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Stress protein-induced immunosuppression: inhibition of cellular immune effector functions following overexpression of haem oxygenase (HSP 32)

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Abstract: This is the first report on suppression of immune effector functions following upregulation of heat shock protein 32 (HSP 32), known as haem oxygenase (HO-1). Here we evaluated the effect of cobalt-protoporphyrin (CoPP)-induced HO-1 expression on cell-mediated immune responses. Administration of CoPP to CBA mice resulted in overexpression of HO-1 in the spleen, liver and kidneys. *In vitro* measurements of T cell-mediated and NK-cell-mediated cytotoxicity in spleens from CoPP-treated animals demonstrated a severe suppression of their effector functions while administration of Zn-PP or vitamin B_{12} had no effect. Furthermore, CoPP therapy decreased the lymphoproliferative alloresponse and differentiation of cytotoxic T cells. Inhibition of proliferation appeared to be due to cell growth arrest with an increased number of cells staying in G0/G1 phase. Despite the suppressed proliferative response, IL-2 production in the MLR was not inhibited. In contrast, CoPP decreased the production of IL-10, IFN-γ and TNF-α. *In vivo*, CoPP prolonged the survival of heterotopic heart allografts in mice. The immunosuppressive effects following CoPP-mediated upregulation of HO-1 were similar to those observed after peptide-mediated upregulation of HO-1. The results indicate that overexpression of HO results in the inhibition of several immune effector functions and thus provides an explanation for stress-induced immunosuppression.

Introduction

Heat shock proteins (HSP) are highly conserved proteins that are expressed by all prokaryotic and eukaryotic cells. Originally described as proteins expressed in cells following heat stress, it is now established that expression of HSPs can be induced by various stressful stimuli, including oxidative stress and exposure to heavy metals.[1–3]

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The medical importance of stress proteins is apparent from numerous studies on infection, inflammation, autoimmune disease, tumour immunity and organ transplantation immunology. In many experimental models and clinical situations, HSP-responsive lymphocytes have been shown to participate in the immune response.[4–7]

HSPs may also be involved in the regulation of the immune system. Most studies have been performed on members of the HSP 70 family (HSP 70s; HSP 70 and HSC 70). HSP 70s appear to be involved in antigen processing and the assembly of MHC–peptide complexes.[8–11] Anti-HSC 70 antibodies have been shown to inhibit antigen presentation.[9] Induction of

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HSP70 expression following heat stress, resulted in increased antigen-presenting function of B cells.[12] HSC 70 is a key component of a multiprotein complex (foldosome) required for the assembly of the glucocortoid receptor.[13]

By comparison, little is known about HSP 32, an oxygenase, a known function of which is to degrade haem into biliverdin; the latter is subsequently reduced to bilirubin by biliverdin reductase.[14] To date, two isoforms of haem oxygenase (HO-1 and HO-2) have been characterized. The two isoforms are different gene products, they differ in their molecular and biochemical characteristics and their mode of regulation.[15,16] While HO-2 is expressed constitutively, HO-1, also known as HSP 32, is inducible by various forms of stress (heat, UV-radiation, starvation, hypoxia, hyperoxia, ischaemia, GSH-depletion, endotoxins) and has been shown to be upregulated during an inflammatory response and acute allograft rejection.[17–20] Upregulation of HO-1 activity has been shown to protect cells from oxidative injury.[21]

Apart from stress, compounds like haem (Fe-protoporphyrin, FePP), metalloporphyrins and heavy metals have been shown to induce expression of HO-1 *in vivo*.[14,22–29] Unlike haem, certain heavy metal complexes induce HO-1 activity with only a single administration, and the activity remains elevated for several days before returning to basal levels.[14,25] Synthetic metalloporphyrins also induce a rapid and prolonged increase in HO-1 mRNA expression. The effect on HO-1 enzyme activity, however, varies with respect to the chelated metal of the metalloporphyrins.[26] CoPP and FePP result in increased HO-1 activity in various tissues,[20,26] while treatment with ZnPP or MnPP results in decreased activity.[26–28] In addition, differences among various tissues have been observed. Based on their capability to inhibit HO-1 *in vitro* and *in vivo*, metalloporphyrins have been clinically tested to prevent excessive hyperbilirubinaemia in human neonates.[14,26–29]

We used metalloporphyrins for the modulation of HO-1 activity in mice and studied its effect on immune effector functions. We report that administration of CoPP mimicked a stress response and caused an increase in HO-1 activity *in vivo*. This upregulation of HO-1 activity resulted in immunosuppression, while inhibition of HO-1 activity with ZnPP had little effect. A short course of CoPP led to a significant prolongation of heart allograft survival in a murine model.

Materials and methods

Animals

Male, 7–8-week-old CBA/J (H-2^k) and C57BL/6/J (H-2^b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed according to the Animal Welfare Guidelines, Department of Health, California.

Synthetic metalloporphyrins

Metalloporphyrins were purchased from Porphyrin Products Inc. (Logan, UT, USA). They were dissolved in small quantity of 0.2 M NaOH, subsequently adjusted to pH 7.4 with 1 M HCl and diluted in 0.85% NaCl. The stock concentration of metalloporphyrins was 1 mg/ml.

Treatment of animals

Mice were treated by intraperitoneal injection of metalloporphyrins at various dosages and schedules as described in the figure legends.

Detection of HSP32 expression by western blotting

Groups of mice (n = 3) were treated with 20 mg/kg metalloporphyrins. Four and 24 h following treatment, the spleens, kidneys, livers, and hearts of the mice were collected. The tissues were homogenized in 1 ml lysis buffer (50 mM Tris, 5 μM EDTA, 0.5% Triton-100, 5 μg/ml leupeptin, aprotinin, pepstatin A, antipain, chymostatin) and centrifuged at 16 000g for 15 min. The supernatants were stored until analysis at –20°C. For the detection of HSP32, equal amounts of protein were separated by SDS-electrophoresis on 12.5% polyacrylamide gels and transferred to nitrocellulose. Nonspecific binding sites were blocked by overnight incubation of the membranes in PBS, 1% BSA at 4°C. After a short wash with PBS, 0.1% Tween 20 the membrane was incubated with polyclonal rabbit anti-rat HSP 32 or monoclonal anti-rat HSP 70 antibodies (Stressgen, Victoria, BC, USA), diluted 1:5000 in PBS, 0.1% Tween 20. Bound antibody was detected using peroxidase conjugated anti-rabbit or anti-rat antibody (Jackson ImmunoResearch, West Grove, PA, USA). Bound peroxidase was visualized using the SuperSignal™ CL-HRP substrate system (Amersham).

