Protection from Abortion by Heme Oxygenase-1 Up-Regulation Is Associated with Increased Levels of Bag-1 and Neuropilin-1 at the Fetal-Maternal Interface

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Tolerance mechanisms allowing pregnancy success resemble those involved in allograft acceptance. Heme oxygenase (HO) is a tissue-protective molecule, which allows graft acceptance and is known to have antiapoptotic effects on several cell types. We previously reported down-regulated levels of HO-1 and HO-2 in placenta from allopregnant mice undergoing abortion. In this study, we analyzed whether the up-regulation of HO-1 by cobalt-protoporphyrin (Co-PP) during implantation window can rescue mice from abortion. Induction of HO-1 by Co-PP treatment prevented fetal rejection, whereas the down-regulation of HOs by zinc-protoporphyrin application boosted abortion. The beneficial effect of HO-1 induction was not related to a local shift to Th2-profile or to a change in the NO system. Interestingly, the expression of the antiapoptotic/cytoprotective molecule Bag-1 as well as the levels of neuropilin-1, a novel marker for T regulatory cells, were up-regulated after Co-PP treatment. Our data strongly support a very important role for HO-1 in fetal allotolerance and suggest that HO-1 might be protective by up-regulating tissue protective molecules, i.e., Bag-1, and by activating T regulatory cells rather than by changing the local cytokine profile. The Journal of Immunology, 2005, 175: 4875–4885.

The mechanisms involved in pregnancy maintenance, i.e., the mechanisms leading to acceptance of the semiallogeneic fetus by the maternal immune system, are still not fully understood. The fetus has been considered as a naturally occurring semiallograft (1), and the tolerance mechanisms involved in pregnancy maintenance are thought to be similar as in long-time graft acceptance. Breaking of the natural tolerance at the fetal-maternal interface might provoke fetal rejection by the maternal immune system, i.e., immunological spontaneous abortion.

The cytoprotective enzyme heme oxygenase (HO) has been proposed to play a critical role in graft acceptance (2). HO is the rate-limiting enzyme in the heme catabolism, being free iron, biliverdin, and carbon dioxide, the main products of its action. Biliverdin is then converted into bilirubin by biliverdin reductase. Accumulation of free heme, which is very toxic, leads to tissue inflammation and injuries (3, 4). HO is therefore responsible, at least in part, for the avoidance of these injuries (3, 4). Three forms of HO are known: 1) HO-1, a stress-responsive protein that can be induced by various oxidative agents, inflammatory cytokines, heat shock, heavy metals, and ultra violet radiation (4–8); 2) HO-2, which is thought to be not inducible—however, we and others have shown that HO-2 is inducible in reproductive tissues, i.e., in placental and decidual tissue (7, 9, 10); and 3) HO-3, which might have a regulatory role in heme-dependent cellular processes (11).

It has been very nicely shown that the expression of HO is necessary for graft survival (12, 13). Furthermore, induction of HO-1 in endothelial cells inhibits inflammatory responses (14, 15), and HO-1 overexpression alleviates graft-vs-host disease (16, 17). We therefore hypothesized that the role of HO during pregnancy maintenance may resemble the role of HO in graft acceptance. In this regard, we were recently able to show that HO-1 and HO-2 are down-regulated at the fetal-maternal interface from mice undergoing immunological abortion when compared with normal pregnant mice (10). Furthermore, boosting abortion with sonic stress or IL-12 application down-regulated the HO-1 and HO-2 expression at the murine fetal-maternal interface (9). Interestingly, HO-1 and HO-2 were also down-regulated at the fetal maternal interface from patients undergoing spontaneous abortion during the first trimester, as compared with age-matched women undergoing normal pregnancies (18).

Treatment with cobalt-protoporphyrin (Co-PP), which is known to up-regulate HO-1 expression, showed positive effects on long-term graft acceptance (16, 17, 19) and might therefore be an interesting approach for avoiding murine abortion. In this study, we hypothesized that up-regulating HO levels at the fetal-maternal interface from mice undergoing abortion would prevent fetal rejection possibly by modifying the Th1/Th2 production, by interfering with the NO synthase (NOS) system, or by preventing tissue...
damage, all these mechanisms are known to be associated with both the HO-1 pathway and pregnancy outcome. For testing our hypothesis, we up- or down-regulated HO-1 by injecting abortion-prone mice with a single dose of Co-PP or zinc-protoporphyrin (Zn-PP) during implantation window. Interestingly, up-regulation of HO-1 by Co-PP was able to prevent abortion, whereas Zn-PP treatment boosted it. The protective effect of HO-1 was not related to a shift in the Th1:Th2 production or to a modification in the NOS system. HO-1 overexpression up-regulated the levels of the cytoprotective molecule Bag-1 as well as the levels of neuropilin-1, a marker for regulatory T cells (Tregs). Our data suggest that HO-1 prevents abortion by preventing tissue damage and by stimulating Treg pathways.

