully restores only IL-8 secretion but has weaker effect on IL-6 response. Thus, the present results: (i) support HO-1 activity to be relevant to negatively control production of pro-inflammatory cytokines, and (ii) underline implication of HO-1 in mediating Tau-Cl inhibitory action.

Keywords: Interleukins (IL) – Fibroblast-like synoviocytes (FLS) – Rheumatoid arthritis (RA) – Heme oxygenase-1 (HO-1) – Taurine chloramine (Tau-Cl)

Introduction

Heme oxygenase-1 (HO-1) acts as a critical defender of cellular homeostasis. Expression of this enzyme is up-regulated in response to various forms of injury or insults and is associated with marked cytoprotection. HO-1 catabolizes the degradation of heme to carbon monoxide (CO), iron (Fe^{2+}) and biliverdin (BV), which is subsequently reduced to bilirubin (BR). The products of heme degradation (CO, BV, BR) mediate many of the anti-inflammatory, antiapoptotic, antioxidant, and immune-modulatory effects attributed to HO-1 activity (Kirkby and Adin, 2006; Ryter et al., 2006).

Overexpression of HO-1 accompanies many pathological conditions, including ischemia, atherosclerosis, and inflammation (Ryter et al., 2006). Rheumatoid arthritis (RA) is a chronic autoimmune, inflammatory, systemic disease characterized by progressive joint destruction, deformity and disability, associated with significant morbidity and increased mortality. By contrast, osteoarthritis (OA) is a debilitating, progressive joint disease associated with the aging process. Despite this, accumulating evidence supports contribution of inflammation also to the symptoms and progression of OA (Bonnet and Walsh, 2005). Consistently with more inflammatory features of RA, HO-1 protein was reported to be expressed more abundantly in the lesions of synovial tissue from RA than OA patients (Kobayashi et al., 2006). In both of these diseases, fibroblast-like synoviocytes (FLS), that localize in lining layer of synovial membrane, are implicated in inflammation and joint tissue destruction (Stebulis et al., 2005; Huber et al., 2006; Wang et al., 2006). These cells are the major source of pro-inflammatory cytokines (IL-6 and IL-8), that contribute to the pathological processes (Malemud, 2004; Cecil et al., 2005; McInnes and Schett, 2007). Taurine chloramine (Tau-Cl) was reported to normalize several pathogenic functions of RA FLS, e.g. proliferation of these cells responsible for synovial membrane hyperplasia, synthesis of tissue degrading enzymes and inflammatory mediators, as well as IL-6 and IL-8 (Kontny et al., 1999, 2003, 2006b, 2007; Kim et al., 2007). Interestingly, endogenously expressed or chemical-

ly induced HO-1 has also been reported to inhibit pro-inflammatory cytokine production by rheumatoid synoviocytes (Kobayashi et al., 2006). Therefore, we ask the question whether in these cells HO-1 activity mediates anti-inflammatory properties of Tau-Cl. To this aim the effects of Tau-Cl, hemin and HO-1 inhibitor on the expression of HO-1 and/or cytokine production, respectively, were investigated.

Materials and methods

Patients, synovial samples and synoviocyte cultures

Synovial tissue were obtained from the knee joints at the time of joint surgery or synovectomy, performed as a normal part of clinical care, from 20 RA patients (18F/2M) who fulfilled criteria of the American College of Rheumatology for the classification of RA at stages III to IV, and 7 patients with osteoarthritis (5F/2M). The mean ± SEM of age for RA and OA patients were 49.4 ± 2.9 and 63.3 ± 4.8, respectively. The study was approved by the local Ethics Committee. FLS were isolated and cultured in vitro as describe previously (Kontny et al., 1999). To trigger IL-6 and IL-8 production FLS (15×10^3 cells/ml/well) were stimulated with 1 ng/ml of recombinant human interleukin-1β (IL-1β) (R&D Systems, Abingdon, U.K.). Cells cultured in medium alone were used as a control. Sodium salt of N-chlorotaurine (Tau-Cl), prepared as described previously (Gottardi and Nagl, 2002), or bovine hemin (Sigma) were added to the cell cultures at physiologically relevant concentrations (200–400 μM and 25–100 μM, respectively) together with the stimulus. The inhibitor of HO-1 (Zinc(II) Deuteroporphyrin IX-2, 4-bisethyleneglycol – ZnDP; Alexis Biochemicals, San Diego, U.S.A.) was added to the cell cultures at 3 μM concentration, and 60 min before the treatment with IL-1β and tested compounds.

