# Carbon Monoxide Protects Pancreatic β-Cells From Apoptosis and Improves Islet Function/Survival After Transplantation

**Lukas Günther,[1] Pascal O. Berberat,[1] Manabu Haga,[1] Sophie Brouard,[3] R. Neal Smith,[2] Miguel P. Soares,[1] Fritz H. Bach,[1] and Edda Tobiasch[1]**

**Pancreatic islets transplanted to treat autoimmune type 1 diabetes often fail to function (primary nonfunction), likely because of islet β-cell apoptosis. We show that carbon monoxide (CO), a product of heme oxygenase activity, protects β-cells from apoptosis. Protection is mediated through guanylate cyclase activation, generation of cyclic GMP (cGMP), and activation of cGMP-dependent protein kinases. This antiapoptotic effect is still observed when β-cells are exposed to CO for 1 h before the apoptotic stimulus. In a similar manner, mouse islets exposed to CO for just 2 h function significantly better after transplantation than islets not exposed to CO. These findings suggest a potential therapeutic application for CO in improving islet function/survival after transplantation in humans. *Diabetes* 51:994–999, 2002**

Transplanted islets may fail to function in the initial period after transplantation (primary nonfunction), be rejected, or fail because of recurrence of the autoimmune process underlying type 1 diabetes. In all these cases, apoptosis of β-cells contributes to the failure of the transplanted islets (1,2).

We and others (3–6) have hypothesized that the expression of antiapoptotic genes in islets may ameliorate the above problems, a hypothesis supported by the observation that A20, an antiapoptotic gene, protects β-cells from apoptosis (3), and ex vivo gene transfer of bcl-2, another antiapoptotic gene, was cytoprotective for transplanted islets (5).

Another antiapoptotic gene is heme oxygenase-1 (HO-1) (7), the rate-limiting enzyme in the catabolism of heme to yield equimolar amounts of carbon monoxide (CO), free iron, and biliverdin, the latter two leading to expression of ferritin and bilirubin, respectively (8). HO-1 expression is antiapoptotic and cytoprotective against oxidative stress in a variety of cell types in vitro (7,9–12) and in vivo (7,13). Presumably, the ability of HO-1 to suppress apoptosis contributes critically to its capacity to suppress a variety of inflammatory conditions, including rejection of transplanted organs (7). The observation that CO alone (in the absence of HO-1) can in some systems mediate the same effects as HO-1 suggests that at least some of the protective effects of HO-1 are mediated through the generation of CO (14–17).

We examined whether CO acts in a cytoprotective manner in pancreatic islet β-cells and tested whether CO treatment of islets could enhance islet survival and function after transplantation. We found that exogenous CO protects the murine insulinoma cell line βTC3 and murine islets of Langerhans from apoptosis via cyclic GMP (cGMP) and involves activation of cGMP-dependent protein kinases (cGKs). Brief exposure of purified mouse islets to CO before transplantation as a sole treatment results in significantly improved functional performance after transplantation to a diabetic syngeneic recipient.

## RESEARCH DESIGN AND METHODS

**Animals.** Male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were housed in accordance with guidelines from the National Institutes of Health. The experiments were approved by the Institutional Animal Care and Use Committee.

**Cell cultures.** The murine insulinoma cell line βTC3 (DSMZ, Braunschweig, Germany) was cultured in Dulbecco's Modified Eagle's Medium (Life Technologies, Grand Island, NY) supplemented with 2 mmol/l L-glutamine, 100 units/ml penicillin G, 100 units/ml streptomycin, and 10% FCS (Life Technologies).

**Crystal violet staining.** βTC3 cell lines were seeded at $2 \times 10^5$ cells, washed once with 500 μl PBS, and stained with 200 μl 0.05% crystal violet in 20% ethanol for 10 min at room temperature. To elute stain from cells, 100 μl 50% acetic acid was added to each well, and 50 μl were transferred into 96-well microtiter plates and read with a plate reader (EL 340 biokinetics reader; Bio-Tek Instruments, Winooski, VT) at 562 nm.