Materials and Methods

Animals and samples collection

We conducted our experiments using the well-established abortion-prone combination CBA/J × DBA/2J (20). CBA/J × BALB/c mating was included as a group of normal pregnant animals. BALB/c male mice were purchased from Bundesinstitut für Gesundheitswesen Verbraucherschutz und Veterinaermedizin. Female CBA/J and male DBA/2J mice were obtained from Charles River Laboratories. Animal care and experimental procedures were followed according to institutional guidelines and confirmed to the requirements of the state authority for animal research conduct (LaGeSi 0070/03, Berlin, Germany). The animals were maintained in our barrier animal facility with a 12-h light-dark cycle and received water and food ad libitum. The data presented in this study are derived from three independent experiments. Female CBA/J mice were paired with BALB/c or DBA/2J males. Twice a day the females were checked for the presence of a vaginal plug and separated if mated. The day of plug detection was considered as day 0 of pregnancy. The abortion-prone mice were randomly separated into three groups, which received the following treatments: the first group (n = 10) was treated with PBS; the second group (n = 6) received 5 mg/kg Co-PP (Sigma-Aldrich); and the third group (n = 5) received 40 mg/kg Zn-PP (Frontier Scientific as described elsewhere (16).

For flow cytometry, decidual and spleen tissue were obtained from pregnant and non-pregnant mice by replacing the first Ab by BSA or by incubating the Ab with the corresponding blocking peptide. All of the sections were analyzed under the light microscope by two independent and blind observers, and the expression levels were set by using scores from 0 (no staining) to 6 (very intense staining) as described before (10).

Western blot (WB)

Sample treatment and protein separation were performed following our standard protocol (10). Ten micrograms of protein was resuspended in loading buffer and then loaded on polyacrylamid gels (12% gel for HO-1 and HO-2, 10% gel for Bag-1, and 7% gel for iNOS and eNOS). Proteins were then transferred to nitrocellulose membranes (Bio-Rad) at 4°C (10). Membranes were blocked for 2 h in TBS containing 5% milk powder. The blots were then incubated with first Abs as follows: dilution 1/1000 for HO-1 (Alexis Biochemicals), 1/5000 for HO-2 (StressGen Biotechnologies), iNOS (Santa Cruz), and eNOS (Santa Cruz Biotechnology), and 1/200 for Bag-1, bcl-2, or bcl-xL (Santa Cruz Biotechnology) ON at 4°C. After washing, the membranes were incubated with a biotinylated goat anti-rabbit secondary Ab dilution 1/5000 (DakoCyto) for 2 h at RT. After washing with PBS, 0.1% Tween 20, the blots where incubated for 30 min with avidin-biotin peroxidase complex solution. Positive bands were detected by chemiluminescence (Amersham Biosciences) after exposing the membrane onto Kodak Biomax MR Imaging film (Sigma-Aldrich). β-actin (Abcam) was used as housekeeping gene. The intensity of the bands was quantified by using the ImageQuant TL program (Amersham Biosciences).

Measurement of caspase-3 activity

For protein extraction, the frozen placenta was resuspended in 500 μl of lysis-buffer containing 200 mM HEPES (pH 7.4; Fluka), 1% CHAPS (Fluka), 50 mM DTT (Sigma-Aldrich), and 20 mM EDTA (Sigma-Aldrich). Protein concentration was measured in the supernatants by using the BioRad Protein Assay (Bio-Rad). The caspase-3 activity was evaluated by taking into account the micromoles of p-nitroaniline released per minute per volume.

TUNEL

The ethanol-fixed placenta sections were embedded in paraffin as described by Saint-Marie (23). The immunohistochemistry (IHC) was conducted using our standard protocol. The Abs used were as follows: polyclonal rabbit anti-HO-1 (Alexis Biochemicals) diluted 1/100; polyclonal rabbit anti-HO-2 (StressGen Biotechnologies) diluted 1/500; polyclonal rabbit anti-inducible NOS synthree (iNOS) diluted 1/25 (Santa Cruz Biotechnology); polyclonal rabbit anti-endothelial NOS synthree (eNOS) diluted 1/50 (Santa Cruz Biotechnology); and polyclonal rabbit anti-Bag-1, anti-Bcl-2, and anti-Bcl-xL (all from Santa Cruz Biotechnology) all diluted 1/100. First Abs were applied ON at 4°C, diluted in 5% BSA in TBS. As secondary Ab, a horseradish goat anti-rabbit IgG (DakoCytomation) was used. TUNEL staining (Diaminobenzidine (DakoCytomation) was used to detect positive cells for HO-2, eNOS, Bag-1, and Bcl-2. Negative controls were performed by replacing the first Ab by BSA or by incubating the Ab with the corresponding blocking peptide. All of the sections were analyzed under the light microscope by two independent and blind observers, and the expression levels were set by using scores from 0 (no staining) to 6 (very intense staining) as described before (10).
Total RNA from each sample was quantified by spectrophotometry, portioned at a concentration of 1 µg/µl, and kept at −80°C until use.

**Real-time RT-PCR**

Two micrograms of RNA was used for cDNA synthesis, adding dNTPs (2.5 230 mM; Amersham Biosciences), DNase I (2 U/ml; Stratagene), and RNase inhibitor (40 U/ml, 231; Promega), and incubated at 75°C for 5 min. After adding reverse transcriptase (200 U/ml; Amersham) and RNase inhibitor, the mix was incubated at 42°C for 60 min followed by 5 min at 94°C. For real-time PCR, 2 µl (for TNF-α, TGF-β, and HO-1) or 1 µl (for Bag-1 and Bcl-2) of cDNA was used as starting volume to amplify the DNA. PCR-Mastermix (6.25 µl; Eurogentec), 3 µl of the primer mix, 0.5 µl of the fluorescent probes, and RNase-free water were added to a final volume of 13 µl. The amplification reactions were performed on the ABI Prism 7700 Sequence Detection System (PerkinElmer Applied Biosystems) as follows: 2 min at 50°C, followed by an initial denaturation step of 10 min at 95°C, and 40 cycles of 15 s at 95°C, and 1 min at 60°C. β-actin was used as housekeeping gene. For amplifying iNOS, eNOS, neuropilin-1, and IDO, SYBR green technology was used as follows: 1 µl of cDNA, 6 µl of SYBR green, 50 mmol/µl fluoresceine, 3 µl of primer mix probes, and RNase-free water were added to a final volume of 13 µl. The amplification reaction was performed with the i-Cycler (Bio-Rad). As housekeeping gene, β-actin was used. To confirm specificity of the amplified product, a conventional PCR is conducted using our standard protocol. The PCR products are then evaluated by gel electrophoresis to determine the m.w. and to confirm the absence of other bands. When using SYBR green technology, the specificity of the desired gene products is documented with melting curve analysis. The amount of mRNA was calculated as 2−ΔΔCt in both cases. Primers and probe sequences can be supplied after request.