Measurement of HO-activity and bilirubin levels

Mouse spleen samples were homogenized on ice in a Tris–HCl lysis buffer (pH 7.4) containing 0.5% Triton X-100 and protease inhibitors (see above). Samples were frozen in small aliquots until use. Biliverdin reductase was purified from rat liver as described.[30] Spleen homogenate (100 μl) was mixed with 0.8 mM NADPH, 0.8 mM glucose-6-phosphate, 1.0 unit G-6-P dehydrogenase, 1 mM $MgCl_2$ and 10 μl of biliverdin reductase at 4°C. The reaction was initiated by the addition of haemin (20 μl of 2.5 mM) and the mixture was incubated at 37°C in the dark for 30 min. At the end of the incubation period, any insoluble material was centrifuged and supernatants were analysed for bilirubin concentration as described (Sigma Diagnostics, kit #552, St Louis, USA).[31] Controls included spleen samples in the absence of the NADPH-generating system and all components of the reaction mixture in the absence of spleen homogenates.

Measurement of cytokine concentrations by ELISA

Concentrations of IL-2, IFN-γ, IL-10 and TNF-α were determined by sandwich ELISA. The following antibodies were used as capture antibodies: anti-IL-2 mAb: clone JES-1A12, anti-IFN-γ: clone R46A2, anti-IL-10 mAb: clone JES5-2A5, anti-TNF-α mAb: clone MP6-XT22. The following biotinylated antibodies were used for the detection of captured cytokines: anti-IL-2 mAb: clone JES-5H4, anti-IFN-γ: clone XMG1.2, anti-IL-10 mAb: clone SXC-1, polyclonal anti-TNF-α antiserum. All antibodies were purchased from Pharmingen (San Diego, CA, USA). Capture antibodies were coated onto ninety-six wells Maxisorb flat bottom plates (Nunc, Denmark) at 2 μg/ml in 0.1 M Na_2HPO_4 pH 9, overnight. Plates were washed three times with PBS, 0.05% Tween solution to remove unbound antibodies and remaining binding sites were blocked with 1% BSA/PBS/Tween. Following the addition of culture supernatants, the plates were incubated 3 h at 4°C. Captured cytokines were detected using biotinylated mAb (1 μg/ml) and streptavidin-conjugated HRP (Jackson ImmunoResearch, West Grove, PA, USA). Bound HRP was detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction was stopped by addition of 1 M HCl solution and absorbance was measured at 490 nM.

Inhibition TNF-α production by macrophages
The macrophage cell line RAW 264.7 was cultivated in RPMI 1640 10% FCS. TNF-α production was stimulated by the addition of LPS (10 μg/ml). HO activity was upregulated by the addition of CoPP at the indicated concentration. Cells were cultivated for 24 h and the TNF-α concentration in the culture supernatant was determined by ELISA.

Flow cytometric analysis of spleen cell subpopulations
Freshly isolated spleen cells were isolated with the following monoclonal antibodies diluted 1:100 in PBS/1%BSA: PE-conjugated hamster anti-murine CD3ε IgG, clone 145-2C11; FITC-conjugated rat anti-mouse CD4 IgG, clone RM4-5; PE-conjugated rat anti-mouse CD8 IgG, clone 53-6.7; FITC-conjugated hamster anti-mouse TCR IgG, clone H57-597; FITC-conjugated rat-anti-mouse Mac-1 IgG, clone m1/70; and PE-conjugated rat anti-mouse B220 IgG, clone RA3-6B2. Antibodies of identical isotypes were used as controls. All antibodies were from Pharmingen (San Diego, CA, USA). After incubation for 45 min at 4°C the cells were washed and fixed in 1% paraformaldehyde in PBS. Flow analysis was performed using a FACSCAN flow cytometer.

Measurement of cellular DNA content
The cellular DNA content was measured as described.[32] Spleen cells were stimulated with Con A at 2 μg/ml for 48 h in R-10 medium (RPMI 1640, 10% FCS) at 37°C. After incubation, cells were washed twice in PBS containing 0.1% glucose and subsequently fixed overnight in 70% alcohol at 4°C. Fixed cells were washed and stained with propidium iodide (50 μg/ml PI, 100 U/ml RNAse A in PBS, 0.1% glucose for 30 min at room temperature. Stained cells were then analysed by FACScan flow cytometry.

Concanavalin-A stimulation and mixed lymphocyte culture
Mouse (CBA) spleen cells (2×10^5/well) were stimulated with 40 ng/ml anti-TCR monoclonal antibody (clone H57-597; Pharmingen, San Diego, USA) in 96-well, round-bottomed tissue culture plates (Nunc, Denmark) using R-10 medium. For a mixed lymphocyte culture, CBA spleen cells were used as responders and B6 or Balb/c spleen cells as stimulators. Stimulator spleen cells were pre-treated with 0.5 mg/ml mitomycin-C (Calbiochem, La Jolla, Ca, USA) for 20 min at 37°C. Subsequently, the cells were washed three times to remove excess mitomycin-C. CBA spleen cells (2×10^5/well) were cultured with an equal number of mitomycin-C treated B6 cells in R-10 medium for periods of 2–5 days. Twenty-four hours before harvesting, 1 μCi of [^{3}H]-Tdr was added to the culture. The degree of thymidine incorporation was measured using a TopCount microplate scintillation counter (Packard, Downers Grove, IL, USA). Results were expressed as CPM ± 2SD.

NK-cell activity
Yac-1, a mouse lymphoma induced by inoculation of Moloney leukemia virus into a newborn A/Sn mouse, was used as the NK-cell target. Yac-1 cells were labelled with 0.1 mCi ^{51}Cr in 100 μl PBS for 1 h at 37°C. Two thousand ^{51}Cr-labelled Yac- 1 cells were incubated with freshly isolated, Ficoll-Paque purified CBA spleen cells at effector:target ratios of 200:1, 100:1, 50:1 and 25:1 in V-shaped bottom plates in RPMI-1640 (Sigma) supplemented with 10% FBS (R-10 medium) for 4 h at 37°C, 5% CO_2. For the determination of maximal release, 1% triton X-100 was added to separate wells. Following incubation, the release of ^{51}Cr into 75 μl of the supernatant was determined by measuring radioactivity using a TopCount scintillation counter (Packard, Downers Grove, IL, USA). The degree of cell lysis was calculated using the following formula:

$$\% \text{ lysis} = \frac{CPM_{experimental} - CPM_{spontaneous}}{CPM_{total} - CPM_{spontaneous}} \times 100$$

Cytotoxic T cell activity
Cytotoxic T-cells were generated in a 5-day mixed lymphocyte culture of 3×10^6 CBA spleen cells with 3×10^6 mitomycin-treated B6 spleen cells in 24-well plates (Nunclon Delta) using R-10 medium. Subsequently, the cells were harvested, washed and incubated with ^{51}Cr-labelled EL4 ($H2^b$) mouse lymphoma cells. EL4 cells were routinely subcultured once every 3 days. Labelling was performed with 0.1 mCi ^{51}Cr in 100 μl PBS for 1 h at 37°C. Effector (E) and target (T) cells were added into V-shaped tissue culture plates (Nunclon Delta) at an E:T ratio of 20:1 and centrifuged for 3 min to increase the cellular contact. For the determination of maximal release, 0.1% triton X-100 was added to separate wells. Following the incubation cell lysis was determined as described above.

