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Ebselen and Cytokine-Induced Nitric Oxide Synthase Expression in Insulin-Producing Cells

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ABSTRACT. Interleukin-1 (IL-1) may be a mediator of β-cell damage in insulin-dependent diabetes mellitus (IDDM). The IL-1 mechanism of action on insulin-producing cells probably includes activation of the transcription nuclear factor κB (NF-κB), increased transcription of the inducible form of nitric oxide synthase (iNOS) and the subsequent production of nitric oxide (NO). Reactive oxygen intermediates, particularly H_2O_2, have been proposed as second messengers for NF-κB activation. In the present study, we tested whether ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a glutathione peroxidase mimicking compound, could counteract the effects of IL-1β, H_2O_2 and alloxan in rat pancreatic islets and in the rat insulinoma cell line RINm5F (RIN cells). Some of these experiments were also reproduced in human pancreatic islets. Ebselen (20 μM) prevented the increase in nitrite production by rat islets exposed to IL-1β for 6 hr and induced significant protection against the acute inhibitory effects of alloxan or H_2O_2 exposure, as judged by the preserved glucose oxidation rates. However, ebselen failed to prevent the increase in nitrite production and the decrease in glucose oxidation and insulin release by rat islets exposed to IL-1β for 24 hr. Ebselen prevented the increase in nitrite production by human islets exposed for 14 hr to a combination of cytokines (IL-1β, tumor necrosis factor-α and interferon-γ). In RIN cells, ebselen counteracted both the expression of iNOS mRNA and the increase in nitrite production induced by 6 hr exposure to IL-β but failed to block IL-1β-induced iNOS expression following 24 hr exposure to the cytokine. Moreover, ebselen did not prevent IL-1β-induced NF-κB activation. As a whole, these data indicate that ebselen partially counteracts cytokine-induced NOS activation in pancreatic β-cells, an effect not associated with inhibition of NF-κB activation. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1703–1709, 1996.

KEY WORDS. ebselen; nitric oxide; insulin-dependent diabetes mellitus; pancreatic islets; interleukin-β; hydrogen peroxide

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is an anti-inflammatory and antioxidant seleno-organic compound that has been extensively studied in the last decade. The particular interest in this drug resulted from the early observation that ebselen mimics GSH[‖] activities [1]. The reaction catalyzed is the reduction of one hydroperoxide at the expense of thiol. The specificity for substrates ranges from hydrogen peroxide and smaller organic hydroperoxides to membrane-bound phospholipid and cholesterol hydroperoxides [2]. This agent also inhibits the enzymatic activity of isolated iNOS [3], but it is not known if it affects iNOS expression. A number of cytoprotective actions of ebselen have been reported, not only against hydroperoxides but also against a variety of drugs known to trigger oxidative stress [4]. Because many inflammatory diseases, including diabetes mellitus, may be associated with oxidative stress, the therapeutic use of antioxidants such as ebselen seems of interest. Some favorable characteristics of ebselen for future therapeutic use are low toxicity and lack of known adverse effects, metabolic stability due to several cycles of inter conversions and biologic activity residing not only in ebselen itself but in some of its metabolites [4].

Cytokines, particularly IL-1β, have been proposed as potential mediators of β-cell damage in IDDM [5, 6]. *In vitro* exposure of rodent islets to human IL-1β, alone or in combination with TNF-α and IFN-γ, induces suppression and damage to β-cells [5–7]. The deleterious effects of IL-1β in rat islets are related to generation of the radical NO [8–10]. NO forms iron nitrosyl complexes with enzymes containing FeS, such as the Krebs cycle enzyme aconitase, leading to

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‖ *Abbreviations:* IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; NO, nitric oxide; iNOS, inducible form of nitric oxide synthase; ROIS, reactive oxygen intermediates, NF-κB; nuclear factor κB; IDDM, insulin-dependent diabetes mellitus; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; IκB, inhibitory factor κB; PDTC, pyrrolidine dithiocarbamate; BSA, bovine serum albumin; FCS, fetal calf serum; RIN cell, rat insulinoma cell line RINm5F; EMSA, electrophoretic mobility shift assay.

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inhibition of aconitase activity [9], a decrease in glucose and aminoacid metabolism [11, 12] and a decrease in ATP generation [7]. Human islets are more resistant to the suppressive effects of cytokines and NO than are rodent islets [13], and it remains unclear whether NO is the major mediator of cytokine effects in these cells [13–15]. Besides NO, oxygen radicals produced during pancreatic inflammation may also contribute to β-cell death and the development of IDDM [6, 16]. In line with this hypothesis, treatment with antioxidants prevents diabetes in some rodent models of the disease [6, 16].

Although the ultimate mechanism of action of IL-1β on insulin-producing cells remains to be clarified, IL-1β effects depend on gene transcription and protein synthesis [17, 18]. One of the IL-1β-stimulated genes in β-cells encodes iNOS [19], and activation of the transcriptional regulator NF-κB is a necessary step for iNOS mRNA expression in these cells [20–22]. NF-κB is located in the cytoplasm as an inactive complex with IκB. Following activation, IκB is phosphorylated and degraded, thus allowing NF-κB to translocate to the nucleus [23]. Cytokines, including IL-1β, activate one or more signal transduction pathways, leading to the activation of protein kinase(s) that phosphorylates IκB [23]. One of these pathways may involve generation of ROIS, in particular H_2O_2 [24, 25]. The H_2O_2 scavenger PDTC prevents NF-κB activation [26] and iNOS transcription in different cell types [27, 28], including insulin-producing cells [21, 29]. However, this reactive oxygen model of NF-κB activation may be restricted to certain cell types [30].

