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Islet β-Cell Apoptosis Triggered *in Vivo* by Interleukin-1β Is Not Related to the Inducible Nitric Oxide Synthase Pathway: Evidence for Mitochondrial Function Impairment and Lipoperoxidation

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IL-1β is recognized as an effector cytokine contributing to islet β-cell destruction during diabetes. We have previously shown *in vitro* that IL-1β induces nitric oxide (NO) and β-cell damage. Here, we show that IL-1β administration *in vivo* to Wistar rats transiently increases manganese superoxide dismutase activity, whereas inducible NO synthase is not detected, and the levels of nitrate+nitrate do not change. Moreover, a significant decrease of mitochondrial aconitase, leading to a rise of hydroperoxides, and islet β-cell apoptosis, involving caspase-3 and -8, is observed. Analysis of adhesion molecules in β-cells showed that intercellular adhesion molecule-1 is highly expressed 48 h after IL-1β administration and that this is concomitant to the fall of manganese superoxide dismutase activity. Thus, IL-1β exerts a proapoptotic effect *in vivo* through mitochondrial enzyme alteration, which is not related to the inducible NO synthase pathway, and dysregulates the immune system through the up-regulation of adhesion molecules. (*Endocrinology* 144: 4264–4271, 2003)

IL-1β-INDUCED CYTOTOXICITY in pancreatic β-cells *in vitro* (1), potentiated by other inflammatory mediators such as TNF-α and interferon-γ (IFN-γ) (2, 3), has been ascribed to increased nitric oxide (NO) release (4). In fact, the gene encoding for the inducible NO synthase (iNOS) is induced by IL-1β or IL-1β plus IFN-γ treatment in rodent and human islets, respectively (4). Human β-cells die by apoptosis, whereas cytokines lead to both necrosis and apoptosis in rat and mouse β-cells (2). It has been suggested that the necrotic component in rodent islets is attributable to NO-induced mitochondrial impairment and consequent decreased ATP production. Instead, human islets have better antioxidant defenses that preserve glucose oxidation and ATP production, allowing cells to complete the apoptotic program after the death signal triggered by cytokines (3).

Reactive oxygen species (ROS), free radicals, and other mitochondrial intermediates control the cytotoxic and gene-regulatory effects of cytokines on β-cells, thus providing bidirectional communication between mitochondria and nucleus. In fact, it has been widely demonstrated that inflammatory cytokines trigger complex signaling cascades, often resulting in excessive ROS production at the mitochondrial level with damage to cellular components. Manganese superoxide dismutase (MnSOD), a vital antioxidant enzyme localized into the mitochondrial matrix, acts as a cellular defense to detoxify these ROS. Islet β-cells contain extremely low levels of superoxide dismutase (SOD) (5, 6); and, with multiple low-dose streptozocin-induced diabetes, these levels are even further decreased (7). Administration of free radical scavengers partially protect islet β-cells from both alloxan- and streptozotocin-induced damage (8, 9).

Several cytokines, such as TNF-α, elicit elevations of both mRNA and protein levels of MnSOD (10). Administration of recombinant IL-1β induces MnSOD expression in rat pancreatic islets (11, 12). An increase in the level of this antioxidant enzyme has been shown to be cytoprotective, even if the pathways determining MnSOD expression are still unclear.

The effect of IL-1β, when administered *in vivo*, is more complex, and contradictory effects have been reported (for a review, see Ref. 13). Systemically administering low doses of IL-1β to Bio Breeding rats resulted in a significant reduction of diabetes incidence, whereas high doses of the same cytokine accelerated the disease (14). Conversely, systemic administration of a wide variety of cytokines has been demonstrated to both prevent and suppress diabetes, irrespective of their effect *in vitro*. This is not surprising, because systemic administration of a given cytokine may affect the production and action of other cytokines, resulting in changes different from those induced by the endogenous cytokine (13).

Furthermore, circulating forms of intercellular adhesion molecule (cICAM)-1 have been detected in human serum

Abbreviations: ABTS, 2,2′-Azinobis-(3-ethylebenzothiazoline-6-sulfonic acid; cICAM, circulating form of intercellular adhesion molecule; FOX, ferrous oxidation; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; MnSOD, manganese superoxide dismutase; NO, nitric oxide; ROOH, lipid hydroperoxide in xylene orange; ROS, reactive oxygen species; SOD, superoxide dismutase; TRAP, total plasma antioxidant capacity.

(15), and its enhancement has been described in several inflammatory and immune diseases (16–18). Recently, it has been postulated that cICAM-1 expression on cell membranes and in serum is regulated by different and independent mechanisms via proteolytic cleavage (19). In fact, it has been demonstrated that adhesion molecules, such as ICAM-1, are up-regulated by cytokines (20) or by SODs *in vitro* (21).

To further address the IL-1β *in vivo* effects in rat islet β-cells, we administered exogenous IL-1β in the nearest arterial supply to the pancreas and examined the outcome on purified islet cells. We found that IL-1β induces stimulation of MnSOD activity and concomitant disruption of mitochondria with activation of caspase-3 and -9.

Materials and Methods

In vivo treatment

Wistar rats, 10–12 wk old, were housed in our facility, under standard laboratory conditions, with free access to food and water. For *in vivo* administration, rats were anesthetized and a midline incision made. The major trunk of the pancreatic-duodenal artery was evidenced; and each animal received, through this artery, an infusion of 5.0 ml human IL-1β (280 U/ng), at a concentration of 4.0 μg/kg body weight (Sigma, Milan, Italy) (22). Similar volumes (5 ml) of vehicle were administered to the control animals. The animals were killed 12 h, 24 h, and 48 h later. In a first series of experiments, observations were made up to d 5; but, because of the fact that a large number of animals were affected by a pancreatitis, because of the combination of the use of cytokine and the abdominal surgical intervention, we decided to stop our observation at 48 h (d 2).