**Expression plasmids.** The β-galactosidase expression vector (Clontech Laboratories, Palo Alto, CA) was cloned into the pcDNA3 vector (17) (Invitrogen, Carlsbad, CA). A 1.0-kbp X*ho*I-H*ind*III fragment encoding the full-length rat HO-1 cDNA was cut from the prHO-1 vector (18) and subcloned into the pcDNA3 vector.

**Transient transfections.** βTC3 cell lines were seeded at $3 \times 10^5$ cells in 16-mm wells and transfected 15–20 h later using Lipofectamine plus reagents (Life Technologies) according to the manufacturer's instructions. Total DNA was maintained constant using empty pcDNA3 vector. The percentage of viable cells was assessed by normalizing the percentage of viable cells to the number of control-transfected cells without the apoptotic stimulus (100% viability) (7,17).

**Flow cytometry.** βTC3 cultures were incubated with recombinant tumor necrosis factor-α (TNF-α) (500 units) (R&D Systems, Minneapolis, MN) for 24 h, and islets were stimulated with TNF-α (5,000 units/ml) (R&D Systems) and cycloheximide (CHX) (Sigma, St. Louis, MO) (50 μg/ml) for 48 h (6). βTC3

From the [1]Immunobiology Research Center, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; the [2]Immunopathology Unit, Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts; and [3]INSERM U437, ITERT, Nantes, France.

Address correspondence and reprint requests to Fritz H. Bach, Immunobiology Research Center, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115. E-mail: fritzhbach@aol.com.

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F.H.B. is the Lewis Thomas Professor of Surgery at Harvard Medical School and is a paid consultant for Novartis Pharma.

8-Br–cGMP, 5′-cyclic-monophosphate–cyclic GMP; cGK, cyclic GMP–dependent protein kinase; CHX, cycloheximide; cGMP, cyclic guanine monophosphate; HO-1, heme oxygenase-1; IL, interleukin; ODQ, 1H[1,2,4]oxadiazolo[4,3-α]quinoxalin-1; SnPPIX, tin protoporphyrin IX; TNF-α, tumor necrosis factor-α.

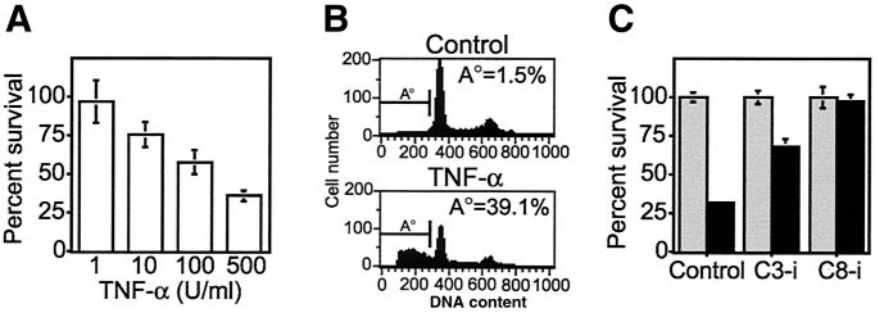


**FIG. 1. TNF-α induces apoptosis in β-cells. *A*: βTC3 cells were treated with increasing concentrations of TNF-α. Viable cells were stained 24 h after activation by crystal violet. The extinction was measured at 562 nm and normalized to untreated cells. *B*: βTC3 cells were treated with TNF-α, stained by propidium iodide 24 h later, and analyzed for DNA fragmentation (FACScan). *C*: βTC3 cells were co-transfected with a β-gal expressing vector (pcDNA3/β-gal) plus control (pcDNA3). When indicated, cells were treated with the caspase-3 inhibitor Z-DEVD-FMK (C3–i) or the caspase-8 inhibitor IETD-CHO (C8-i). Gray histograms represent untreated β-cells, and black histograms represent β-cells treated with TNF-α for 24 h. Results shown are the means ± SD from duplicate wells taken from one representative experiment of three.**

