Heme Oxygenase-1 Protects Pancreatic β Cells From Apoptosis Caused by Various Stimuli

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ABSTRACT

Background: **Several problems can occur after allogeneic islet transplantation: primary nonfunction, rejection, and the recurrence of autoimmune disease, which involve attack by the recipient's cytokines, T cells, natural killer cells, and monocytes on the donor's β cells, which leads to β-cell destruction. Recent studies have revealed that loss of transplanted islets is caused mainly by apoptosis. Heme oxygenase-1 (HO-1) is one of the antiapoptotic genes up-regulated under stress conditions. The aim of this work was to investigate any mechanisms of HO-1–mediated protection of β cells from apoptosis.**

Methods: **Apoptosis was assessed by comparison of viable transfected cells with and without apoptotic stimuli, and with and without HO-1 overexpression. Activation and function of p38 mitogen-activated protein kinase were determined using the specific inhibitor SB203580.**

Results: **We have shown that HO-1 mediates antiapoptotic effects in β cells. The percentage of apoptotic cells after stimulation with tumor necrosis factor α decreased from 75% without HO-1 to 5% when HO-1 was overexpressed. Our data indicate that HO-1 acts as a signal terminator of tumor necrosis factor α–induced apoptosis by modulation of the p38 mitogen-activated protein kinase pathway.**

Conclusions: **Profound cell stress that occurs in islets after transplantation, as well as at the onset of diabetes, results in β-cell loss through apoptosis. Protection of β cells by HO-1 improves their survival in vitro after various proapoptotic stimuli, suggesting that HO-1 suppresses one or several signaling pathways leading to apoptosis. We hypothesize that our in vitro findings can be extrapolated to the in vivo situation, and we propose that expression of HO-1 in islets may illuminate a valuable new approach to improving diabetes treatment. (J Investig Med 2001;49:566–571) Key Words: diabetes mellitus • heme oxygenase-1 • apoptosis • p38 mitogen-activated protein kinase • beta cells**

INTRODUCTION

The pathophysiology of insulin-dependent diabetes mellitus (IDDM, type I diabetes) is characterized by dysfunction and death of insulin-producing β cells in the pancreatic islets of Langerhans.[1] At an early stage of the disease, referred to as insulitis, histologic findings show progressive mononuclear cell invasion of the islets, which persists for several weeks to months before severe β-cell destruction occurs.[2,3] In autoimmune nonobese diabetic mice, β cell insulitis has been linked to activated macrophages and T cells that secrete soluble mediators, such as oxygen-free radicals, NO, and cytokines[4–6]. Insulitis has been specifically associated with increased expression of proinflammatory type 1 cytokines, such as interleukin (IL)-1β, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α.[4,6,7] There is compelling evidence that the effect of TNF-α upon β-cell function and apoptosis is mediated by the local production of NO, which follows the expression of inducible NO synthase by β cells and other surrounding cell types.[8–10] Apoptosis is the main form of β-cell destruction in nonobese diabetic mice,[11] and there is evidence that β-cell death in the early stages of human type 1 diabetes also occurs because of apoptosis.[12,13]

The Edmonton protocol[14] and recent improvements to it make islet transplantation a promising treatment for IDDM, although several problems remain to be overcome.

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Early dysfunction of transplanted islets (primary nonfunction) and rejection are major pitfalls in the successful re-establishment of glucose homeostasis. Recent studies have shown that apoptosis is the main cause for loss of transplanted islets.[15,16] Under in vitro conditions, exposure of human, rat, or mouse β-cells to IL-1β, in combination with IFN-γ and/or TNF-α, induce severe functional suppression and cell death by apoptosis.[17–21]

In the past decade several genes were discovered that are involved in the regulation of apoptosis. The expression of pro- and antiapoptotic genes is regulated tightly through several signal transduction pathways. The mitogen-activated protein kinase (MAPK) pathway, which is activated by exogenous factors such as proinflammatory cytokines or cell stress, holds a pivotal position in pathways that signal apoptosis.[22] Up-regulation of pro- or antiapoptotic genes results in apoptosis and in cell protection, respectively.[23–26]

Very little is known about physiological mechanisms of defense against apoptosis in islets. Heme oxygenase-1 (HO-1) has been shown to be up-regulated in islets under stress conditions, such as during islet isolation before transplantation[27] or after islet and β-cell cytokine treatment with IL-1β and IFNγ.[28] The ability of protective genes such as HO-1 to prevent apoptosis may account in large measure for their cytoprotective function.