Islet preparation

At the end of treatment, each pancreas was removed and suspended in buffered Hanks' solution at 4 C, dissected free from extraneous fat, and minced with scissors. Tissue was incubated at 37 C with vigorous shaking for 15–20 min in 5 ml Hanks' solution containing 1.6 mg/ml type V collagenase (Sigma). After centrifugation (500 g, 15 min), the pellet was washed twice. Islets were then isolated on a Ficoll gradient. We obtained isolated islets with a purity more than 90% yields of at least 1,500 islets/ml medium. In addition, untreated animals were used for islet isolation and culture with IL-1β (3 ng/ml). Briefly, the islets were precultured at 37 C in a 5% CO_2 humidified air atmosphere for 3–6 d in RPMI 1640 medium (Sigma), supplemented with 10% fetal calf serum. Free-floating islets, 300 per 3 ml medium, were then cultured for another 2 d with 3 ng/ml human recombinant IL-1β (Sigma). At the end of the *in vitro* experiments, islets were collected, and RNA was extracted for RT-PCR analysis. The rationale of these experiments, made in triplicate, was to obtain a positive control for both MnSOD and iNOS mRNA transcripts and for comparison with the *in vivo* results.

Insulin levels

Insulin levels from peripheral blood were examined using RIA kits (Bio-Rad, Milan, Italy). Values are expressed as mM.

Fasting blood glucose levels

Blood glucose levels were determined using One-Touch profile (Lifescan Inc., Milpitas, CA) daily. Rats were considered hyperglycemics when their blood glucose levels were higher than 12 mM but lower than 15 mM and diabetics when their blood glucose levels exceeded 15 mM in two successive determinations.

MnSOD activity

In triplicate experiments, isolated islets were homogenized (Ultra Turrax mechanical blender) in 100 vol of 10 mM phosphate buffer (pH 7.4), supplemented with 30 mM KCl. Briefly, the homogenates were sonicated for 1 min at 4 C with a Branson B12 sonicator and left for 30 min to allow solubilization of the enzyme. After centrifugation at 20,000 × *g* for 30 min at 4 C, the supernatants were removed and stored at −70 C. MnSOD activity was measured using the RanSOD kit (Randox, Crumlin, Antrim, UK). This method uses xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity was measured by percentage inhibition of xanthine to water and molecular oxygen. The results are given as units per milligram of protein, a unit being the degree of inhibition of the reaction. The intra- and interassay coefficients of variation were, respectively, less than 2 and 4.5%. Because of the absence of hemoglobin, there was no need to correct the enzyme activities. The detection limit was 2 U/mg.

Nitrite+nitrate levels

In triplicate experiments, at least 150 islets, belonging to *in vivo* IL-1β-treated animals, were incubated for 30 min at 37 C in 5% CO_2 in 300 μl Krebs-Ringer-Bicarbonate (KRB) buffer [25 mM HEPES (pH 7.4), 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM D-glucose, 0.1% BSA]. Nitrite/nitrate was measured after conversion of nitrate to nitrite with Aspergillus nitrate reductase (Sigma). After mixing 0.1 ml supernatant with 0.1 ml Griess reagent (equal parts of 1.3% sulfanilamide in 60% acetic acid and 0.1% naphthyl-ethylene-diamine HCl in water) and incubating for 10 min at room temperature, nitrite was measured at 540 nm in a Gilford spectrophotometer and compared with a standard curve with known nitrite levels. The intra- and interassay coefficients of variations were less than 15%, and the detection limit was 1 μM.

Total plasma antioxidant capacity (TRAP)

The assay, originally described by Rice-Evans and Miller (1994) (23) is based on the quenching of the ABTS (2,2′-azinobis-(3-ethylebenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) by antioxidants. In the method, $ABTS^{\cdot+}$ is produced by the interaction of ABTS with ferrylmyoglobin radical species, generated by the activation of metmyoglobin with H_2O_2. In our assay procedure, ABTS (150 μM), and plasma (25 μl) were mixed together, and the reaction was started by the addition of H_2O_2 (75 μM). $ABTS^{\cdot+}$ formation was continuously monitored by absorbance increase at 734 nm, at 20 C. The delay or inhibition-time between the addition of H_2O_2 (time zero) and the onset of absorbance increase ($ABTS^{\cdot+}$ formation) was measured. All the reagents were dissolved in phosphate buffer treated with Cheelex-100 and containing DTPA (0.1 mM) to prevent any metal-catalyzed oxidation. The assay was standardized using Trolox, a water-soluble vitamin E analog. Experiments were performed in triplicate, and the intra- and interassay coefficients of variation for this method were 6.5 and 8.6%, respectively.

Lipid peroxidation products

Plasma lipids were extracted using a modification of the method of Folch *et al.* (1957) (24). A mixture of 3.8 ml of 2:1 (vol/vol) chloroform-methanol was added to 0.2 ml plasma. The mixture was vigorously mixed (using a vortex) for 2 min, and then 1.0 ml distilled water, acidified to pH 2.5 with 0.1 N HCl, was added. After agitation with a vortex for 2 min, the suspension was centrifuged at 3000 rpm for 5 min at 4 C. The lower chloroform lipid layer was removed, vacuum-dried in a Savant RC 100 Speed-Vac concentrator (Savant Instruments, Farmingdale, NY), and resuspended in 100 μl HPLC-grade methanol for hydroperoxide measurement. The hydroperoxide content of plasma was determined with the ferrous oxidation (FOX) Version II assay for lipid ROOHs (lipid hydroperoxide in xylene orange) (FOX2) (25). This technique relies on the rapid hydroperoxide-mediated oxidation of Fe (2) under acidic conditions. Fe (3) forms a chromofore with xylenol orange, which absorbs strongly at 560 nm. FOX2 reagent was prepared by dissolving xylenol orange and ammonium ferrous sulfate in 250 mM H_2SO_4 to final concentrations of 1 and 2.5 mM, respectively. One volume of this concentrated reagent was added to 9 vol HPLC-grade methanol containing 4.4 mM BHT to make the working reagent, which comprised 250 μM ammonium in 90% (vol/vol) methanol. The working reagent was routinely calibrated against a solution of H_2O_2 of known concentration. Aliquots (90 μl) of plasma lipid extracts in HPLC-grade methanol were transferred into 1.5-ml microcentrifuge vials. Triphenylphosphine in meth-

anol (10 μl of 10 mM) was added to the blank samples to selectively reduce ROOHs to hydroxyl derivatives having no reactivity with Fe (2). Methanol (10 μl) was added to the test sample. All vials were then vortex-mixed and incubated at room temperature for 30 min before the addition and mixing of 900 μl FOX2 reagent. After incubation at room temperature for a further 30 min, the vials were centrifuged at 12,000 × *g* for 10 min. The absorbance of the supernatant was then read at 560 nm. Hydroperoxide content in the plasma samples was determined as a function of the mean absorbance difference of samples with and without elimination of ROOHs by triphenylphosphine. Experiments were performed in triplicate, and the intra- and interassay coefficients of variation for this method were 5.0 and 6.8%, respectively.

