Article

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PILEGGI, A, *et al*.

Abstract

Transplantation of islets of Langerhans represents a viable therapeutic approach for the treatment of type 1 diabetes. Unfortunately, transplanted islets are susceptible to allogeneic recognition and rejection, recurrence of autoimmunity, and destruction by local inflammation at the site of implantation. The last of these phenomena might not only result in functional impairment and death of islet cells but could also contribute to amplifying the subsequent specific immune response. Induction of islet cell protection against inflammation could therefore be postulated to be a powerful means to improve overall graft fate. Heme oxygenase-1 (HO-1) has been described as an inducible protein capable of cytoprotection via radical scavenging and apoptosis prevention. The purpose of the present study was to analyze whether HO-1 upregulation in a beta-cell line and in freshly isolated murine islets could result in protection from apoptosis and improve in vivo functional performance. HO-1 upregulation was induced reproducibly with protoporphyrins and was correlated with protection from apoptosis induced in vitro with proinflammatory [...]

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Heme Oxygenase-1 Induction in Islet Cells Results in Protection From Apoptosis and Improved In Vivo Function After Transplantation

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Transplantation of islets of Langerhans represents a viable therapeutic approach for the treatment of type 1 diabetes. Unfortunately, transplanted islets are susceptible to allogeneic recognition and rejection, recurrence of autoimmunity, and destruction by local inflammation at the site of implantation. The last of these phenomena might not only result in functional impairment and death of islet cells but could also contribute to amplifying the subsequent specific immune response. Induction of islet cell protection against inflammation could therefore be postulated to be a powerful means to improve overall graft fate. Heme oxygenase-1 (HO-1) has been described as an inducible protein capable of cytoprotection via radical scavenging and apoptosis prevention. The purpose of the present study was to analyze whether HO-1 upregulation in a β-cell line and in freshly isolated murine islets could result in protection from apoptosis and improve in vivo functional performance. HO-1 upregulation was induced reproducibly with protoporphyrins and was correlated with protection from apoptosis induced in vitro with proinflammatory cytokines or Fas engagement. Furthermore, in vivo HO-1 upregulation resulted in improved islet function in a model of marginal mass islet transplantation in rodents. Strategies aimed at inducing HO-1 upregulation might result in improved success in islet transplantation. *Diabetes* 50:1983–1991, 2001

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CHX, cyclohexamide; CO, carbon monoxide; CoPP, cobaltic protoporphyrin IX chloride; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FePP, ferriprotoporphyrin IX chloride; HBSS, Hanks' balanced salt solution; HO-1, heme oxygenase-1; IEN, islet equivalent number; IFN-γ, interferon-γ; IL, interleukin; MT, median time; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; RT, reverse transcription; STZ, streptozotocin; TNF-α, tumor necrosis factor-α; WB, Western blot.

Type 1 diabetes results from the autoimmune destruction of pancreatic β-cells (1). Conventional therapy, based on the administration of exogenous insulin, often is characterized by the occurrence of systemic complications, including angiopathy and neuropathy. Improvement in metabolic control, through intensive insulin therapy, results in decreased incidence and progression of complications, but it is often associated with the occurrence of severe hypoglycemic episodes (2). Conversely, transplantation of islets of Langerhans, when successful, is characterized by excellent metabolic control in the absence of hypoglycemia (3).

Transplanted islets are susceptible not only to rejection and recurrence of autoimmunity but also to damage mediated by nonspecific inflammatory events that occur in the microenvironment early after transplantation. Early inflammation at the site of implantation leads to generation and release of biological mediators, such as cytokines and oxygen radicals that can damage islet cells, inducing either functional impairment or death (4–8). Islet β-cells are in fact exquisitely sensitive to the noxious effects of selected proinflammatory mediators and oxidative stress (9–11). Inflammation can not only functionally impair or reduce the mass of implanted islets but can also result in the amplification of the subsequent immune response in a transplantation setting. In the transplanted islets, the induction of cytoprotective genes that are capable of scavenging oxygen radicals and preventing the toxic effects of proinflammatory cytokines might therefore promote early function (12–16).

Heme oxygenase-1 (HO-1) has been identified as a ubiquitous stress protein induced in many cell types by various stimulants, such as hemolysis, inflammatory cytokines, oxidative stress, heat shock, heavy metals, and endotoxin (17–22). HO-1 is the rate-limiting enzyme in the heme degradative pathway that catalyzes the oxidation of heme into biliverdin, carbon monoxide (CO), and free iron (23). Biliverdin is readily converted by biliverdin reductase into the bile pigment bilirubin, a powerful antioxidant (24), and free iron stimulates the production of ferritin. Induction of HO-1 results in protection from cytokine-induced apoptosis and oxidative stress in selected in vitro and in vivo models (25–28). The current working hypothesis on the mechanisms of action of HO-1 suggests that, in addition to

the antioxidant properties of bilirubin, production of CO might prevent apoptosis via induction of Mn-superoxide dismutase (29). Furthermore, the cytoprotective properties of HO-1 have been linked to the extrusion and sequestration (by ferritin) of cellular free iron (28,30), which is toxic because it contributes to the formation of free radicals, with consequent damage to DNA, proteins, and lipids (31). HO-1 induction in islets of Langerhans can be obtained in vitro by means of selected stimuli (32,33) and might protect from interleukin-1β (IL-1β)–mediated noxious effects (34–36).

The aim of the present study was to assess whether induction of HO-1 expression in islets of Langerhans and in the murine insulinoma βTC3 cell line could be achieved reproducibly, confer protection against pro-apoptotic stimuli, and improve graft function in vivo. The data that we present in this article indeed support the hypothesis that induction of HO-1 upregulation in β-cells and islets confers protection from noxious stimuli, resulting in reduced susceptibility to apoptosis and improved in vivo functional performance. Overexpression of cytoprotective molecules such as HO-1 might prove useful to modify positively the overall outcome of islet transplantation.

