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Original Research Communication

β-Cells, Oxidative Stress, Lysosomal Stability, and Apoptotic/Necrotic Cell Death

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ABSTRACT

Reactive oxygen intermediates (ROI) may be involved in the destruction of pancreatic β-cells during the development of insulin-dependent diabetes mellitus (IDDM). To investigate the possible role of lysosomes in this process, normal mouse β-cells were cultured as monolayers at D-glucose concentrations of 1.6 (pronounced crinophagy), 11 or 28 m*M* (minimal crinophagy), subjected to a low level of oxidative stress and returned to standard culture conditions. Some cultures were exposed to desferrioxamine (Des) before the oxidative stress. As a result of such stress, many of the cells' lysosomes ruptured with consequent apoptosis or necrosis. Cells kept at 1.6 m*M* glucose were rich in secretory granules, showed crinophagy/autophagy, were very sensitive to oxidative stress, and had the least stable lysosomes. Cells kept at 28 m*M* glucose did not show crinophagy, contained fewer secretory granules, were less sensitive to oxidative stress, and had more stable lysosomes. Des-treated cells behaved almost as cells not exposed to oxidative stress at all. The findings suggest that iron may occur together with zinc within the secretory granules and that it sensitizes crinophagic lysosomes to oxidative stress. The stress that was applied in this study may be comparable to what occurs within the vicinity of activated macrophages during autoimmune insulitis. Antiox. Redox. Signal. 1, 305–315.

INTRODUCTION

OXIDATIVE STRESS, which is a cellular imbalance between pro- and anti-oxidant factors toward a nonreducing state, seems to play an important role in many disease processes, including insulin-dependent (Type I) diabetes mellitus (IDDM) (Sinclair *et al.*, 1990; Rabinovitch, 1992; Horio *et al.*, 1994). It has also been reported that in several cell types reactive oxygen species (ROS), which are generated under oxidative stress or produced by inflammatory cells, may induce a variety of cellular degenerative alterations, including apoptosis and necrosis (Takasu *et al.*, 1991; Dypbukt *et al.*, 1994; Sandström *et al.*, 1994; Hellquist *et al.*, 1997; Brunk and Svensson, 1999). In contrast to apoptosis, which is a controlled, relatively slow, energy- and gene expression-requiring degenerative process within an intact plasma membrane, necrosis is an "accidental," rapid event with cellular swelling and uncontrolled rupture resulting from major injuries and severe ATP depletion (Wyllie *et al.*, 1980; Bursch *et al.*, 1990; Kerr *et al.*, 1994).

Selective apoptotic death of β-cells, which is associated with infiltration of mononuclear cells into the pancreatic islets (insulitis), is a

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characteristic feature of early IDDM (Foulis *et al.*, 1986; Itoh *et al.*, 1993; Delaney *et al.*, 1997; O'Brien *et al.*, 1997; Augustein *et al.*, 1998). Locally produced cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), or interferon-γ (IFN-γ) have been considered responsible together with ROS and nitric oxide (NO) (Campbell *et al.*, 1988; Horio *et al.*, 1994; Kaneto *et al.*, 1995; Mandrup-Poulsen, 1996, Rabinovitch *et al.*, 1996). β-Cells appear to be particularly vulnerable to oxidative stress, partly due to their low levels of key ROS scavenging enzymes (Grankvist *et al.*, 1981; Malaisse *et al.*, 1982; Horio *et al.*, 1994; Lenzen *et al.*, 1996; Kubisch *et al.*, 1997; Tiedge *et al.*, 1997).

Recently, it has been shown that limited lysosomal damage, involving the rupture of only a few lysosomes, results in reparative autophagocytosis, moderate rupture leads to apoptotic cell death, whereas extensive rupture gives rise to necrosis (Brunk *et al.*, 1995a,b, 1997; Öllinger and Brunk, 1995; Hellquist *et al.*, 1997; Brunk and Svensson, 1999; Neuzil *et al.*, 1999). Modest leak of lysosomal proteases, such as the cysteine protease cathepsin B, may induce classical apoptosis by proteolytic activation of the caspase system (Deiss *et al.*, 1996; Ishisaka *et al.*, 1998; Vancompernolle *et al.*, 1998), whereas a more complete leak would result in necrosis through uncontrolled cellular degradation, including that of pro-caspases.