Analysis of HO-1 expression by Western blotting

Expression of HO-1 was analyzed by Western blotting after 24 and 48 h of cell treatment (4×10^5 cells/4 ml/well). Total protein fraction was prepared as previously described (Kontny et al., 2006b). Samples containing 40 μg of protein were separated on 12% SDS-PAGE denaturating gel, transferred onto the PVDF membranes (Bio-Rad, U.S.A.), and probed with monoclonal antibodies specific for human HO-1 isoenzyme (Stressgen Bioreagents, Canada) or α-tubulin (Sigma). Expression of α-tubulin was evaluated to normalize the protein levels in the samples (Kontny et al., 2003). Detection was performed with peroxidase conjugated goat anti-mouse IgG and the chemiluminescence reagents ECL (SuperSignal West Femto Maximum Sensitivity Substrate), both from Pierce Biotechnology, Rockford, IL. The band corresponding to analyzed proteins were densitometrically scanned using Kodak ID Image Analysis Software (Eastmond Kodak, Rochester, NY).

Analysis of gene expression using real-time PCR

Total RNA was isolated from cells using QIAamp®RNA Blood Mini Kit (QIAGEN, Germany). The cDNA was generated from the RNA with reverse transcriptase (Invitrogen). Panels of primers specific for human HO-1 and for the housekeeping gene encoding GAPDH were purchased from IBB PAN Service for Oligonucleotides Sequencing (Warsaw, Poland). Real-time PCR was performed using a SYBR green, 2× DyNAmo HS Master Mix (FINNZYMES), and the data was analyzed by Rotor-Gene 6000 program (Corbett Research). Briefly, 2 μl of cDNA, 5 μl of SYBR green, 1 μl of primer pair for HO-1 (forward 5′-CAA CATCCAGCTCTTTGAGG-3′, reverse 5′-GGCATAAAGCCCTACAG CAA-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward 5′-TGGTATCGTGGAAGGACTCATGAC-3′, reverse 5′-ATGCC AGTGAGCTTCCCGTTCAGC-3′), supplemented with 2 μl of DEPC water (total volume equal 10 μl) were incubated at 50 °C for 2 min and at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 15 sec and 72 °C for 20 sec. Under these conditions, the rate of amplification exceeded the threshold and was within the expotential rate. Gene expression levels in the individual samples were calculated on the basis of the standard curve. The data were normalized to the expression of the house keeping gene encoding GAPDH in the same samples.

Measurement of cytokine production

Concentrations of IL-6 and IL-8 were determined in culture supernatants collected 24 h after stimulation. The IL-6 and IL-8 specific enzyme-linked immunosorbent assays (ELISA) were performed as described previously (Kontny et al., 1999). Recombinant human IL-6 and IL-8 (R&D Systems) were used for preparation of standard curves. Goat polyclonal neutralizing antibodies specific for human IL-6 and IL-8 (R&D Systems) were used to capture cytokines from samples. Then cytokines were detected with either IL-6- or IL-8-specific rabbit polyclonal antibody (Sigma and R&D Systems, respectively). Finally horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin and 0-phenylenediamine dihydrochloride (OPD) as a substrate (both from Sigma) were applied to develop enzymatic reaction. Optical density was measured at 492 nm using an automatic ELISA reader (LP-400, Diagnostics Pasteur, Marnes-La-Coquette, France). The detection limit for IL-6 and IL-8 was 39 pg/ml and 31.25 pg/ml, respectively.

Statistical analysis

Repeated-measures analysis of variance (ANOVA), followed by the Tukey's test, was applied to evaluate the effects of the stimuli and tested compounds. Results are expressed as the mean ± SEM. *P* values less than 0.05 were considered significant.