RESEARCH DESIGN AND METHODS

Animals. Male C57BL/6 mice were purchased from Hilltop Lab Animals (Scottdale, PA), kept in the University of Miami Virus Antibodies Free animal facility, and used in compliance with National Institutes of Health and Institutional Animal Care and Use Committee guidelines. Islet donors were killed at 12 weeks of age. Recipients (7–8 weeks old) were rendered diabetic by intravenous administration of 200 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO) freshly dissolved in citrate buffer, and they were transplanted 7 days after STZ administration, only after two consecutive nonfasting blood glucose readings of >250 mg/dl were obtained from whole blood.

In vitro culture of the βTC-3 cell line. The murine insulinoma βTC-3 cell line (βTC3; DSMZ, Braunschweig, Germany) was cultured at 37°C, 5% CO_2, in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, supplemented with 15% horse serum (Gibco-BRL) and 2.5% fetal calf serum (FCS; HyClone, Logan, UT). Aliquots of 2.5×10^5 cells were harvested after 24 h of culture under different experimental conditions, snap-frozen, and stored at −80°C until assayed for RNA and protein expression. Additional samples were harvested for fluorescence-activated cell sorter (FACS) analysis of DNA content (see below).

Isolation of islets of Langerhans. Murine islets were isolated as described previously (37). Briefly, animals were killed under general anesthesia, and the pancreas was exposed and injected with Hanks' balanced salt solution (HBSS; Mediatech, Herndon, VA) containing 0.8 mg/ml collagenase type V (Sigma) via the main bile duct until distension was achieved. Digestion was performed at 37°C for 17 min with gentle shaking and terminated by the addition of cold RPMI–10% FCS and 2 mmol/l L-glutamine (Mediatech). Mechanical disruption of the pancreas was achieved by passages through needles of decreasing gauge until release of islets was observed under a microscope; the tissue was filtered through a 450-μm mesh, and islets were purified on Euro-Ficoll (Mediatech) gradients by centrifugation at 900*g* for 11 min, routinely yielding preparations of >90% purity. Islet purity was assessed by dithizone (Sigma) staining, and the islets were counted and scored for size. An algorithm was used for the calculation of a 150-μm-diameter islet equivalent number (IEN) (38). Islets were cultured overnight at 37°C in CMRL medium-10% FCS, 2 mmol/l L-glutamine and 25 mmol/ml HEPES (Mediatech).

In vitro induction of HO-1. Ferriprotoporphyrin IX chloride (FePP hemin, Porphyrin Products, Logan, UT) and cobaltic protoporphyrin IX chloride (CoPP; Sigma) were dissolved in NaOH 0.1 N and diluted 1:1 in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}; pH was adjusted to 7.4, and the solution was sterilized by filtration. Islets or βTC3 cells were incubated at 37°C for 24 h with selected concentrations of FePP or CoPP. Control cells were cultured with the same volume of vehicle. After a 24-h incubation with or without protoporphyrins, islets were either transplanted into diabetic recipients or collected in PBS, centrifuged at 800*g* for 5 min, dried, snap-frozen, and stored at −80°C until assayed for RNA and protein expression.

For analyzing the kinetics of HO-1 upregulation, islets were cultured for 24 h with or without 100 μmol/l FePP and subsequently incubated in the absence of the protoporphyrin for up to 6 days. HO-1 expression was evaluated by Western blot (WB) analysis on samples collected 1, 3, and 6 days after induction.

Induction of apoptosis in βTC3 cells. After a 24-h incubation in the presence or absence of increasing concentrations of FePP (10, 40, 70, and 100 μmol/l), βTC3 cells were cultured with 10 units/ml of murine recombinant IL-1α (Sigma) and 100 units/ml of murine recombinant interferon-γ (IFN-γ; Gibco-BRL) to induce the expression of Fas on the cell surface (39). A pro-apoptotic stimulus at that point was delivered via engagement of Fas by the hamster anti-mouse agonistic anti-Fas antibody JO2 (PharMingen) at 10 μg/ml, followed by 10 μg/ml protein G (Sigma) to induce cross-linking (40). FePP was maintained in the culture medium for the entire protection/induction protocol. Cells cultured in the absence of apoptosis-inducing stimuli (induced by FePP or not) were used as controls.

Induction of apoptosis in islets of Langerhans. Islets were cultured overnight after isolation. They were then incubated for 24 h in the presence or absence of 100 μmol/l FePP. A pro-apoptotic signal then was delivered by addition of 5,000 units/ml murine recombinant tumor necrosis factor-α (TNF-α; R&D) and 50 μg/ml cyclohexamide (CHX; Sigma) for an additional 48 h. Control islets were treated similarly in the presence or absence of FePP, without TNF-α and CHX. Cells were harvested at the end of the culture and assayed for DNA content via FACS analysis (see below).

Analysis of HO-1 expression by WB. Frozen pellets of βTC3 cells or islets were resuspended in 150 μl of lysis buffer (330 mmol/l NaCl, 50 mmol/l Tris-HCl [pH 7.6], 0.5% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mmol/l phenylmethylsulfonyl fluoride, and 1.8 mg/ml iodoacetamide, all from Sigma) with intermittent vortexing. Samples were centrifuged at 15,700*g* for 15 min at 4°C, and the pellets were discarded. Protein concentration of the supernatants was assessed with micro BCA kit (Pierce, Rockford, IL). Twenty micrograms of proteins were resuspended in loading buffer, boiled for 3 min, and separated on 12% polyacrylamide gel. Recombinant rat HO-1 protein (StressGen Biotechnologies, Victoria, Canada) and bovine muscle actin (Sigma) were loaded as controls. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA) overnight at 4°C.

Immunoprobing was achieved in blocking buffer with a rabbit polyclonal anti–HO-1 antibody (1:2,000; StressGen) and a rabbit anti-actin antibody (1:3,000; Sigma) for 1 h. A horseradish peroxidase–conjugated goat anti-rabbit IgG (1:3,000; Biorad) was used as secondary antibody in a 1-h incubation. Positive signals were revealed with the addition of a chemiluminescent substrate (Super Signal West Pico; Pierce) and exposure on X-ray film (Pierce). Relative quantities of HO-1 protein were determined using a densitometer (FluorChem digital imaging system, Alphaeasy FC 32-bit 1-D analysis software, Alpha Innotech, San Leandro, CA) and normalized according to the actin content of individual samples.