In this context, it is of interest to characterize further the effects of both ROIS and antioxidant agents on insulin-producing cells. In the present study, we investigated the effects of ebselen on cytokine-induced iNOS expression and NO formation by insulin-producing cells. The protective action of this agent against H_2O_2 and alloxan-induced β-cell dysfunction was also examined.

MATERIALS AND METHODS

Cytokines and Chemicals

Human recombinant IL-1β was a kind gift from Dr. K. Bendtzen (Laboratory of Medical Immunology Rigshospitalet, Copenhagen, Denmark). The cytokine was produced by Immunex (Settle, WA, U.S.A.) and had biological activity of 5×10^7 U/mg. Recombinant human IFN-γ and murine TNF-α (bioactivities of 6.7 U/ng and 10 U/ng, respectively) were purchased from AMS Biotechnology (Sollentuna, Sweden). The chemicals were obtained from the following sources: culture medium RPMI 1640 and FCS from Northumbria Biologicals (Cramlingthon, U.K.); BSA from Miles Laboratories (Slough, U.K.); D-(U-^{14}C)glucose from Amersham (Amersham, U.K.); ebselen from INC Biomedicals (Aurora, OH, U.S.A.); agarose from FMC Bioproducts (Rockland, ME, U.S.A.); Nylon Magnagraph Transfer Membrane for Micron Separation (Westboro, MA, U.S.A.). All other chemicals of analytical grade were obtained from E. Merck (Darmstadt, Germany) or from Sigma Chemical (St. Louis, MO, U.S.A.).

Tissue Culture and Experimental Treatments

Pancreatic rat islets were isolated by collagenase digestion from adult male Sprague-Dawley rats bred in a local colony (Biomedical Centre, Uppsala, Sweden). The islets were cultured free-floating in medium RPMI 1640 containing 11.1 mM glucose and supplemented with 10% (v/v) of FCS [31].

After 5–10 days in culture, groups of 50–100 islets were transferred to new culture dishes containing the same medium as described above. Culture dishes allocated to the experimental groups were supplemented with IL-1β (25 U/mL) or H_2O_2 (200–400 μM) in the presence or absence of ebselen (20 μM, diluted in DMSO, 0.25%, v/v) added 1 hr before cytokine exposure. This ebselen concentration was selected following dose-response studies (see Results), showing that higher concentrations of the drug impaired β-cell function. Moreover, ebselen concentrations in the range of 5–20 μM have been previously shown to significantly decrease iNOS enzymatic activity in endothelial cells [3]. After culture for 6 and 24 hr (IL-1β) or 2 hr (H_2O_2), the islet glucose oxidation rates were evaluated and/or the medium collected for nitrite and insulin measurements. In a separate series of experiments, islets exposed or not exposed to ebselen (20 μM) for 1 hr were transferred to dishes containing Krebs-Ringer bicarbonate buffer supplemented with 2 mg/mL BSA and 10 μM HEPES (KRBH) and glucose 2.8 mM and incubated for another 30 min in presence or absence of ebselen (20 μM). After this, the islets were exposed to alloxan (2.0 mM) or to vehicle only (citrate buffer) for another 30 min [32] and islet glucose oxidation rates were evaluated.

Human pancreata were excised from adult heart-beating organ donors, transported to the Central Unit of the β-Cell Transplant (Medical Campus, Vrije Universiteit Brussels, Brussels, Belgium) and the islets isolated as previously described [33]. The islets were subsequently sent by air from Brussels to Uppsala, Sweden. In Uppsala, the human islets were cultured free-floating in medium RPMI 1640 containing 5.6 mM glucose and supplemented with 10% FCS (v/v), benzylpenicillin (100 U/mL) and streptomycin (0.1 mg/mL), and the medium was changed every 2 days [33]. After 5–6 days in culture, the medium was supplemented with a combination of cytokines (IL-1β, 50 U/mL; IFN-γ, 1000 U/mL; TNF-α, 1000 U/mL) [13] in presence or absence of ebselen (20 μM) and the culture continued for an additional 14 hr. This combination of cytokines was used because IL-1β alone, under conditions that significantly increase nitrite production by rodent islets, does not induce iNOS activity in human islets [13]. Subsequently, the medium was collected for nitrite measurement and the islets retrieved for DNA content determination.

RIN cells were originally obtained from Dr. Å. Lernmark (Seattle, WA, USA). Growing RIN cells were trypsinized

and subcultured in RPMI 1640 and supplemented with 10% (v/v) FCS, as previously described [34]. Exposure of RIN cells to IL-1β (25 U/mL) in the presence or absence of ebselen (20 μM) was performed in RPMI 1640 medium for 6 hr. This time point was selected following previous studies because of the maximal expression of iNOS mRNA after 6 hr exposure to IL-1β [35, 36]. In some experiments, the exposure time was prolonged to 24 hr. After this, medium was retrieved for nitrite determination and the cells used for Northern blot analysis. Cell viability was assessed by the trypan blue exclusion method. In another series of experiments, RIN cells were exposed to IL-1β (25 U/mL) for 20 min in the presence or absence of ebselen and their nuclear proteins extracted for electrophoretic mobility shift assay.