In a number of studies concerning the cytotoxic effects of oxidative stress, we have found that the resulting cell damage is mediated through intralysosomal, iron-catalyzed, Fenton-type oxidative reactions, with ensuing destabilization of membranes that surround lysosomes (Zhang *et al.*, 1992; Brunk *et al.*, 1995a,b; Öllinger and Brunk, 1995; Garner *et al.*, 1998; Brunk and Svensson, 1999). Such destabilization results in a release into the cytosol of lysosomal contents, including a number of proteases, with consequent cellular degeneration or even cell death. Due to their normal content of redox-active, low-molecular-weight iron, lysosomes appear to be particularly sensitive to oxidative stress (Zhang *et al.*, 1992, 1996; Brunk *et al.*, 1995a,b; Öllinger and Brunk, 1995; Garner *et al.*, 1998). The characteristic lysosomal content of iron is a consequence of the normal and constantly ongoing autophagocytotic degradation of a variety of metalloproteins, such as ferritin and cytochromes, within the acidic vacuolar apparatus (Brunk *et al.*, 1992).

The secretory granules of β-cells contain zinc (Howell *et al.*, 1969; Orci *et al.*, 1986) and, probably, iron as well (Juntti-Berggren *et al.*, 1987). These two elements are close in the periodic table, are to some degree similar, and, thus, it may be difficult for the cells to discriminate between them (Coleman and Matrone, 1969; Awai *et al.*, 1979). Following iron overloading of rats and during hemachromatosis in humans, there is an early and pronounced iron uptake in β-cells with ensuing cellular destruction and diabetes (Rahier *et al.*, 1987; Lu *et al.*, 1991; Lu and Hayashi 1994). Extensive crinophagy/autophagy within β-cells during prolonged periods of low blood glucose levels might lead to an enrichment of lysosomal iron and, thus, a further increased susceptibility to oxidative stress.

The aim of the present study was to evaluate the relationship between oxidative stress, crinophagy/autophagy of secretory granules, lysosomal damage, and cell death (apoptosis/necrosis) of normal mouse β-cells.

MATERIALS AND METHODS

Materials

The following materials were purchased from the sources indicated: acridine orange (AO) from BDH (Poole, UK); RPMI 1640 culture medium, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin from Gibco BRL (Paisley, UK); hydrogen peroxide (H_2O_2) and propidium iodide (PI) from Sigma-Aldrich (Steinheim, Germany); sodium cacodylate (dimethylarsinic acid sodium salt) from Merck (Darmstadt, Germany); desferrioxamine mesylate (Des) from Ciba AG (Basel, Switzerland); osmium tetroxide (OsO_4) from Johnson Matthey (Royston, UK); glutaraldehyde (Ga) and Epon-812 from Fluka AG (Buchs, Switzerland); Hoechst 33342 (Ho342) from Calbiochem-Novabiochem (La Jolla, CA).

Preparation and culture of pancreatic islets cells

Islets of Langerhans were isolated from collagenase-digested pancreata of NMRI mice

(B&K, Universal, Sollentuna, Sweden) that were precultured for 1–2 days in RPMI 1640 medium with 10% vol/vol FBS and with the glucose concentration adjusted to 11 mM (Andersson, 1978). A single-cell suspension was then prepared by dissociation of the islets in 0.25% trypsin in PBS for 3 min at 37°C.

Small sized, moderately dense monolayers were obtained by seeding the cells in microtiter (10-mm diameter) cylinders (1×10^5 cells/cylinder in 200 μl of medium) positioned either directly on the bottom of 35-mm Petri plastic dishes (Costar, Cambridge, MA), or on round coverslips placed in such dishes. The cells were then cultured for another 17 hr at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium with 10% vol/vol FBS, 2 m*M* L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

D-Glucose concentrations of the media were adjusted to 1.6, 11, or 28 m*M*, the rationale being that it is known that mouse islet cells maintain their specific function best at 11 m*M* D-glucose, whereas crinophagic/autophagic activity is high at 1.6 m*M*, and exocytosis of secretory granules at 28 m*M* D-glucose (Andersson *et al.*, 1974; Landström *et al.*, 1988).