Results

Expression of HO-1 at the mRNA and protein levels

Consistently with previous report of others (Kobayashi et al., 2006), endogenous expression of HO-1 protein in RA FLS was higher (81,896 ± 18,211 arbitrary units; $n = 12$) than in OA FLS (53,262 ± 22,938 arbitrary units; $n = 6$) but did not reach statistical significance because the expression level of this enzyme was variable among the cells lines from both groups of patients. Real-time PCR (Fig. 1) and Western blot (Fig. 2) analysis revealed that IL-1β slightly diminished, while Tau-Cl and hemin significantly raised the levels of both mRNA encoding HO-1 and HO-1 protein. Treatment of the cells with 300 μM of Tau-Cl showed tendency to increase HO-1 protein to higher level in RA (205,054 ± 52,442 arbitrary units; $n = 12$) than OA FLS (105,963 ± 28,794 arbitrary units; $n = 6$), but the difference was not statistically significant. In the cells treated simultaneously with IL-1β and Tau-Cl, the effect of the latter compound prevailed (Figs. 1 and 2C). Expression of HO-1 protein in Tau-Cl-treated cells

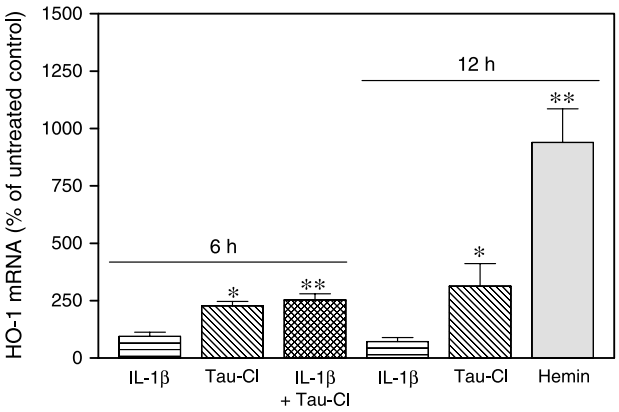

Fig. 1. Hemin and taurine chloramine (Tau-Cl) raise transcription of gene encoding heme oxygenase-1 (HO-1). Cells were cultured in culture medium alone (untreated control) or in medium supplemented with 1 ng/ml of IL-1β, 400 μM of Tau-Cl or 100 μM of hemin. Expression of HO-1 mRNA was evaluated at indicated time points by real-time RT-PCR, as described in Materials and methods. Values are the mean ± SEM of 4 experiments in which FLS from 4 different RA patients were used. $^{*}P = 0.05$–0.01 and $^{**}P = 0.01$–0.001 for untreated control versus cells treated as above

was similar at 24 and 48 h (data not shown), while hemin raised the level of this enzyme up to 48 h (Fig. 2D). Therefore, similarly to known effect of hemin, also Tau-Cl is able to up-regulate transcription of gene coding for HO-1 and the expression of this enzyme at the protein level.

The effects of Tau-Cl, hemin and the inhibitor of HO-1 activity on the cytokine production

Unstimulated FLS secreted moderate amounts of IL-6 and IL-8 (318 ± 88 pg/ml and 1415 ± 330 pg/ml, respectively), while stimulation with IL-1β raised the production of both cytokines significantly (5167 ± 900 pg/ml and 20590 ± 250 pg/ml, respectively; $0.0001 < P < 0.001$). As shown in Fig. 3, hemin damped cytokine secretion by FLS from all tested patients ($n = 4$) and exerted stronger inhibitory effect on IL-8 than IL-6. Conversely, Tau-Cl inhibited more potently IL-6 than IL-8 secretion. Pre-treatment of the cells with the inhibitor of HO-1 activity (ZnDP) almost completely reversed restraining effect of hemin. Similarly in Tau-Cl treated cells ZnDP completely restored secretion of IL-8 (Fig. 3B). However the secretion of IL-6 was restored only partially and occurred in 33% (4/12) cell cultures (Fig. 3A; group A). Although the secretion of IL-6 by FLS from this patient group was inhibited by Tau-Cl more strongly than the response of FLS from the other patients (Fig. 3A; compare group A and B) the difference (~75% vs 60%) did not reach statistical significance. In every group the distribution of RA (75%) and OA (25%) patients was similar.

These results show that HO-1 activity negatively regulates production of both cytokines and mediates inhibitory effect of Tau-Cl exerted mostly on IL-8 secretion, while being less relevant to Tau-Cl inhibition of IL-6 secretion.