Measurements of Medium Insulin and Nitrite Levels and Islet Glucose Oxidation and DNA Content

For nitrite determinations, triplicate aliquots of the medium (100 μL) were collected and the nitrite determined as previously described [36]. Insulin in the medium was measured by radioimmunoassay according to Heding [37].

For measurements of islet glucose oxidation rates, triplicate groups of 10 islets were transferred to glass vials containing D-(U-$_{14}$C)glucose and nonradioactive glucose to a final concentration of 16.7 mM glucose and a specific activity of 0.5 mCi/mMol; islet glucose oxidation was determined as previously described [38]. Islet DNA content was measured as described by Hinegardner [39].

Northern Blot Analysis

For Northern blot analysis, total RNA was isolated from 10^7 RIN cells by using an RNA Isolation Kit (RNAeasy for total RNA extraction, Qiagen, CA, USA). Following extraction, the RNA samples (20 μg) were electrophoresed on a 1% agarose gel containing formaldehyde. After acridine orange staining of the gel to assure similar sample loading, the RNA was transferred to a nylon membrane [35] and hybridized to a ^{32}P-labeled cDNA probe coding for the inducible form of NOS cloned from a murine macrophage cell line [40] (a kind gift from Dr. J.M. Cunningham, Hematology-Oncology Division, Harvard Medical School, Boston, MA, USA). Hybridization to human GAPDH (cDNA probe obtained from American Type Culture Collection, Rockville, MD, USA) or 18 S ribosomal RNA (performed with a labeled rat genomic DNA clone [41], kindly provided by Dr. S. M. Morris, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA) were used as internal controls. Hybridization and autoradiography were performed as previously described [35]. The blots were evaluated by densitometric scanning with a Quick Scan Jr. Densitometer (Helena Laboratories, Beaumont, TX, USA).

EMSA

Three times 10^6 RIN cells were exposed for 20 min to IL-1β with or without ebselen (added 1 hr before IL-1β) and washed twice with cold PBS, and the nuclear protein fractions were extracted (for validation of the method and a detailed description of protein extraction and EMSA, see [20]). For the EMSA, a double-stranded 26-mer oligonucleotide containing the kB binding site 5′-AGCTTCAGAGGGGACTTTCCGAGAGG [42], produced by Dr. J. Seibt (Department of Immunology, Uppsala University, Uppsala, Sweden), was used. The oligonucleotide was labeled with [^{32}P] dCTP by using a Megaprime labeling kit (Amersham International, Alyesbury, UK) and extracted once with an equal volume of phenol/chloroform/isoamylalcohol (25/25/1, v/v). As negative control, a 100–1000-fold excess of nonlabeled oligonucleotide was used. Before incubation with the oligonucleotide, nuclear fractions (5–10 μg) were denatured with formamide (27%). The fractions were then allowed to react with the nucleotide for 30 min at room temperature in a solution containing 10 mM Tris (pH 7.5), 0.2% deoxycholic acid, 40 mM NaCl, 1 mM EDTA, 1 mM bis-mercaptoethanol, 4% glycerol, 2 μg polydeoxyinosinic acid and 0.1 μg DNA (14,000 cpm). The samples were then separated on 5% nondenaturing polyacrylamide gels in 0.5 × TBE. Band intensities were quantified by densitometric scanning as described above and values were corrected for the amount of loaded protein. Protein concentrations were determined by using the Bradford reagent [43].

Statistical Analysis

Data are presented as means ± SEM, and groups of data were compared by using paired or unpaired Student's t-test as appropriate. When multiple comparisons were performed, the data were evaluated by analysis of variance and the critical P values were corrected according to the Bonferroni method [44]. When experiments were performed in triplicate, the mean of the triplicate incubations was always considered as one separate observation.

RESULTS

In initial experiments, the possible direct effects of ebselen on insulin-producing cells were evaluated. Ebselen at concentrations 5–20 μM (24 hr exposure) had no effects on rat islet glucose oxidation rates and insulin release or RIN-cell viability (data not shown), but ebselen at 50–100 μM significantly reduced glucose oxidation (55–69%) by rat islets and viability (20%) of RIN cells.

Because 20 μM ebselen had no deleterious effects on insulin-producing cells, in a second series of experiments we exposed rat pancreatic islets or RIN cells to cytokines for 6 hr in the presence or absence of 20 μM ebselen. Ebselen prevented the increase in nitrite production (used as an index of NO production) induced by IL-1β by both isolated rat islets and RIN cells (Table 1). The decrease in nitrite production by IL-1β-stimulated RIN cells caused by ebselen was accompanied by inhibition of iNOS mRNA expression (Fig. 1). Inhibition of iNOS mRNA in the eb-

TABLE 1. Effects of IL-1β and/or ebselen on nitrite production by rat islets (pmol/hr × islet) and RIN cells (pmol/hr × 10^6 cells)

IL-1β (U/mL)	Ebselen (μM)	Nitrite Production	
		Rat Islets	RIN Cells
0	0	1.12 ± 0.15*	155 ± 36*
25	0	2.16 ± 0.27	480 ± 19
0	20	1.12 ± 0.12*	246 ± 14*
25	20	1.32 ± 0.16*	195 ± 25*

Rat islets or RIN cells were incubated for 1 hr with or without ebselen. IL-1β was then added to the culture dishes, the islets or cells were incubated for another 6 hr and the medium was retrieved for nitrite measurement. Results are mean ± SEM of 6 (rat islets) or 7 (RIN cells) separate experiments.