Oxidative stress

Following culture for 17 h at the different concentrations of D-glucose (as described above), the cells were exposed to 30 μ*M* H_2O_2 in 1.5 ml of Hanks' balanced salt solution (HBSS), or to HBSS alone (control), for 15 min at 37°C. The cells were then rinsed in HBSS at 37°C and returned to their respective media for another 3 hr. Some cultures that were maintained in 11 m*M* D-glucose were pretreated for 1.5 hr with 1 m*M* Des, a potent iron chelator, before being subjected to oxidative stress.

Assessment of apoptosis and necrosis

The percentages of viable, apoptotic, or necrotic cells were estimated 3 hr after the exposure of H_2O_2. The cells were first stained with the DNA binding dyes Ho342 (20 μg/ml) at 37°C for 10 min and then with PI (10 μg/ml) for 10 min at 20°C (Dive *et al.*, 1992; Delaney *et al.*, 1997). Ho342 permeates the plasma membrane freely and thus stains the DNA in all cells blue, whereas PI, being a highly polar compound, does not penetrate cells with preserved plasma membranes and, consequently, adds a pink color to the DNA of necrotic cells only.

The cells were examined in a fluorescence Microphot-SA microscope (Nikon, Japan) using excitation light of 340–380 nm and a 510-nm barrier filter. A minimum of 500–600 cells were counted and classified as viable (normal-sized blue nuclei), apoptotic (pycnotic blue nuclei), or necrotic (pink nuclei).

Lysosomal membrane stability

Lysosomal membrane stability was evaluated by the AO uptake-test (Brunk *et al.*, 1995a). The culture medium was withdrawn 3 hr after exposure of the cells to H_2O_2, and the cells (growing as moderately dense monolayers on coverslips) were exposed to acridine orange (AO, 5 μg/ml) in complete culture medium for 15 min at 37°C and then rinsed for a few minutes in HBSS. Control cells were exposed to HBSS, but not to H_2O_2, before AO staining. At each occasion, 100 randomly selected AO-treated cells/sample were evaluated by static fluorometry, using a Nikon microscope equipped with an G filter cube (green light) and an extra 630-nm barrier filter as previously described (Olejnicka *et al.*, 1997).

To obtain optical sections through the cells, allowing the demonstration of lysosomes without overlaying disturbances, the islet cells were also studied by confocal scanning microscopy using LSM 410 equipment (Carl Zeiss, Jena, Germany) according to Olejnicka *et al.* (1997).

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as previously described (Olejnicka *et al.*, 1998), although on a selected part of the material only (due to limited number of cells). In brief, following culture at 1.6 or 28 m*M* D-glucose for 20 hr, the cells were fixed in 2% cacodylate-buffered (0.1 m*M*) Ga supplemented with 0.1 *M* sucrose, pH 7.2, for 2 hr at room temperature, post-fixed in 1% OsO_4 in 0.15 m*M* cacodylate buffer for 1 hr, stained *en bloc* with 2% uranyl acetate in 50% ethanol overnight, dehydrated in a graded series of ethanol, and embedded *in situ* in Epon 812. Ul-

trathin sections, cut parallel with the growth substratum and post-stained with lead citrate, were examined in a JEOL 1200 EX transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

Statistical analysis

All experiments were performed in triplicate unless otherwise stated. Data are expressed as means ± SD. The Mann-Whitney test was used for the evaluation of statistical differences between groups. $*p < 0.05$ values versus the controls were considered significant ($**p < 0.01$).

RESULTS

H_2O_2 cytotoxicity

Exposure of cells, incubated for 17 hr in a medium containing 1.6 m*M* D-glucose, to 30 μM H_2O_2 in HBSS at 37°C for 15 min resulted in about 30% viable, 30% apoptotic, and 40% necrotic cells, as evaluated by the combined Ho342 and PI intravital staining method, after another 3 hr at standard culture conditions. In contrast, cells incubated for the same period at 11 or 28 m*M* D-glucose showed lower values for both apoptotic and necrotic cells. Cells incubated at 11 m*M* D-glucose and pretreated with 1 m*M* Des in the same medium for 1.5 hr before the exposure to oxidative stress were additionally better preserved (Fig. 1).