cells or islets were harvested, dispersed, fixed in 70% ethanol, and suspended into DNA staining buffer (PBS, pH 7.4, containing 0.1% Triton X-100, 0.1 mmol/l EDTA, 50 μg/ml propidium iodide, and 50 mg/ml RNase A). DNA content was analyzed on a FACScan (Becton Dickinson, Palo Alto, CA). Cells with DNA content ≥2 n were scored as viable; cells with a hypoploid DNA content (<2 n, termed A°) were scored as apoptotic. To exclude debris and cell fragments, all events with a FL-2 area profile below that of chicken erythrocyte nuclei were excluded from analysis.

**Cell treatment and reagents.** Murine recombinant TNF-α (R&D Systems) was dissolved in PBS and 1% BSA and added to the culture medium (17.5 ng/ml = 500 units) 24 h after transfection. The caspase-3 inhibitor Z-DEVD-FMK and the caspase-8 inhibitor IETD-CHO (Calbiochem, San Diego, CA) were dissolved in DMSO (Sigma) and added to the culture medium (10 and 1 μmol/l, respectively) 2 h before treatment with TNF-α. Tin protoporphyrin IX (SnPPIX) (Porphyrin Products, Logan, UT) was dissolved (10 mmol/l) in 100 mmol/l NaOH and added 6 h after transfection to the culture medium (50 μmol/l). The guanylyl cyclase inhibitor 1H[1,2,4]oxadiazolo[4,3-α]quinoxalin-1 (ODQ) (Calbiochem) was dissolved in DMSO and added to the culture medium (100 μmol/l) 6 h after transfection. The cGMP analog 8-bromoguanosine-3′-5′-cyclic-monophosphate (8-Br–cGMP) (Sigma) was dissolved in water and added to the culture medium (10 μmol/l) 30 min before induction of apoptosis. The protein kinase G inhibitor KT5823 (Calbiochem) was dissolved in DMSO and added to the culture medium (1.6 μmol/l) 6 h after transfection.

**CO exposure.** Cells and islets were exposed to 1% CO in compressed air balanced with 5% $CO_2$, as described elsewhere (15). Islets were incubated in RPMI medium presaturated with CO (4°C overnight, 1% CO, 5% $CO_2$) for 2 h at 37°C, while treatment with 1% CO, 5% $CO_2$ was continued.

**Induction of diabetes.** Recipient C57BL/6 mice (8 weeks of age) were rendered diabetic by a single intraperitoneal injection of 220 mg/kg (19) streptozotocin (Sigma) freshly dissolved in citrate buffer. Mice were transplanted if two consecutive nonfasting blood glucose levels >350 mg/dl were obtained from whole blood.

**Islet isolation.** Pancreatic islets of Langerhans (C57BL/6 mice) were provided by the Islet Core Laboratory of the Juvenile Diabetes Research Foundation Center for Islet Transplantation at Harvard Medical School and isolated as described previously (20).

**Syngeneic marginal mass islet transplantation.** Using a dissecting microscope, 250 islets of 150–250 μmol/l in diameter were hand-picked. Islets were transplanted under the kidney capsule as described previously (19). The same numbers of control and treatment animals were transplanted from each islet preparation. Animals were anesthetized with ketamine (0.9 mg/20 g body wt) and xylazine (0.1 mg/20 g body wt) administered as a single intraperitoneal dose.

**Graft functional outcome analysis.** Graft function was defined when the first of 3 consecutive days of nonfasting blood glucose levels <200 mg/dl was reached. The primary end point of the experiment was defined as time to normoglycemia.

**Statistical analysis.** Blood glucose data are summarized as the means ± SD of mice receiving untreated or treated islets. Time to recovery of islet function was calculated using Kaplan-Meier life tables and differences between groups tested using a log-rank test, with the three islet preparations treated as separate strata in the analysis and the median time to recovery, with 95% CI reported.