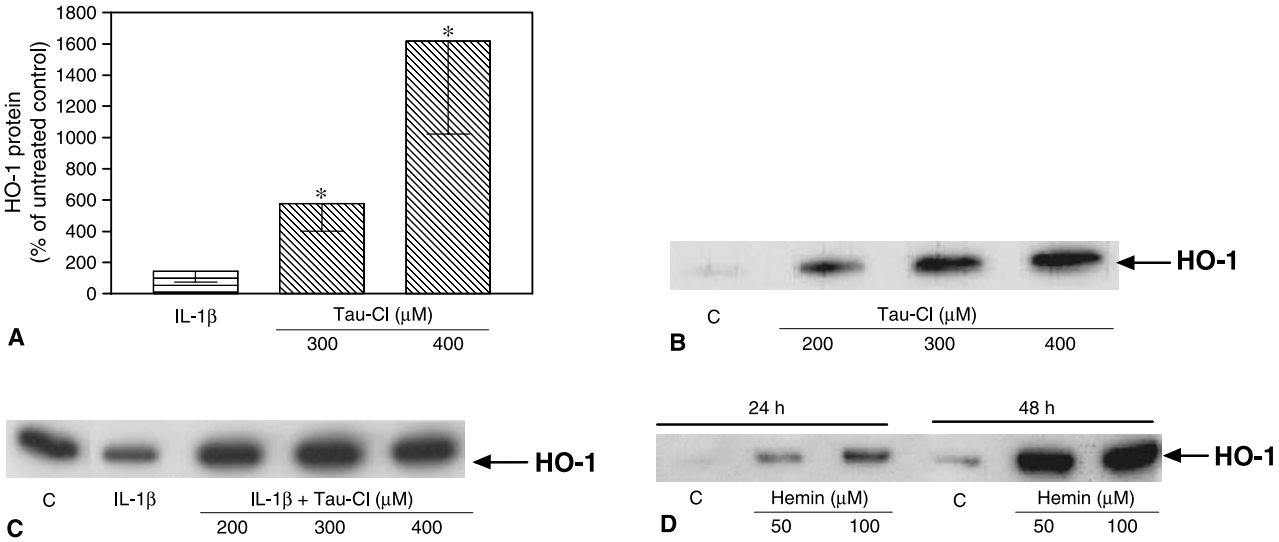

Fig. 2. Hemin and taurine chloramine (Tau-Cl) increase the expression of heme oxygenase-1 at the protein level. Fibroblast-like synoviocytes from different rheumatic patients (n) were cultured for 24 (**A**–**D**) and 48 (**D**) hours in culture medium alone (untreated control – C) or in medium supplemented with 1 ng/ml of IL-1β or with different concentrations of Tau-Cl or hemin. Expression of HO-1 was evaluated by Western blotting, as described in Materials and methods. (**A**) The bands representing HO-1 protein were densitometrically scanned. Results are expressed as a percentage of the HO-1 level from untreated control cells. Values are the mean ± SEM of 7 or 18 experiments (for IL-1β and 400 or 300 μM concentration of Tau-Cl, respectively). $^{*}P = 0.05$–0.01 for untreated control versus Tau-Cl treated cells. (**B**–**D**) Representative Western blots of 3, 4 performed