Mitochondrial aconitase levels

Islets (at least 3000/condition) were dispersed into individual cells with dispase (0.25 mg/ml) in Ca^{2+}- and Mg^{2+}-free Hanks' solution (15 min at 31 C), filtered (60-μm nylon screen), and placed in medium CMRL-1066 (Life Technologies, Inc., Milan, Italy) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin. They were then incubated (18 h at 37 C); isolated by centrifugation (800 g, 4 C); resuspended in buffer (5 ml of 250 mM sucrose, 20 mM HEPES, 10 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EGTA, pH 7.4); permeabilized with digitonin (0.007%, 30 min on ice); agar-isolated by centrifugation; lysed by treatment with Triton X-100, 30 mM NaCl, 30 mM Tris-Cl, pH 7.4; and centrifuged (5000 × *g*, 4 C, 15 min). Supernatant aconitase activity was measured spectrophotometrically (340 nm, 21 C in 20 mM citrate, 0.5 mM NADP, 0.5 mM $MnCl_2$, 50 mM Tris-Cl, pH 7.4, 1 U isocitrate dehydrogenase; total volume 1 ml). Experiments were done in triplicate. Intra- and interassay coefficients of variation for this method were, respectively, less than 3% and less than 6.5%. Values are expressed as (pmol/100 islets) × (hour ± SD).

SOD and iNOS mRNA levels

Total RNA was extracted from 200–250 rat islets by homogenization in 4 M guanidinium thiocyanate containing 17 mM sodium *N*-laurylsarcosinate, 25 mM citrate buffer, 0.1 M 2-mercaptoethanol, and a 30% aqueous emulsion of 0.1% of Antifoam A (Sigma). RNA was precipitated with ethanol, pelleted, and reextracted with 8 M guanidine hydrochloride:0.5 M EDTA (19:1). After pelleting and drying, samples were extracted twice with phenol:chloroform (1:1) and precipitated with ethanol. cDNA synthesis was carried out from total RNA with Superscript reverse transcriptase kit (Life Technologies, Inc.), using oligo (dt)12–18 and Moloney murine leukemia virus reverse transcriptase (20 U) in a 25-μl reaction at 37 C for 1.5 h. The solution containing cDNA was diluted 30, 90, and 270 times in sterile water. Semiquantitative PCR amplification was carried out on the cDNA from each animal using 3 μl of each dilution of cDNA in a 20-μl reaction with 80 ng of each primer, 0.25 mM of each deoxynucleotide triphosphate, 2.5 μCi of (α-^{32}P) deoxycytidine triphosphate (3,000 Ci/mmol; DuPont NEN Life Science Products, Milan, Italy), 1 U AmpliTaq (Perkin-Elmer/Cetus, Monza, Italy), and 3 mM $MgCl_2$. The oligonucleotide primer sequences were: for MnSOD, 5′-ATTAACGCGCAGATCATGCAG-3′(forward) and 5′-TTTCAGATAGTCAGGTCTGACGTT-3′ (reverse); and for iNOS, 5′-AGCTTCTGGCACTGAGTAAAGATA-3′ (forward) and 5′-TTCTCTGCTCTCAGCTCCAAG-3′ (reverse). Glyceraldehyde-3-phosphate dehydrogenase housekeeping gene in rat islets was used as a positive control; the primers were: 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). The RT-PCR analyses were made in triplicate using a PTC-100 thermal cycler (MJ Research, Watertown, MA). The amplification products were separated on a 1.5% agarose gel and stained with ethidium bromide and were compared with DNA reference markers. As specified above, the *in vitro* experiments were used to obtain a positive control for both MnSOD and iNOS mRNA transcripts. The intensities of the bands were quantified in an Ultrascan XL Enhanced Laser densitometer (LKB, Bromma, Sweden) and expressed in arbitrary units of OD.

Determination of caspase activation

Islets were treated with ribonucleases for different time-intervals. At the end of treatment, colorimetric protease assays kits for caspase-3, -8, and -9 were used (Alexis Biochemicals, San Diego, CA). These assays, made on islet extracts, are based on the spectrophotometric detection of the chromophore p-nitroanilide after cleavage from the labeled substrate. The p-chromophore p-nitroanilide light emission is quantitated by spectrophotometric determinations at 400 or 405 nm. Briefly, the caspase-3/CPP32 kit assays the activity of caspase-3 that recognizes the amino acid sequence DEVD-pNA; the Flice/caspase-8 kit assays the activity of caspase-8 that recognizes the sequence IETD; the caspase-9/Mch6 kit assays the activity of caspase-9 that recognizes the sequence LEHD. Comparison of the absorbance of pNA from a treated sample with an untreated control allowed the determination of the increase in caspase activity. For each time point, results from three independent experiments were averaged.

Apoptosis evaluation

The TUNEL technique was used to detect DNA strand breaks *in situ*. Islets, double-stained with fluorescein isothiocyanate and PI, were fixed on glass slides with 50% glycerol in PBS. Fluorescence was monitored with a Leica TCS NT laser scanning confocal microscope (Leica, Lasertechnik, Heidelberg, Germany), with excitation from the 488-nm line of an argon/krypton laser. Fluorescence emission was detected with a band-pass filter (Chroma Technology, Brattleboro, VT) centered at 530 nm for fluorescein isothiocyanate and above 590 nm for PI. Several confocal images were used for continuing the number of apoptotic cells. In each condition, a minimum of 1000 cells from three to eight different isolations was counted.

cICAM-1 levels

Soluble ICAM-1 activity was assayed using the ELISA CD54 kit (Endogen, Woburn, MA). Blood samples from each animal were collected from the retroorbital plexus and processed following the kit's instructions. Values were expressed as nanograms per milligram.