**Statistical analysis**

Because our data do not have a normal distribution, all of the data are expressed as median ± 75% quartiles (box plots) or medians (tables). Please note that outliers and extreme values (represented by stars and circles) are included in the statistical analysis. The differences among the

### Table I. Co-PP treatment augmented the HO-1 and HO-2 protein expression at the fetal-maternal interfacea

|          | NPb | SA | Co-PP | Zn-PP |
|----------|-----|----|-------|-------|
| HO-1     |     |    |       |       |
| Decidua  | 0.50| 1.25| 1.00  | 2.00  |
| Giant cells | 4.00| 1.75 (NP*) | 2.00   | 2.25 (NP*) |
| Spongiotrophoblasts | 3.50| 2.00 (NP*) | 4.00 (SA*) | 2.75 |
| Labyrinthic cells | 3.25| 2.50 (NP*) | 3.50 (SA**) | 2.75 |
| HO-2     |     |    |       |       |
| Decidua  | 1.25| 1.00| 0.75  | 1.00  |
| Giant cells | 4.25| 3.50 (NP*) | 5.00 (NP*,SA**) | 4.25 (SA*,Co-PP*) |
| Spongiotrophoblasts | 3.00| 1.75 (NP*) | 3.50 (SA*) | 2.75 (Co-PP*) |
| Labyrinthic cells | 3.00| 1.75 (NP*) | 3.00 (NP*,SA**) | 3.00 (SA*,Co-PP*) |

a Data represent the intensity of the staining analyzed as explained in Material and Methods and are shown as medians. Statistical analysis was done by using the nonparametric Kruskall-Wallis test followed by Mann-Whitney U test. *, p < 0.05 and **, p < 0.01 when compared to other experimental group (indicated in parentheses).

b NP, Normal pregnant mice; SA, PBS-treated abortion-prone mice.
different groups were calculated using the nonparametric Kruskall-Wallis test, followed by the Mann-Whitney U test for two groups using the 11.5 version of the SPSS computer program. In all of the cases, \( p < 0.05 \) was considered statistically significant.

**Results**

**Co-PP application up-regulated HO-1 expression at the fetal-maternal interface**

We analyzed the placental and decidual HO protein expression 10 days after treatment with Co-PP or Zn-PP, namely on day 14 of pregnancy, and compared these results to those of the control groups using different methods, namely IHC and WB. As reported previously, HO-1 and HO-2 were expressed in all placental cells types as well as in decidual cells (9, 10). When analyzing the intensity of the staining, we confirmed a significant down-regulation in the HO-1 and HO-2 expression at the fetal-maternal interface from abortion-prone mice compared with normal pregnant mice (Table I) (10). Interestingly, we observed that the treatment of abortion-prone mice with Co-PP led to a significant augmentation in the HO-1 expression in spongiotrophoblasts and labyrinthic cells and to an augmentation in giant cells. Furthermore, treatment with Co-PP provoked an augmentation of HO-2 in giant cells, spongiotrophoblasts, and labyrinthic cells. HO-2 expression was significantly up-regulated in giant cells from Co-PP-treated mice when compared with the PBS-treated abortion group. These data on up-regulated HO-1 after Co-PP treatment nicely support findings from other groups in transplanted organs (16). HO-2 was not found to be up-regulated after Co-PP in other tissues; however, we already reported that HO-2 can be up- or down-regulated at the fetal-maternal interface (9, 10, 18). These data on augmented HO-2 protein expression at the fetal-maternal interface suggest that HO-2 is being differently regulated at the fetal-maternal interface than in other tissues. By WB, we confirmed an augmentation in the HO-1 but also in the HO-2 protein expression after Co-PP treatment, as shown in Fig. 1A (HO-1) and Fig. 1B (HO-2). Furthermore, Zn-PP treatment increased HO-1 and HO-2 expression in several cell types (Table I), which could be due to the late time point of the analysis (10 days after treatment) probably induced by a feedback mechanism due to inhibition of HO activity. Increased levels of HO-1 were also reported in other cell types after tin-protoporphyrin treatment, which was also described to act as an HO-1 inhibitor (24, 25). However, this single treatment appears to be sufficient to boost abortion rate as indicated below, indicating that the blocking of HO-1 during implantation window is being pregnancy-deleterious. Again, the IHC data on HOs after Zn-PP (shown in Table I) were confirmed by WB (Fig. 1).