Detection of apoptosis by cell-cycle analysis. βTC3 cells were collected by trypsinization (0.05% Trypsin, 0.53 mmol/l EDTA; Gibco-BRL) of cultured aliquots at the indicated time points after apoptosis induction. Islets were dispersed in single-cell suspensions by a brief incubation in trypsin/EDTA. Islet cells and βTC3 cells were resuspended in HBSS–50% fetal bovine serum, fixed in ice-cold 70% ethanol, and stored at 4°C. Cell suspensions were incubated for 30 min at 4°C in PBS containing 0.1% Triton X-100, 1 mg/ml glucose (Sigma), 100 μg/ml propidium iodide (PI; Sigma), and 100 units/ml RNase A (Sigma). Cells were analyzed by flow cytometry on a linear scale using a Becton-Dickinson FACS (San Diego, CA), considering as apoptotic the cells within the distinct sub-G_1 peak, in which DNA condensation and fragmentation result in decreased PI staining (16,41).

RT-PCR analysis. HO-1 mRNA steady-state levels were assessed by quantitative reverse transcription (RT)–polymerase chain reaction (PCR) using the LightCycler instrument (Roche-Boehringer Mannheim). RNA extraction was performed using the RNA NOW-LM kit (Biogentex, Seabrook, TX). Synthesis of first-strand cDNA was performed from DNase-treated RNA (DNase I; Gibco-BRL), using the SuperScript II RT kit (Gibco-BRL), with 25 ng/μl oligo$(dT)_{12–18}$ as primer. The RT product was amplified by PCR in the LightCycler, which allows real-time quantification of the PCR product, based on the incorporation of a fluorescent dye in the neosynthesized DNA and its measurement at the end of each PCR cycle. The relative level of the initial transcript copy number in each cDNA sample was calculated as described previously (42). PCR conditions were 1 min at 94°C, 35 cycles of 1 s at 94°C, 9 s at 68°C, and 20 s at 70°C.

Initial starting concentrations of cDNA were determined by arbitrary units, and the ratio of HO-1/β-actin levels was used to normalize the different samples. Oligonucleotide primers for β-actin (forward, 5′-GAT GAC CCA GAT CAT GTT TG-3′; reverse, 5′AGG CTG GAA GAG TGC CTC A-3′) and murine HO-1 (forward, 5′-TCC CAG ACA CCG CTC CTC CAG-3′; reverse, 5′-GGA TTT GGG GCT GCT GGT TTC-3′) were used. Negative RT samples were always run in parallel for each sample to exclude genomic DNA contamination.

Marginal islet mass transplantation. Isolated islets were handpicked in aliquots of ~100–125 each under a dissection microscope and assessed for islet diameter to calculate the IEN. The number was adjusted in each aliquot according to calculated IEN and weight of the recipient to obtain grafts of 5,000 IEN/kg body wt. Islets then were transplanted under the kidney capsule as described elsewhere (37). Transplantation of a marginal mass of syngeneic islets leads to reversal of diabetes, with a measurable delay from the time of implantation. This model allows the analysis of the beneficial effects of islets and/or transplant microenvironment manipulations that result in improvement of graft function and shorter time to diabetes reversal.

Graft function analysis. Blood glucose levels were measured daily after transplantation on whole-blood samples. Graft function was defined as occurring on the first of 5 consecutive days of nonfasting blood glucose of <200 mg/dl. Mice that remained hyperglycemic for >100 days were considered to have failed and were killed. To verify that the reversal of diabetes was not due to residual function of the native pancreas, we performed survival nephrectomy after return to normoglycemia. Time to normoglycemia was recorded as the primary end point of the experiment.

HO-1 induction in vivo. CoPP was dissolved in NaOH 0.1 N and diluted 1:1 in 0.9% NaCl; the pH was adjusted to 7.4, and the solution was sterilized via filtration. Recipients were given vehicle alone or 20 mg/kg CoPP i.p. on days –1, 1, 3, 5, and 7 after marginal islet mass transplantation. For assessing whether HO-1 upregulation was achieved by administration of CoPP, two groups of C57BL/6 mice received a syngeneic islet transplant of ~600 IEN under the kidney capsule and were treated on day –1 and day 1 with vehicle alone or CoPP. Animals were killed on day 2 postoperation, and the grafts were dissected free from the kidney and snap-frozen for RT-PCR analysis of HO-1 expression.

Statistical analysis. The Statistica software package (Statsoft, Tulsa, OK) was used for statistical analysis. Data are expressed as median and range or mean ± SD or SE whenever appropriate. Variables were compared using the Student's t test. Kaplan-Meier analysis was performed for diabetes reversal determination, and differences were assessed with Mantel-Cox log rank test on the animals that became normoglycemic. Time to normoglycemia is expressed as median (interquartile range). $P < 0.05$ was considered significant.

RESULTS

HO-1 upregulation in βTC3 cells by protoporphyrins. The first experimental question that we wanted to address was whether HO-1 induction could be obtained reproducibly in the β-cell line βTC3 by incubation with iron protoporphyrin, a well-described HO-1–inducing agent in other cells (17). WB analysis showed that treatment of βTC3 cells with FePP for 24 h induced substantial upregulation of HO-1 protein expression in a dose-dependent manner (Fig. 1*A*). HO-1 upregulation was quantified by densitometry-based analysis of protein expression levels after normalization with β-actin content of each sample. HO-1 upregulation was 1.6-, 2.3-, 2.5-, and 2.4-fold over baseline at 10, 40, 70, and 100 μmol/l, respectively. The highest protein expression was obtained when FePP was used at concentrations between 70 and 100 μmol/l (Fig. 1*B*).