ICAM-1 expression on islet cells

Samples from the tail of each pancreas were collected and kept frozen in liquid nitrogen. Randomly selected cryocut sections were stained by the avidin-biotin peroxidase indirect staining method. The monoclonal antibody anti-ICAM-1 was a mouse antirat (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was biotinylated goat anti-mouse antibody. As a negative control, the primary Ab was replaced with goat nonimmune serum. Sections of 5-μm thickness were examined for semiquantitative analysis. The immunoreactive cells on alternate sections were determined at a magnification of ×400 using an eyepiece with a square-ruled grid with a total area of 0.062 mm^2 and were counted with the M4 image analysis system (Imaging-Brock University, St.

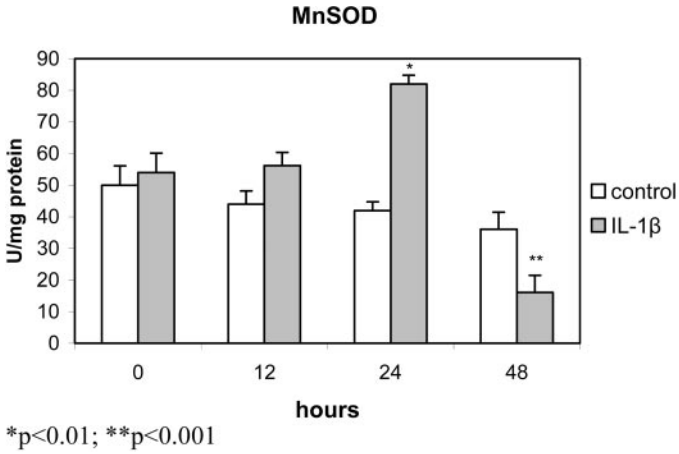

FIG. 1. Effect of *in vivo*-administered IL-1β on islet cell MnSOD activity. Kinetics of MnSOD activity performed on purified pancreatic islets from rats exposed to IL-1β. Results are shown as mean values ± SD and are representative of three independent experiments. *, $P < 0.01$; **, $P < 0.001$.

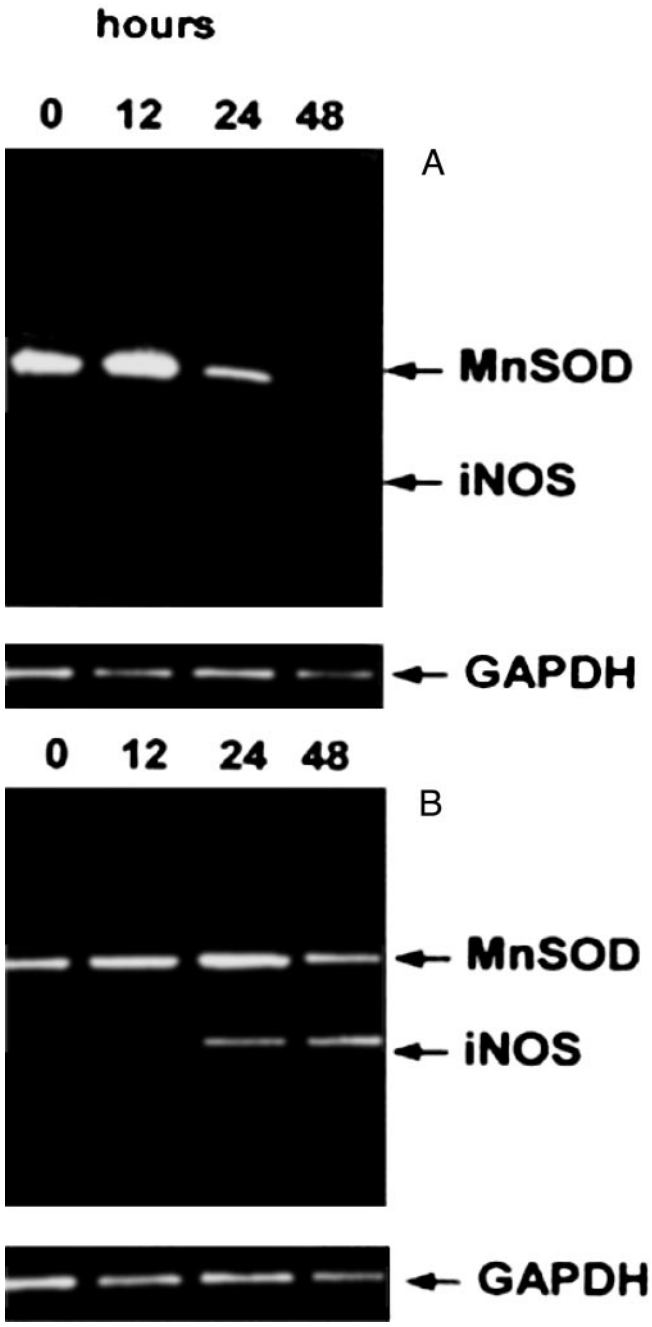

FIG. 2. IL-1β regulates SOD and iNOS mRNA levels on pancreatic islet cells. A, RT-PCR analysis of MnSOD and iNOS mRNA transcripts on islet homogenates purified from animals exposed to IL-1β; B, mRNA transcripts of MnSOD and iNOS in control pancreatic islets treated *in vitro* with IL-1β. Loading controls were performed by detecting glyceraldehyde-3-phosphate dehydrogenase. One representative of three independent experiments is shown.

Catherine, Ontario, Canada) in 60 different areas. This allowed the calculation of immunoreactive cells/mm^2 ± SEM. Three different researchers carried out the observations blindly.

Statistical analysis

Student's t test and ANOVA (when appropriate) were used for statistical analyses. P values < 0.05 were considered significant.