**The up-regulation of HO-1 by Co-PP application during implantation window prevented abortion**

As already described in the literature, the abortion rate of DBA/2J-mated CBA/J females treated with PBS was significantly higher than in the PBS-treated BALB/c-mated CBA/J females. To analyze the effect of HO-1 up- or down-regulation on pregnancy outcome, we treated abortion-prone mice with Co-PP or Zn-PP on day 4 of pregnancy, i.e., during implantation window. Co-PP treatment, which successfully up-regulated HO-1, was effective in preventing fetal rejection as shown in Fig. 2A. Accordingly, the Zn-PP treatment significantly boosted the abortion rate of abortion-prone mice from 20 to 30%, further confirming a negative effect of HO-1 down-regulation or absence during implantation window on pregnancy outcome. We performed a second set of experiments, sacrificing the animals 4 days after treatment, i.e., on day 8 of pregnancy; however, the abortion rate of these animals could not be registered, because it has been not possible to distinguish between resorption and healthy implantation sites, as already described in literature (20, 22). The implantation rates were comparable among all groups (Fig. 2B).

**FIGURE 2.** Co-PP treatment prevented fetal rejection, whereas Zn-PP treatment boosted abortion rates in abortion-prone animals. DBA/2J-mated CBA/J females presented increased abortion rate compared with BALB/c-mated CBA/J females on day 14 of pregnancy. Co-PP application significantly diminished the abortion rate, whereas Zn-PP application boosted abortion (A). The implantation rates were comparable among all groups (B). Data are presented as median ± 75% quartiles in box plots due to their non-Gaussian distribution. Outliers and extreme values are represented by circles and asterisks and are included in the statistical analysis. Statistical significances were evaluated by the nonparametric Kruskall-Wallis test followed by the Mann-Whitney U test between two particular groups. *, \( p < 0.05 \); **, \( p < 0.01 \). C depicts the left uterus of an abortion-prone animal, bearing two healthy fetuses along with a rejected one, also known as resorption.
FIGURE 3. Co-PP application did not modify the TNF-α:IL-10 (A) or IFN-γ:IL-4 (B) ratio in spleen or the TNF-α mRNA levels in deciduial tissue (C) on day 14 of pregnancy. Surprisingly, the Th1:Th2 cytokine ratio was not modified in Co-PP-treated animals when compared with PBS-treated abortion-prone animals (A and B). Zn-PP-treated animals presented down-regulated Th1:Th2 ratio in spleen as analyzed by flow cytometry after PMA/ionomycin stimulation (A and B). Furthermore, slightly elevated TNF-α mRNA levels were observed in placental tissues from the Co-PP-treated group as compared with all other groups, as analyzed by using real-time RT-PCR and indicated by 2^ΔΔCt (C). Data are presented as median ± 75% quartiles in box plots due to their non-Gaussian distribution. Outliers and extreme values are represented by circles and asterisks and are included in the statistical analysis. Statistical significances were evaluated by the nonparametric Kruskall-Wallis test followed by the Mann-Whitney U test between two particular groups. *, p < 0.05; **, p < 0.01.

The positive effect of HO-1 on pregnancy outcome was not related to a Th2 shift at the fetal-maternal interface

As previous studies reported (26–31), the Th1:Th2 ratio plays an important role in pregnancy outcome. We therefore measured Th1 and Th2 cytokine secretion by spleen and decidual immune cells after therapy with Co-PP or Zn-PP by flow cytometry and compared these results from those obtained from normal pregnant or abortion-prone mice, aiming to investigate whether the positive effect of HO-1 up-regulation on pregnancy outcome was accompanied by a shift to a protective cytokine profile. Real-time RT-PCR was further used to analyze cytokine mRNA levels in whole tissue, meaning cytokines produced by immune and nonimmune cells as, for example, trophoblasts. No significant changes could be observed in the TNF-α:IL-10 or IFN-γ:IL-4 ratio by spleen cells in abortion mice compared with normal pregnant mice, as shown in Fig. 3, A and B, respectively, on day 14 of pregnancy. No significant changes could be observed after Co-PP treatment compared with the PBS-treated abortion-prone group on day 14 of pregnancy. Contrary to our expectations, the TNF-α:IL-10 ratio was significantly down-regulated in the Zn-PP-treated group when compared with all of the other groups at this time point (Fig. 3A). No changes could be observed for IFN-γ:IL-4 ratio after Zn-PP application (Fig. 3B). Decidual cells from abortion mice produced more TNF-α and IFN-γ than normal pregnant mice, as already known (10). These data are shown in Table II. Treatment with Co-PP could diminish the TNF-α production but was not able to up-regulate the levels of IL-10 or IL-4 (Table II). Surprisingly, the animals treated with Zn-PP showed increased production of Th2 cytokines (IL-10 and IL-4) and decreased Th1 cytokine secretion (Table II). Real-time RT-PCR confirmed tendencies upon an augmentation in the TNF-α mRNA levels in the decidua after Co-PP treatment (Fig. 3C).