Analysis of the steady-state levels of mRNA expression assessed via quantitative RT-PCR showed, in accordance with the WB data, that HO-1 mRNA was increased between 3.45- and 3.75-fold over baseline in cells that were treated with FePP at 70 and 100 μmol/l and between 2.14- and 2.31-fold in cells that were treated with the lower concentrations of FePP (Fig. 1*B*). Similar results were obtained when CoPP was used to induce HO-1 upregulation, albeit with lower efficiency of HO-1 induction (not shown).

HO-1 upregulation in islets of Langerhans by iron protoporphyrin. The second set of experiments was aimed at the analysis of HO-1 upregulation in isolated murine islets. Islets were cultured in the presence or absence of 100 μmol/l FePP. This concentration was chosen on the basis of the results obtained in βTC3 cells. After 24 h of induction, WB analysis demonstrated overexpression of HO-1 (Fig. 2*A*). WB densitometry allowed quantification of HO-1 overexpression in the treated samples that showed a 6.3-fold increase over untreated samples in protein level (Fig. 2*B*). Quantitative analysis of HO-1 mRNA steady state levels performed via RT-PCR showed a 5.8-fold increase in treated islets when compared with control islets (Fig. 2*B*).

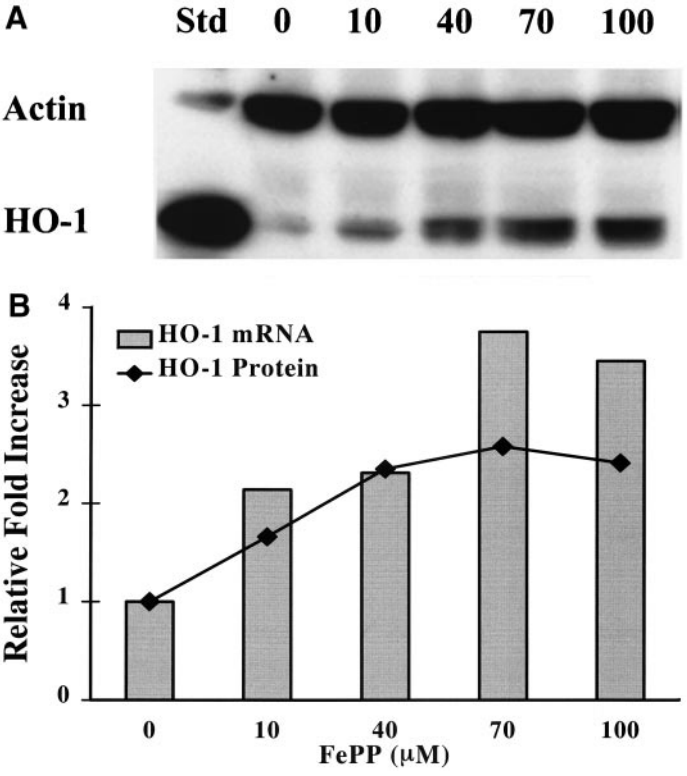

FIG. 1. ***A*: WB analysis of HO-1 protein expression in βTC3 cells. βTC3 cells were cultured in the presence of the indicated concentrations of FePP (from 0 [control] to 100 μmol/l) for 24 h. At the end of the culture period, proteins were extracted and immunoblotted with antibodies specific for HO-1 and β-actin. Standards (Std) were loaded in lane 1 as positive controls (see RESEARCH DESIGN AND METHODS for details). Data are representative of three individual experiments. *B*: Quantitative analysis of HO-1 protein and mRNA expression in βTC3 cells. βTC3 cells were cultured in the presence of the indicated concentrations of FePP (*x* axis, from 0 [control] to 100 μmol/l) for 24 h. At the end of the culture period, separate aliquots were used for quantitative RT-PCR and for densitometry-based quantitative analysis of HO-1 protein expression after immunoblotting. Data are expressed as relative fold increase over control (untreated) cells.**

Analysis of the kinetics of HO-1 expression after 24 h of stimulation was performed by WB analysis at 24, 72, and 120 h after removal of the stimulus. Maximal expression of HO-1 was observed immediately after the 24-h stimulation period, followed by a rapid decrease of expression at the subsequent time points (not shown). At 120 h after FePP removal, HO-1 expression had returned to prestimulation baseline levels. Consistent results were obtained in islets isolated from numerous species, including rat, neonatal pig, dog, nonhuman primates, and humans (data not shown).

FePP-induced HO-1 upregulation protects the βTC3 cells from Fas-mediated apoptosis. We next investigated whether FePP-induced HO-1 upregulation in βTC3 could confer protection from Fas-mediated apoptosis.

