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An Imidazoline Compound Completely Counteracts Interleukin-1β toxic Effects to Rat Pancreatic Islet β Cells

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Abstract

Background: *In vitro* studies have demonstrated that interleukin (IL)-1β decreases insulin and DNA contents in pancreatic islet β cells, causing structural damage, that it is toxic to cultured human islet β cells and that it is able to induce apoptosis in these cells.
Materials and Methods: Isolated rat islets of Langerhans were exposed *in vitro* to interleukin (IL)-1β and either the imidazoline compound RX871024 (RX) or/and M40403, an Mn-containing superoxide dismutase mimetic (MnSODm).
Results: Insulin secretion, on days 1, 2 and 3 after challenge with 3 ng/ml of IL-1β, was almost abolished and this was accompanied by an early increase in MnSOD activity. By days 2 and 3, SOD activities were lower than those of untreated controls and NO significantly increased by day 2. Moreover, IL-1β induced a significant increase in MnSOD transcripts, while iNOS mRNA appeared by days 2 and 3 when MnSOD mRNA was absent. RX blocked all toxic effects of IL-1β by maintaining insulin secretion and islet β cell phenotype, including the inhibition of nonspecific proteins and of iNOS induction. In contrast, the MnSODm, failed to counteract iNOS induction as well as the reduced insulin secretion.
Conclusions: In summary, our findings stress that IL-1β-induced suppression of insulin secretion may be related to iNOS induction in β cells and that RX can reverse this effect, by maintaining insulin secretion. Oppositely, the MnSODm is not able to restore IL-1β-suppressed insulin secretion. Hence, imidazoline compounds may protect β cells against damage caused by IL-1β-induced free oxygen and nitrogen radicals.

Introduction

In vitro studies have demonstrated that interleukin (IL)-1β decreases insulin and DNA contents in pancreatic islet β cells, ultimately causing structural damage (1–3). IL-1β has also been shown to be toxic to cultured human islet β cells and to induce apoptosis in these cells (4,5). The cytotoxic effect of IL-1β is potentiated by other cytokines such as tumour necrosis factor and interferon γ (6,7).

Free radicals formed in islet β cells during immunoinflammatory diabetogenesis may be involved in cytokine-mediated toxicity (8). For example, administration of superoxide dismutase (SOD), the first cellular defense against toxic free radicals, protects β cells from damage induced by alloxan and/or streptozotocin (9–13). In addition, β cells contain low levels of SOD, and these further decrease in mice rendered diabetic by treatment with multiple low-doses of streptozotocin (9,14–17).

Recombinant IL-1β induces MnSOD in rat pancreatic islets, possibly through a direct action on gene transcription (18). IL-1β also damages mitochondrial DNA in NOD mouse β cells, and this is at least in part reduced by aminoguanidine administration, suggesting that inhibition of the inducible form of nitric oxide synthase (iNOS) blocks IL-1β-induced damage to these cells (19,20). Indeed, it has been shown that endogenous NO induced by IL-1β in rat islets causes significant DNA damage (21). On the other hand, it has been demonstrated that imidazoline compounds, which are known to promote insulin secretion and raise diacylglycerol levels in rat pancreatic islets, may protect against IL-1β-induced β-cell apoptosis (22–24).

In particular, compounds with an imidazoline moiety are known to promote insulin secretion and release by blocking ATP-dependent K^+ channels, with a subsequent increase of cytoplasmic free Ca^2 concentration (22). These compounds for both their effects on insulin and Ca^{2+} concentration are of high interest because disturbances in Ca^{2+} homeostatic control can trigger apoptosis (25). On the other hand,

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IL-1β among the cytokines is the main mediator of β-cell dysfunction (26) and acts upon islet β cell by triggering numerous signals, including NO formation, protease activity and insulin secretion in the cell (27).

Since it is still uncertain to what extent free radicals may contribute to the toxic effect of IL-1β on β cells, we studied the effects of an imidazoline compound and an Mn-containing superoxide dismutase mimetic (MnSODm) on the *in vitro* responses of isolated rat islets to IL-1β with emphasis on the effects on the transcriptional levels and activities of MnSOD, CuZnSOD and total SOD, and expression of iNOS and nitric oxide production.

Materials and Methods

Isolation and In Vitro Culture of Islets

Wistar rats, aged 10–12 weeks, were housed in our facility under standard laboratory conditions with free access to food and water. The islets were isolated as previously described (17). Each pancreas was removed and suspended in buffered Hank's solution at 4°C, then dissected free from extraneous fat and minced with scissors. The tissue was incubated at 37°C with vigorous shaking for 15–20 min in 5 ml of Hank's solution, containing 1.6 mg/ml of type V collagenase (Sigma, Milan, Italy). After centrifugation (500 g, 15 min), the pellet was washed twice and resuspended in 3 ml Hank's solution in a Petri dish. The islets were isolated on a Ficoll gradient and then handpicked using a stereomicroscope and transferred with a Pasteur pipette (14). The islets were precultured at 37°C in a 5% CO_2 humidified air atmosphere for 3–6 days in RPMI 1640 medium (Sigma), supplemented with 10% fetal calf serum. Free-floating islets, 300 per 3 ml medium, were then cultured for another 3 days with or without 3 ng/ml of IL-1β (Sigma). The islets were also incubated with or without 150 μM the imidazoline compound RX871024 (RX) (Reckitt and Colmar, Kingston upon Hull, UK) (24) or 100 μM of M40403 (Metaphore Pharmaceuticals, St. Louis, MI, USA), a low molecular weight MnSODm, which removes superoxide anions and attenuates several parameters of inflammation (28).