The action of HO-1 yields three products: biliverdin, free iron, and CO.[29,30] Each of these three products has been shown to have different cytoprotective effects. Biliverdin is rapidly catabolized to bilirubin, which is a potent antioxidant that prevents oxidative damage.[30] Iron leads to the induction of ferritin, which has been shown to protect endothelial cells from damage mediated by activated neutrophils and H_2O_2[31]. Carbon monoxide that is derived from HO-1 protects endothelial cells from TNF-α–mediated apoptosis.[32] The expression of antiapoptotic genes might be a powerful therapeutic tool for several different problems that are related to IDDM. In comparison to the expression of the endogenous counterpart, HO-1 gene transduction of β cells can offer the advantage of immediate overexpression and protection.

The aim of this work was to investigate the molecular basis of the protective effect of HO-1 in β cells and the possible use of this gene as a therapeutic agent in IDDM. We demonstrate that HO-1 has an antiapoptotic effect on β cells that are exposed to various proapoptotic stimuli and that this effect is mediated through its enzymatic function. In addition, we show that HO-1 suppresses β-cell apoptosis through a mechanism that is dependent on the activation of the p38-MAPK signal transduction pathway.

METHODS

Cell Culture

The murine βTC3 insulinoma cell line (DSMZ, Braunschweig, Germany) was cultured as described in Efrat et al (1988).[33]

Expression Plasmids

The β-galactosidase gene (Clontech Laboratories, Palo Alto, Calif) was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, Calif), as described before.[34] The full-length rat HO-1 cDNA (a kind gift from Augustine Choi, Pittsburgh, Pa) was subcloned into the pcDNA3 vector under the control of the cytomegalovirus enhancer–promoter (pcDNA3–HO-1).[32] The murine Bcl-2 cDNA expression vector (a kind gift from R. Gerard, University of Texas Southwestern, Dallas) has been described elsewhere.[35]

Transient Transfections

The βTC3 cells were transiently transfected with LipofectamineReagents™ (Life Technologies, Carlsbad, Calif), as described elsewhere.[32] All experiments were carried out 24 hours after transfection. β-galactosidase–transfected cells were detected, and the percentage of viable cells was assessed by evaluating the number of β-galactosidase–expressing cells that retained their normal morphology.[34,36] The number of random fields counted was determined to have a minimum of 200 viable transfected cells per control well. The percentage of viable cells was normalized for each DNA preparation to the number of transfected cells counted in the absence of the apoptosis-inducing agent (100% viability). All experiments were performed at least three times in duplicate.

Cell Treatment and Reagents

βTC3 apoptosis was induced by etoposide (2.5 μM in DMSO, 8 hours) (Calbiochem-Novabiochem Corp, La Jolla, Calif) or by serum deprivation (0.1% FCS for 24 hours). Cobalt protoporphyrin (CoPPIX) and tin protoporphyrin (SnPPIX) (10 mM; Porphyrin Products Inc, Logan, Utah) were dissolved in 100 mM NaOH and stored at -20°C until they were used. Metalloporphyrins (50 μM) were added to the culture medium 6 hours after transfection. Murine recombinant TNF-α (R&D Systems, Minneapolis, Minn) was dissolved in PBS 1% bovine serum albumin and added to the culture medium (17.5 ng/mL = 500 U) 24 hours after transfection. The p38 MAPK–inhibitor pyridinyl imidazol SB203580 (Calbiochem-Novabiochem Corp)[37] was dissolved in DMSO and added to the culture medium (1–50 μM) 6 hours after transfection.