Having found a contrary effect on cytokine production/expression as expected after Zn-PP treatment, which boosted abortion rate, we wondered whether this was an artifact due to a too late time point of analysis. We hypothesized that on day 14 of pregnancy (thus 10 days after treatment with Co-PP or Zn-PP), the immune system may try to counteract the detrimental effects of Zn-PP by up-regulating Th2 cytokines and down-regulating Th1 cytokines. For confirming or refusing our hypothesis, a different time point (day 8 of pregnancy) of sample collection was chosen in a further set of experiments. When analyzing the TNF-α:IL-10 ratio and the IFN-γ:IL-4 ratio by spleen cells on day 8 of pregnancy, we were able to confirm the results we obtained on day 14

| Cytokines | NP | SA | Co-PP | Zn-PP |
|-----------|----|----|-------|-------|
| TNF-α | 1.83 | 4.25 | 2.85 | 1.07 (SA*:Co-PP**) |
| IFN-γ | 2.46 | 3.05 | 3.87 | 0.90 (SA*:Co-PP**) |
| IL-10 | 1.72 | 2.19 | 1.90 | 3.93 |
| IL-4 | 0.99 | 1.54 | 0.95 | 2.78 |

a Th1:Th2 production by immune decidual cells on day 14 of pregnancy

The production of Th1 or Th2 cytokines was analyzed by flow cytometry in isolated decidual lymphocytes. Data are shown as medians (percentage of positive cells). Statistical analysis was done by using the nonparametric Kruskall-Wallis test followed by Mann-Whitney U test. *, p < 0.05 and **, p < 0.01 when compared to other experimental group (indicated in parentheses).

b NP, Normal pregnant mouse; SA, PBS-treated abortion-prone mouse.
of pregnancy (Fig. 4, A and B): In the Zn-PP-treated group, a down-regulation of the TNF-α:IL-10 ratio and the IFN-γ:IL-4 ratio in spleen compared with the abortion-prone group could be observed. Interestingly, the same tendencies in the Th1:Th2 ratios for flow cytometry could be observed when analyzing pooled decidual cells obtained on day 8 of pregnancy with the χ² method (data not shown). We pooled decidual immune cells from day 8 of pregnancy (at least three animals each time) because the quantity of immune cells obtained from single animals is not enough to process the samples by flow cytometry. These results were also confirmed at the mRNA levels by real-time RT-PCR, because on day 8 of pregnancy (4 days after treatment) the levels of TNF-α in decidual after Zn-PP application were down-regulated (Fig. 4C). Having found the same results on Th1:Th2 cytokine production/expression on day 14 or 8 of pregnancy, meaning 10 or 4 days after treatment, we wondered whether the Th3 cytokine TGF-β might play a role in the Co-PP-mediated protection and/or in the Zn-PP-mediated worsening of abortion. TGF-β1 levels were measured by real-time RT-PCR, which revealed significantly elevated levels of this molecule on day 14 of pregnancy after Zn-PP treatment (Table III). On day 8 of pregnancy, we observed no changes in TGF-β1 mRNA levels (Table III), concluding that TGF-β1 is being up-regulated 10 days after Zn-PP treatment as part of the counteracting mechanisms following the tissue injury caused by the Zn-PP treatment. All together, our results suggest that Co-PP is being pregnancy beneficial despite Th1 cytokine augmentation, whereas Zn-PP is detrimental for pregnancy outcome, although it shifts the cytokine profile to a Th2/Th3 one. Moreover, our results on cytokine production and expression show that the therapeutic effects of Co-PP are not related to the changes on the Th1:Th2 ratio and other pathways might be activated, which contribute to pregnancy success after Co-PP treatment.

Therapy with Co-PP had no influence on the iNOS/eNOS expression at the fetal-maternal interface

We recently proposed an interaction between HO-1 and enzymes from NOS system during pregnancy at the placental level (10). Diminished eNOS and iNOS expression could be observed in abortion-prone mice compared with normal pregnancy mice as already described (10). In this article, we analyzed the effect of HO-1 up- or down-regulation on the NOS expression in placental or decidual tissue. Protein expression of iNOS and eNOS was augmented in decidual cells as well as giant cells after Co-PP treatment as analyzed by IHC (Table IV). However, when quantifying by WB, this augmentation after Co-PP treatment was found to be statistically not significant (eNOS/β-actin: group 1, 1.375; group 2, 0.89; group 3, 1.12; and group 4, 1.16 and iNOS/β-actin: group 1, 1.92; group 2, 1.41; group 3, 1.45; and group 4, 1.86. All data are shown as medians). Real-time RT-PCR analysis showed only a slight down-regulation of iNOS after Co-PP and Zn-PP compared with normal pregnant mice (Table V). These results indicate that HO-1 up- or down-regulation is not affecting the NOS expression at the fetomaternal interface and might therefore play no important role in the HO-1-mediated pregnancy protection.

| Table III. TGF-β1 mRNA levels as measured by real-time RT-PCR in placental tissue from 14- or 8-day pregnant animals |
|---|---|---|---|---|
| | NP | SA | Co-PP | Zn-PP |
| Day 14 of pregnancy | 0.18 | 0.25 | 0.29 | 1.63 (NP*) |
| Day 8 of pregnancy | n.d. | 0.63 | 0.32 | 0.82 |

* Data are shown as medians (25%EQR). Statistical analysis was done by using the nonparametric Kruskall-Wallis test followed by Mann-Whitney U test.
* * p < 0.05 when compared to other experimental group (indicated in parentheses).
* NP, Normal pregnant mice; SA, PBS-treated abortion-prone mice; n.d., not done.
HO-1 up-regulation led to slightly diminished apoptosis, while significantly up-regulating the levels of the cytoprotective molecule Bag-1

HO-1 was described to have antiapoptotic properties, and the Co-PP treatment was reported to up-regulate tissue-protective molecules (12, 15, 32). In this study, we analyzed the effect of up- or down-regulation of HO-1 on apoptosis rate as well on the expression of several molecules known to be tissue protective. After treatment with Co-PP the caspase-3 activity, which is known to be a reliable hallmark for apoptosis, was slightly diminished. This was, however, nonsignificant (Fig. 5A). On day 8 of pregnancy, the caspase-3 activity was comparable among all of the experimental groups (Fig. 5B). Interestingly, the levels of caspase-3 activity on day 8 were ~10 times higher than on day 14, which might mirror the remodeling processes taking place after implantation and during placental growth. On day 14, on the contrary, the placenta is already formed and does not further grow, whereas the fetus grows very fast (22). The analysis of apoptotic cells as measured by TUNEL assay on day 14 of pregnancy reveals that the apoptosis rate is tendentially down-regulated in the Co-PP-treated group compared with the PBS-treated abortion group (Fig. 5C), which again indicates an antiapoptotic or tissue-protective effect of HO-1 up-regulation at the fetal-maternal interface.