Insulin Levels and Rate of Insulin Secretion

Total insulin levels tested in triplicates in each supernatant were quantitated in mU/ml using a RIA kit (Bio Rad, Milan, Italy), whereas insulin synthesis and release were tested by the procedure described by Ling et al. (29). Briefly, islets were washed with Ham's medium F-10, containing 0.5% BSA and 10 mM HEPES. Labeling was carried outat 37°C in multiwell plates (50 islets in 400 μl of medium) using Ham's F-10 medium, containing10 mM glucose, 1% bovine serum albumin (BSA), 2 mM glutamine, 50 μM IBMX, and 250 μCi/ml L-(3,5-^{3}H) tyrosine (Fluka, Milan, Italy). Insulin synthesis was maximal under these conditions, and total ^{3}H-labelled insulin immunoreactivity thus represents total proinsulin synthesis during the 2 h-incubation period. Supernatants were collected after centrifugation and assayed for secreted ^{3}H-labelled insulin; the rate of insulin secretion was expressed as 10^3 dpm$\cdot 10^3$ islets$^{-1} \cdot 2$ h^{-1}. The islets were washed in Ham's F-10 medium, supplemented with 1 mM L-tyrosine and extracted in 1 ml of 2 mM acetic acid, containing 0.25% BSA. The cell extracts were assayed for ^{3}H-labelled protein, ^{3}H-labelled insulin, and total immunoreactive insulin; the islet insulin content was expressed as ng/10^3 islets.

SOD Activities

Isolated islets were homogeneized (Ultra Turrax mechanical blender) in 100 volumes of 10 mM phosphate buffer (pH 7.4), supplemented with 30 mM KCl, as previously described (30). 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. SOD activities (total SOD, CuZnSOD and MnSOD) were measured in triplicates 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 (INT) to form a red formazan dye. The superoxide dismutase activity was measured by percentage inhibition of xanthine to water and molecular oxygen. The results are given as U/mg of protein, a unit being the degree of inhibition of the reaction. The intra-assay coefficient of variation was <2%. Due to the absence of hemoglobin, there was no need to correct the enzyme activities. The detection limit was 2 U/mg and total immunoreactive insulin; the islet insulin content was expressed as ng/10^3 islets.

Hydroperoxide Levels

Supernatant lipids were extracted, and 3.8 ml of 2:1 (vol/vol) chloroform-methanol mixture was added to 0.2 ml of supernatant. The mixture was vigorously mixed (using a vortex) for 2 min and then 1.0 ml of distilled water, and 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 3,000 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, USA), and resuspended in 100 μl HPLC-grade methanol for hydroperoxide measurement. The hydroperoxide content of supernatant was determined with the FOX Version II assay for lipid ROOHs (FOX2). 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 mmol/l H_2SO_4 to final concentrations of 1 and 2.5 mmol/l, respectively. One volume of this concentrated reagent was added to 9 vol of HPLC-grade

methanol containing 4.4 mmol/l BHT to make the working reagent, which comprised 250 μmol/L 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. TPP in methanol (10 μl of 10 mmol/L) was added **to** the blank samples to selectively reduce ROOHs to hydroxy 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 of 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 supernatant samples was determined as a function of the mean absorbance difference of samples with and without elimination of ROOHs by TPP. Intra- and interassay coefficients of variation for this method were 5.0 and 6.8%, respectively.

Nitrite+nitrate Levels

Isolated islets (150/well) were incubated for 30 min at 37°C in 5% CO_2 in 300 ml KRB medium (25 mM HEPES, pH 7.4, 115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM D-glucose, 0.1% bovine serum albumin). 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 of known nitrite levels. All determinations were made in triplicates. The intra- and interassay coefficients of variations were $<$15%; the detection limit was 2 pmol/islet.

MnSOD and iNOS mRNA Levels

In each of three different experiments, total RNA was extracted from 200–250 rat islets by homogenization in 4 M guanidinium thiocyanate, containing 17 mM sodium N-lauryl-sarcosinate, 25 mM citrate buffer, 0.1 M 2-mercapto ethanol, and a 30% aqueous emulsion of 0.1% of Antifoam A (Sigma). RNA was precipitated with ethanol, pelleted, and re-extracted with 8M guanidine hydrochloride: 0.5 M EDTA (19:1). After pelleting and drying, the 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, Gibco BRL, Milan, Italy), using oligo $(dt)_{12-18}$ and Moloney murine leukaemia virus reverse transcriptase (20 U) in a 25 ml reaction at 37°C for 1.5 hours. The solution containing cDNA was diluted 30, 90, and 270 times in sterile water. Semiquantitative, reverse transcriptase polymerase chain reaction (RT-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 dNTP, 2.5 μCi of (α-^{32}P) dCTP (3,000 Ci/mmol; DuPont-NEN, Milan, Italy), 1 U of AmpliTaq (Perkin-Elmer/Cetus, Monza, Italy), and 3 mM $MgCl^2$), as previously described (31,32).