* Significantly different ($P < 0.05$, paired t-test) from the group exposed to IL-1β in the absence of ebselen.

selen-treated RIN cells was not total. In a second series of experiments, RIN cells were exposed to ebselen and/or IL-1β for 24 hr before RNA extraction (experimental conditions as in Fig. 1). After 24 hr, iNOS expression (results in O.D. of iNOS/GAPDH; mean ± SEM of three separate experiments) was control and ebselen, 0; IL-1β, 2.7 ± 0.4; ebselen + IL-1β, 2.7 ± 1.3. Thus, the observed inhibitory effect of ebselen on IL-1β-induced iNOS expression observed after 6 hr (Fig. 1) was not maintained when the observation time was extended to 24 hr.

Ebselen also prevented the increase in nitrite production by human islets exposed to a combination of cytokines (IL-1β, 50 U/mL; TNF-α, 1000 U/mL; and IFN-γ, 1000 U/mL). After a 14-hr period of cytokine exposure, the medium nitrite accumulation values (pmol/μg DNA × hr) were control, 1.6 ± 0.6*, cytokines, 9.3 ± 0.6; ebselen, 2.3 ± 1.1* and cytokines + ebselen, 3.6 ± 1.6* (n = 4; $P < 0.05$ vs. cytokines, paired t-test).

Because NF-κB is implicated in the transcriptional regulation of IL-1β-induced iNOS and mRNA, we studied ebselen effects on IL-1β-stimulated translocation of NF-κB from the cytoplasm to the nucleus in RIN cells. Figure 2 shows that 20 min of IL-1β exposure increased nuclear NF-κB binding. Addition of ebselen 1 hr prior to the cytokine did not counteract this increased nuclear binding.

We also evaluated whether ebselen induced long-term (24 hr) protection against the deleterious effects of IL-1β on functional parameters of isolated rat islets (Table 2). In agreement with the 24-hr observations for RIN cells, ebselen failed to prevent the IL-1β-induced increase in nitrite production and the decrease in glucose oxidation and insulin release. To test whether the lack of ebselen protection over 24 hr was due to loss of drug activity in the culture medium, new 24-hr experiments were performed (experimental conditions as in Table 2), including medium change and addition of fresh ebselen and IL-1β after the initial 12 hr of exposure. Under these conditions, glucose oxidation rates (pmol/10 islets × 90 min; mean ± SEM; n = 4) were control, 656 ± 122; ebselen, 678 ± 78; IL-1β, 338 ± 119; and ebselen + IL-1β, 309 ± 104. These results are similar to