Because the caspase-3 activity rates indicate that the tissue is not undergoing increased apoptosis after Co-PP treatment despite high Th1 levels, on the contrary an antiapoptotic tendency could be observed, we wondered whether the HO-1 up-regulation may stimulate the expression of tissue-protective molecules, like Bag-1, Bcl-1, Bcl-2, or Bcl-xL. From all of the molecules we analyzed, only Bag-1 was significantly up-regulated after treatment with Co-PP when compared with the PBS-treated abortion group on a WB analysis, suggesting a tissue-protective effect of HO-1 up-regulation during implantation (Fig. 6A). Furthermore, the treatment with Zn-PP down-regulated the expression of Bag-1 (Fig. 6A). Real-time RT-PCR also revealed a slight up-regulation of Bag-1 after Co-PP treatment (Fig. 6B). The protein or mRNA levels of the other measured antiapoptotic/cytoprotective molecules were not influenced by Co-PP or Zn-PP treatment (data not shown). All together, these data indicate that the up-regulation of HO-1 by Co-PP might protect fetal tissue from being rejected by maternal immune system by up-regulating the expression of Bag-1, a cytoprotective molecule.

Table IV.  Expression of iNOS and eNOS at the fetal-maternal interface as analyzed by IHC on day 14 of pregnancy

|            | NP  | SA  | Co-PP | Zn-PP |
|------------|-----|-----|-------|-------|
| iNOS       |     |     |       |       |
| Decidua    | 1.75| 2.00| 3.25  | 2.50  |
| Giant cells| 0.25| 0.00| 0.75  | 0.75  |
| Spongiothrophoblast | 1.50| 1.00| 1.25  | 1.75  |
| Labyrinthic cells | 1.00| 0.50| 1.25  | 1.00  |
| eNOS       |     |     |       |       |
| Decidua    | 3.75| 1.75| 2.50  | 2.75  |
| Giant cells| 0.50| 0.50| 1.00  | 0.50  |
| Spongiothrophoblast | 1.00| 1.00| 1.75  | 1.25  |
| Labyrinthic cells | 1.00| 1.50| 1.75  | 1.25  |

a Data represent the intensity of the staining analyzed as explained in Material and Methods. Data are shown as medians. Statistical analysis was done by using the nonparametric Kruskall-Wallis test followed by Mann-Whitney U test. *, p < 0.05 when compared to other experimental group (indicated in parentheses).

Recently, Choi et al. (33) have proposed that Foxp3, present exclusively in Treg, can induce HO-1 expression. No reports exist on the effects of HO-1 up-regulation on Treg function or number. In this study, we analyzed the number of CD4+CD25+ cells in decidual tissue after Co-PP or Zn-PP treatment and found no differences as compared with the PBS-treated abortion-prone group (data not shown). This could be, however, due to the fact that CD25 is also marking activated cells and might not be an accurate marker for Tregs at the site of activation (34). Therefore, we measured the mRNA levels of foxp3 and neuropilin-1, both markers for Treg activity (35), at the fetal-maternal interface. The levels of Foxp3 mRNA were very low; therefore, we refrained from making any conclusion of these data (data not shown). Interestingly, neuropilin-1 mRNA, a very novel marker for Tregs (35), was diminished in abortion-prone mice compared with normal pregnant mice, suggesting diminished Treg levels in accordance with our previous observations (34). Furthermore, neuropilin-1 mRNA levels were significantly augmented after Co-PP treatment on day 14 of pregnancy (Fig. 7). This suggests an augmentation in the Tregs number or activity at the fetal-maternal interface following HO-1 augmentation.

Discussion

CBA/J female mice spontaneously show high abortion rates when mated with DBA/2J males. However, if mated with other H-2<sup>d</sup>-bearing male like BALB/c, no abortion can be observed (20). The high abortion rates observed in this model were therefore thought to be MHC-restricted, minor loci dependent (20). However, other features, like different expression of determined molecules in the F<sub>1</sub> tissue, namely the placenta, could be responsible for the enhanced abortion rate. In this regard, we reported low levels of HO-1 and HO-2 at the fetal-maternal interface from DBA/2J-mated CBA/J mice undergoing abortion (10). It remains to be elucidated whether the low HO-1 levels are a consequence of the fetal tissue damage or the cause of abortion (i.e., due to a different genetic background in the F<sub>1</sub> tissue). Accordingly, Denschlag et al. (36) observed that a microsatellite polymorphism of the HO-1 gene (leading to low levels of HO-1 expression) is associated with