The oligonucleotide primer sequences for Mn-SOD were:

5′-ATTAACGCGCAGATCATGCAG-3′ (forward), and
5′ TTTCAGATAGTCAGGTCTGACGTT-3′ (reverse)

The oligonucleotide primer sequences for CuZn-SOD were:

5′-TTCGAGCAGAAGGCAAGCGGTGAA-3′
(forward), and
5′ AATCCCAATCACACCACAAGCCAA-3′ (reverse).

The oligonucleotide primer sequences for iNOS were:

5′-AGCTTCTGGCACTGAGTAAAGATA-3′ (forward), and
5′TTCTCTGCTCTCAGCTCCAAG-3′ (reverse).

Cyclophilin was used as a positive control; the primers were:

5′-GACAGCGAAACTTTCCGTGC-3′ (forward), and
5′-TCCAGCCACTCAGTCTTGG-3′ (reverse).

The RT-PCR analyses were made in triplicate using a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA), in which samples underwent a 10-min initial denaturing step to activate the DNA polymerase, followed cycling 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C for 35 cycles. Each gene of interest was amplified along with an internal control gene (cyclophilin). The amplification products, in a first set of experiments were separated on a 1.5% agarose gel containing ethidium bromide, and compared with DNA reference markers, in order to validate the RT-PCR analysis of each gene and to adjust the PCR conditions (ie., number of cycles). In the subsequent experiments, done in triplicates, PCR products were separated on a 6% polyacrylamide gel in Tris borate EDTA (TBE) buffer. Band intensities were quantified with an Ultrascan XL Enhanced Laser densitometer (LKB, Bromma, Sweden) and expressed in arbitrary units of optical density (OD). The amount of each product was expressed relative to the internal control gene. PCRs were performed on RT-negative samples to exclude genomic DNA contamination for each cDNA preparation.

MnSOD and iNOS Protein Synthesis

Proteins were analyzed by Western blot at the end of each experiment. Newly synthesized proteins were labelled at 37°C for 2 h in 200 μl Ham's F10 medium, containing 10 mM glucose, 1% BSA, 2 mM glutamine, 1 mM arginine and 50 μCi/ml L-(3,5-^{3}H)-tyrosine (Invitrogen, Milan, Italy). After culture, the islets were washed and extracted for 3 H-labelled protein as mentioned above. Islets were sonicated in 50 μl 5% SDS, 5% β-mercaptoethanol, 80 mM TrisCl (pH 6.8), 5 mM EDTA, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride, run on 10% SDS-polyacrylamide gels, and then electrically transferred to nitrocellulose filters. The filters were incubated with the following antibodies to rat proteins: rabbit anti-iNOS, mouse anti-heat shock protein 70 (HSP70) (1:1,000) (Sigma), rabbit anti-HO-1 (all from Sigma), rabbit anti-MnSOD (kind gift of Laboratory "L. Califano", University of Naples, Federico II°, Naples, Italy), rabbit anti-GLUT2 (Dako, Milan, Italy), rabbit anti-pancreatic and duodenal homeobox gene (PDX)-1, or goat anti-β actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-linked Ig were used as secondary antibodies, and the bound peroxidase activity was detected by enhanced chemiluminescence (Amersham, Bucks, Buckinghamshire, UK). The intensities of the bands were quantitated in an Ultrascan XL Enhanced Laser Densitometer (LKB, Bromma, Sweden) and expressed in arbitrary units of optical density (OD).

Statistical Analysis

Student's t test and ANOVA were used for statistical analyses. P values <0.05 were considered statistically significant.

Results

Effects of IL-1β, RX871024 and M40403 on Total Islet Insulin Secretion

Insulin secretion was almost blocked by 3 ng/ml of IL-1β both with regards to the pre-experimental level ($p < 0.001$) and those on days 1, 2 and 3 of the corresponding untreated controls (Fig. 1). The imidazoline compound RX, but not the MnSODm M40403, reversed this effect and, indeed, significantly ($p < 0.001$) increased the insulin levels compared to controls. When both the imidazoline compound and the SODm were added to IL-1β cultured islets, the total islet insulin secretion was slightly lower with respect to the levels found after the use of RX by alone, but higher than those observed using the SODm.

Effects of IL-1β, RX871024 and M40403 on Islet SOD Activities

As shown in Figure 2, the MnSOD activities in rat islets challenged with IL-1β increased considerably on day 1 ($p < 0.001$ compared to day 0 and corresponding day 1 control), but decreased significantly by days 2 and 3 compared to their respective controls. In contrast, IL-1β induced significantly lower levels of CuZnSOD already by day 1 ($p < 0.001$ compared to day 0 and control), and these levels remained significantly lower than those of controls. Total SOD declined considerably after 3 days of IL-1β treatment to levels considerably below those of controls ($p < 0.001$ compared to day 0 and controls).