## RESULTS

**TNF-α induces apoptosis in βTC3 cells.** TNF-α induced high levels of cell death in the insulinoma cell line βTC3 (21) (Fig. 1*A*). DNA fragmentation was demonstrated (Fig. 1*B*), suggesting that TNF-α induces β-cell death through apoptosis. TNF-α–mediated apoptosis was strictly dependent on the activation of caspase-8 and partially dependent on that of caspase-3 (Fig. 1*C*).

**CO protects β-TC3 cells from apoptosis to a similar extent as HO-1.** Overexpression of HO-1 protected βTC3 cells transiently transfected with a HO-1 expression vector from TNF-α–mediated apoptosis (6) (87% survival vs. 33% in control) (Fig. 2*A*). Blocking HO-1 activity by SnPPIX (22) suppressed the antiapoptotic effect (Fig. 2*A*), suggesting that one or more of the end products of heme catabolism by HO-1, i.e., iron, bilirubin and/or CO, is required for its antiapoptotic function.

When the action of HO-1 was suppressed by SnPPIX, CO exposure suppressed TNF-α–mediated apoptosis to a similar extent as HO-1 (Fig. 2*A*). Exposure to exogenous CO alone protected from TNF-α–induced apoptosis (11.7 vs. 20.3% in apoptotic cells vs. controls not exposed to CO) (Fig. 2*B*, DNA fragmentation analysis). Similarly, β-cell apoptosis induced by etoposide or serum starvation was suppressed by CO exposure (Fig. 2*C*).

**Exogenous carbon monoxide protects murine islets from apoptosis.** Exposure to CO for 24 h protected isolated murine (C57/BL6) islets of Langerhans from TNF-α plus CHX-mediated apoptosis (11.7 vs. 20.3% in apoptotic cells vs. controls not exposed to CO), as assayed by DNA fragmentation (Fig. 3*A*) or histological analysis (Fig. 3*B*).

**The antiapoptotic effect of carbon CO is mediated via guanylate cyclase activation and signals through cGK.** Inhibition of soluble guanylate cyclase (sGC) activity by oxadiazoloquinoxalin (ODQ) suppressed the antiapoptotic effect of CO, suggesting that sGc is a mediator for CO (Fig. 4*A*), as also found in fibroblasts (12). The cGK activator/cGMP analog, 8-Br–cGMP, suppressed βTC3 apoptosis to a similar extent as CO (Fig. 4*B*). Inhibition of cGKs by KT5823 suppressed the antiapoptotic effect of exogenous CO (Fig. 4*C*), suggesting involvement of one or more cGKs.

**Exogenous CO provides antiapoptotic protection under various protocols.** We exposed βTC3 cells to CO for different time periods (1–24 h) immediately after the addition of TNF-α and tested for apoptosis 24 h later. One