RESULTS

HO-1 Protects β Cells from TNF-α–Mediated Apoptosis

We tested different proinflammatory cytokines and cytokine combinations, which included IL-1β, IL-1β plus IFNγ, TNF-α alone, and TNF-α plus IFNγ, for their abilities to induce dose-dependent apoptosis in the murine insulinoma cell line βTC3. In our hands TNF-α alone showed the best concentration dependency (data not shown). We then used a similar experimental approach to investigate whether transient overexpression of HO-1 would protect βTC3 from undergoing apoptosis. βTC3 cells were transiently co-transfected with varying amounts of the pcDNA3–HO-1 and the pcDNA3–β-galactosidase expression vectors. For comparison, the cells were also co-transfected with either empty pcDNA3 expression vector or the Bcl-2 expression vector with the pcDNA3–β-galactosidase expression vector. We found that the overexpression of HO-1 dose-dependently protected βTC3 cells from TNF-α–induced apoptosis (Figure 1). The protective effect of HO-1 was equal to that observed for the prototypical antiapoptotic protein Bcl-2.[35,38] Transfection of βTC3 with 10 to 50 ng of HO-1 expression vector resulted in optimal protection: 3 to 6% apoptotic cells in the presence of HO-1 versus 70 to 80% apoptotic cells in the vector control. When higher concentrations were used, HO-1 expression became cytotoxic and the protective effect vanished, a phenomenon also described with other cell types.[39] Thus, in all subsequent experiments, 25 ng DNA of pcDNA3–HO-1 expression vector per 3×10^5 cells was used for transient transfections. Expression of HO-1 in βTC3 cells was confirmed by western blot (data not shown).

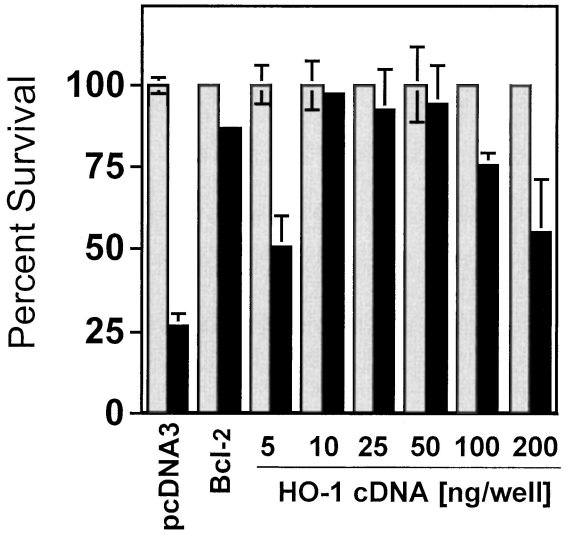

Figure 1. Expression of HO-1 suppresses β-cell apoptosis in a dose-dependent manner. βTC3 were co-transfected with a β-galactosidase (pcDNA3–β-galactosidase)-expressing vector plus control vector (pcDNA3), Bcl-2, or increasing amounts (5–200 ng/3 ×10^5 cells/well) of pcDNA3–HO-1, as indicated. Gray histograms represent untreated β cells and black histograms represent β cells treated with TNF-α (500 U/mL). Results shown are the mean±standard deviation from duplicate wells taken from one representative experiment out of three.

HO-1 Protects β Cells from Other Proapoptotic Stimuli

We hypothesized that HO-1 protection is specific for TNF-α–mediated apoptosis or apoptosis induced by other proapoptotic stimuli, such as serum deprivation or the chemical etoposide. To test our hypothesis, cultured β cells were transiently co-transfected with β-galactosidase plus HO-1 or control (pcDNA3) expression vectors and tested for apoptosis. Stimulation of β cells by serum deprivation caused widespread apoptosis of control β cells (60–70% apoptotic β cells) (Figure 2). HO-1 overexpression improved β-cell survival to 20 to 40% apoptotic cells. Similar results were obtained with the proapoptotic stimulus etoposide, which caused 55 to 60% apoptosis in our in vitro model. HO-1 overexpression enhanced cell survival to 10 to 20% apoptotic β cells. β-cell survival was not affected by the concentration of the solvent (DMSO) for etoposide used in this experiment (data not shown).