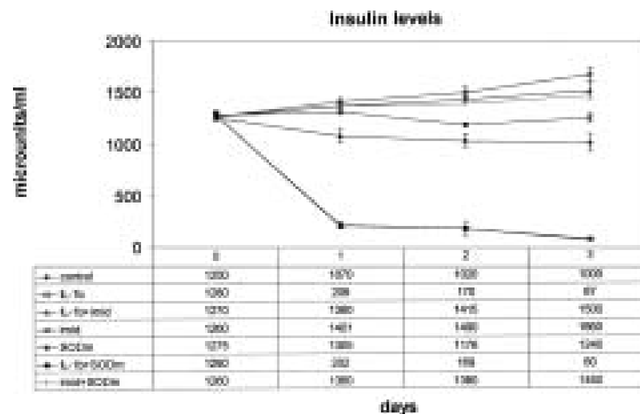


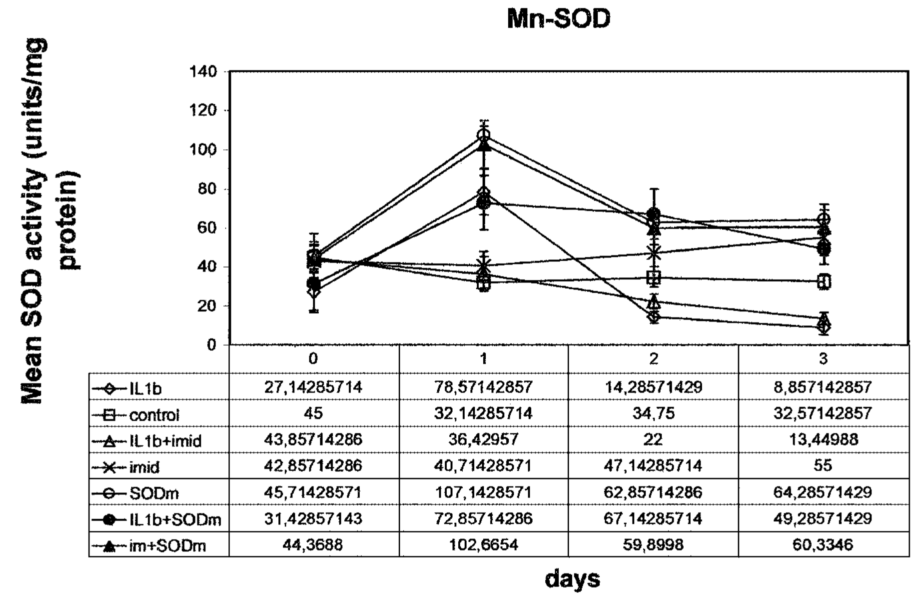
Fig. 1. Effects of IL-1β, RX871024 and M40403 on total islet insulin secretion. Islets were isolated from Wistar rats and treated with vehicle alone as controls, IL-1β, RX871024, IL-1β + RX871024, M40403, IL-1β + M40403 or IL-1β + RX871024 + M40403. Data are shown as means ± SD; n = 7. *$p < 0.001$ compared with controls.

Figure 2 also shows that while RX by itself failed to alter the SOD levels, the imidazoline compound counteracted the effect of IL-1β on all SOD levels throughout the course of the studies. When both imidazoline and the SODm were used together with IL-1β, MnSOD activity resulted enhanced with values comparable to those found using the SODm alone.

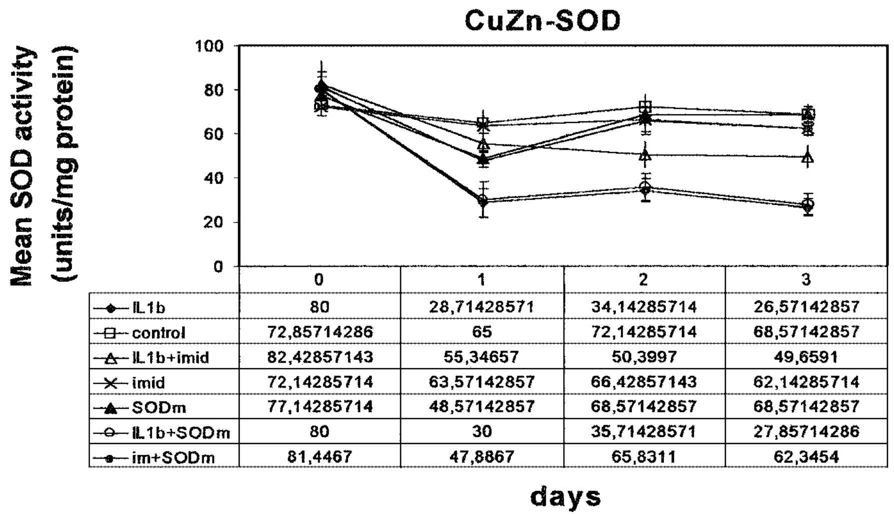
We have also tested the effects of IL-1β on lipid peroxidation by detecting hydroperoxide levels in the supernatants. In this study, the hydroperoxides remained low during the first two days of incubation and treatment with the cytokine (Fig. 3). Then they dramatically increased, up to the end of the culture period (day 6); $p < 0.0001$ vs controls and days 0–2). The imidazoline compound does not exert effects upon lipoperoxidation and, when added to the cytokine, completely counteracts its effects upon free radicals and hydroperoxides (Fig. 3). Oppositely, the SODm is not able to counteract the effects of IL-1β, and, in addition, by itself, is capable of increasing hydroperoxides ($p < 0.001$ vs day 0 and controls).