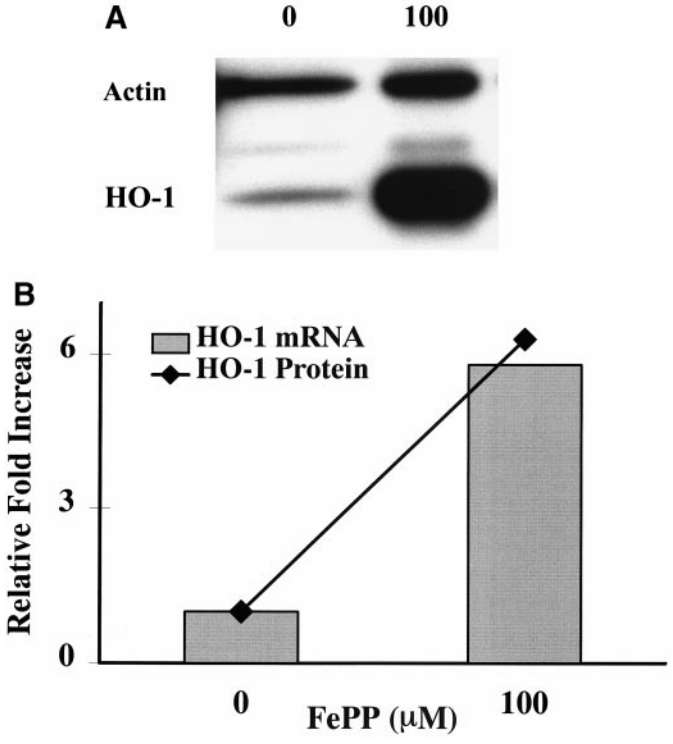

FIG. 2. *A*: **WB analysis of HO-1 protein expression in freshly isolated murine islets. Islets were cultured in standard conditions for 24 h in the presence of the indicated concentrations of FePP (0 [control] and 100 μmol/l). At the end of culture, proteins were extracted and immunoblotted with an anti–HO-1 and an anti–β-actin antibody. Data are representative of three separate experiments.** *B*: **Quantitative analysis of HO-1 protein and mRNA expression in freshly isolated murine islets. Islets were cultured for 24 h in the presence of the indicated concentrations of FePP (0 [control] and 100 μmol/l). At the end of the culture, separate aliquots of islets were harvested for densitometry-based quantification of HO-1 protein expression on immunoblots (line) and for quantitative RT-PCR of HO-1 mRNA steady-state levels (□). Data are expressed as protein and mRNA fold increase over control. Data are representative of at least three individual experiments.**

βTC3 is an insulinoma cell line that, similarly to islet cells, undergoes Fas-mediated apoptosis upon induction of Fas with cytokines and treatment with an agonistic antibody (11,40).

βTC3 cells were incubated for 24 h in the presence or absence of increasing concentrations of FePP to induce HO-1 upregulation. Subsequently, they were incubated in the presence of IL-1α (10 units/ml) and IFN-γ (100 units/ml) to induce surface expression of Fas. FePP was not removed from the culture. βTC3 cells do not express sufficient surface Fas receptors to be susceptible to Fas-mediated apoptosis unless stimulated in the presence of low cytokine concentrations. After 24 h of incubation in the presence of the indicated cytokines, βTC3 cells were treated with an agonistic anti-Fas antibody. At 48 h after delivery of the pro-apoptotic stimulus, cells were assayed for apoptosis by analysis of DNA content, which showed that the percentage of apoptotic cells (within the sub-G_1 peak) inversely correlated with the concentration of FePP in the media, suggesting a dose-dependent protective effect of FePP-induced HO-1 upregulation (Fig. 3). Apoptosis induction averaged 12.5 ± 2.0% in the unprotected (control) cells. Induction of HO-1 upregulation by FePP treatment afforded βTC3 cells dose-dependent protection that reached statistical significance when FePP was given at 70 and 100 μmol/l (% protection = 29 ± 7.5 at 70 μmol/l, $P < 0.01$, and 42 ± 5 at 100 μmol/l, $P < 0.001$ vs. control, respectively).

FePP-induced HO-1 upregulation protects islets from TNF-α–mediated apoptosis. Isolated murine islets were cultured for 24 h in the presence or absence of FePP (100 μmol/l), followed by delivery of an apoptosis-inducing stimulus. Apoptosis was induced by incubation of the islet preparations in the presence of TNF-α (5,000 units/ml) and CHX (50 μg/ml). This treatment consistently leads to occurrence of measurable apoptosis within 48 h from delivery of the stimulus; analysis of apoptosis was therefore performed 48 h after incubation with TNF-α and CHX. At this time point, single-cell suspensions were obtained by trypsin incubation of islets. FACS analysis of islet cells was performed on samples stained with PI to identify cells in the sub-G_1 peak. Figure 4 shows the results of a representative experiment. Incubation of islets with FePP before delivery of the pro-apoptotic stimulus resulted in a decreased percentage of apoptotic cells (Fig. 4*D*), as compared with the control sample (not pretreated with FePP) (Fig. 4*B*). Average percentage of protection in three separate experiments was 40.25 ± 10.48 ($P < 0.05$, paired t test). Controls (islets treated with or without FePP but not treated with pro-apoptotic stimuli) are included for comparison and shown in Fig. 4*A* and *C*.

HO-1 induction in islet grafts results in shorter time to normoglycemia in a syngeneic marginal islet mass model. The next question that we wanted to address was whether HO-1 upregulation resulted in a cytoprotective effect on islets transplanted in vivo. We selected a model of syngeneic transplantation of a marginal islet mass. In this setting, a suboptimal mass of islets is transplanted into syngeneic recipients rendered diabetic by STZ administration. Implantation of a marginal mass of islets results in delayed reversal of hyperglycemia. Manipulations of the islet preparation or the implant microenvironment that either make islets less susceptible to inflammatory

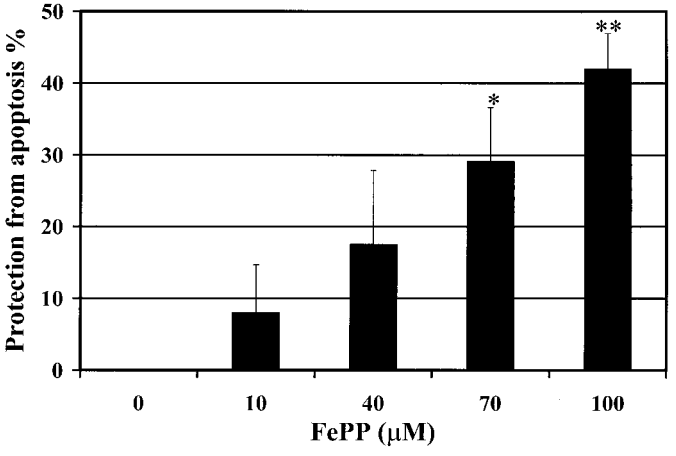

FIG. 3. FePP-induced HO-1 upregulation results in partial protection from cytokine and Fas-mediated apoptosis induction in βTC3 cells. βTC3 cells were cultured in the presence of the indicated concentrations of FePP (from 0 [control] to 100 μmol/l) 24 h before addition of cytokines and anti-Fas antibody. They then were incubated sequentially with low doses of cytokines and with an anti-Fas antibody to induce apoptosis. FePP was not removed from the culture. Data are expressed as percentage protection, as determined by analysis of the sub-G_1 peak of DNA content. Bars represent mean ± SE of six separate experiments. Apoptosis induction averaged 12.5 ± 2.0% in the control (0 FePP) cells. *$P < 0.01$ and **$P < 0.001$ versus control cells.