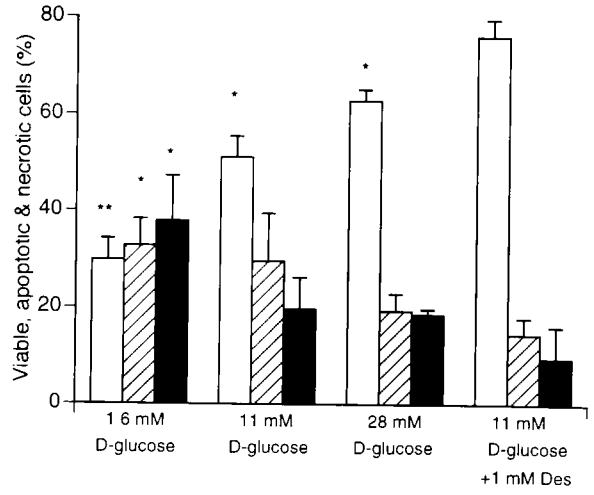

FIG. 1. Apoptotic and necrotic changes of β-cells were evaluated by the use of a combined Ho342 and PI intravital staining technique. Following culture in media with 1.6, 11, or 28 m*M* glucose for 17 hr and an ensuing exposure to 30 μM H_2O_2 for 15 min in HBSS, the cells were returned to their respective media and, 3 hr later, stained with Ho342 followed by PI. Some cells incubated at 11 m*M* glucose were exposed to 1 m*M* Des for 1.5 hr before being subjected to oxidative stress (for details see the Materials and Methods section). Cells with normal-sized blue nuclei, pycnotic blue nuclei, or enlarged pink nuclei correspond to viable (□), apoptotic (▨), or necrotic cells (■), respectively. Values for viable, apoptotic, or necrotic cells are all compared to those obtained for cells exposed to 11 m*M* glucose + 1 m*M* Des before the oxidative stress. Results are given as means ± SD ($n = 3$).

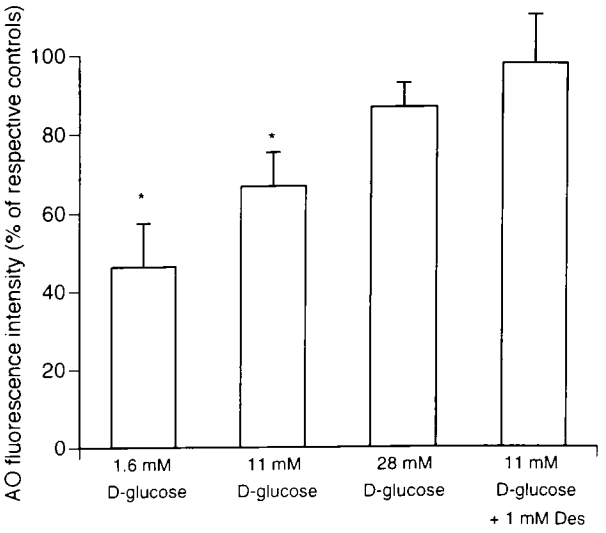

FIG. 2. Lysosomal stability, measured as degree of AO-induced red, granular fluorescence following the exposure to 30 μM H_2O_2 in HBSS at 37°C for 15 min with ensuing culture at standard conditions for another 3 hr. The cells were initially incubated for 17 hr in media with either 1.6, 11, or 28 m*M* glucose. Some cells kept at 11 m*M* glucose were exposed to 1 m*M* Des for 1.5 hr before being subjected to oxidative stress (for details see the Materials and Methods section). In each experiment, at least 100 cells were measured at each point of time. Comparison between the results (against values for cells exposed to 11 m*M* glucose + 1 m*M* Des before the oxidative stress) was made using multiple nonlinear regression analysis. Fluorescence intensities for each group are expressed as percentage of their control values (cells exposed to HBSS without H_2O_2) mean values ± SD ($n = 4$).

In cultures grown at 11 (± Des) or 28 D-glucose for 17 hr, approximately 10% apoptotic/necrotic cells were normally found, whereas cells grown at 1.6 m*M* D-glucose showed somewhat higher numbers of apoptotic/necrotic cells.