Effects of IL-1β, RX871024 and M40403 on Islet Nitric Oxide Production

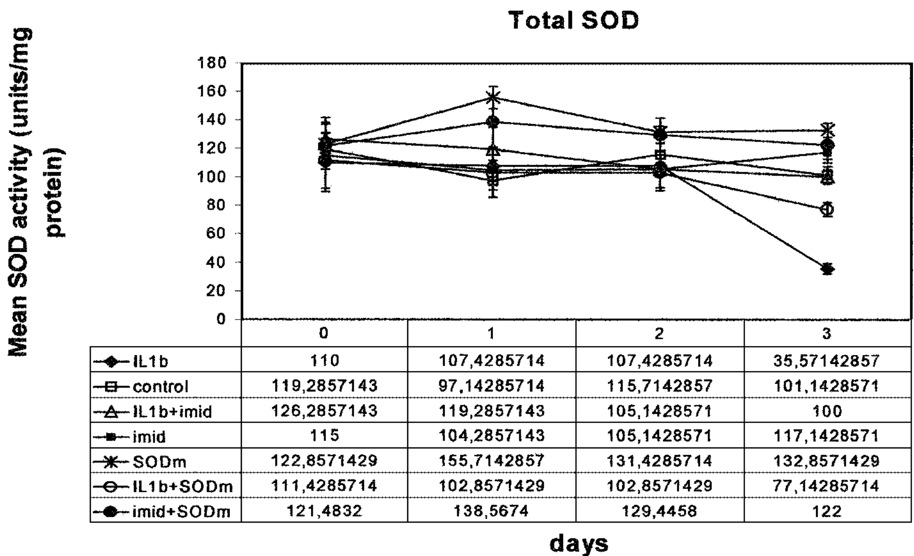
As shown in Figure 4, the levels of nitrite+nitrate increased dramatically by days 2 and 3 ($p < 0.001$ compared to day 0 and controls). This effect was blocked by simultaneous exposure to RX.



	0	1	2	3
IL1b	27,14285714	78,57142857	14,28571429	8,857142857
control	45	32,14285714	34,75	32,57142857
IL1b+imid	43,85714286	36,42957	22	13,44988
imid	42,85714286	40,71428571	47,14285714	55
SODm	45,71428571	107,1428571	62,85714286	64,28571429
IL1b+SODm	31,42857143	72,85714286	67,14285714	49,28571429
im+SODm	44,3688	102,6654	59,8998	60,3346



	0	1	2	3
IL1b	80	28,71428571	34,14285714	26,57142857
control	72,85714286	65	72,14285714	68,57142857
IL1b+imid	82,42857143	55,34657	50,3997	49,6591
imid	72,14285714	63,57142857	66,42857143	62,14285714
SODm	77,14285714	48,57142857	68,57142857	68,57142857
IL1b+SODm	80	30	35,71428571	27,85714286
im+SODm	81,4467	47,8867	65,8311	62,3454



	0	1	2	3
IL1b	110	107,4285714	107,4285714	35,57142857
control	119,2857143	97,14285714	115,7142857	101,1428571
IL1b+imid	126,2857143	119,2857143	105,1428571	100
imid	115	104,2857143	105,1428571	117,1428571
SODm	122,8571429	155,7142857	131,4285714	132,8571429
IL1b+SODm	111,4285714	102,8571429	102,8571429	77,14285714
imid+SODm	121,4832	138,5674	129,4458	122

Fig. 2. Effects of IL-1β, RX871024 and M40403 on islet SOD activities. The experiments were carried out as in Fig. 1.

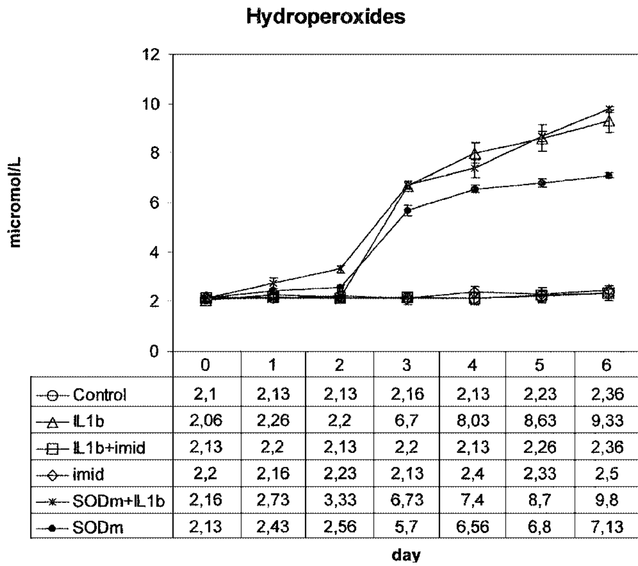


Fig. 3. Effects of IL-1β, RX871024 and M40403 on hydroperoxide levels. The experiments were carried out as in Fig. 1.

Effects of IL-1β, RX871024 and M40403 on SOD and iNOS mRNA Levels

RX by itself failed to induce MnSOD, CuZnSOD or total SOD transcripts, both when compared to the pre-experimental transcript levels and to those in controls (data not shown). The compound also failed to induce iNOS mRNA. On the other hand, M40403 by itself induced transcripts of MnSOD (OD = 4.1) as well as of iNOS, though in low amounts (OD = 1.35); OD for cyclophilin mRNA was 4.2.