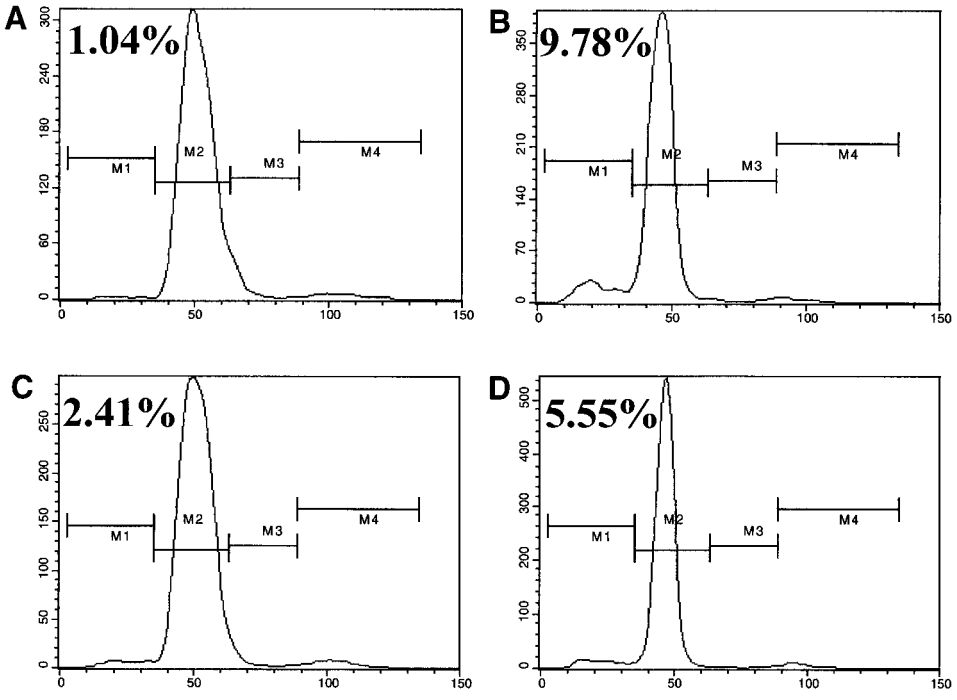

FIG. 4. FACS analysis of DNA content in isolated islet cells. Freshly isolated islets were cultured in the presence of 100 μmol/l of FePP (*C* and *D*) or with medium alone (*A* and *B*) for 24 h. They then were treated with TNF-α and cyclohexamide (*B* and *D*) or medium (*A* and *C*) for an additional 48 h. DNA content was assessed by FACS analysis of single-cell suspension obtained via trypsinization after permeabilization and staining with PI. Percentage values of cells comprised in the sub-G_1 peak (apoptotic cells) are reported in each panel (M1). Data are representative of three separate experiments (see RESEARCH DESIGN AND METHODS for further details).

mediators or make the microenvironment less prone to nonspecific activation result in reduced time to normoglycemia after transplantation (6,37). Moreover, the functional performance of the islets can be assessed in the absence of the confounding effects of rejection and/or recurrence of autoimmunity. In these experiments, 5,000 IEN/kg body wt were implanted in each recipient mouse. The number of implanted islets was selected on the basis of extensive titration experiments performed previously in our laboratory (37). Implantation of this mass of insulin-producing tissue in the control group led to reversal of hyperglycemia in 79.3% (23 of 29) of the recipients, with a median time (MT) to normoglycemia of 48.0 ± 30.0 days (Fig. 5).

We first asked whether in vitro induction of HO-1 upregulation in isolated islets could improve their in vivo function. The first experimental group of animals (n = 8) therefore received islets that were cultured in the presence of 100 μmol/l FePP for 24 h before implantation. Animals in this group returned to normoglycemia, with an MT of 36.0 ± 28.9 days. This suggests that FePP-induced HO-1 upregulation before transplantation does not impair islet function and possibly improves their performance, leading to reversal of diabetes in both a shorter time than that observed in the control group and in a higher percentage of the animals, although without statistical significance (P = NS, Mantel-Cox test) (Fig. 5*A*).

In the second experimental group of animals, HO-1 upregulation was induced in vivo via administration of CoPP (20 mg/kg body wt i.p.) in the peritransplantation period. Administration of CoPP has been described to allow sustained HO-1 expression in selected tissues in vivo (43,44), and our preliminary experiments showed that acute CoPP treatment led to a significant increase in HO-1 mRNA in islets grafted under the kidney capsule as early as 48 h after implantation (Fig. 5). HO-1 mRNA steady-state levels were 7.78-fold higher in the grafts obtained from CoPP-treated animals than in controls (4.93 ± 1.73 vs. 0.63 ± 0.32, respectively; $P < 0.001$) (Fig. 6).

The animals in this group received intraperitoneal injections of CoPP on days –1, 1, 3, 5, and 7 and were transplanted with unmanipulated islets. Of the recipients, 14 of 16 (87%) returned to normoglycemia, with an MT of 33.0 ± 30.4 days (P = NS vs. control, Mantel-Cox test) (Fig. 5*B*).

The last experimental group received in vivo CoPP treatment and in vitro FePP-treated islets. The schedule of in vivo CoPP treatment was identical to that of the previous group, and in vitro FePP treatment of isolated islets was identical to that described for the first experimental group. Of the recipient animals, 9 of 10 (90%) returned to normoglycemia, with an MT of 18.0 ± 28.3 days, showing a statistically highly significant improvement in the performance of the grafts (P = 0.006, Mantel-Cox test; Fig. 5*C*). The general health status of all recipients (including CoPP-treated) seemed to be good during the entire time frame of the experiment.