The Antiapoptotic Function of HO-1 Depends on its Enzymatic Activity

We investigated whether the antiapoptotic effect of HO-1 requires its enzymatic activity by blocking such

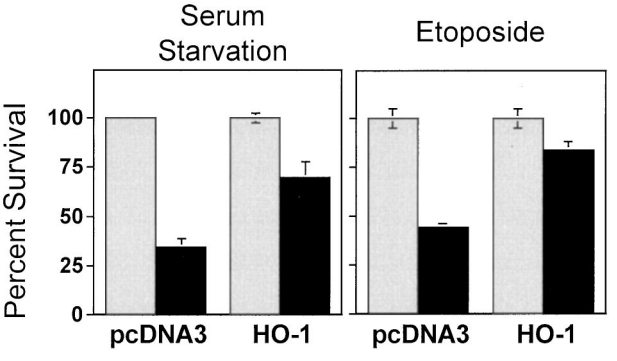

Figure 2. Expression of HO-1 suppresses β-cell apoptosis induced by serum starvation or etoposide. βTC3 were co-transfected with the pcDNA3–β-galactosidase expressing vector plus control (pcDNA3) or pcDNA3–HO-1 at a concentration of 5 to 200 ng/3 × 10^5 cells/well as indicated. Gray histograms represent untreated β cells and black histograms represent β-cell subjected to either serum deprivation (0.1% FCS) or that were treated with etoposide (2.5 μM). Results shown are mean±standard deviation from duplicate wells taken from one representative experiment out of three.

activity with tin protoporphyrin IX (SnPPIX), a specific HO-activity inhibitor.[40] Inhibition of HO-1 enzymatic activity by SnPPIX suppressed the antiapoptotic effect of HO-1 (Figure 3C). As expected, SnPPIX did not affect the antiapoptotic effect of Bcl-2 (Figure 3B). Cobalt protoporphyrin (CoPPIX), which has a similar molecular structure to SnPPIX but which does not inhibit HO enzymatic activity[40] did not suppress the antiapoptotic effect of HO-1. β-cell survival per se was not affected by the SnPPIX and CoPPIX concentrations used in these experiments (Figure 3A).

The Mechanism by which HO-1 Prevents β-cell Apoptosis Includes p38 MAPK Activation

The p38 MAPK pathway is known to be activated via TNF-α in various cell systems. Earlier results from our laboratory have revealed that the protection of endothelial cells by CO, a product of HO-1 action, acts via this pathway. Subsequently, we have investigated whether the protective effect of HO-1 in β cells is dependent on this pathway as well. To do so we blocked the action of p38 MAPK with pyridinyl imidazol SB203580, a specific inhibitor for two of the four p38 MAPK isoforms: α and β.[41] The antiapoptotic action of HO-1 (0–10% apoptotic cells) was suppressed when p38 MAPK–activation was inhibited by SB203580 (55% apoptotic cells with 20 μM SB203580). This effect was dose-dependent in that increasing concentrations of the inhibitor decreased the antiapoptotic effect of HO-1 (Figure 4). Inhibition of p38 MAPK activation per se did not sensitize β cells to TNF-α–mediated apoptosis (data not shown).

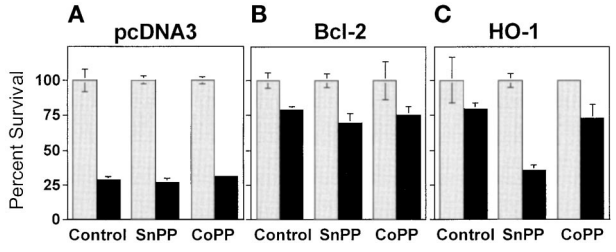

Figure 3. *The antiapoptotic effect of HO-1 requires its enzymatic activity. βTC3 were co-transfected with β-galactosidase, control (pcDNA3), pcDNA3–HO-1, or Bcl-2–expressing vectors. As indicated, transfected β cells were either left untreated (Control) or were treated (6 h after transfection) with tin protoporphyrin (SnPP, 50 μM), a specific inhibitor of HO-1. Cobalt protoporphyrin (CoPP, 50 μM) was used as a control treatment. Gray histograms represent untreated β cells and black histograms represent β cells treated with TNF-α (500 U/mL). Results shown are mean±standard deviation from duplicate wells taken from one representative experiment out of three.*