As shown in Figure 5A, IL-1β induction of Mn-SOD mRNA was most pronounced on day 1 with a more than 3-fold increase in intensity measured by image analysis ($p < 0.01$ vs. day 0). The MnSOD transcripts disappeared by day 3. The CuZnSOD mRNA patterns gradually diminished to barely measurable levels by day 3. In contrast, the transcript for iNOS was absent on day 1 but gradually became very pronounced on day 3, at a time where signs of structural damage of IL-1β are detectable (2,3). Thus, when IL-1β is added to isolated islets in culture, induction of iNOS takes place only when MnSOD activity is undetectable and at a time where the toxic effect of IL-1β becomes apparent.

As shown in Figure 5B, islets cultured with both IL-1β and RX showed an intense MnSOD expression, also on days 2 and 3, where MnSOD expression induced by IL-1β alone is waning (Fig. 5A). This shows that the imidazoline compound is capable of maintaining MnSOD expression even in the presence of IL-1β. Figure 5B also shows that RX suppressed IL-1β induction of iNOS by days 2 and 3.

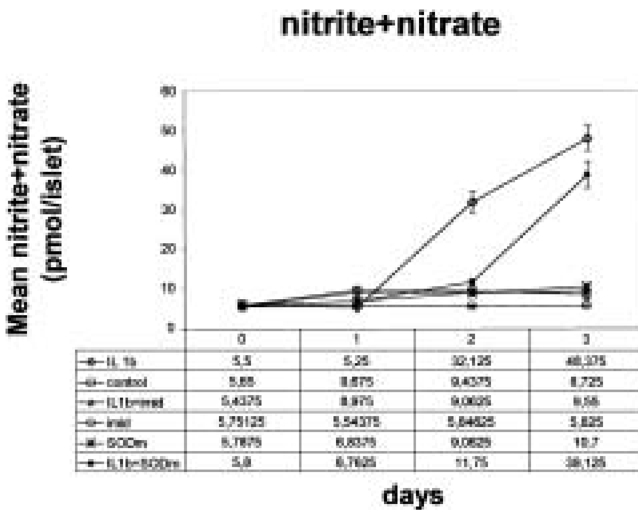


Fig. 4. Effects of IL-1β, RX871024 and M40403 on islet nitric oxide production. The experiments were carried out as in Fig. 1.

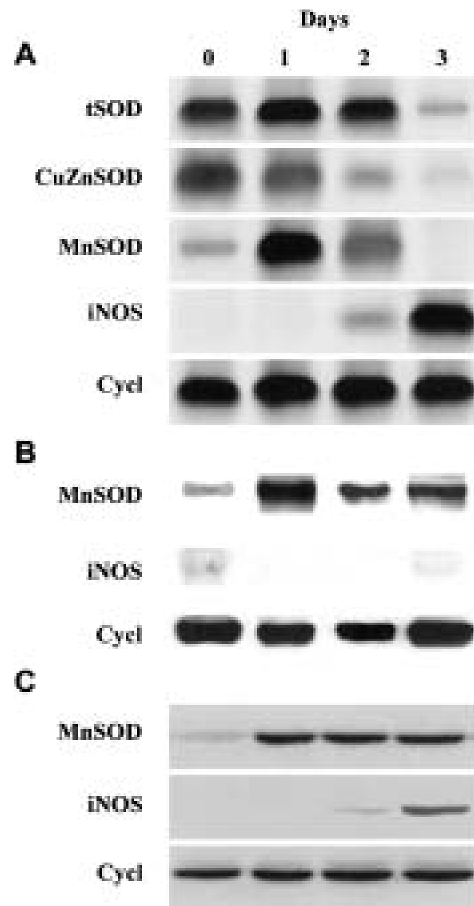


Fig. 5. Effects of IL-1β, RX871024 and M40403 on SOD and iNOS mRNA levels. The experiments were carried out as in Fig. 1. The panels show representatives of three independent experiments measuring transcripts of total SOD (tSOD), CuZnSOD, MnSOD and iNOS measured by RT-PCR in the panel A and transcripts of MnSOD and iNOS in panels B and C. Transcripts for cyclophilin (Cycl) were used as controls. **(A)** Cultures exposed to IL-1β. **(B)** Cultures exposed to IL-1β + RX871024. **(C)** Cultures exposed to IL-1β + M40403.

Table 1. Effects of IL-1β, RX871024 and M40403 on insulin content and rate of insulin secretion

Treatment	Islet Insulin Content (ng/10^3 cells)	Rate of Insulin Secretion (10^3 dpm$\cdot 10^3$ cells$^{-1} \cdot 2h^{-1}$)
a) Controls	16.5 ± 2.4	15.0 ± 0.9
b) +IL-1β	10.5 ± 2.6 ($p < 0.05$ vs. a)	11.0 ± 0.9 ($p < 0.01$ vs. a)
c) +IL-1β + RX871024	18.1 ± 3.4 ($p < 0.05$ vs. b)	18.6 ± 3.6 ($p < 0.02$ vs. b)
d) +IL-1β + M40403	11.0 ± 1.7 ($p < 0.05$ vs. c)	11.5 ± 1.6 ($p < 0.05$ vs. c)
e) +IL-1β + RX871024 + M40403	12.4 ± 2.2 ($p < 0.05$ vs. c)	13.6 ± 2.4 ($p < 0.05$ vs. c)

Data are means ± SD of three independent experiments, each performed in triplicate.