Table V.  mRNA levels of iNOS and eNOS at decidua and placenta as analyzed by real-time RT-PCR on day 14 or 8 of pregnancy

|            | NP  | SA  | Co-PP | Zn-PP |
|------------|-----|-----|-------|-------|
| eNOS       |     |     |       |       |
| 14 days pregnancy | 0.041| 0.021| 0.058| 0.129 |
| 8 days pregnancy | 0.025| 0.030| 0.020| 0.016 |
| iNOS       |     |     |       |       |
| 14 days pregnancy | 0.098| 0.098| 0.092| 0.098 |
| 8 days pregnancy | 0.001| 0.009| 0.009| 0.002 |

a Data are shown as medians (2<sup>−ΔΔCt</sup>). Statistical analysis was done by using the nonparametric Kruskall-Wallis test followed by Mann-Whitney U test.

b NP, Normal pregnant mice; SA, PBS-treated abortion-prone mice; n.d., not done.
Mann-Whitney evaluated by the nonparametric Kruskall-Wallis test followed by the and are included in the statistical analysis. Statistical significances were corrected for their non-Gaussian distribution. Outliers and are represented by asterisks TUNEL ginal and not statistically significant augmentation in the number of DNA fragmentation by TUNEL staining in placental cells revealed a mar-

In this study, we first show that the application of Co-PP signif-

icantly up-regulates HO-1 and marginally up-regulates HO-2 at the fetal-maternal interface. Most interestingly, this treatment di-
mirrored abortion rates. Increased levels of HO-1 following a sin-
gle Co-PP treatment were also effective in inhibiting early inflam-
matory events following allograft transplantation and prevented therefore chronic graft rejection (16). In our study, animals receiv-
ing a single dose of Zn-PP, which is known to inhibit not only HO-1 but also HO-2 (41), displayed only a slight down-regulation in the HO-2 protein and mRNA levels, maybe because the dele-
terious effect in tissues is being rapidly counteracted by compens-
atory mechanisms as already reported when using another inhib-
itor of HO-1 activity, tin-protoporphyrin (24, 25). The single Zn-PP injection was, however, effective in vivo, because it boosted abortion, confirming for the first time that the lack/down-regula-
tion of HOs during implantation is not being compatible with suc-

cessful pregnancy. All together, our in vivo data support the initial hypothesis that HO expression at the fetal-maternal interface is clue in protecting the fetus from abortion.

Having confirmed the in vivo effects of HOs up-regulation, we analyzed the mechanisms involved in HO-mediated fetal protec-
tion. The popular Th1:Th2 paradigm proposes that the up-regula-
tion of proinflammatory cytokines, mainly TNF-\(\alpha\), IFN-\(\gamma\), and IL-6, would lead to fetal rejection (10, 21, 26 –29). High levels of Th2 (IL-4 and IL-10) or Th3 cytokines (TGF-\(\beta\), on the contrary, would be associated with a successful pregnancy (30, 31, 39 –41). However, the validity of this paradigm was recently questioned (42–44). Increased Th1 levels associated with low HOs levels could be observed in rejected allografts (17). Accordingly, we re-
ported that in abortion mice, the HO down-regulation was associ-
ated with increased Th1:Th2 ratio (10), leading us to speculate a possible connection between these two pathways during preg-
nancy. In this study, we analyzed the Th1 and Th2 cytokine pro-
duction by isolated decidual immune cells from all animals on day 14 of pregnancy. Contrary to our expectations, Co-PP treatment did not shift the cytokine profile to a Th2 one. Very surprisingly, Zn-PP treatment, which boosted abortion, significantly down-regu-
ulated the Th1:Th2 ratio, a situation supposed to be pregnancy-
protective. Because the animals were sacrificed 10 days after treat-
ment, we first hypothesized that these data might not reflect the in vivo situation, probably because of the late time point, in which the immune system might already have counteracted the negative ef-
fects of the Zn-PP application by augmenting the production of Th2 cytokines. For testing this, we repeated the experiments but sacrificing the mice on day 8 of pregnancy, i.e., 4 days after treat-
ment and immediately before the abortion onset. The same cyto-
kine pattern could be confirmed, which refutes the original hy-
thesis: a down-regulation of the Th1:Th2 ratio in spleen and decidua was observed in the Zn-PP-treated group compared with the abortion-prone control group. Furthermore, the levels of TNF-\(\alpha\) after Zn-PP application were down-regulated 4 days after treatment. Because we found the same results on Th1:Th2 cytokine production/expression on day 14 or 8 of pregnancy, meaning 10 or 4 days after treatment, we suppose that the immune system is acting very quickly in response to the injury provoked by Zn-PP treatment. We wondered whether other molecules, i.e., the Th3 cytokine TGF-\(\beta\), might play a role in the HO-1-mediated protec-
tion. TGF-\(\beta\) was detected in murine decidua and described to have immunosuppressive properties, thus contributing to the normality of allogeneic pregnancy (39 –41). Furthermore, TGF-\(\beta\) 2 and 3 are absolutely necessary for embryonic survival (45). We measured TGF-\(\beta\) 1 mRNA levels at the fetal-maternal interface and found significantly elevated levels of it on the day 14 of pregnancy (10 days after Zn-PP injection). Four days after treatment, no changes in TGF-\(\beta\) 1 mRNA levels could be observed compared with the PBS-treated abortion-prone controls. These results suggest that TGF-\(\beta\) 1 is not playing a role in the HO-1-mediated protection and is being up-regulated after Zn-PP treatment just as part of the counteracting mechanisms following tissue injury. All together,
our results suggest that Co-PP treatment is being pregnancy-beneficial despite Th1 cytokine augmentation, whereas Zn-PP treatment is detrimental for pregnancy outcome, although it shifts the cytokine profile to a Th2 one. The therapeutic effects of Co-PP are not related to the changes on the Th1/Th2/Th3 system, thus other pathways might be activated and will be discussed below.