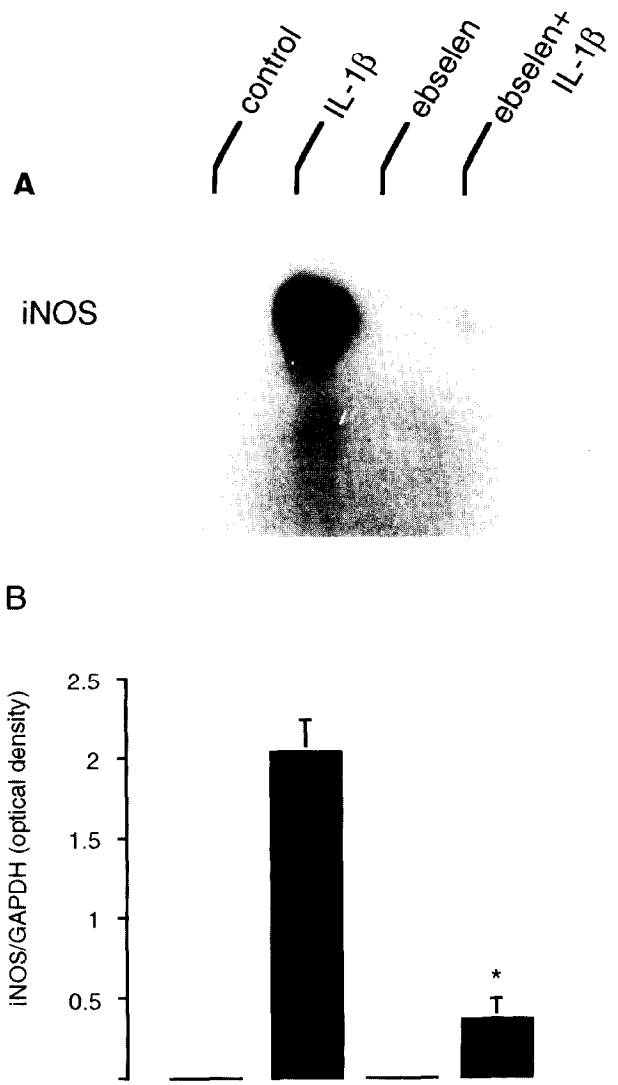

FIG. 1. Effects of IL-1β (25 U/mL) and/or ebselen (20 μM) on iNOS mRNA expression in RIN cells. RIN cells were incubated for 1 hr with or without ebselen. IL-1β was then added to the culture dishes, and the cells were incubated for another 6 hr before harvesting for RNA extraction and Northern blot analysis. Sequential hybridization was performed with cDNAs encoding for iNOS, GAPDH and 18S. (A) Northern blot representative of four separate experiments. (B) Northern blots similar to those shown in A were evaluated by densitometric scanning and expressed as arbitrary units of optical density (O.D.) and corrected for GAPDH O.D. Results are means ± SEM of four experiments. *Significantly different ($P < 0.01$, unpaired t-test) from IL-1β. Correction of the iNOS O.D. values by 18S O.D. provided similar results (data not shown).

those shown in Table 2 and suggest that ebselen degradation is not a likely explanation for the lack of protection against IL-1β over 24 hr.

To investigate further the potential actions of ebselen against oxidative stress in insulin-producing cells, we tested whether the agent could protect rat islets against the suppressive effects of H_2O_2 or alloxan. Alloxan is a compound that damages β-cells, mainly by oxygen free radical genera-

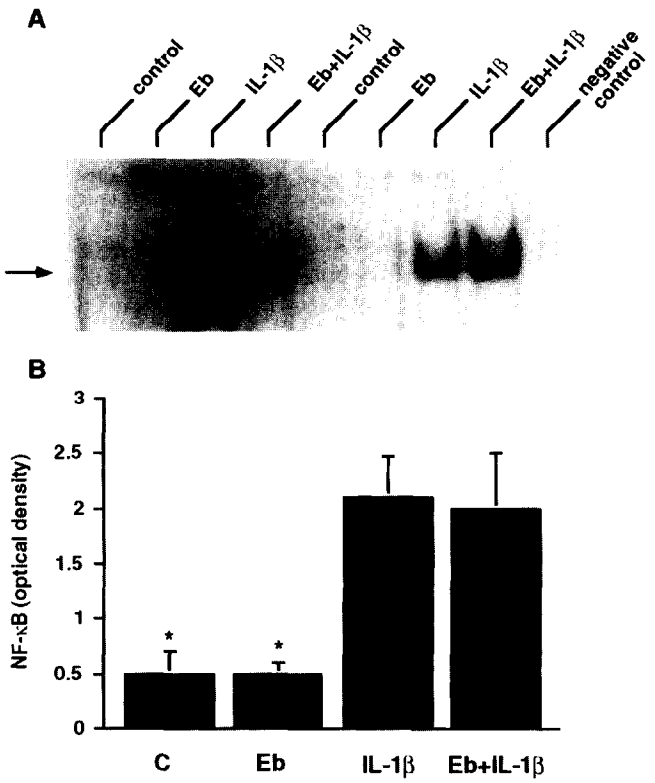

FIG. 2. Effects of IL-1β (25 U/mL) and/or ebselen (20 μM) on NF-κB binding activity. RIN cells were incubated for 1 hr with or without ebselen. IL-1β was then added to the culture dishes and the cells were incubated for another 20 min before protein extraction for EMSA. (A) An autoradiograph showing two separate experiments. The negative control contained a 100-fold excess of nonlabeled oligonucleotide. (B) Autoradiographs similar to that shown in A were evaluated by densitometric scanning and expressed in arbitrary units of optical density/μg protein. Results are mean ± SEM of seven separate experiments. *$P < 0.01$ vs. IL-1β.

tion [45]. Ebselen induced significant protection against short-term H_2O_2 or alloxan exposure, as demonstrated by the higher glucose oxidation rates in the rat islets exposed to H_2O_2 + ebselen or alloxan + ebselen, as compared to the islets exposed to H_2O_2 or alloxan only ($P < 0.05$, Table 3).

There are data indicating that high concentrations of DMSO, the solvent used to dilute ebselen, by itself protects insulin-producing cells against the effects of some toxins [46]. Thus, we tested whether the observed protective effects of ebselen against H_2O_2 and alloxan could be due to DMSO (0.25%, v/v, the same amount used to dilute ebselen in the experiments described above). DMSO alone did not protect against H_2O_2 (400 μM) or alloxan (2.0 mM), as indicated by the low glucose oxidation rates of islets exposed to H_2O_2 + DMSO or alloxan + DMSO, results similar to those observed in islets treated with H_2O_2 or alloxan alone (data not shown).

DISCUSSION

The present results confirm previous observations that IL-1β induces iNOS mRNA expression, NO production and β-cell dysfunction in rodent insulin-producing cells [8, 9, 19] and that a combination of the cytokines IL-1β, TNF-α and IFN-γ increases NO production by human pancreatic islets [13, 14].

NF-κB may regulate IL-1-induced iNOS transcription in rat insulin-producing cells [20–22] and in human pancreatic islets [47]. Several postreceptor signal transduction pathways have been proposed for NF-κB activation in different cell types, including protein kinase and proteinase activation, ceramide generation and production of ROIS [48, 49]. In fact, a universal model for NF-κB activation involving ROIS has been proposed [25]. However, this hypothesis has been challenged by observations suggesting that ROIS-induced NF-κB activation is cell specific [30] and that conclusions as to the role of ROIS may depend on the type of antioxidant used [50]. Ebselen is an effective reductant of hydroperoxides [4] and, if ROIS are necessary for NF-κB activation, this effect could lead to reduced IL-1-induced NF-κB activation and consequent decreased iNOS expression in insulin-producing cells.