Detection of fluorescein-labelled allogeneic spleen cells
Freshly isolated spleen cells from B6 or CBA mice were labelled with fluorescein as described.[33] Briefly, B6 spleen cells were incubated with 30 μg/ml fluorescein isothiocyanate (Sigma) dissolved in PBS for 20 min at 37°C. Following labelling, cells were washed twice in PBS/3% BSA and once in PBS. Subsequently, 4×10^7 cells in PBS were injected via the tail vein into CBA mice. As a control, an equal number of FITC-labelled CBA spleen cells were injected into CBA mice. After 2 days, the mice were sacrificed and the number of FITC-labelled spleen cells was quantified by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA, USA).

Heterotopic heart transplantation
Abdominal heterotopic heart transplantation was performed as previously described by Ono and Lindsey.[34] B6 mice were used as the heart donors and CBA mice were used as recipients. Heart recipients were treated daily with various amounts of metalloporphyrins. Metalloporphyrin was administered intraperitoneally beginning on day 0 until day 4 post-transplantation or until rejection. Transplanted hearts were monitored daily by direct palpation, and rejection was defined as termination of palpable cardiac contractility. Results were expressed as percentage graft survival at a given post-operative period.

Statistics
The significance of differences were determined using the unpaired Student's *t*-test or the Mann–Whitney U test, as appropriate. Values of $p < 0.05$ were considered significant.

Results

Increased HO-1 expression in CoPP-treated mice
Metalloporphyrins have been shown to induce HO-1 in rats (reviewed by Maines[14]). We studied the effect of CoPP and ZnPP administration on HO-1 expression in mice. Also, we

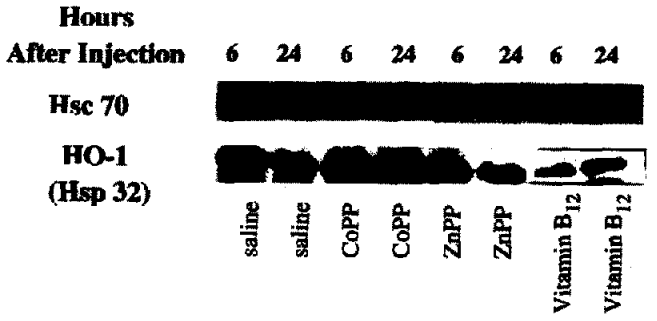

Figure 1 Analysis of HO-1 protein expression. Six hours (lanes 1, 3, 5, 7) and 24 h (lanes 2, 4, 6, 8) after treatment of mice with saline (lane 1 and 2), CoPP (lane 3 and 4), ZnPP (lane 5 and 6) and vitamin B_{12} (lane 7 and 8) spleen cell homogenates were prepared and separated by SDS-gelelectrophoresis. Following protein transfer to nitrocellulose, HSC 70 and HSP 32 were detected using polyclonal antisera and HRP-conjugated secondary antibodies.

examined vitamin B12, a cobaltporphyrin complex with different side chains than CoPP. Six and 24 h after injection of 20 mg/kg the expression of HO-1 in spleen cells was investigated by western blotting (Figure 1). CoPP injection resulted in increased HO-1 expression that could be observed after 24 h. In contrast, ZnPP and vitamin B12 did not alter the expression of HO-1. As a control, we analysed the expression of HSC 70 in the same tissue extracts. Neither CoPP nor ZnPP or vitamin B12 administration influenced HSC 70 levels in spleen cells. Additional analysis using liver and kidney cells demonstrated an increased expression of HO-1 in these tissues as well (data not shown).

Increased HO-1 expression correlated with increased HO-1 activity in spleen cell homogenates. HO-1 activity in spleen from untreated control animals was 19.2 ± 0.7 nmoles bilirubin/min/spleen compared to 27.3 ± 2.5 nmoles bilirubin/min/spleen in CoPP-treated animals. This corresponds to a 42% increase in HO activity. Increased HO activity has been shown to result in elevated bilirubin levels in rat serum. A similar increase in bilirubin was observed in sera from CoPP-treated mice but not in ZnPP-treated or vitamin B_{12}-treated animals (Figure 2).

Flow cytometric analysis of splenocytes from CoPP-treated mice

The phenotype of splenocytes from CoPP-treated animals was analysed by flow cytometry on days 2 and 6 after CoPP (20 mg/kg) administration on days 0 and 1. Compared to saline-treated control animals, the spleen of CoPP-treated animals contained a significantly reduced number of $TCR\alpha\beta^+$ cells

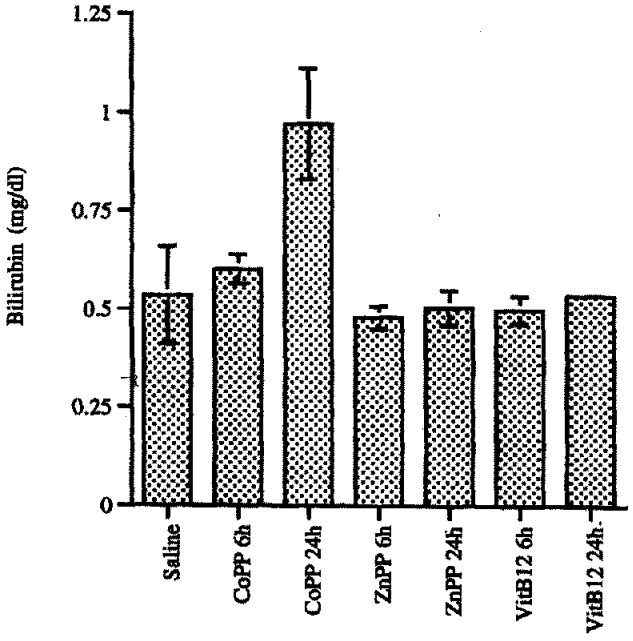

Figure 2 Determination of serum bilirubin levels. Animals were treated with saline, protoporphyrins or vitamin B12. Six and 24 h later bilirubin levels in serum were determined. Levels in CoPP-treated animals were significantly increased 24 h after CoPP injection ($p < 0.01$).