As shown in Fig. 5C, islets cultured with both IL-1β and M40403 showed a moderate but largely unaltered MnSOD expression on days 1 to 3. M40403 was unable to counteract the IL-1β stimulation of iNOS by days 2 and 3. Adding to IL-1β-cultured islets both the imidazoline compound and the SODm, the results (data not shown) were comparable with those obtained using only the SODm.

Effects of IL-1β, RX871024 and M40403 on Islet Synthesis and Release of Insulin

As shown in Table 1, IL-1β significantly decreased both insulin content and rate of secretion. This effect was counteracted and even overcompensated by RX. The table also shows that M40403 was unable to counteract the suppressive effect of the cytokine and that the use of both the imidazoline compound and of the SODm only partially reduces the effects of RX.

Effects of IL-1β, RX871024 or M40403 on MnSOD and iNOS Protein Synthesis

As shown in Table 2, IL-1β increased the synthesis of both MnSOD and iNOS proteins, and addition of RX slightly (MnSOD) or completely reversed (iNOS) this effect. Moreover it decreased the levels of GLUT2 and PDX-1 proteins. When M40403 was added to these cultures, the levels of MnSOD were even higher than in cultures with IL-1β alone, and the levels of iNOS were significantly elevated compared to those in cultures of IL-1β-exposed islets added RX alone. Interestingly, the imidazoline compound RX was able to maintain at high levels the expression of islet β cell specific proteins, including GLUT2 and PDX-1: in fact their levels were always comparable to those found in controls or slightly higher, further demonstrating such a protective effect on islet β cells. The latter is of some evidence in order to understand the mechanism of action of RX. Actually it exerts its activity upon islet β cells by blocking two main IL-1β effects, including insulin impairment and production of islet β cell non-specific proteins. Both abilities of the imidazoline compound in counteracting those toxic effects of IL-1-β are addressed to preserve the normal islet β cell phenotype. When the SODm and the imidazoline are used together, the effects on the protein synthesis are almost comparable to those obtained using the SODm alone.

Discussion

SOD is a group of enzymes known to protect 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 peroxidases. By removing superoxide, SOD prevents the formation of the highly toxic hydroxyl radical, the reaction of which 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 at least part of the toxicity of IL-1β on islet β cells (8,11,12), and these cells contain relatively low levels of SOD (14). Furthermore, streptozotocin-induced diabetes as well as the spontaneous development of type 1 diabetes in animal models are associated with even lower levels of SOD, and administration of scavengers has been found to counteract this reduction (13,15–17).

As inhibition of β cell functions may be related to IL-1β's ability to induce reactive oxygen species, the induction of MnSOD has been thought as part of the initial β cell response to the cytokine. IL-1β also increases the production of nitric oxide, and inhibitors of nitric oxide have been shown by some investigators to counteract the effects of IL-1β on β cells (33,34). It is however still an open question whether nitric oxide is an important mediator of the toxic actions of IL-1β, although recent studies show that stable

Table 2. Effects of IL-1β, RX871024 or M40403 on MnSOD and iNOS protein synthesis

Treatment	MnSOD (OD)	INOS (OD)	β-actin (OD)
a) Controls	20 ± 1.3	0	11.8 ± 0.4
b) +IL-1β	45.8 ± 3.1 ($p < 0.001$ vs. a)	17.5 ± 2.2 ($p < 0.001$ vs. a)	12.7 ± 2.1
c) +IL-β + RX871024	37 ± 2.4 ($p < 0.02$ vs. b)	0.3 ± 0.5 ($p < 0.001$ vs. b)	12.5 ± 2.0
d) +IL-1β + M40403	64 ± 6.1 ($p < 0.02$ vs. b)	13.2 ± 3.0 ($p < 0.02$ vs. b)	12.7 ± 2.1
e) +IL-1β + RX871024 + M40403	62 ± 7.8 ($p < 0.002$ vs. c)	9.8 ± 2.2 ($p < 0.002$ vs. b)	12.2 ± 2.0
Treatment	**GLUT2 (OD)**	**PDX-1 (OD)**	**β-actin (OD)**
a) Controls	34 ± 2.5	29 ± 3.2	12.6 ± 0.6
b) +IL-1β	10.1 ± 1.1 ($p < 0.001$ vs. a)	6.5 ± 0.8 ($p < 0.001$ vs. a)	12.8 ± 1.1
c) +IL-1β + RX871024	39 ± 2.0 ($p < 0.001$ vs. b)	30.3 ± 1.5 ($p < 0.001$ vs. b)	12.0 ± 1.0
d) +IL-1β + M40403	16.4 ± 2.1 ($p < 0.01$ vs. b)	11.6 ± 1.2 ($p < 0.002$ vs. b)	13.2 ± 1.5
e) +IL-1β + RX871024 + M40403	21.8 ± 3.8 ($p < 0.01$ vs. C)	18.2 ± 2.0 ($p < 0.01$ vs. c)	13.2 ± 1.8

Data are means ± SD of three independent experiments, each performed in triplicate.

expression of MnSOD reduces the production of nitric oxide and prevents IL-1β-induced damage to insulinoma cells (31). It has been also suggested that activation of SOD and subsequent removal of superoxide might also reduce the accumulation of nitric oxide-derived toxic products induced by IL-1β (18,35). Other data indicate that nitric oxide may be toxic to cells by combining with superoxide (36). This would lead to the formation of peroxynitrite anions, which decompose when protonated into the toxic hydroxyl radical. CuZnSOD seems to be involved in the scavenging of superoxide anions as well. In contrast to MnSOD, however, it does not appear to be associated with nitric oxide induced toxicity (20,31).