A regulatory interaction between the HO system and the NOS system has been already suggested (46). The expression of iNOS and eNOS was described in placental cells (47–49), and both seem to play a role in the peri-implantation development of the mouse (48). We previously observed a down-regulation in iNOS and eNOS protein in animals undergoing abortion, which also showed low HO-1 and HO-2 levels (10). We therefore hypothesized that the protective effect of HOs in pregnancy might be mediated by the production of large quantities of NO (49). We show in this study that treatment with Co-PP and Zn-PP has no direct influence on the production of iNOS or eNOS at the fetal-maternal interface, because we only found an augmentation in iNOS and eNOS expression in giant cells and decidual cells by IHC, but we were not able to confirm this by WB or at mRNA levels by real-time RT-PCR.

The data obtained in this study suggest that the NOS system is not being influenced by HO-1 up- or down-regulation at the fetal-maternal interface.

HO-1 is also believed to have antiapoptotic and tissue-protective properties. Induction of HO-1 expression by heme protects endothelial cells from TNF-α-mediated apoptosis (12, 14). The mechanisms by which HO-1 protects the cells from undergoing apoptosis are not clear yet. Brouard et al. (15, 50) showed that CO, one metabolite of HO activity, might protect from apoptosis through the activation of NF-κB. In the present study, we observed that the induction of HO-1 by Co-PP only slightly diminished the caspase-3 activity, which is an accurate marker of apoptosis, contradicting previous results in a different system (51). Furthermore, the slightly diminished number of apoptotic cells after Co-PP therapy suggests that HO-1 might protect the fetal tissue from apoptosis/necrosis. For confirming this, we analyzed the mRNA levels of antiapoptotic/cytoprotective molecules. In this context, we found out that Bag-1, a molecule able to suppress apoptosis and protect tissues from injury (52, 53), is up-regulated after Co-PP treatment. These data strongly suggest that Bag-1 overexpression following HO-1 up-regulation is directly implied in the avoidance of abortion. Bag-1 was already described to be up-regulated after HO-1 overexpression by gene therapy in tolerant grafts (32) as well as at the fetal-maternal interface (M. L. Zenclussen, unpublished observations). Since the first demonstration of Bag-1 as an antiapoptotic molecule (54), its overexpression has been shown to inhibit apoptosis induced by a wide range of inducers in various cell types (reviewed in Ref. 52). Bag-1 is supposed to suppress apoptosis upstream of caspase activation by interacting with Bcl-2 family members (55). Townsend et al. (57) have shown that Bag-1 has a novel cytoprotective function, i.e., protecting cardiac myocytes from apoptosis induced by simulated ischemia/reperfusion, mediated via association with HSC70/HSP70. Which mechanisms are involved in the cytoprotective role of Bag-1 in our system is unknown.

It seemed also interesting for us to analyze whether the protective effects of HO-1 on pregnancy outcome could be explained by the activation of novel pathways, i.e., the up-regulation of the enzyme IDO or the activation of pregnancy-induced Treg cells. It had been suggested that IDO might be indispensable for pregnancy outcome (58). However, novel experiments reveal that mating combinations involving H2-matched or -mismatched IDO-deficient parents presented completely normal pregnancies (59). These results suggest that the importance of the IDO system in pregnancy...
outcome has been overestimated. In our study, the levels of IDO were very low at the fetal-maternal interface and comparable among all groups, discarding an effect of HO-1 on this system (data not shown). Furthermore, we recently demonstrated that Tregs are essential for pregnancy outcome (34). Choi et al. (33) proposed that Foxp3 can induce HO-1 expression, suggesting a connection between both pathways. However, no reports exist on the effects of HO-1 up-regulation on Treg function or number. In this study, we analyzed the number of CD4+CD25+ cells in decidual tissue after Co-PP or Zn-PP treatment and found no differences as compared with the PBS-treated abortion-prone group (data not shown), which is maybe due to the fact that CD25 is also marking activated cells and might not be an accurate marker for Tregs at sites of activation (34). We therefore measured the mRNA levels of neuropilin-1, a novel marker for Treg activity (35). Neuropilin-1 levels were diminished in abortion-prone mice compared with normal pregnant mice, suggesting diminished Treg levels in accordance with our previous observations (34). Interestingly, neuropilin-1 mRNA levels were significantly augmented after Co-PP treatment on day 14 of pregnancy. This suggests an augmentation in the Tregs number or activity at the fetal-maternal interface following HO-1 augmentation. Although Bruder et al. (35) present very convincing data that neuropilin-1 expression correlates with foxp3 expression and with suppressor functions, further reports from other groups are necessary to confirm that we are in the presence of a solid and specific marker for Tregs. If confirmed, our data strongly suggest an activation of the Treg pathway after HO-1 up-regulation.

Summarizing, our data indicate that the overexpression of HO-1 after Co-PP treatment prevents abortion, whereas the lack or down-regulation of HO-1 and HO-2 by Zn-PP application boosted abortion in mice. Accordingly to our data, the positive effect of HO-1 overexpression is not mediated by a shift into a protective cytokine profile or by interaction of HO-1 with NO-associated pathways. Our data rather suggest that HO-1 up-regulation stimulates the expression of Bag-1 at the fetal-maternal interface, which acts as a cytoprotective molecule, avoiding tissue injury.

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Disclosures

The authors have no financial conflict of interest.

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