The present data show that ebselen prevents the increase in nitrite production by rat islets exposed for 6 hr to IL-1β. These effects of ebselen were reproduced in RIN cells ex-

TABLE 2. Long-term effects of IL-1β and/or ebselen on isolated rat islet glucose oxidation (pmol/10 islets × 90 min), nitrite production (pmol/islet × hr) and insulin accumulation into the medium (ng/islet × 24 hr)

IL-1β (μM)	Ebselen (μM)	Glucose Oxidation	Nitrite Production	Insulin Accumulation
0	0	704 ± 190	1.04 ± 0.10	213 ± 32
0	20	569 ± 91	1.08 ± 0.13	297 ± 64
25	0	185 ± 26*†	2.19 ± 0.46*†	127 ± 14*†
25	20	165 ± 32*†	2.14 ± 0.34*†	93 ± 23*†

Isolated rat islets were exposed for 1 hr to ebselen or pure RPMI medium. IL-1β was then added and the islets were cultured for another 24 hr. After this period, the medium was removed for insulin and nitrite measurements. Rates of glucose oxidation were measured in triplicate groups of 10 islets incubated for 90 min in KRBH buffer (without albumin) supplemented with D-(U-^{14}C)glucose. Results are the mean ± SEM of five separate experiments.

* Significantly different ($P < 0.05$) from IL-1β 0 + ebselen 0 and † IL-1β 0 + ebselen 20 (ANOVA).

TABLE 3. Effects of H_2O_2, alloxan and/or ebselen on isolated rat islet glucose oxidation (pmol/10 islets × 90 min)

	Ebselen (μM)	Glucose Oxidation
H_2O_2 (μM)		
0	0	553 ± 46 (9)
200	0	402 ± 60 (4)
400	0	301 ± 11 (9)*
0	20.0	410 ± 46 (7)
200	20.0	355 ± 64 (4)
400	20.0	429 ± 36 (7)
Alloxan (mM)		
0	0	981 ± 87 (7)
2.0	0	354 ± 46 (7)†
0	20.0	798 ± 52 (7)
2.0	20.0	629 ± 98 (7)

Isolated rat islets were cultured for 1 hr in RPMI medium in the presence or absence of ebselen. In the alloxan experiments, the islets were transferred to KRBH buffer supplemented with albumin and 2.8 mM glucose and exposed or not exposed to ebselen for another 30 min. Islets were subsequently treated for 30 min with alloxan or vehicle (citrate buffer, 0.15%, v/v). In the H_2O_2 experiments, the drug or vehicle (distilled H_2O, 4%, v/v) was added to the culture medium and the islets incubated for another 2 hr. Rates of glucose oxidation were measured in triplicate groups of 10 islets incubated for 90 min in KRBH buffer (without albumin) supplemented with D-(U-^{14}C)glucose and 16.7 mM nonradioactive glucose. Results are mean ± SEM of the number of experiments indicated in parentheses.

* $P < 0.05$ vs. H_2O_2 0 + ebselen 0.

† $P < 0.05$ vs. alloxan 0 + ebselen 0 (ANOVA).

posed to IL-1β for 6 hr and in human pancreatic islets exposed to a combination of IL-1β, IFN-γ and TNF-α for 14 hr. The reduction in NO production determined by ebselen in RIN cells was associated with a decrease in iNOS mRNA expression but not with an inhibition of IL-1-induced NF-κB activation. This effect indicates that ebselen's effects on NO generation may result from either an interference with transcriptional control of the iNOS gene at a level distal to NF-κB activation or with accelerated degradation of iNOS mRNA. These findings are in line with the recent observations that some antioxidant agents decrease iNOS expression in vascular smooth muscle cells without affecting NF-κB activation [50].

We reported that PDTC, an ROIS scavenger, prevents NF-κB activation and iNOS expression in rodent and human islet cells [21, 29, 47], pointing to a possible role for H_2O_2 in the pathway by which cytokines activate NF-κB and induce iNOS expression in insulin-producing cells. However, there are data to suggest that H_2O_2 generation is not the main mediator of these effects of cytokines. Preliminary observations suggest that H_2O_2 alone neither induces NF-κB activation [49] nor increases iNOS mRNA expression in RIN cells (data not shown). Moreover, it has not been possible to detect H_2O_2 generation in rat pancreatic islets exposed to IL-1β [49]. If H_2O_2 is not a key element in iNOS signal transduction, this may explain why IL-1β-induced NF-κB activation was not counteracted by ebselen and why this agent was not able to prevent iNOS expression and nitrite production by RIN cells and rat islets exposed for longer periods (24 hr) to IL-1β. Clearly, further studies are required to sort out the role of H_2O_2 in IL-1-induced iNOS expression and to determine which component of the signal transduction pathway for cytokine-induced iNOS expression in β-cells is affected by ebselen and other antioxidant agents.