(20–22% versus 33% in controls) (Table 1). A reduction of $CD3^+$ cells was also observed. However, this reduction was less pronounced than the decrease in $TCR\alpha\beta^+$ cells. The ratio of $CD4^+$ to $CD8^+$ T cell in CoPP-treated animals compared to saline-treated controls remained unchanged. The number of $B220^+$ cells and $Mac\text{-}1^+$ macrophages increased following CoPP administration. Similar results were obtained with splenocytes from ZnPP-treated mice, suggesting that these changes are not due to modulation of HO activity (Table 1).

Defective lymphoproliferative response in CoPP-treated mice

All known inducers of HO-1 inhibit HO-1 activity *in vitro*. The presence of enzyme inhibitors in *in vitro* assays can lead to the inhibition of HO activity, even upon upregulation of HO-1 gene transcription and elevated HO-1 protein levels. Therefore, results with metalloporphyrins in *in vitro* experiments can be quite different from results obtained following *in vivo* administration of HO-1 inhibitors. Our focus, therefore, was on *in vivo* effects, which in general may be more relevant in studying immunosuppression.

Table 1 Phenotypic analysis of splenocytes from CoPP-treated or ZnPP-treated mice

	CD4	CD8	CD3	TCR	B220	Mac-1	CD4/CD8
Saline-treated ($n = 12$)	21.10 ± 2.0	12.85 ± 1.5	37.36 ± 5.5	33.54 ±3.9	55.20 ±3.4	3.72 ±1.3	1.65 ± 0.2
CoPP-treated							
Day 2 ($n = 9$)	14.35 ± 2.2[b]	8.96 ± 1.5[b]	28.15 ± 1.5[b]	21.40 ± 2.2[b]	62.17 ± 4.9[b]	7.33 ± 4.2[b]	1.61 ± 0.1
Day 6 ($n = 3$)	16.96 ± 1.4[b]	10.28 ± 1.8[a]	29.31 ± 1.9[a]	20.30 ± 3.9[b]	54.83 ± 2.0	4.47 ± 0.6	1.67 ± 0.2
ZnPP-treated							
Day 2 ($n = 6$)	16.57 ±1.5[b]	8.97 ± 1.5[b]	32.67 ± 2.4	24.41 ± 2.2[b]	65.15 ± 3.4[b]	5.33 ± 1.9[a]	1.88 ± 0.2[a]

The phenotype of mouse splenocytes was analysed 2 and 6 days after CoPP or ZnPP administration. Control animals were treated with saline. The percentage of cells with the corresponding phenotypes is shown. Where 'a' indicates $p < 0.05$, 'b' indicates $p < 0.02$ versus saline treated.

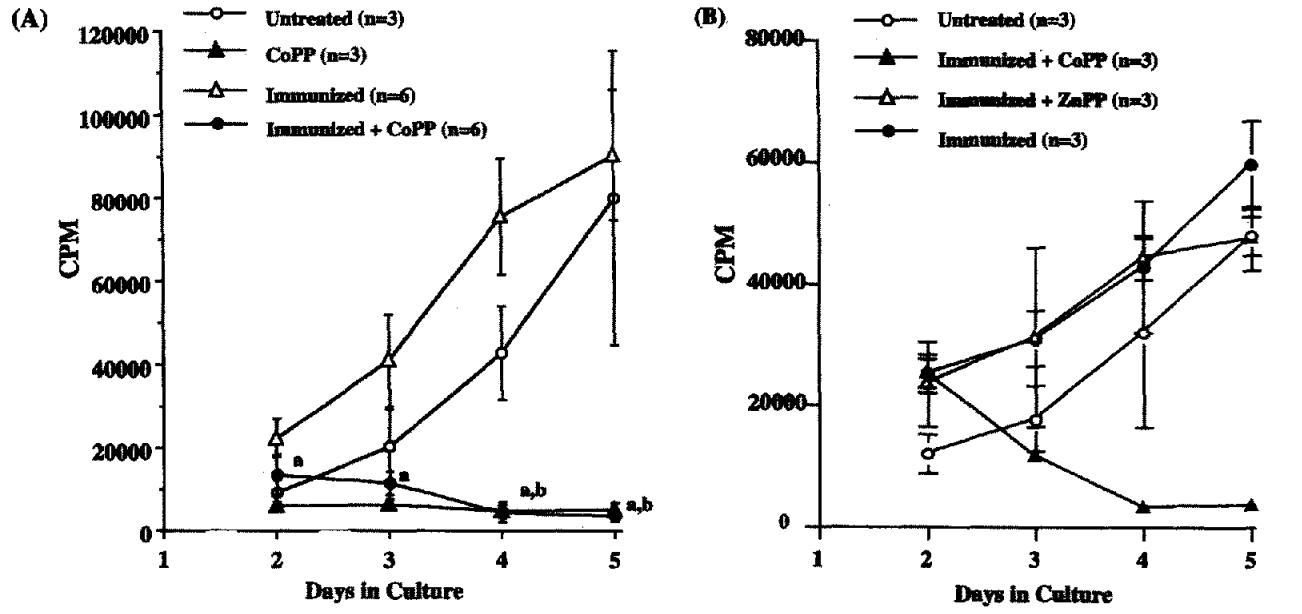

Figure 3 Lymphoproliferative response in CoPP-treated mice. Groups of mice ($n = 6$) were immunized with 4×10^6 B6 spleen cells on day 0. Control mice ($n = 3$) were treated with saline. Subsequently, CoPP was administered at 20 mg/kg. On day 6, the proliferative response of isolated spleen cells to mitomycin-C-treated B6 cells was assayed on day 6 (A). In a second experiment (B), the effect of CoPP and ZnPP (20 mg/kg) was compared. At the indicated time points proliferation was measured by [^{3}H]-thymidine incorporation. The significance of observed differences was analysed using the Student t-test. The 'a' indicates $p < 0.05$ versus immunized; and 'b' indicates $p < 0.05$ versus saline-treated.

The effect of CoPP therapy on a lymphoproliferative response was evaluated in a mixed lymphocyte culture. CBA/J mice were treated once (day 0) or twice (days 0 and 1) with saline or 20 mg/kg CoPP. Five days later (day 6) spleen cells were removed and incubated with mitomycin C-treated B6 stimulator cells. As expected, spleen cells from saline-treated animals proliferated in response to the allogeneic stimulus (Figure 3a). Immunization of CBA/J animals with allogeneic B6 cells on day 0 accelerated the proliferative response by about one day. In both, primed and nonprimed animals, administration of CoPP resulted in a strong inhibition (70–100%) of the lymphproliferative response on days 3, 4, and 5 of a mixed lymphocyte culture. In contrast, no significant difference between spleen cells from treated or untreated animals could be observed on day 2 of the mixed lymphocyte culture. Results with animals treated once or twice were indistinguishable. This suggested that spleen cells from CoPP-treated animals proliferated normally in the early phase of the mixed lymphocyte culture. At later time points, cell growth was arrested.