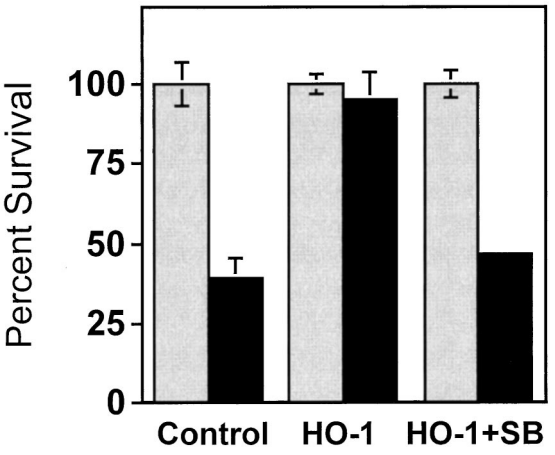

Figure 4. *The antiapoptotic effect of HO-1 requires p38 MAPK activity. βTC3 were co-transfected with β-galactosidase, control (pcDNA3), or HO-1 (pcDNA3–HO-1)-expressing vectors. Transfected β cells were either left untreated (Control) or were treated (6 h after transfection) with increasing concentrations of pyridinyl imidazol SB203580, as indicated (1–50 μM), a specific inhibitor of the p38α and p38β MAPK isoforms. Gray histograms represent untreated β cells and black histograms represent β cells treated with TNF-α (500 U/mL). Results shown are mean± standard deviation from duplicate wells taken from one representative experiment out of three.*

DISCUSSION

The observation that HO-1 can prevent βTC3 cells from undergoing apoptosis that may be induced by different proapoptotic stimuli (Figures 1 and 2) suggests that HO-1 suppresses one or several pivotal signaling pathways that lead to apoptosis. Our finding that a specific inhibitor of HO-1 blocks its antiapoptotic action (Figure 3) suggests that this protective effect probably depends on one or several products of HO-1 action.

The antiapoptotic effect of HO-1 has recently been associated with increased cellular iron efflux through the up-regulation of an iron pump.[42] According to the study in question, HO-1 inhibits apoptosis by limiting the availability of pro-oxidant free-iron to participate in the generation of reactive oxygen species through the Fenton reaction. Reactive oxygen species are well-established components in several signaling cascades that lead to apoptosis.[43] The up-regulation of the iron-storage protein ferritin, as a consequence of the HO-1 action on heme that leads to free-iron generation, may have an effect similar to that of the iron pump. This assumption is based on the ability of ferritin to bind intracellular iron and, therefore, to prevent the formation of free radicals that would damage DNA, proteins, and lipids.[44] The cytoprotective properties of HO-1 have also been linked to bilirubin, a potent antioxidant, which is catabolized from biliverdin by HO-1

action.[30] Carbon monoxide, the third product of HO-1 action on heme may prevent cell death from apoptosis via induction of manganese superoxide dismutase.[45] It has been shown that HO-1–derived CO activates p38 MAPK in macrophages.[46] Our recent studies indicated that HO-1–derived CO can protect endothelial cells from undergoing apoptosis. This protective effect of CO requires the activation of the p38 MAPK signal transduction pathway.[32] We have shown that the antiapoptotic effect of HO-1 is mediated via a transduction pathway that involves the activation of p38 MAPK (Figure 4). This is consistent with other findings which show that activation of p38 MAPK is critical in the regulation of apoptosis for a variety of cell types, including the cervix epithelial cell line HeLa,[24] cardiac myocytes,[26] and lymphoid Jurkat T cells.[47] We suggest that the mechanism by which HO-1 prevents apoptosis differs in different cell types. The three products of HO-1 action probably use different signaling pathways in various cell types. In addition, products of HO-1 action may play pivotal roles in the antiapoptotic effect of HO-1 in different cell types. Its broad antiapoptotic properties make HO-1 particularly interesting as a gene that may potentially be used for therapeutic purposes.

In conclusion, our data indicate that HO-1 acts as an antiapoptotic molecule that can suppress β-cell apoptosis. We show that the mechanism of action of HO-1 involves the activation of the p38 MAPK pathway. Given the specificity of the SB203580 inhibitor for the α and β isoforms of p38 MAPK,[41] we conclude that at least one of these two isoforms is involved in the protective effect of HO-1 in β cells.

We suggest that our in vitro findings can be extrapolated to the in vivo situation and that HO-1 can act as a protective gene that suppresses apoptosis in vivo. We assume that HO-1 can prevent a series of inflammatory reactions that are associated with β-cell apoptosis during the onset of diabetes, as well as in islet graft rejection.

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