Results

Systemic treatment with IL-1β significantly altered islet MnSOD activity (Fig. 1), In fact, activity in freshly purified rat islet cells was significantly enhanced after 24 h 82 ± 2.73 U/mg protein; $P < 0.01$) as compared with cells purified from control animals (42 ± 9.95 U/mg protein). Thereafter, enzyme levels decreased considerably (16 ± 5.47 U/mg protein at 48 h; $P < 0.001$ *vs.* 12 h).

Supporting evidence for this transient activation of MnSOD, followed by a statistically significant decrease, was obtained at the transcriptional level. RNA samples extracted from purified islet-cell homogenates were analyzed by RT-PCR. We found that MnSOD mRNA levels were 2-fold higher at 12 h (7.4 ± 0.23 OD; $P < 0.001$), as compared with levels at time 0 (4.5 ± 0.15 OD). Interestingly, MnSOD mRNA levels at 24 h were not similar to levels at time 0 (3.32 ± 0.21 OD) and were barely or not detectable at 48 h (Fig. 2A and Table 1A). Similarly, analysis of rat islet cells exposed *in vitro* to 3 ng/ml IL-1β demonstrated an up-regulation of MnSOD mRNA levels at 24 h (4.62 ± 0.17 OD) and a down-regulation at 48 h (2.1 ± 0.1 OD *vs.* 3.48 ± 0.10 OD; $P < 0.001$) (Fig. 2B and Table 1B). Furthermore, after *in vivo* IL-1β administration, iNOS transcripts, assessed on purified islet, were always undetectable (Fig. 2A), whereas they were clearly observed after *in vitro* exposure to IL-1β for 24 h (Fig. 2B). Thus, it was hypothesized that IL-1β mediated its effects by disruption of mitochondrial-enzyme activity in islet β-cells. Therefore, we next investigated whether IL-1β modulates mitochondrial aconitase activity essential for Kreb's cycle function. After 48 h of *in vivo* administration, IL-1β induced a considerable decrease in mitochondrial aconitase activity in dispersed pancreatic islet cells (4 ± 0.6 pmol/100 islets·h, $P < 0.0001$), as compared with control cells (22 ± 4 pmol/100 islets·h) (Fig. 3A). These data suggest that disruption of mitochondria of islet cells could be directly dependent on IL-1β secreted by pancreas-infiltrating macrophages associated with β-cell depletion during the acute phase of diabetes.

To rule out the possibility that NO contributes to rat islet cell injury during diabetes, islet cells from *in vivo* IL-1β-treated animals were analyzed for the levels of nitrite+nitrate, as an indirect sign of nitrogen radicals and iNOS involvement. As can

TABLE 1A. ODs (arbitrary units) *in vivo*

Hours	0	12	24	48
Mn SOD	4.5 ± 0.15	7.4 ± 0.23[a]	3.32 ± 0.21	
iNOS				
GAPDH	3.2 ± 0.11	3.0 ± 0.08	3.1 ± 0.01	2.9 ± 0.05

Values are averaged and representative of three different experiments.
[a] $P < 0.001$.

TABLE 1B. ODs (arbitrary units) *in vivo*

Hours	0	12	24	48
Mn SOD	3.48 ± 0.10	3.96 ± 0.11	4.62 ± 0.17	2.1 ± 0.1[a]
iNOS			1.82 ± 0.08	2.24 ± 0.08[a]
GAPDH	3.2 ± 0.05	3.1 ± 0.08	3.1 ± 0.13	2.9 ± 0.14

Values are averaged and representative of three different experiments.
[a] $P < 0.001$.