In a second experiment, the effect of CoPP and ZnPP on lymphocyte proliferation was compared (Figure 3b). While CoPP inhibited lymphocyte proliferation, ZnPP had no effect. These results suggested that upregulation of HO-1 expression was sufficient for the observed reduction of the lymphoproliferative response, while inhibition of HO had no effect.

Effects of CoPP therapy on cytokine production

The diminished proliferative response following CoPP administration was not due to a decreased production of IL-2. The amounts of produced IL-2 were determined using culture supernatants from the mixed lymphocyte cultures (Figure 4). Similar amounts of IL-2 were detected in the cultures on days 2 and 3. In cultures using splenocytes from CoPP-treated animals the IL-2 concentration continued to increase on days 4 and 5, while the IL-2 concentration decreased in cultures using splenocytes from untreated animals. This suggests that decreased proliferation resulted in a lower consumption of produced IL-2.

While CoPP treatment of mice had no effect on the secretion of IL-2, it resulted in the inhibition of IFN-γ and IL-10 production in mixed lymphocyte cultures (Figure 4). With splenocytes from saline-treated animals the IL-10 concentration in culture supernatants was about 80 pg/ml on day 2 and increased to about 600 pg/ml on day 5. In contrast, the IL-10 content in culture supernatants of splenocytes from CoPP-treated animals was only 200 pg/ml on day 6. A similar observation was made with IFN-γ (Figure 4). With splenocytes from saline-treated animals, the IFN-γ concentration increased from about 1 ng/ml on day 2 to about 13 ng/ml on days 4 and 5. With splenocytes from CoPP-treated mice, the IFN-γ concentration on days 4 and 5 was about 5 ng/ml.

The TNF-α concentration in mixed lymphocyte culture supernatants was too low to be evaluated with our ELISA assay (<50 pg/ml). Therefore, the effect of HO upregulation or inhibition on TNF-α production was evaluated using the macrophage cell line RAW 264.7 (Figure 5). While these cells produce no detectable TNF-α, in the absence of LPS, the TNF-α concentration in the culture supernatant increased to about 0.8 ng/ml upon overnight incubation with LPS (10 μg/ml). Upregulation of HO activity through addition of CoPP to the culture medium, resulted in a dose-dependent inhibition of LPS induced TNF-α production. Maximum inhibition (75%) was observed at 50 μg/ml CoPP.

Cell-growth arrest following CoPP therapy

Inhibition of cell proliferation following overexpression of HO-1 has also been observed in transfected human epithelial cells.[21] Slower cell proliferation appeared to be due to the arrest of cell growth in G0/G1 phase. Upon inhibition of HO-1 using ZnPP, the cell growth accelerated to a normal level.[21] The effect of CoPP treatment on cell growth was analysed using lymphocytes

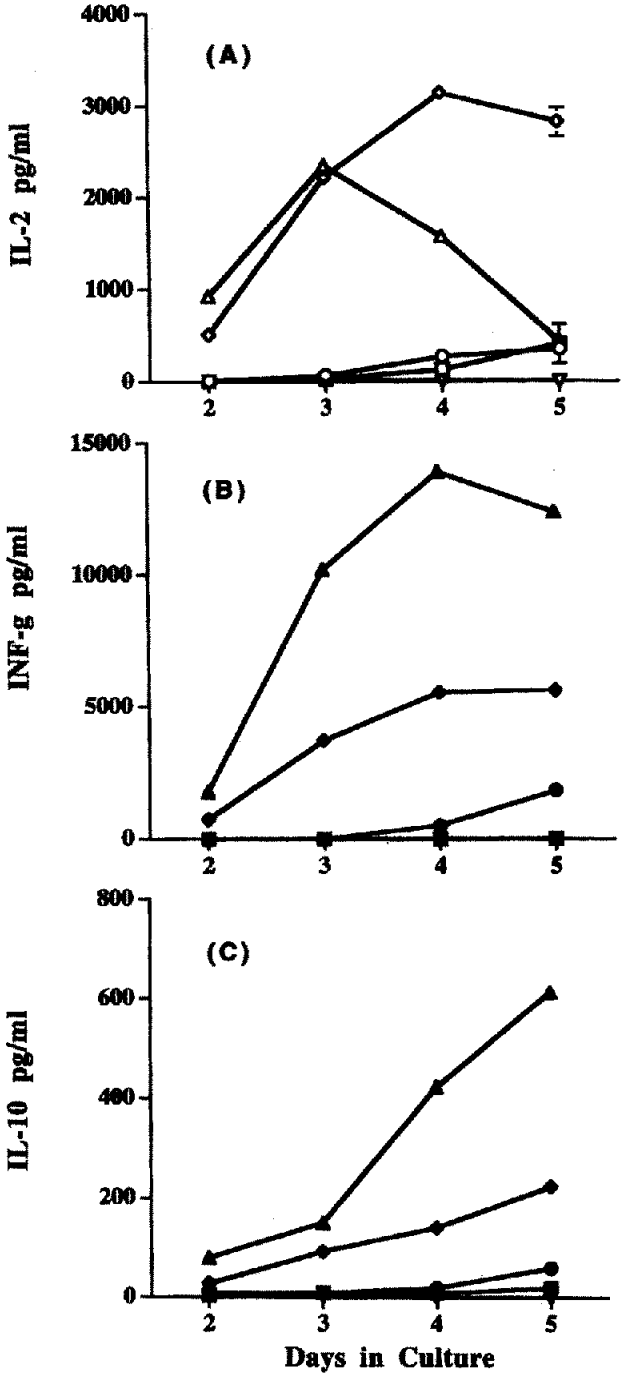

Figure 4 Cytokine concentration in mixed lymphocyte culture supernatants. CBA splenocytes from untreated animals (triangles) or CoPP-treated animals (diamonds) were stimulated with mitomycin-C-treated B6 cells and incubated for 5 days. The IL-2 (A), INF-γ (B) and IL-10 (C) concentration in the culture supernatants was determined on days 2–5. As controls, the supernatants from cultures containing only B6 cells (inverted triangle), splenocytes from untreated CBA mice (circles) or CoPP-treated CBA mice (squares) were assayed.

following stimulation with Concanavalin A (ConA). Similar to the observation with mixed lymphocyte cultures, splenocyte proliferation was inhibited by 50% when animals had been treated with CoPP (20 mg/kg) 2 days before removal of the spleen. The number of cells in G0/G1 phase was analysed after 2 days of stimulation with ConA. With splenocytes from untreated animals ($n = 3$) the frequency of cells in G0/G1, S and G2/M phases were 52.3 ± 2.3%, 17.9 ± 0.5% and 16.5 ± 0.6%, respectively. In contrast, cultures with splenocytes from CoPP-treated animals ($n = 3$) contained a significantly higher number of cells in G0/G1 (59.2 ± 1.3%), and a lower number of cells in S (13.7 ± 0.6%) and G2/M (13.4 ± 1.3%) phases ($p < 0.05$). No significant differences were observed using splenocytes from ZnPP-treated animals (data not shown).