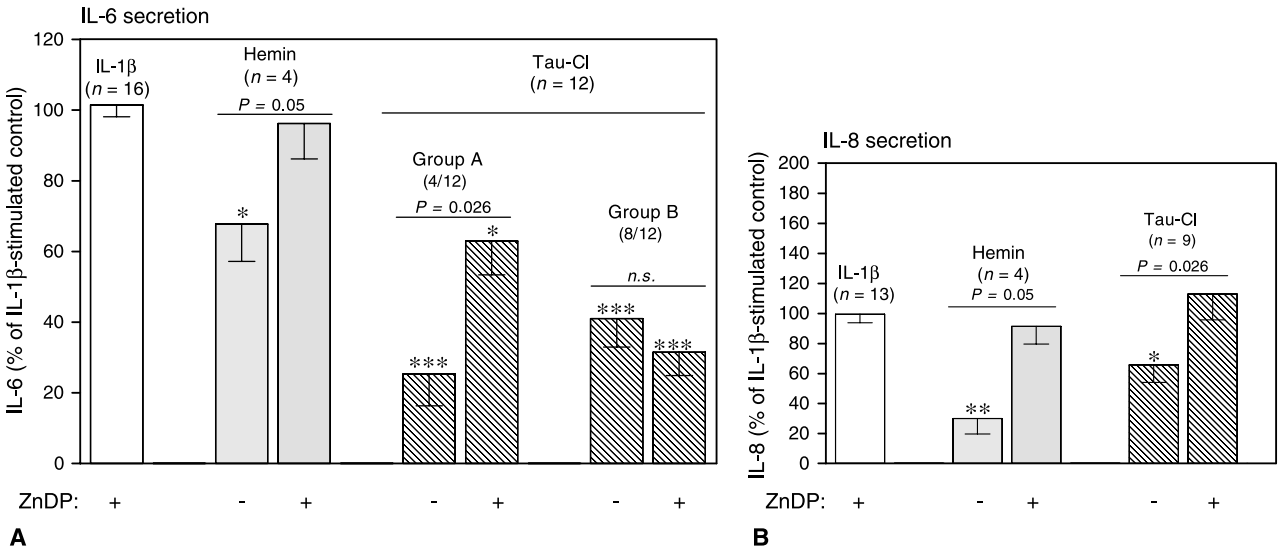

Fig. 3. The restoration of hemin and taurine chloramine inhibited cytokine secretion by the inhibitor of heme oxygenase-1 activity (ZnDP). Fibroblast-like synoviocytes (FLS) from different rheumatic patients (*n*) were stimulated for 24 h with IL-1β (1 ng/ml) in the absence (IL-1β-stimulated control) or presence of either hemin (100 μM) or Tau-Cl (400 μM). The inhibitor of HO-1 activity (ZnDP) was added at 3 μM concentration, 60 min before stimulation of the cells. Concentrations of IL-6 (**A**) and IL-8 (**B**) were measured in the culture supernatants by cytokine specific ELISA (see Material and methods). Results are expressed as a percentage of the cytokine concentrations from IL-1β – treated control cells. Values are the mean ± SEM of several experiments (*n*). In FLS from some patients ZnDP restored Tau-Cl inhibited IL-6 response (Group A) but was ineffective in the others (Group B). The number of patients in each group per total number of patients is shown in parentheses (see Results for details). Asterisks above the bars indicate statistically significant differences in comparison to the treatment with IL-1β alone ($^{*}P = 0.05$–0.01; $^{**}P = 0.01$–0.001; $^{***}P = 0.001$–0.0001). Differences between cells cultured in the absence and presence of ZnDP are shown as the *P* values above the bars (*n.s.* – not significant)

Discussion

The increased synthesis of the HO-1 protein is thought to occur as a general response to stress in biological systems. In cell culture models HO-1 is induced by a broad spectrum of chemical and physical stress agents (e.g. reactive oxygen and nitrogen species, thiol reactive substances, heavy metals, abnormal oxygen tension) as well as growth factors, cytokines and hormones (Ryter et al., 2006). Our previous reports showing up-regulation of HO-1 protein in Tau-Cl treated murine macrophages (Olszanecki and Marcinkiewicz, 2004; Marcinkiewicz et al., 2006), and present results supporting this observation in FLS (Fig. 2), add Tau-Cl to a list of the potent inducers of this enzyme. It is well known that regardless of cell type and inducing factor, the up-regulation of HO-1 depends largely on transcriptional activation of the gene encoding this enzyme (*hmox1*) (Ryter et al., 2006; Alam and Cook, 2007). Consistently, the present results reveal Tau-Cl to act at the transcriptional level of *hmox1* gene expression (Fig. 1). Multiple response elements and corresponding transcription factors, including the members of the major stress-responsive families (nuclear factor-erythroid 2 – Nrf2; heat shock factor 1 – Hsf-1; activator protein 1 – AP-1, and nuclear factor – κB – NFκB), regulate transcription of *hmox1* gene (Ryter et al., 2006; Alam and Cook, 2007), and the StRE (stress-responsive element) – Nfr2 (transcriptional activator) – Bach1 (transcriptional repressor) axis plays a critical regulatory role. Displacement of Bach1 from StRE releases transcriptional repression under basal conditions and in response to heme and hemoproteins. On the other hand, the redox regulation of *hmox1* gene critically depends on the oxidative modification of cytoplasmic factor, Keap1, which under basal conditions binds and retains Nrf2 in the cytoplasm for subsequent ubiquitination and proteosomal degradation. Oxidation of Keap1 releases Nrf2, permitting its nuclear translocation and activation of *hmox1* gene transcription (Ryter et al., 2006). Although we did not investigate the mechanism of Tau-Cl triggered *hmox1* gene transcription, it is unlikely that AP-1 and NFκB are implicated, because in RA FLS Tau-Cl diminishes these transcription factors activities (Kontny et al., 2000; Kim et al., 2007). Regarding NFκB, Tau-Cl was reported to oxidize and stabilize its inhibitor (IκBα), preventing IκBα protein degradation (Miyamoto et al., 2003). Similar mechanism seems to be responsible for nuclear accumulation of p53 tumor suppressor, resulting in the cell-cycle arrest of Tau-Cl treated RA FLS (Kontny et al., 2006b). Therefore, it is