DISCUSSION

HO-1 and HO-2 are the rate-limiting enzymes in the catabolism of heme into bilirubin, free iron, and CO (17). HO-1 is inducible, whereas HO-2 is expressed constitutively. Analyses of HO-1–deficient mice suggest that this gene regulates iron homeostasis while acting as a cytoprotective gene with potent anti-inflammatory and anti-apoptotic properties (45,46). Similar findings were described recently in a case report of HO-1 deficiency in humans (47). The molecular mechanisms responsible for the cytoprotective effects of HO-1 remain largely unknown. The current view is that HO-1 has diverse effects that are associated with the different end products of heme catabolism by HO-1 (bilirubin, free iron, and CO). Bilirubin is a potent antioxidant (24) and, as with other antioxidants, may inhibit the generation of reactive oxygen species, a

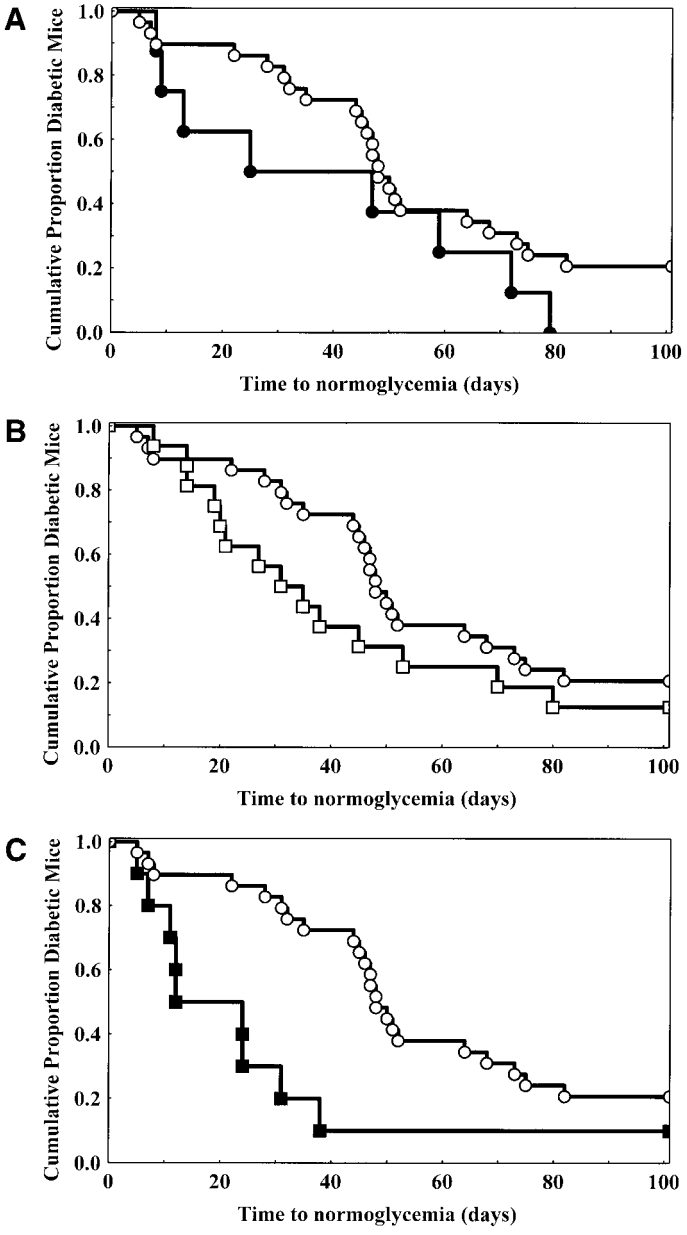

FIG. 5. Time course of return to normoglycemia in mice that received a syngeneic marginal mass islet graft. Chemically induced C57BL/6 mice received 5,000 syngeneic IEN/kg under the kidney capsule. Blood glucose levels were monitored to ascertain reversal of diabetes. Control animals (○; n = 29) received unmanipulated islets and vehicle. MT to return to normoglycemia was 48 ± 30 days. *A*: Time curve to normoglycemia of recipient animals that received FePP-treated islets (●; n = 8). MT to normoglycemia was 36 ± 28.9 days (P = NS vs. controls). *B*: Time to normoglycemia of mice that received untreated islets and were treated in vivo with CoPP (□; n = 16; see RESEARCH DESIGN AND METHODS). MT to normoglycemia in this group was 33 ± 30.4 days (P = NS vs. controls). *C*: Time to normoglycemia in mice that received FePP-treated islets and were treated in vivo with CoPP (■; n = 10). MT to normoglycemia was 18 ± 28.3 days (P = 0.006 vs. controls).

well-established component of the signaling pathways that lead to the upregulation of proinflammatory genes and to apoptosis (48). Heme catabolism by HO-1 also releases free iron, which has the potential to exacerbate the cytotoxic effects of reactive oxygen species (28,30,31). However, generation of intracellular free iron upregulates the expression of ferritin (28,30), which has a high capacity to store free iron (30). Ferritin has been shown to protect endothelial cells from activated neutrophils as well as H_2O_2-mediated cytotoxicity (30), suggesting that some of the effects of HO-1 may be mediated by ferritin (28,30).

CO may also have anti-inflammatory effects (49). As with nitric oxide, CO plays an important role in regulating vasomotor tone during acute inflammation, promoting vasorelaxation (50,51) and suppressing platelet aggregation (52). These actions may contribute to the cytoprotective effects of HO-1 in vivo because vasorelaxation allows maintenance of blood flow at sites of inflammation, and inhibition of platelet aggregation would prevent thrombosis that otherwise leads to anoxia and tissue necrosis.