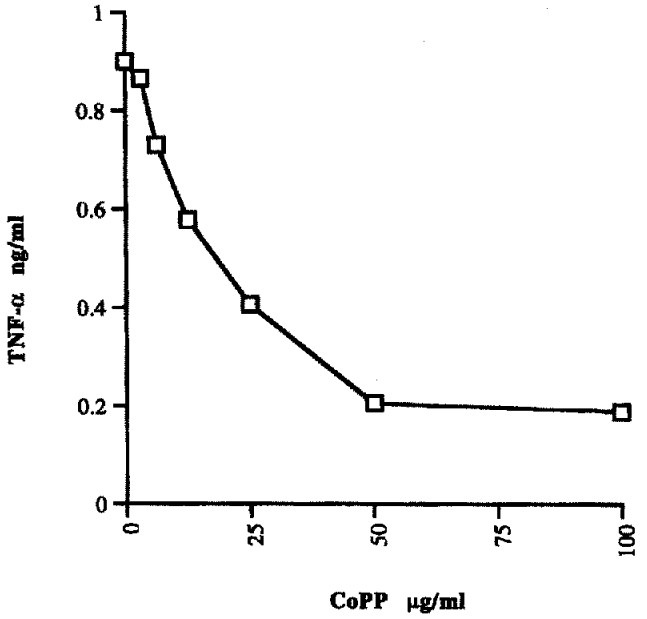

Figure 5 TNF-α concentration in macrophage culture supernatant. RAW 264.7 cells were cultured in the presence of LPS (10 μg/ml) with the indicated amounts of CoPP. Following a 24 h incubation the TNF-α concentration in culture supernatants was determined by ELISA. Without addition of LPS RAW 264.7, cells did not produce detectable amounts of TNF-α.

Reduction of T-cell-mediated cytotoxicity in CoPP-treated mice

Administration of CoPP not only inhibited lymphoproliferation but also cell-mediated cytotoxicity. CBA/J mice were immunized with allogeneic B6 cells on day 0, and treated with 20 mg/kg CoPP on day 0, days 0 and 1, or days 0 to 3. On day 6, spleen cells were removed and used as effector cells in a cytotoxicity assay. EL4-cell ($H\text{-}2^b$) were used as targets. A single administration of CoPP on day 0 had little effect (data not shown). However, in comparison to saline-treated control animals, CoPP treatment on days 0 and 1 resulted in a significant reduction in cytotoxic activity of the effector cells ($p < 0.05$) (Figure 6). Additional treatment of immunized animals on days 2 and 3 completely abrogated spleen cell-mediated cytotoxicity ($p < 0.01$ versus control). No significant inhibition of T-cell-mediated cytotoxicity was observed following administration of ZnPP (data not shown).

Inhibition of NK-cell activity in CoPP-treated mice

The effect of CoPP therapy on NK-cell-mediated cytotoxicity was evaluated following intraperitoneal administration of 10 mg/kg or 20 mg/kg for 4 consecutive days (days 0–3). Control groups were treated with saline or ZnPP. NK-cell mediated cytotoxicity was measured on day 4 using Yac-1 cells as targets. CoPP therapy

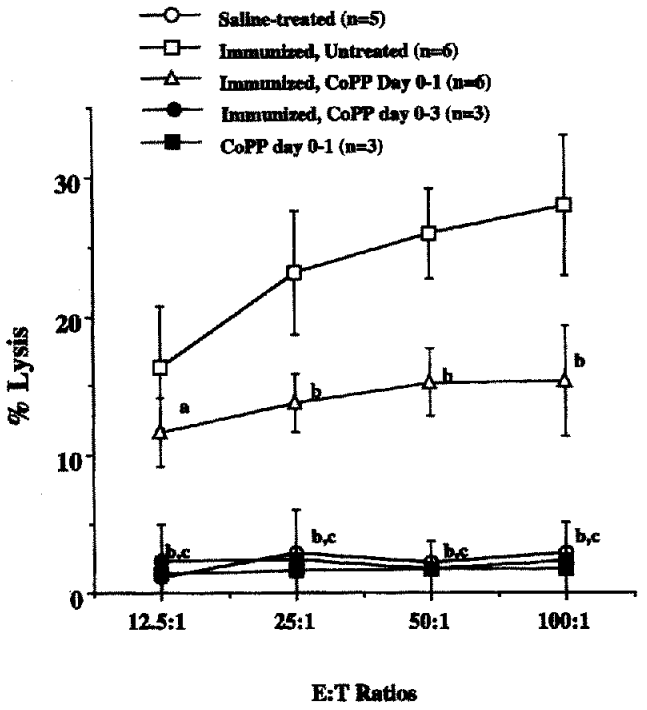

Figure 6 Inhibition of T-cell-mediated cytotoxicity. Groups of mice (n = 3–6) were immunized with 4×10^6 B6 spleen cells on day 0. Control mice were treated with saline. Subsequently, CoPP was administered at 20 mg/kg on days 0 and 1, or on days 0 to 3. On day 6, spleen cells were assayed in a cytotoxicity assay using EL4 as target cells. The percentage of lysis at several different effector:target ratios is shown. The significance of observed differences was analysed using Student's t-test. Where 'a' indicates $p < 0.05$ versus immunized, untreated; 'b' indicates $p < 0.01$ versus immunized, untreated; and 'c' indicates $p < 0.01$ versus immunized, CoPP day 0–1.