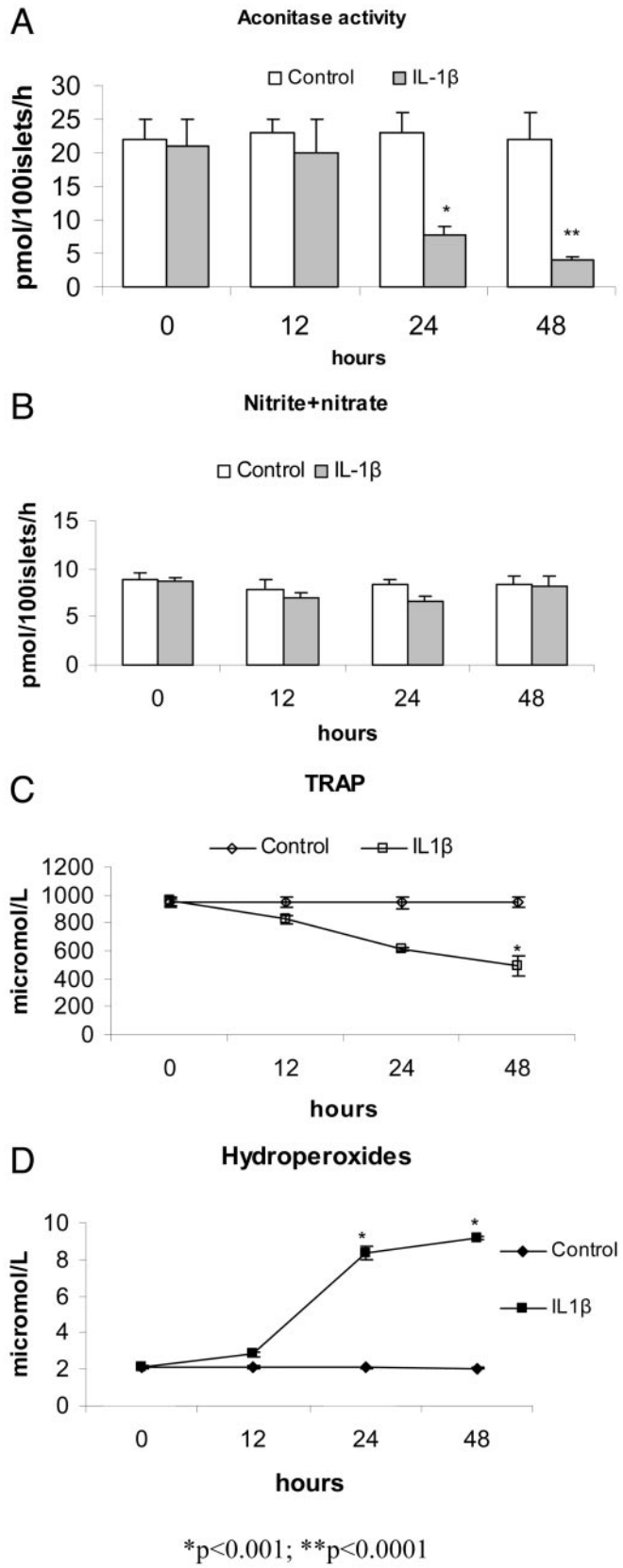

FIG. 3. Mitochondrial aconitase and total antioxidant capability are modulated by *in vivo* IL-1β administration. A, Time course of mitochondrial aconitase activity performed on dispersed pancreatic islet cells from controls and IL-1β-treated rats. B, Nitrite+nitrate levels detected in samples as in A. C, Evaluation of TRAP, at different time points, on plasma collected from rats after *in vivo* IL-1β administration. D, Analysis of hydroperoxide activities on plasma harvested from control and IL-1β-treated animals. Data are mean ± SD of three independent experiments. *, $P < 0.001$; **, $P < 0.0001$.

be observed in Fig. 3B, islet cells were not reactive for nitrite+nitrate, demonstrating that nitrogen radicals are not directly involved in rat islet β-cell destruction.

To better understand the degree of islet β-cell survival and function, including the ability of these cells to counteract oxidative radical injuries, levels of some antioxidant parameters were measured in the plasma of *in vivo* IL-1β-treated rats. TRAP remained unmodified up to 12 h, whereas it significantly decreased at 24 h (606.6 ± 11.5 μM) and 48 h (490 ± 75.5 μM; $P < 0.001$), as compared with control animals at the same time points (943.3 ± 37.8 μM and 950 ± 36.1 μM, respectively) (Fig. 3C). Similarly, the antioxidant ability of hydroperoxides in the plasma, as an index of ongoing lipid peroxidation, was greatly increased at 24 and 48 h ($P < 0.001$ *vs.* controls) (Fig. 3D).

Furthermore, *in vivo* islet β-cell function was followed by monitoring glucose and insulin levels in the peripheral blood of animals exposed to exogenous IL-1β. *In vivo* IL-1β administration substantially increased blood glucose levels after 24 h (8.08 ± 0.16 mM) and 48 h (10.06 ± 0.3 mM, $P < 0.001$ *vs.* controls and time 0) and concomitantly reduced insulin levels (89.8 ± 6.7 mM and 73 ± 9.89 mM, respectively, $P < 0.0001$ *vs.* controls and time 0), further demonstrating the significant impairment of islet β-cell function. (Fig. 4).

The ability of IL-1β to induce β-cell destruction by the apoptotic machinery was tested, on purified pancreatic islets, by a colorimetric protease assay for the analysis of caspase-3, -8, and -9 activity. *In vivo* IL-1β treatment induced significant activity of both caspase-3 and -9, whereas negligible caspase-8 activity was detected (Fig. 5A). In particular, the increased activity of caspase-3 and -9 was detected early, at 12 h and 24 h ($P < 0.001$ *vs.* controls and time 0) and increased dramatically at 48 h (15.36 ± 0.56 mM for caspase-3 and 14.83 ± 0.68 mM for caspase-9, $P < 0.0001$ *vs.* controls and time 0), whereas apoptosis analysis showed an abundant number of apoptotic events at 48 h (70.1 ± 0.4 cell %; $P < 0.0001$) (Fig. 5B). These data confirm that IL-1β indirectly participates in the induction of the mitochondrial apoptotic mechanism in rat islet β-cells.

We further studied the possible effects exerted by the cytokine on the immune system, by monitoring the activity of adhesive molecules. According to our data, cICAM-1 levels, assessed on blood samples from the retroorbital plexus, did not significantly change up to 24 h (Fig. 6A); whereas, at the same time point, MnSOD activity was high (Fig. 1). In contrast, at 48 h, ICAM-1 levels were significantly enhanced, up to values considerably higher than those found in controls and in IL-1β-treated animals at 12 and 24 h (278 ± 23 ng/ml; $P < 0.001$) (Fig. 6A). To determine ICAM-1 expression more accurately, cryostat sections of pancreas from IL-1β-treated animals were analyzed by immunohistochemistry. We found that ICAM-1 was scarcely expressed on islet cells from IL-1β-treated animals at time 0 but potently up-regulated at 24

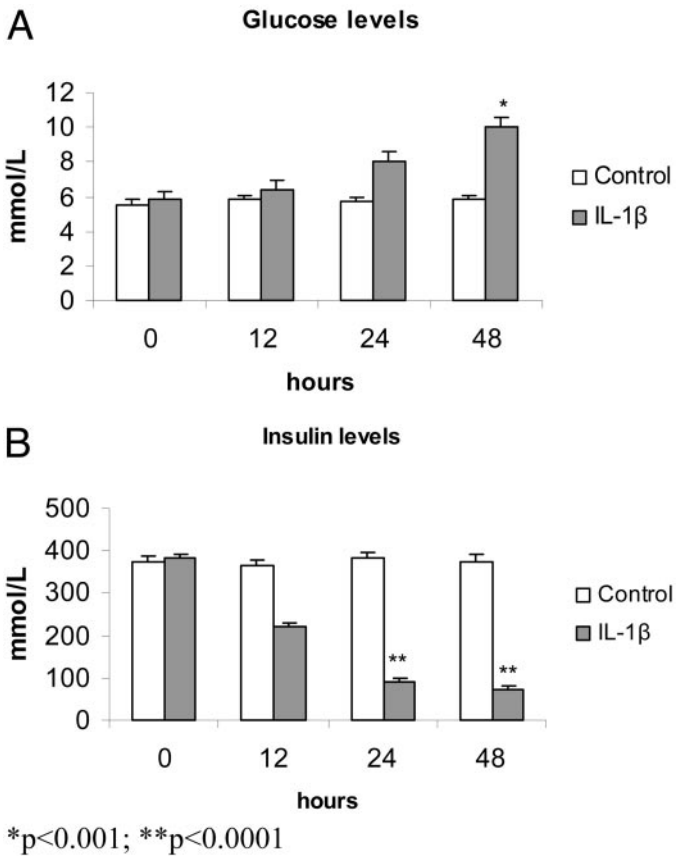

FIG. 4. IL-1β *in vivo* exposure regulates islet β-cell function. Evaluation of glucose (A) and insulin levels (B) on peripheral blood collected from control and IL-1β administered *in vivo* to animals for 0, 12, 24, and 48 h. Data are mean ± SD of three independent experiments. *, $P < 0.001$; **, $P < 0.0001$.