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References

1. Wendel A, Fausel M, Safayhi H, Tiegs G and Otter R, A novel seleno-organic compound—II. Activity of PZ 51 in relation to glutathione peroxidase. *Biochem Pharmacol* **33:** 3241–3245, 1984.
2. Sies H, Ebselen: A glutathione peroxidase mimic. *Method Enzymol* **234:** 476–482, 1994.
3. Hattori R, Inoue R, Sase K, Eizama H, Kosuga K, Ayoama T, Masayasu H, Kawai C, Sasayama S and Yui Y, Preferential inhibition of inducible nitric oxide synthase by ebselen. *Eur J Pharmacol* **267:** R1–R2, 1994.
4. Schewe T, Molecular actions of ebselen—An antiinflammatory antioxidant. *Gen Pharmacol* **26:** 1153–1169, 1995.
5. Bendtzen K, Immune hormones (cytokines): Pathogenic role in autoimmune arthritis and endocrine diseases. *Autoimmunity* **2:** 177–189, 1989.
6. Mandrup-Poulsen T, Helqvist TS, Wogensen LD, Molvig J, Pociot F, Johanennsen J and Nerup J, Cytokines and free radicals as effector molecules in the destruction of pancreatic β-cells. *Curr Topics Microbiol Immunol* **164:** 169–193, 1990.
7. Sandler S, Eizirik DL, Svensson C, Strandell E, Welsh M and Welsh N, Biochemical and molecular actions of IL-1 on pancreatic β-cells. *Autoimmunity* **10:** 241–253, 1991.
8. Southern C, Schulster D and Green IC, Inhibition of insulin secretion by interleukin-1β and tumor necrosis factor-α via an L-arginine dependent nitric oxide generation mechanism. *FEBS Lett* **276:** 42–44, 1990.
9. Welsh N, Eizirik DL, Bendtzen K and Sandler S, Interleukin-1β induced nitric oxide production in isolated rat pancreatic islets requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase. *Endocrinology* **129:** 3167–3173, 1991.
10. Corbett JA, Lancaster JR Jr, Sweetland MA and McDaniel ML, Interleukin-1β induced formation of EPR detectable iron nytrosil complexes in islets of Langerhans. Role of nitric oxide in IL-1β induced inhibition of insulin secretion. *J Biol Chem* **266:** 21351–21354, 1991.
11. Eizirik DL, Interleukin-1-induced impairment in pancreatic islets oxidative metabolism is potentiated by tumor-necrosis factor. *Acta Endocrinol* **119:** 321–325, 1988.
12. Eizirik DL, Sandler S, Holberg A, Bendtzen K, Sener A and Malaisse WJ, Differential sensivity to β-cell secretagogues in rat islets exposed to human interleukin-1β. *Endocrinology* **125:** 752–759, 1989.
13. Eizirik DL, Sandler S, Welsh N, Cetkovic-Cvrlje M, Nie-