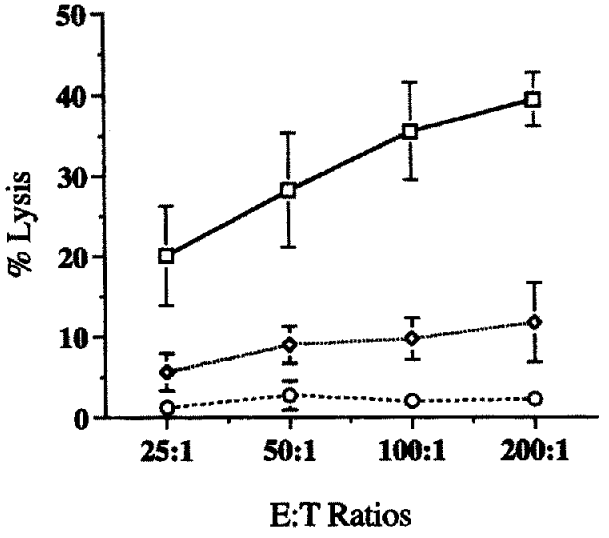

Figure 7 Inhibition of NK-cell-mediated cytotoxicity. Mice (n = 6) were treated with CoPP on days 0–3. Animals were injected with 0 mg/kg (squares), 10 mg/kg (diamonds), or 20 mg/kg (circles). On day 4, NK-cell-mediated cytotoxicity in spleen cell preparations was determined using YAC target cells. The percentage of lysis at several different effector:target ratios is shown.

resulted in a dose-dependent inhibition of cytotoxicity (Figure 7). Administration of 20 mg/kg completely abrogated cell-mediated killing of Yac-1 cells. Administration of ZnPP had no significant effect on NK-cell-mediated cytotoxicity (data not shown).

Increased persistence of injected allogeneic spleen cells in CoPP-treated mice

The rapid elimination of allogeneic cells in mice has been shown to be NK-cell-mediated.[32] Because we observed a reduction of NK-cell-mediated cytotoxicity in spleen cell populations from CoPP-treated animals, we tested the effect of CoPP therapy on the survival of allogeneic cells *in vivo* (Table 2). C57/B16 ($H\text{-}2^b$) spleen cells were labelled with fluorescein and injected into CBA/J mice. Recipient mice were treated with CoPP at 10 mg/kg and 20 mg/kg doses on the day of cell transfer and the following day. On day 2, the spleens were harvested and the number of surviving allogeneic cells was monitored by flow cytometry. As a control, an equivalent number of syngeneic CBA/J spleen cells were injected into CBA recipients. In these animals, the number of fluorescin-labelled spleen cells on day 2 was 7.4 ± 0.7% (Table 2). In the allogeneic combination, where B6 spleen cells were injected into CBA/J recipients, the number of surviving cells decreased significantly to 2.3 ± 0.6%. As shown, treatment of CBA/J recipients of B6 spleen cells with 10 mg/kg or 20 mg/kg of CoPP resulted in a significant increase in surviving B6 spleen cells (3.3 ± 0.2%, $p = 0.0002$, and 3.8 ± 1.0%, $p = 0.0042$, respectively). Treatment of mice with ZnPP had no effect on the survival of allogeneic cells (2.7 ± 0.08%).

Prolongation of heart allograft survival following CoPP-treatment

The effect of HO-1 overexpression was also evaluated in a mouse heart allograft model. CBA recipients ($H\text{-}2^k$) of B6

Table 2 Persistence of FITC-conjugated allogeneic spleen cells *in vivo*

Group	Dose (mg/kg/day)	Number	Mean ± SD	p-value*
B6-FITC → CBA				
PBS-treated		8	2.311 ± 0.58	
CoPP	10	9	3.342 ± 0.23	0.0002
CoPP	20	6	3.837 ± 1.04	0.0042
ZnPPR	20	3	2.670 ± 0.08	0.3282
CBA-FITC → CBA				
PBS-treated		5	7.386 ± 0.71	

FITC-conjugated splenocytes were injected into CBA recipients. The mice were treated with PBS, CoPP or ZnPP. After 2 days the percentage of FITC-labelled splenocytes in CBA spleen was measured by flow cytometric analysis.
*Statistical analysis comparing experimental groups to PBS-treated control group.

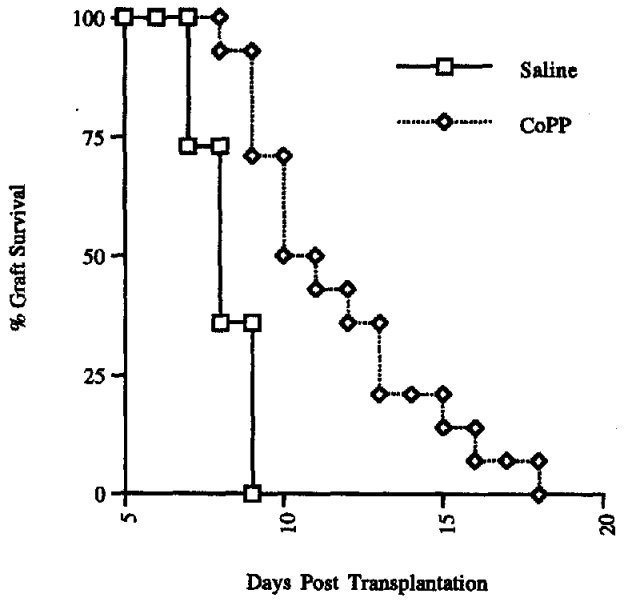

Figure 8 Prolongation of heart allograft survival. CBA recipients of vascularized B6 heart allografts were treated with saline (n = 11) or CoPP (20 mg/kg) (n = 14) on days 0–1. Graft survival, expressed in % survival, was monitored daily by palpation.

(H-2^b) hearts were treated on days –1 and 0 with 20 mg/kg CoPP. Compared to untreated control animals, mean graft survival time in CoPP-treated animals was significantly prolonged (11.7 ± 2.8 vs. 8.0 ± 1.4, $p < 0.001$) (Figure 8).

Discussion

The present study describes for the first time suppression of immune effector functions following upregulation of HSP32, commonly known as HO-1. *In vivo* expression of HO-1 is very responsive to various forms of stress, including heat shock, ischaemia, GSH depletion, UV-radiation, hypoxia, hyperoxia and disease states (reviewed by Maines[14]). The stress-induced upregulation of this enzyme can be mimicked by administration of the synthetic metalloporphyrin CoPP.[22,26]

Upregulation of HO-1 activity has been shown to protect cells from oxidative injury.[21,35] By degrading haem, a pro-oxidant contained in all intracellular haemproteins, and by generating bilirubin, an anti-oxidant,[35–37] induction of HO may provide an anti-oxidant protection. Bilirubin scavenges peroxyl radicals *in vitro* as efficiently as alpha-tocopherol, which is regarded as the most potent anti-oxidant against lipid peroxidation.[36,37] In addition, upregulation of HO-1 was shown to inhibit an inflammatory response.[17]