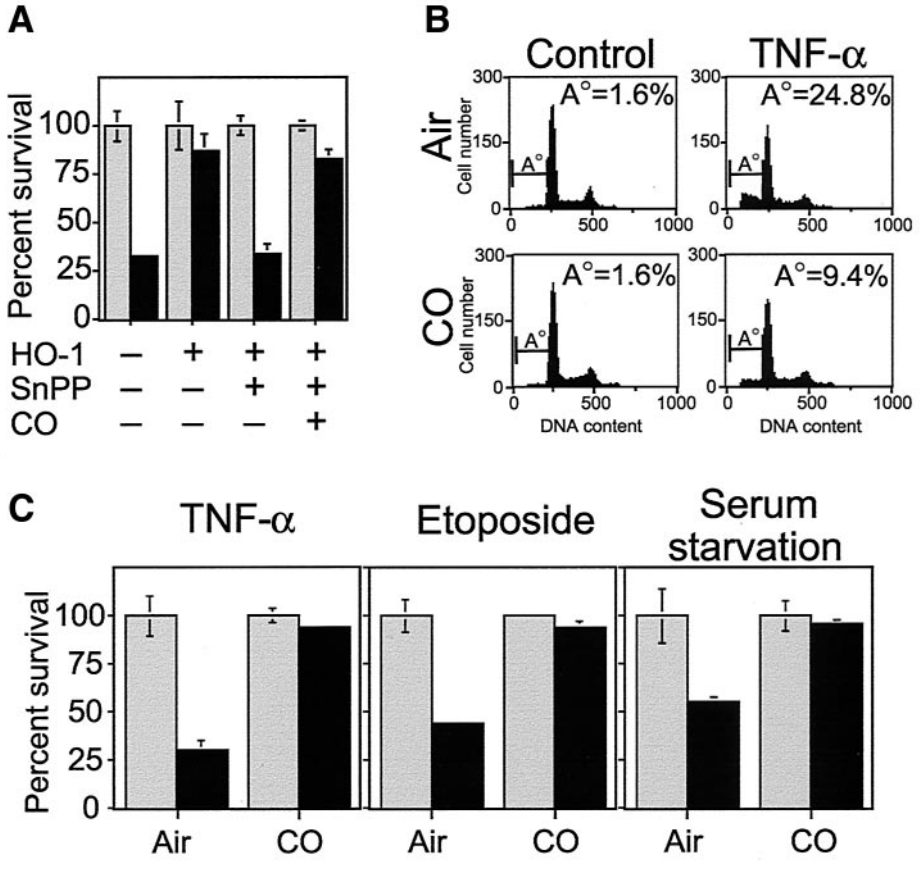


**FIG. 2. Exogenous CO protects β-cells from apoptosis. *A*: Exogenous CO can substitute for HO-1 when HO-1 activity is blocked. βTC3 cells were co-transfected with a β-gal expressing vector plus control or HO-1 (33). When indicated, HO-1 enzymatic activity was inhibited by SnPP. When indicated, β-cells were exposed to exogenous CO (1%) as described earlier (15). Gray histograms represent untreated β-cells, and black histograms represent β-cells treated with TNF-α. Results shown are the means ± SD from duplicate wells taken from one representative experiment of three. *B*: Exogenous CO protects β-cells from apoptosis in the DNA fragmentation analysis. βTC3 cells were treated with TNF-α. Directly after stimulation, βTC3 were exposed to exogenous CO (15) for 24 h. Control βTC3 cells were treated in the same manner but not exposed to CO. After 24 h, cells were stained by propidium iodide and analyzed for DNA fragmentation on a FACScan. *C*: Exogenous CO protects β-cells from apoptosis in the absence of HO-1. βTC3 cells were transfected with β-gal expressing vectors and were exposed to exogenous CO (15). Gray histograms represent untreated β-cells, and black histograms represent β-cells treated with TNF-α or etoposide or subjected to serum deprivation as indicated. Results shown are the means ± SD from duplicate wells taken from one representative experiment of three.**

hour of CO exposure was sufficient to prevent β-cell apoptosis (Fig. 5*A*).

We exposed β-cells to CO for 1 h, 0.5–12 h after induction of apoptosis by TNF-α. CO still suppressed β-cell apoptosis 2 h after TNF-α stimulation (Fig. 5*B*).

Further, 1 h of preincubation with CO prevented β-cell apoptosis (data not shown). Lastly, 1 h of preincubation with CO still prevented β-cell apoptosis in cells stimulated with TNF-α 2–3 h after the end of the 1-h treatment with CO (Fig. 5*C*). Thus, relatively brief treatment with CO