Islet transplants are susceptible not only to the occurrence of rejection and recurrence of autoimmunity in type 1 diabetes patients, but also to nonspecific inflammatory events that take place at the site of implantation, which might contribute significantly to graft failure. Activation of the graft microenvironment and carryover of heterologous proteins and lipopolysaccharide have been postulated to represent major contributors to this inflammatory process (36,53,54). Proinflammatory cytokines and free oxygen radicals are released in situ and might exert deleterious effects on the implanted islets, which are exquisitely sensitive to inflammation (9–11). Two consequences might derive from this local perturbation. The first is functional impairment of the islets that lose glucose-stimulated insulin release. The second is the induction of islet cell apoptosis. Clearly, both of these phenomena can participate in (or contribute to) graft failure. In the present study, we collected data that suggest that both phenomena (functional impairment and apoptosis) can be prevented by HO-1 upregulation in islet cells. First, both a β-cell line and primary isolated islets show increased resistance to pro-apoptotic stimuli when HO-1 upregulation is induced. Second, analysis of restoration of normoglycemia in a marginal mass system suggests a beneficial effect of HO-1 upregulation obtained by protoporphyrin induction on islets and recipients. The latter observation is likely linked

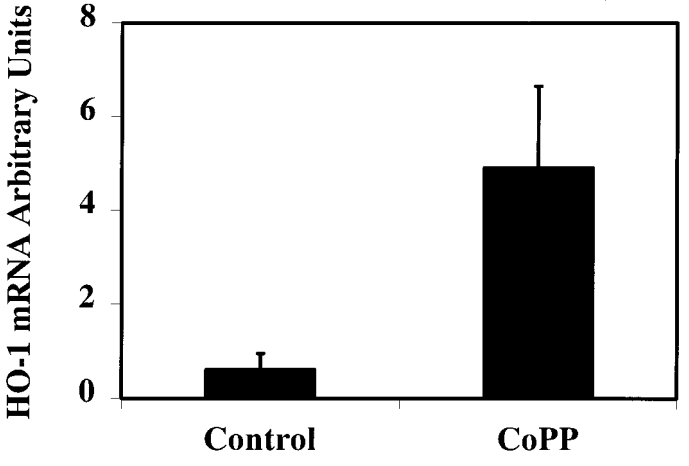

FIG. 6. Quantitative analysis of HO-1 mRNA expression in islet graft. Chemically induced diabetic C57BL/6 mice were treated with CoPP (20 mg/kg body wt) or saline on days –1 and 1 and received a syngeneic islet graft under the kidney capsule on day 0. The kidneys bearing the graft were obtained on day 2, and quantitative RT-PCR for HO-1 mRNA expression was performed on the grafts. Data show that in the treated group, CoPP treatment of the recipient leads to a 7.78- to 8-fold increase of HO-1 mRNA steady levels versus control (CoPP, n = 4; control, n = 3; P < 0.001). Data are expressed as arbitrary units of HO-1 mRNA normalized to β-actin.

to prevention (or reduction) of the described functional impairment observed at the site of implantation. Although prevention of apoptosis as the mechanism that mediates this phenomenon cannot be ruled out, a more likely explanation relies on reduction of the functional impairment, because eventually most grafts mediate return to normoglycemia in both control and treated animals. Upregulation of cytoprotective genes, HO-1 in particular, might therefore be a very advantageous strategy to promote immediate function and survival of a larger number of implanted islets. This ultimately might lead to the need for a substantially lower islet mass to revert diabetes in transplant recipients. This is a finding of enormous value in view of two facts: first, organ availability for transplantation is scarce; second, the demand for islet transplantation is likely to grow exponentially in the near future because of the report of the remarkable rate of success in recent clinical trials (3).

Prevention of apoptosis was analyzed in our in vitro system using defined models of apoptosis induction. We are aware that this might represent a relative limitation to infer a generalized cytoprotective effect of HO-1 upregulation on apoptosis prevention. It is possible that other pathways of apoptosis induction might not be influenced by HO-1 upregulation. Nonetheless, the use of cytokines (which are produced in situ after implantation) and the use of engagement of the Fas pathway of apoptosis are likely to represent fundamental phenomena of apoptosis induction after islet transplantation (11,38,55,56).

Also, administration of CoPP has been demonstrated to have pleiotropic effects, including a generic anti-inflammatory effect via targeting of tissue macrophages (43,57,58). It cannot be excluded that CoPP administration might therefore exert its beneficial effects on islet function via multiple mechanisms (59). Also, protoporphyrins have been shown to exert modulatory (enhancing) effects on glucose-stimulated insulin secretion (34,35,60,61), a mechanism that might correlate to the observed amelioration of the glucose profiles in the treated mice. Conversely, CoPP indeed leads to remarkable upregulation of HO-1 mRNA expression in the grafted islets, suggesting this as an important mechanism underlying the protective effects observed in vivo.

It is conceivable that similar phenomena of microenvironment activation may occur in the scenario of allogeneic grafts. They are likely to lead to function of a reduced mass of islets, and they also might contribute to enhancement of the subsequent immune recognition of the implanted tissue through alteration of the surface antigenic molecules expressed by islet cells. Intervening on the first phase of inflammation via the induction of cytoprotective genes might therefore result in the added bonus of reducing the subsequent specific immune recognition. In accordance with this hypothesis, data were presented recently on a positive effect of HO-1 upregulation on the survival of allogeneic heart transplants in rats (62). Further optimization of HO-1 induction protocols (timing and dose of protoporphyrins administration) might result in additional benefit in model systems of islet transplantation. Also, humoral immunity may contribute to graft loss via antibody binding to alloantigens and complement activation (63). Furthermore, complement activation has been shown to play an important role in the failure of islet grafts in the absence of alloantibodies, via activation mediated by the exposure of extracellular matrix proteins on the islet surface (64). HO-1 upregulation has been clearly shown to protect cells from the antibody/complement–mediated injury in vascularized xenografts (65,66), and it is conceivable, therefore, that a similar protective effect will be observed in islets exposed to alloantibodies and complement activation.

In summary, we report on the protective effects of HO-1 upregulation on the function and survival of insulin-producing β-cells. Strategies aimed at HO-1 upregulation might result in better outcome in islet transplantation.

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