mann A, Geller DA, Pipeleers DG, Bendtzen K and Hellerström C, Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. *J Clin Invest* **93:** 1968–1974, 1994.
14. Corbett JA, Sweetland MA, Wang JL, Lancaster JR Jr and McDaniel ML, Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proc Natl Acad Sci USA* **90:** 1731–1735, 1993.
15. Rabinovitch A, Suarez-Pinzon WL, Strynadka K, Schultz R, Lackey JRT, Warnock GL and Rajotte RV, Human pancreatic islet β-cell destruction by cytokines is independent of nitric oxide production. *J Clin Endocrinol Metab* **79:** 1058–1062, 1994.
16. Rabinovitch A, Roles of cytokines in IDDM pathogenesis and islet β-cell dysfunction. *Diabetes Rev* **1:** 215–240, 1993.
17. Hughes JH, Colca JR, Easom RA, Turk J and McDaniel ML, Interleukin-1 inhibits insulin secretion from isolated rat pancreatic islets by a process that requires gene transcription and mRNA translation. *J Clin Invest* **86:** 856–863, 1990.
18. Eizirik DL, Bendtzen K and Sandler S, Short exposure of rat pancreatic islets to interleukin-1β induces a substantial but reversible impairment in β-cell function. This effect is influenced by protease activation, gene transcription and protein synthesis. *Endocrinology* **128:** 1611–1616, 1991.
19. Eizirik DL, Cagliero E, Björklund A and Welsh N, Interleukin-1β induces the expression of an isoform of nitric oxide synthase in insulin-producing cells, which is similar to that observed in activated macrophages. *FEBS Lett* **3:** 249–252, 1992.
20. Saldeen J and Welsh W, Interleukin-1β induced activation of NF-κB in insulin-producing RINm5F cells is prevented by the protease inhibitor Nα-*p*-tosyl-L-lysine chloromethylketone. *Biochem Biophys Res Commun* **203:** 149–155, 1994.
21. Bedoya FJ, Flodström M and Eizirik DL, Pyrrolidine dithiocarbamate prevents IL-1-induced nitric oxide synthase mRNA, but not superoxide dismutase mRNA, in insulin-producing cells. *Biochem Biophys Res Commun* **210:** 816–822, 1995.
22. Kwon G, Corbett JA, Rodi CP, Sullivan P and McDaniel ML, Interleukin-1β-induced nitric oxide synthase expression by rat pancreatic β-cells: Evidence for the involvement of nuclear factor κB in the signalling mechanism. *Endocrinology* **138:** 4790–4795, 1995.
23. Thanos D and Maniatis T, NF-κB: A lesson in family values. *Cell* **80:** 529–532, 1995.
24. Schmidt KN, Amstad P, Cerutti P and Baeuerle PA, The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-κB. *Chem Biol* **2:** 13–22, 1995.
25. Schulze-Osthoff K, Loss M and Baeuerle PA, Redox signaling by transcription factors NF-κB and AP-1 in lymphocytes. *Biochem Pharmacol* **50:** 735–741, 1995.
26. Schreck R, Meir B, Männel D, Dröge W and Baeuerle PA, Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J Exp Biol Med* **175:** 1181–1194, 1992.
27. Xie Q-W, Kashiwabara Y and Nathan C, Role of transcription factor NFκB/Rel in induction of nitric oxide synthase. *J Biol Chem* **269:** 4705–4708, 1994.
28. Eberhardt W, Kuns D and Pfeilschifter J, Pyrrolidine dithiocarbamate differentially affects interleukin 1 beta and cAMP-induced nitric oxide synthase expression in rat mesangial cells. *Biochem Biophys Res Commun* **200:** 163–170, 1994.
29. Flodström M, Niemann A, Bedoya FJ, Morris SM and Eizirik DL, Expression of citrulline-nitric oxide cycle in rodent and human pancreatic β-cells: Induction of argininosuccinate synthase by cytokines. *Endocrinology* **136:** 3200–3206, 1995.
30. Brennan P and O'Neil LA, Effects of oxidants on nuclear κB activation in three different cell lines: Evidence against a universal hypothesis involving oxygen radicals. *Biochim Biophys Acta* **1260:** 167–175, 1995.
31. Anderson A, Isolated mouse pancreatic islets in culture: Effects of serum and different culture medium on insulin production of the islets. *Diabetologia* **14:** 397–404, 1978.
32. Eizirik DL and Sandler S, Functional restoration of cultured mouse pancreatic islets after in vitro exposure to alloxan. *Pharmacol Toxicol* **63:** 396–399, 1988.
33. Eizirik DL, Korbutt GS and Hellerström C, Prolonged exposure of human pancreatic islets to high glucose concentrations in vitro impairs the β-cell function. *J Clin Invest* **90:** 1263–1268, 1992.
34. Sandler S, Bendtzen K, Eizirik DL, Sjöholm A, and Welsh N, Decreased cell replication and polyamide content in insulin-producing cells after exposure to human IL-1β. *Immunol Lett* **22:** 267–272, 1989.
35. Eizirik DL, Björklund A and Welsh N, Interleukin-1-induced expression of nitric oxide synthase in insulin-producing cells is preceded by c-*fos* induction and depends on gene transcription and protein synthesis. *FEBS Lett* **317:** 62–66, 1993.
36. Niemann A, Björklund A and Eizirik DL, Studies on the molecular regulation of the inducible form of the nitric oxide synthase (iNOS) in insulin-producing cells. *Mol Cell Endocrinol* **106:** 151–155, 1994.
37. Heding LG, Determination of total serum insulin (IRI) in insulin treated patients. *Diabetologia* **8:** 260–266, 1972.
38. Eizirik DL, Welsh M, Strandell E, Welsh N and Sandler S, Interleukin-1β depletes insulin messenger ribonucleic acid and increases the heat shock protein hsp70 in mouse pancreatic islets without impairing the glucose metabolism. *Endocrinology* **127:** 2290–2297, 1990.
39. Hinegardner RT, An improved fluorimetric assay for DNA. *Anal Biochem* **39:** 197–201, 1971.
40. Lyons RC, Orloff GJ and Cunningham JM, Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J Biol Chem* **267:** 6370–6374, 1992.
41. Katz RA, Mitsialis SA and Guntoka RV, Studies on the methylation of avian sarcoma parvoviruses in permissive and nonpermissive cells. *J Gen Virol* **64:** 429–435, 1983.
42. Siebenlist U, Franzoso G and Brown K, Structure, regulation and function of NF-κB. *Annu Rev Cell Biol* **110:** 405–455, 1994.
43. Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
44. Wallenstein S, Zucker CL and Fleiss JL, Some statistical methods useful in circulation research. *Cir Res* **47:** 1–9, 1980.
45. Malaisse WJ, Alloxan toxicity to the β-cell. A new hypothesis. *Biochem Pharmacol* **31:** 3527–3534, 1982.
46. Eizirik DL and Kisby GE, Cycad toxin induced damage of rodent and human pancreatic islets. *Biochem Pharmacol* **50:** 355–65, 1995.
47. Flodström M, Welsh N and Eizirik DL, Cytokines activate the nuclear factor κB (NF-κB) and induce nitric oxide production in human pancreatic islets. *FEBS Lett* **385:** 4–8, 1996.
48. O'Neil LAJ, Towards understanding of the signal transduction pathway for interleukin-1. *Biochem Biophys Acta* **1266:** 31–44, 1995.
49. Eizirik DL, Flodström M, Karlsen AE and Welsh N, The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* **39:** 875–890, 1996.
50. Hecker M, Preiß C, Schini-Kerth VB and Busse R, Antioxidants differentially effect nuclear factor κB-mediated nitric oxide synthase expression in vascular smooth muscle cells. *FEBS Lett* **380:** 224–228, 1996.