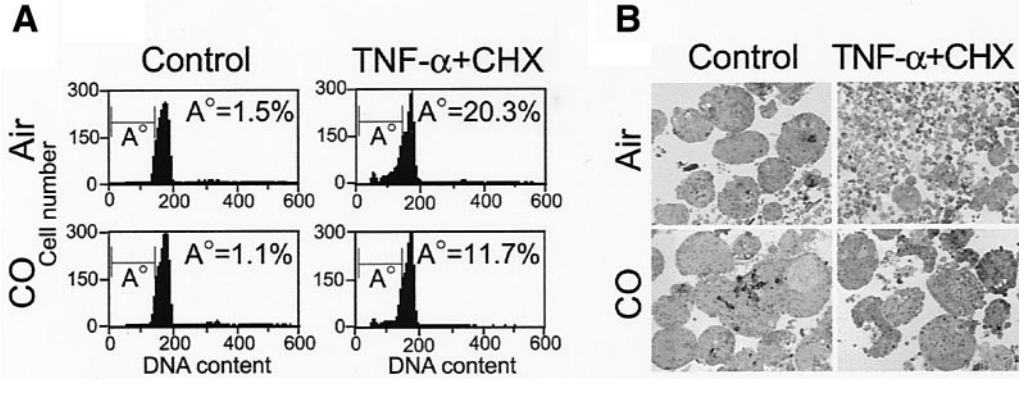


**FIG. 3. Exogenous CO protects murine islets of Langerhans from apoptosis. *A*: Apoptosis was induced in freshly isolated murine islets (C57BL/6) by stimulation with TNF-α and CHX. Immediately after stimulation, islets were exposed to exogenous CO (15) for 24 h. Control islets were treated in the same manner but not exposed to CO. After 48 h, cells were analyzed on a FACScan for DNA fragmentation. This experiment was done twice with indistinguishable results. *B*: Islets were treated as described in Fig. 3*A* and then fixed, embedded in plastic, and stained with toluidine blue for histopathologic evaluation.**

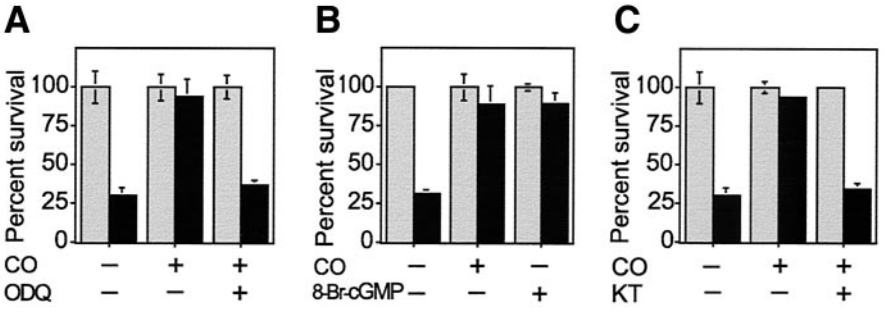


**FIG. 4. CO protects from apoptosis via cGMP. *A*: The antiapoptotic effect of exogenous CO is mediated by guanylate cyclase activation. βTC3 cells were transfected with β-gal expressing vectors and exposed to exogenous CO (1%) (33). Where indicated, βTC3 cells were treated with the guanylyl cyclase inhibitor ODQ. *B*: A cGMP analog can substitute for CO in protecting from apoptosis. βTC3 cells were transfected with β-gal expressing vectors. Where indicated, βTC3 cells were exposed to exogenous CO (15). Where indicated, βTC3 cells were treated with the cGMP analog 8-Br–cGMP but not exposed to CO. *C*: cGK mediate the antiapoptotic effect of CO. βTC3 cells were co-transfected with β-gal–expressing vector. When indicated, βTC3 cells were exposed to exogenous CO (15). When indicated, cells were treated with the protein kinase G inhibitor KT5823 (KT). Gray histograms represent untreated β-cells, and black histograms represent β-cells treated with TNF-α. Results shown are the means ± SD from duplicate wells taken from one representative experiment of three.**

provides an antiapoptotic effect that lasts for an extended time period.