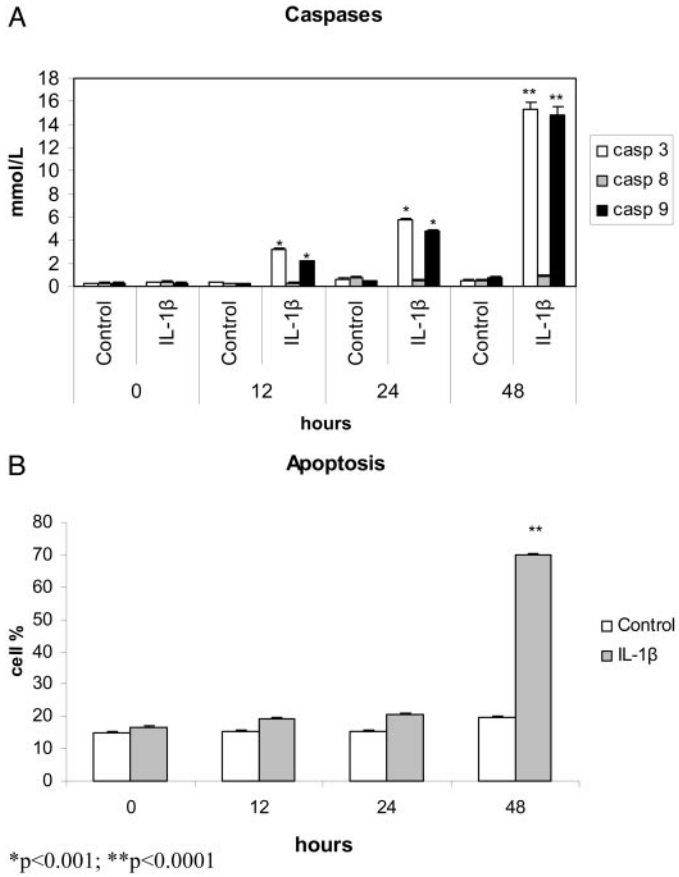

FIG. 5. *In vivo* IL-1β administration induces pancreatic islet cell death. A, Detection of caspase (casp)-3, -8, and -9 activity in freshly purified islets from control and IL-1β-treated rats up to 48 h. B, Cell death percentage of cells treated as in (A) performed by TUNEL assay. Data presented are mean ± SD of three independent experiments. *, $P < 0.001$; **, $P < 0.0001$.

and 48 h (Fig. 6, B and C; Table 2). Thus, IL-1β regulates the mechanism responsible for initiating and amplifying the autoimmune process.

Discussion

Our *in vivo* observations partially agree with previously reported *in vitro* observations (12, 26) of an early, but transient, increase in MnSOD activity after IL-1β administration. In fact, *in vivo* experiments have evidenced a MnSOD induction shorter and less intense than *in vitro* induction (12, 26). MnSOD transcripts after IL-1β challenge *in vivo* were clearly evidenced by RT-PCR after 24 h, but they became undetectable already at 48 h. On the other hand, mRNA transcripts of iNOS were undetectable *in vivo*, whereas they were clearly observed in *in vitro* experiments; similarly, nitrite+nitrate levels were unmodified *in vivo* but significantly increased *in vitro* (26).

The suppression of MnSOD activity *in vivo* at 48 h coincides with the impairment of islet β-cell functions. SOD is a group of enzymes that protects cells against toxic oxygen radicals. These enzymes convert superoxide to molecular oxygen, and hydrogen peroxide is subsequently converted to water and oxygen by catalase and various peroxides. By removing superoxide, SOD prevents the formation of the highly toxic hydroxyl radical that probably explains much of the tissue damage that accompanies superoxide and hydrogen peroxide formation. Indeed, generation of free oxygen radicals has been suggested to mediate IL-1β's action on islet β-cells (8) that normally contain relatively low levels of SOD (5). In fact, streptozotocin-induced diabetes, as well as the spontaneous development of type 1 diabetes in animal models, is associated with lower SOD levels. Administration of scavengers has been found to counteract this reduction (5, 9). It has been recently demonstrated that an improvement of the mitochondrial antioxidant defense status prevents NF-kB activation and iNOS expression in islet β-cells, leading to the thought that MnSOD overexpression may contribute to β-cell survival (27) through the suppression of oxygen free radical formation.

Interestingly, in our experiments, a severe impairment of mitochondrial functions takes place; in fact, mitochondrial aconitase activity was clearly reduced after *in vivo* administration of IL-1β, reinforcing the thought that IL-1β impairs mitochondrial antioxidant enzyme activity. Mitochondrial aconitase is a 4Fe-4S-cluster-containing enzyme involved in the Kreb's cycle and is affected by nicotinamide adenine dinucleotide (NAD+) depletion (28). Moreover, the decrease of TRAP levels, together with the increase of plasma hy-