As shown here, similar to previous observations made in rats,[20,25] administration of CoPP into mice resulted in the upregulation of HO-1 expression and increased enzyme activity. Increased enzyme activity led to elevated bilirubin levels in mouse serum. The overexpression of HO-1, apart from potentially providing protection from oxidative cell injury, resulted in the modulation of several immune effector functions. Following CoPP administration, the number of $TCR\alpha\beta^+$ cells in mouse spleen decreased. The ratio of $CD4^+/CD8^+$ cells remained unchanged, but the ratio of $CD3^+/TCR\alpha\beta^+$ cells decreased indicating that CoPP modulated TCRαβ expression. Similar changes in spleen cell populations were observed following administration of ZnPP, an effective inhibitor of HO activity. These results suggest that the observed changes were not due to modulation of HO activity. In addition, these small changes (20–22% versus 33% in controls, $p < 0.02$) cannot explain the complete inhibition of proliferation in a mixed lymphocyte culture. A one time, CoPP administration resulted in complete inhibition of a proliferative response to an allogeneic stimulus, while administration of ZnPP had no effect. This observation was made with spleen cells from naive and previously sensitized animals. Lack of proliferation was not due to inhibition of IL-2 synthesis. However, CoPP caused an inhibition of IL-10, IFN-γ and TNF-α production. CoPP treatment also inhibited T-cell-mediated and NK-cell-mediated target cell lysis. *In vivo*, the inhibition of NK-cell activity caused a prolonged survival of allogeneic spleen cells in mice. Immunosuppression by HO overexpression also resulted in a significant prolongation of heart allograft survival. Even though CoPP resulted in an almost complete inhibition of immune effector function as measured *in vitro*, the prolongation of allograft survival was moderate (3 days). This may be due to the applied regimen where a high dose of CoPP (20 mg/kg) was administered twice (the day of transplantation and day 1). Further studies on the kinetics of HO expression following CoPP administration may allow optimization of this new mode of immunosuppression.

Immunomodulatory effects similar to those mediated by CoPP were also observed following administration of a recently described immunomodulatory peptide (D2702.75–84 (E > V)) (Table 3).[38] Similar to CoPP, this peptide-inhibited HO activity *in vitro*. In contrast, injection of D2702.75–84 (E > V) or CoPP into mice resulted in upregulation of HO-1 gene transcription and HO activity. HO-1 upregulation following administration of both compounds was associated with inhibition of cell-mediated cytotoxicity and prolongation of heart allograft survival. These results indicate that upregulation of HO-1 is not just associated with immunosuppression but an essential component of stress-mediated immunomodulation. Formal proof of this hypothesis has to await the development of transgenic or 'knock-out' animals.

Table 3 Comparison of D2702.75-84(E > V) and CoPP

Effect	D2702.75-84 (E > V)	Co-protoporphyrin
HO-activity *in vitro*	inhibition	inhibition
HO-activity *in vivo*	upregulation	upregulation
T cell proliferation	inhibition	inhibition
Cytokine production	inhibition	inhibition
NK-cell cytotoxicity	inhibition	inhibition
T cell cytotoxicity	inhibition	inhibition
Graft survival	prolongation	prolongation

Although the mechanism of immunosuppression by HO-overexpression remains unclear, several possible explanations can be envisioned. Degradation of haem by HO results in the depletion of cellular haem and haemproteins[23] and the production of biliverdin, bilirubin and carbon monoxide. Due to the haemprotein nature of NO synthase, induction of HO-1 is likely to modulate NO production, an important effector molecule involved in immune regulation.[39]

Biliverdin, the intermediate compound of haem degradation, has been shown to inhibit human complement *in vitro*.[40] In this context, it is interesting to note that complement deposition has been observed in unsensitized rat allograft recipients.[41] Based on this observation, one may speculate that increased production of biliverdin following overexpression of HO may protect a transplanted organ from complement-mediated cell injury and prolong graft survival.

Bilirubin, one of the end-products of haem degradation, has been shown to inhibit responses of human lymphocytes including phytohemagglutinin-induced proliferation, interleukin-2 production, and antibody-dependent and independent cell-mediated cytotoxicity.[42,43] Such inhibition was observed following incubation of human lymphocytes with bilirubin at concentrations of 6–12 mg/dl and correlated with amounts of intracellular bilirubin.[42] Bilirubin levels of 6–12 mg/dl can be detected in jaundice patients and may explain their increased susceptibility to infection. Bilirubin has also been shown to inhibit several intracellular enzymes such as protein kinase C, cAMP-dependent protein kinase and NADPH oxidase.[44,45] Inhibition of such enzymes may be responsible for the inhibition of the cytolytic machinery of effector cells. Bilirubin levels in serum of CoPP-treated mice were about 1 mg/dl, a concentration that had no effect on immune effector functions in *in vitro* assays (data not shown). However, one cannot exclude the possibility that overexpression of HO resulted in substantially higher intracellular bilirubin levels. Elevated intracellular bilirubin concentrations could cause the inhibition of spleen cell proliferation following CoPP treatment of mice. Additional studies evaluating the intracellular concentration of bilirubin in spleen cells of CoPP treated mice by flow cytometry will be necessary to clarify this issue.[46]

In contrast to the observations made with bilirubin, the reduced proliferative response of spleen cells following CoPP treatment were not associated with a decreased production of IL-2. This may indicate that additional factors may be involved in the inhibition of spleen cell proliferation following HO-overexpression. Carbon monoxide, the gaseous end-product of haem degradation, has been shown to stimulate the production of cGMP via activation of guanyl cyclase.[47,48] The secondary messenger, cGMP, has been implicated in cell growth arrest and the release of tumour necrosis factor-α by activated macrophages.[49] In addition, cGMP is involved in the regulation of various protein kinases, phosphodiesterases and ion channels.[50–54] Interestingly, overexpression of HO-1 in transfected pulmonary epithelial cells has been shown to slow cell division and to increase the numbers of cells in G_0G_1 phase.[18] Inhibition of HO-1 activity by ZnPP reversed this inhibitory effect of HO-1 overexpression. Based on these observations, one could speculate that an increased production of carbon monoxide following HO overexpression may be involved in the inhibition of spleen cell proliferation.

In summary, upregulation of HO activity *in vivo* inhibited several immune effector functions including lymphocyte proliferation and cell-mediated cytotoxicity. The regulation of HO-1 is very sensitive to various forms of stress. The data presented here suggest that immunomodulation following such stressful stimuli are due to the consequent upregulation of HO-1. The mechanism of immunomodulation by HO-1 is not resolved. Our current knowledge of the biological effects of haem degradation products (biliverdin, bilirubin and carbon monoxide) suggest that elevated HO activity effects multiple pathways of immune responses. Upregulation of this enzyme may, therefore, provide novel strategies to modulate immune responses in autoimmune diseases and organ or cell transplantation.

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