**Exposure of murine islets to CO improves islet survival/function after transplantation.** We evaluated whether pre-exposure of islets to CO can improve islet survival and/or function after transplantation. To test this, we transplanted a marginal islet mass under the kidney capsule of diabetic syngeneic recipients. A total of 500 handpicked transplanted C57/BL6 islets led rapidly to normoglycemia [1.5 ± 0.5 days ($n$ = 4)], whereas 250 transplanted islets resulted in a significant delay [14.2 ± 2.9 days ($n$ = 9), data not shown]. Using a 250-islet marginal mass in this system does not involve rejection or recurrence of autoimmune disease (2). The time needed to reach normoglycemia was reduced in a highly significant manner ($P$ = 0.0011), when islets were preincubated for 2 h in medium presaturated with CO [7 days (95% CI 6–8)] as compared with control islets not pre-exposed to CO [14 days (12–18)] (Fig. 6*A* and *B*). In total, three different islet preparations were used for these experiments. There was no statistically significant difference in the time to normoglycemia for CO-treated and untreated islets among these three preparations ($P > 0.25$).

## DISCUSSION

Islet transplantation suffers from the need for high numbers of donor islets (23). Islet apoptosis after transplantation is thought to be caused by stress factors such as hypoxia, pro-inflammatory cytokines, and free radicals released from macrophages in the microenvironment of the transplanted islets (4,19,24,25). The observation that islets undergo primary graft nonfunction when transplanted into syngeneic recipients (26) strongly suggests that nonspecific inflammation plays a major role in this process.

Preventing apoptosis might improve islet survival/function after transplantation, allowing a significant reduction in the number of islets required for this procedure. In the present studies, we have investigated TNF-α–mediated β-cell apoptosis based on the assumption that TNF-α is a potential mediator of β-cell death after transplantation (24,27,28).

Freshly isolated rat islets do not express HO-1, whereas cultured rat islets have increased HO-1 expression (29,30). HO-1 induction by hemin (31) or $CoCl_2$ (30) can protect islets from the inhibitory effects of interleukin (IL)-1β on

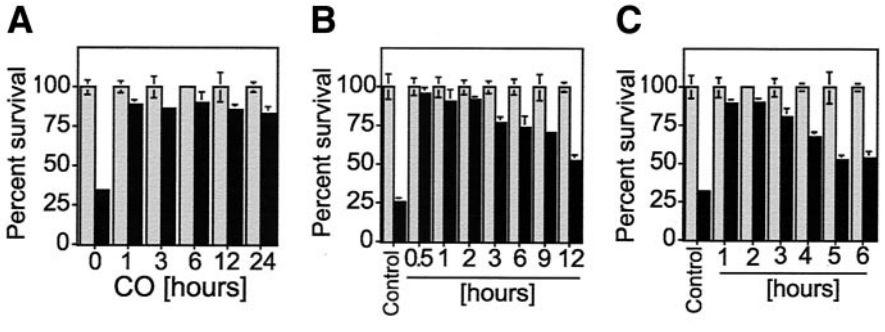


**FIG. 5. The antiapoptotic effect of exogenous CO is protracted. *A*: One hour of CO exposure is sufficient to prevent apoptosis. βTC3 cells were transfected with β-gal–expressing vectors. Apoptosis of β-cells was induced by TNF-α. Immediately after TNF-α activation, cells were exposed to 1% CO (15) for varying periods (0–24 h). Control βTC3 cells were treated in the same manner but were not exposed to CO. Cell survival was determined 24 h after application of TNF-α. *B*: CO protects β-cells after induction of apoptosis. βTC3 cells were transfected with β-gal–expressing vectors. Apoptosis was induced by TNF-α. After varying periods (0.5–12 h, as indicated), βTC3 cells were exposed to 1% CO (15). Control βTC3 cells were treated in the same manner but were not exposed to CO. Cell survival was determined 24 h after application of TNF-α. *C*: Preincubation with CO prevents β-cell apoptosis. βTC3 cells were transfected with β-gal–expressing vectors, and apoptosis was induced by TNF-α. βTC3 cells were pre-exposed to 1% CO (15) for 1 h. Control βTC3 cells were treated in the same manner but were not exposed to CO. Apoptosis was induced by TNF-α 1–6 h after termination of the pre-exposure. Gray histograms represent untreated β-cells, and black histograms represent β-cells treated with TNF-α. Results shown are the means ± SD from duplicate wells taken from one representative experiment of three.**