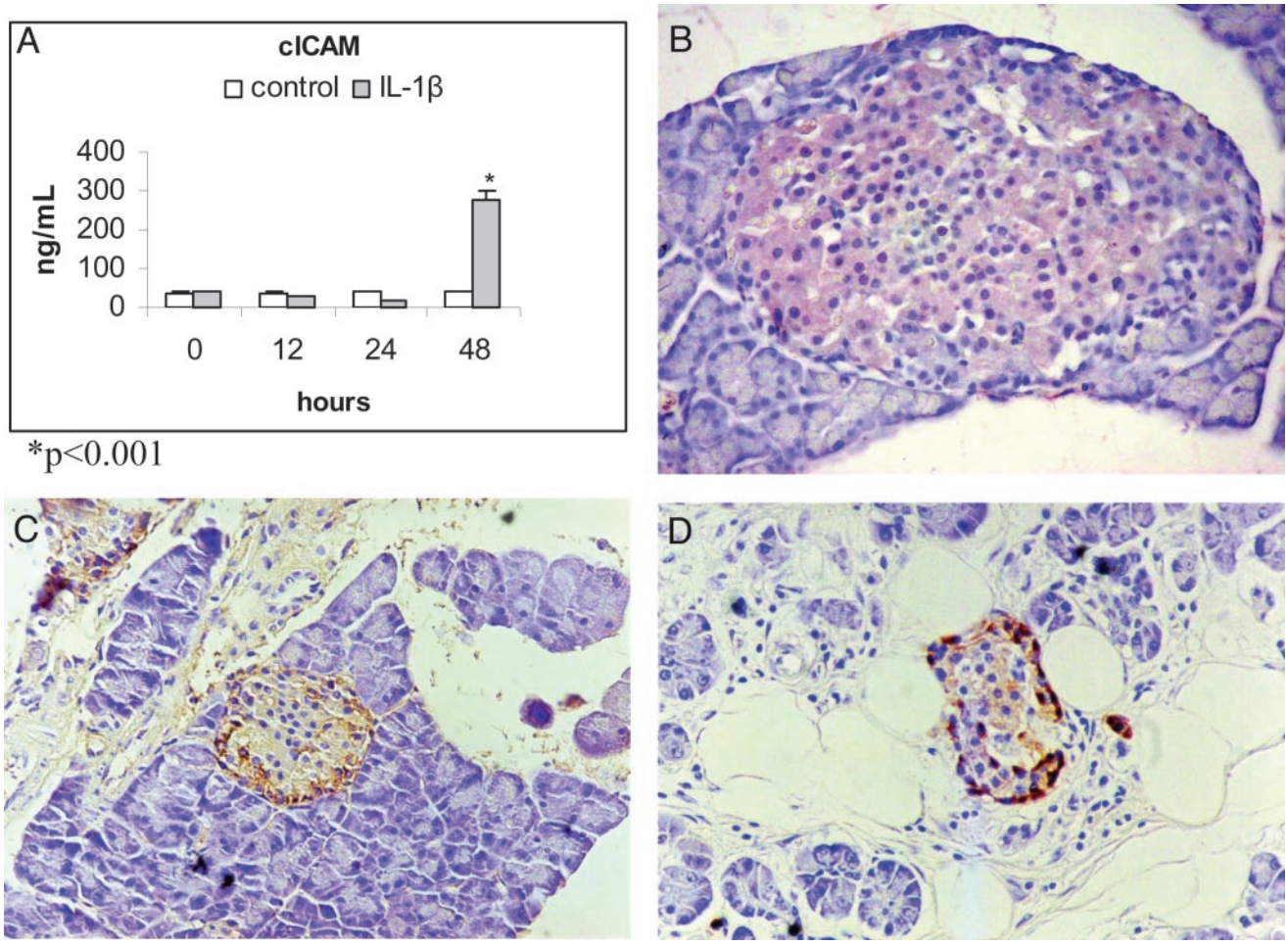

FIG. 6. Expression of ICAM-1 on pancreatic islet cells exposed to IL-1β. A, Kinetics of cICAM-1 levels detected in blood samples from each animal control and IL-1β-treated animals and collected from the retroorbital plexus. B, Immunohistochemical analysis of ICAM-1 on cryostat sections of pancreatic gland from animals exposed to IL-1β for 0 h (B), 24 h (C), and 48 h (C) and revealed by diaminobenzidine (*brown staining*; original magnification, ×300). One representative of three independent experiments is shown.

TABLE 2. ODs (arbitrary units) *in vivo*

Hours	0	12	24	48
ICAM-1			20.4 ± 3.33[a]	12.5 ± 1.77

Values are averaged and representative of three different experiments.

[a] $P < 0.001$.

droperoxide, a signal of ongoing lipoperoxidation, is consistent with the evidence that this cytokine, administered *in vivo*, acts by impairing all antioxidant functions, including those within islet β-cells. The highest levels of lipoperoxidation were quickly reached, and this was soon followed by a significant increase of apoptotic islet cells. Alterations of antioxidant function were detected in plasma in our experiments; moreover, they were mainly not evident until 24 h, whereas the increase in caspase activity and the decrease in insulin levels were already observed within 12 h. We and other researchers have previously reported, in several studies, that oxygen free radicals may contribute to the islet β-cell destruction in type 1 diabetes animal models and have suggested that free radical scavengers significantly increased SOD, catalase, and glutathione peroxidase and reduce the antioxidant status impairment, although they were not able to fully counteract the progression of the disease (5–9, 29). Thus, the impairment of antioxidant function may serve to worsen the alterations that have been initiated by IL-1β, but it does not represent the initiating event.

These above-reported alterations happen without iNOS and nitrite+nitrate changes. This leads to the observation that IL-1β-induced death *in vivo* is not related to the iNOS pathway.

Interestingly, *in vivo* administration of IL-1β up-regulates adhesion molecules. In particular, the levels of cICAM-1 were first decreased after 24 h and were enhanced, together with islet-cell expression of adhesion molecules. Adhesion molecule enhancement has been found in inflammatory and immune disorders (16–18), and up-regulation is clearly associated with ongoing dysregulation of the immune system. The latter is of significance because of the involvement of the cytokine in immunity.

In summary: 1) IL-1β-induced MnSOD activation takes place also when the cytokine is administered *in vivo*, as *in vitro*, although this transient induction, of lower intensity, is already and completely reversed by 48 h; 2) the cytotoxicity

of IL-1β, when administered *in vivo*, does not involve the activation of iNOS; 3) plasma lipid peroxidation and then apoptosis of islet β-cells are observed; 4) caspase-3 and -9 activity was increased very early on, thus the mitochondrial pathway is involved during islet β-cell death; and 5) adhesive molecules are up-regulated in islet cells, leading to the involvement of the immune system.

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