Lysosomal membrane stability

Cells (from 1.6, 11, or 28 m*M* D-glucose-containing media) that were exposed to oxidative

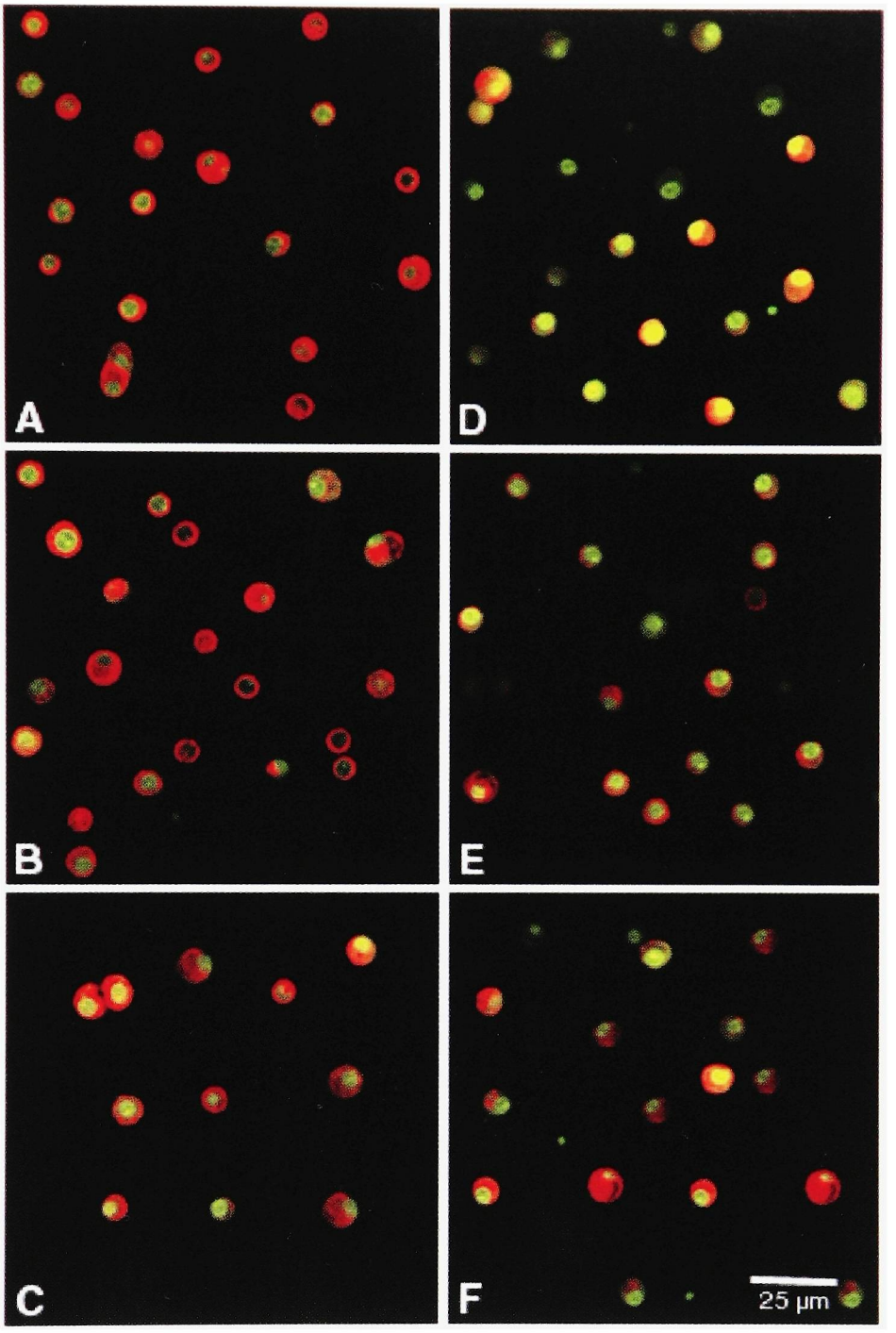

FIG. 3. Confocal scanning laser micrographs (argon laser) of β-cells stained with AO before and after exposure to oxidative stress. Cells were incubated for 17 hr at 1.6 **(A)** or 28 **(B)** m*M* glucose, exposed to HBSS for 15 min, stained with AO, and examined. The cells depicted in **C** were initially grown for 15.5 hr at 11 m*M* glucose and then for another 1.5 hr at the same concentration of glucose but with 1 m*M* Des added. Other cells **(D–F)** were exposed to oxidative stress for 15 min (30 μ*M* H_2O_2 in HBSS) after an initial 17 hr of incubation at different glucose concentrations, rapidly rinsed in HBSS, returned to their original media for another 3 hr, and, finally, stained with AO and examined. The cells depicted in *F* had 1 m*M* Des added to the media during the last 1.5 hr before being exposed to oxidative stress. Note that cells kept at 1.6 m*M* glucose **(D)** are the most affected ones with pronounced signs of lysosomal rupture, while cells grown at 28 m*M* glucose **(E)** are better preserved. Cells protected by Des **(F)** show only small signs of lysosomal destabilization.