very likely that Tau-Cl may trigger *hmox1* gene transcription by modifying expression, subcellular distribution or function of critical regulatory proteins, e.g. Keap1 or Bach1. This suggestion, as well as identification of another signalling molecules modified by Tau-Cl, needs further studies. Accumulating data show this compound to regulate pro-inflammatory mediators synthesis in a stimulus and signaling-pathway-specific manner (Mainnemare et al., 2004; Kim et al., 2006), as well as to exert different biological effects in different cell-types (Kontny et al., 2006a). Thus, the mechanism(s) of Tau-Cl action is an important and still open question.

The present results show that Tau-Cl triggered up-regulation of HO-1 is associated with the inhibitory effect of this compound on pro-inflammatory cytokine secretion, because inhibitor of HO-1 activity (ZnDP) recovers, at least partly, the cytokine responses (Fig. 3). Interestingly, Tau-Cl is more potent inhibitor of IL-6 than IL-8 production (Kontny et al., 1999) (Fig. 3), while hemin, a known inducer of HO-1, exerts opposite effect. Moreover, ZnDP more efficiently counteracts inhibitory effect of Tau-Cl on IL-8 than on IL-6 secretion (Fig. 3). These observations suggest that in FLS HO-1 activity is more relevant to the inhibition of IL-8 than IL-6 synthesis. This difference may result from the ability of IL-6 to regulate HO-1 expression, as it was shown in some cell types both in vitro (Ricchetti et al., 2004; Tron et al., 2006) and in vivo (Tron et al., 2005).

The results of present study confirm observations of others that up-regulation of HO-1 expression and/or HO-1 activity exerts inhibitory effect on IL-6 and IL-8 production in various cell types and represents an important component of protective anti-inflammatory response (Dawn and Bolli, 2005; Mizuno et al., 2005; Ockaili et al., 2005; Shinohara et al., 2005; Kirino et al., 2007; Orozco et al., 2007). Moreover, our results are consistent with so far scarce data, suggesting regulatory role of HO-1 in RA pathogenesis. It has recently been demonstrated that HO-1 reduces production of pro-inflammatory cytokines (TNF-α, IL-6, IL-8) by RA synovial cell lines (Kobayashi et al., 2006), and attenuates inflammation-induced osteoclastogenesis (Zwerina et al., 2005). On the other hand, TNF-α, which plays pivotal role in the pathogenesis of chronic inflammatory diseases, has been shown to down-regulate expression of HO-1 in peripheral blood monocytes of healthy volunteers, while anti-TNF-α therapy of RA patients leads to increased HO-1 mRNA levels and reduced TNF-α synthesis by peripheral blood mononuclear cells (Kirino et al., 2007). Although little is known about the mediators of these HO-1 effects, CO was shown to be responsible for HO-1-dependent suppression of pro-inflammatory cytokine synthesis by RA FLS (Kobayashi et al., 2006), while bilirubin was suggested to mediate protective effect of HO-1 on the bone loss (Zwerina et al., 2005).

Heme oxygenase-1 activity was reported to mediate the anti-inflammatory and immunosuppressive effects of some compounds (Alcaraz et al., 2000; Jun et al., 2006; Ho et al., 2007), including gold agents used in the therapy of RA patients (Kataoka et al., 2001; Kobayashi et al., 2006). Based on our previous (Olszanecki and Marcinkiewicz, 2004; Marcinkiewicz et al., 2006) and present results we demonstrate implication of the same mechanism in Tau-Cl anti-inflammatory action. We also propose that in rheumatoid FLS Tau-Cl inhibits pro-inflammatory cytokine production by two ways: (i) the reduction of crucial transcription factors (NFκB, AP-1) activities (Kontny et al., 2000), and (ii) the up-regulation of HO-1 (present findings). These pathways are more relevant to restrict either IL-6 or IL-8 synthesis, respectively.

Acknowledgements

We want to thank Prof. Marcus Nagl from the Institute of Hygiene and Social Medicine, Leopold-Franzens-University of Innsbruck, Austria, for giving us N-chlorotaurine sodium salt. This work was supported by the grants from the State Committee for Scientific Research of Poland (No. 2 P05A 09129) and the Institute of Rheumatology, Warsaw (No. I/21).

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