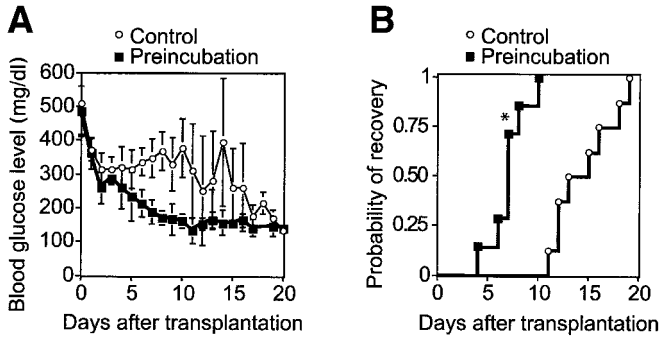


**FIG. 6. Exposure of murine islets to CO improves islet survival/function after transplantation. *A*: A total of 250 freshly isolated and handpicked islets from C57BL/6 mice were incubated in medium presaturated with 1% CO for 2 h at 37°C. Control islets were treated in the same manner but were not exposed to CO. The islets were transplanted under the kidney capsule of the diabetic syngeneic recipients as described before (19). After transplantation, blood glucose levels were determined on a daily basis. A total of 16 animals (8 with pre-exposed islets and 8 controls) were transplanted. One animal receiving pre-exposed islets died on day 3 of non–exposure-related technical reasons and was included in the statistical analysis as censored animal. The primary end point of these experiments was the first day of normoglycemia. Data are shown as the means ± SD. *B*: Probability of recovery (blood glucose level <200 mg/dl) for animals receiving islets pre-exposed with CO or control islets. **P* = 0.001 vs. control.**

glucose-stimulated insulin release and glucose oxidation. HO-1 expression protects these cells from TNF-α–mediated apoptosis (Fig. 2*A*) (6), a finding that may have important therapeutic implications (2,28,32).

We show here that CO protects pancreatic β-cells from apoptosis, as it does in endothelial cells (7,33). CO is an important signaling molecule in several cell types, including islets of Langerhans, where CO has been suggested to stimulate insulin and glucagon release (34). Compared with NO, CO is only a weak activator of sGC (35). However, CO may exert its effects for longer times and thus over longer distances than NO (34,36).

Our findings (Fig. 4*A* and *B*) suggest that cGMP is the main messenger of the antiapoptotic action of CO, as it is in fibroblasts (12). In monocytes (15) and endothelial cells (33), CO effects involve the p38 mitogen-activated protein kinase pathway, which does not appear to be involved in the present studies (data not shown).

We (17) and others (15,34,37) suggest that the protective function of CO may involve both antiapoptotic and anti-inflammatory effects. Low concentrations of CO in macrophages inhibit expression of the lipopolysaccharide-induced pro-inflammatory cytokines, while increasing the expression of IL-10 both in vivo and in vitro (15). In islets of Langerhans, the release of pro-inflammatory cytokines by activated macrophages upregulates inducible nitric oxide synthase in β-cells (25), which is highly cytotoxic for β-cells (38). Exogenous CO also suppresses NO production in islets (37), a potential direct protective effect.

We show here that pre-exposure of islets to CO for 2 h increases their survival and/or function after transplantation in a highly significant manner presumably based on the antiapoptotic and anti-inflammatory effects of CO. Preventing apoptosis that occurs after islet transplantation (32) would improve function of the transplanted islets and reduce the number of islets needed to treat diabetes. Given the above, CO might be a useful therapeutic tool for improving islet transplantation in humans.

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