In this study we have also demonstrated that an increase of hydroperoxides, which are signals of an ongoing lipoperoxidation, takes place, evidencing that the cytokine acts upon islet β cells by involving hydroxyl radicals, which lead to an increase of lipoperoxidation. In particular, we have evidenced that the imidazoline compound is capable of counteracting the effects of the cytokine upon free radicals and hydroperoxides, while the SODm does not. In addition, an increase of lipoperoxidation is also seen when adding the SODm alone to cultures. This demonstrates that an increase in superoxide removal without an increase of catalase and glutathione peroxidase activities, lead to an accumulation of the bioproducts of the MnSOD, namely H_2O_2 and O_2, which can explain the increase in hydroperoxides and lipoperoxidation.

The present *in vitro* observations agree with and extend previous findings of an early increase in MnSOD activity after exposure of isolated islets to IL-1β (18,37,38). We could confirm that MnSOD activity increased transiently upon *in vitro* challenge with 3 ng/ml of IL-1β. However, the activity of the enzyme fell significantly after 2 and 3 days of exposure to the cytokine, coinciding in time with the impairment of β cell functions (11). These observations were substantiated by the pronounced levels of MnSOD transcripts found during the first day of culture with IL-1β and the subsequent gradual decrease to levels below detection. Moreover, IL-1β increased significantly the levels of both iNOS mRNA and nitrite+nitrate only after 2 days of culture, at the time where the MnSOD transcripts disappeared and the enzyme fell to barely detectable levels.

An imidazoline compound, RX871024, completely counteracted all the negative effects of IL-1β, ie., on insulin total levels, rate of secretion and on islet insulin contents, as well as on iNOS and nitrite+ nitrate levels. This agrees with the recently described

ability of RX to inhibit the apoptotic effect of IL-1β by decreasing NO production and by reducing iNOS expression (24). On the other hand it has been also shown that imidazoline compounds by itself do not trigger apoptosis and are not toxic for islet β cells (24). However, RX in our study did not enhance, by itself, MnSOD activity, suggesting that the ability of IL-1β to induce MnSOD is linked to processes not affected by imidazoline. This is further substantiated by the failure of M40403, an Mn-containing superoxide dismutase mimetic (MnSODm) to counteract the cytotoxic effect of the cytokine even though it enhanced MnSOD. Furthermore, RX efficaciously counteracted IL-1β-induced synthesis of iNOS, but not of MnSOD, at the protein level and maintained or slightly enhanced the levels of islet β cell specific proteins, including GLUT2 and PDX-1. Taken together these findings highly evidenced that the imidazoline compound exerted a direct effect upon insulin secretion and β-cell activity and specific function, in agreement with the insulinotropic activity recently found in the GK rat (39). Interestingly, the imidazoline compound RX was able to maintain at high levels the expression of islet β cell specific proteins, including GLUT2 and PDX-1: this is of some relevance to better understand the mechanism of action of the compound. Actually, it exerts its activity upon islet β cells by early blocking IL-1β cytotoxic effects, such as insulin impairment and islet β cell nonspecific protein induction. RX activity is, therefore, addressed to preserve the normal islet β cell phenotype. The latter must be taken into consideration also in view of the anti-inflammatory activity of imidazoline agents recently found by some authors (40). In fact, it has been observed that imidazoline compounds exert their anti-inflammatory activity inhibiting iNOS induction, as well as NO levels, by a direct action on intracellular signal transduction (40).

We can stress that our results have demonstrated that imidazoline, other than promoting insulin production/secretion, was able to counteract the IL-1β-induction of iNOS and NO. Moreover, imidazoline, by maintaining an islet β cell differentiate phenotype, possibly exerted through its insulinotropic activity, seems to be capable to give the cell a lesser susceptibility to cytotoxicity, also through maintaining high MnSOD activity.

The present findings make RX871024 and most likely other imidazoline compounds interesting candidates for the prevention/treatment also of type 1 diabetes mellitus. We are presently studying the *in vivo* effect of the compound, using the NOD mouse model of type 1 diabetes. In summary, IL-1β, at a dosage causing significant inhibition of insulin secretion, induced iNOS expression and nitrite+nitrate production in β cells. An imidazoline compound effectively counteracted these effects, by stimulating insulin production and secretion and possibly by preventing late MnSOD suppression. This may contribute to cellular resistance to the deleterious effect of IL-1β, also by neutralizing superoxide and nitric oxide generation. The results suggest that imidazoline compounds may be used as pharmacological agents to prevent the development of type 1 diabetes.

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