stress, then returned to their respective culture media for another 3 h, and, finally, stained with the weak basic and lysosomotropic fluorochrome AO showed decreased red, granular (lysosomal) fluorescence, reflecting a reduced number of nonruptured lysosomes with retained proton gradients over their membranes. Cells incubated for 17 h at 1.6 m*M* D-glucose before the ensuing exposure to oxidative stress retained about 45% of their normal red fluorescence intensity after another 3 h at standard culture conditions. The decline in red fluorescence was significantly lower in cells kept for the same period of time at 11 m*M* or 28 m*M* D-glucose. Cells that were preincubated for 1.5 h with 1 m*M* Des (in 11 m*M* D-glucose-containing medium) before the oxidative stress showed further preserved lysosomes (Figs. 2 and 3).

Confocal laser scanning microscopy

AO-loaded cells that were not exposed to oxidative stress (from 1.6, 11, or 28 m*M* D-glucose-containing media) displayed a strong distinct red, granular fluorescence in combination with a weak green, nuclear and cytosolic fluorescence as a sign of intact lysosomes, when excited with blue laser light (Fig. 3A–C). Following oxidative stress, the cells showed a less granular red and a stronger diffuse green-yellowish pattern, although this shift took place to varying extents in the different groups of cells. Thus, cells initially kept at 1.6 m*M* D-glucose (Fig. 3D) showed more evidence of alterations compared to those incubated at 28 m*M* (Fig. 3E) and especially more so than cells kept at 11 m*M* D-glucose and pretreated with 1 m*M* Des for 1.5 h before the exposure to oxidative stress (Fig. 3F). The latter group of cells did not change much following exposure to H_2O_2.

Electron microscopy

β-Cells maintained in a culture medium containing 1.6 m*M* or 28 m*M* D-glucose for 20 h differed considerably in their ultrastructure, although some variations occurred within each of the two populations. β-Cells maintained at 1.6 m*M* D-glucose contained numerous secretory granula, whereas their rough endoplasmic reticulum and Golgi complexes were only moderately developed. Crinophagy/autophagocytosis was observed (Fig. 4A–C). In contrast, β-cells incubated at 28 m*M* D-glucose medium were severely degranulated, displayed an extensive synthetic apparatus, but did not show any crinophagy/autophagocytosis (Fig. 4D–F).

DISCUSSION

The main objective of the present study was to investigate the susceptibility of normal β-cells from NMRI mice to the noxious effects of oxidative stress and, in particular, to any possible relationship between crinophagy/autophagy of secretory granules, oxidative stress, lysosomal rupture, and loss of viability (apoptosis/necrosis).

Previously, we have studied the susceptibility of several lines of insulinoma cells (HIT, NIT, and RIN) to oxidative stress and found them to be highly vulnerable, as compared to many other cell types. We also observed that insulinoma cells developed apoptosis following oxidative stress as a consequence of lysosomal burst which, in turn, resulted from intralysosomal iron-catalyzed oxidative reactions (Zhang *et al.*, 1996; Olejnicka *et al.*, 1997, 1998, 1999). In those and other studies on different cell types, it turned out that a limited lysosomal rupture results in apoptosis, whereas a more pronounced lysosomal rupture leads to necrotic cell death (Brunk *et al.*, 1995b, 1997; Brunk and Svensson, 1999; Öllinger and Brunk, 1995; Hellquist *et al.*, 1997; Li *et al.*, 1998; Neuzil *et al.*, 1999; Olejnicka *et al.*, 1999).

In this study, we tried to obtain further evidence for our hypothesis that local oxidative stress within Langerhans' islets may result in β-cell degeneration and IDDM as a consequence of lysosomal rupture. Due to problems in obtaining large numbers of dispersed normal β-cells for monolayer cultures, we had to scale the study down to the critical experiments of primary interest. However, because our present findings are supported by similar findings in insulinoma cells, we suggest that involvement of lysosomes during oxidative stress of insulin-producing cells to be well established.

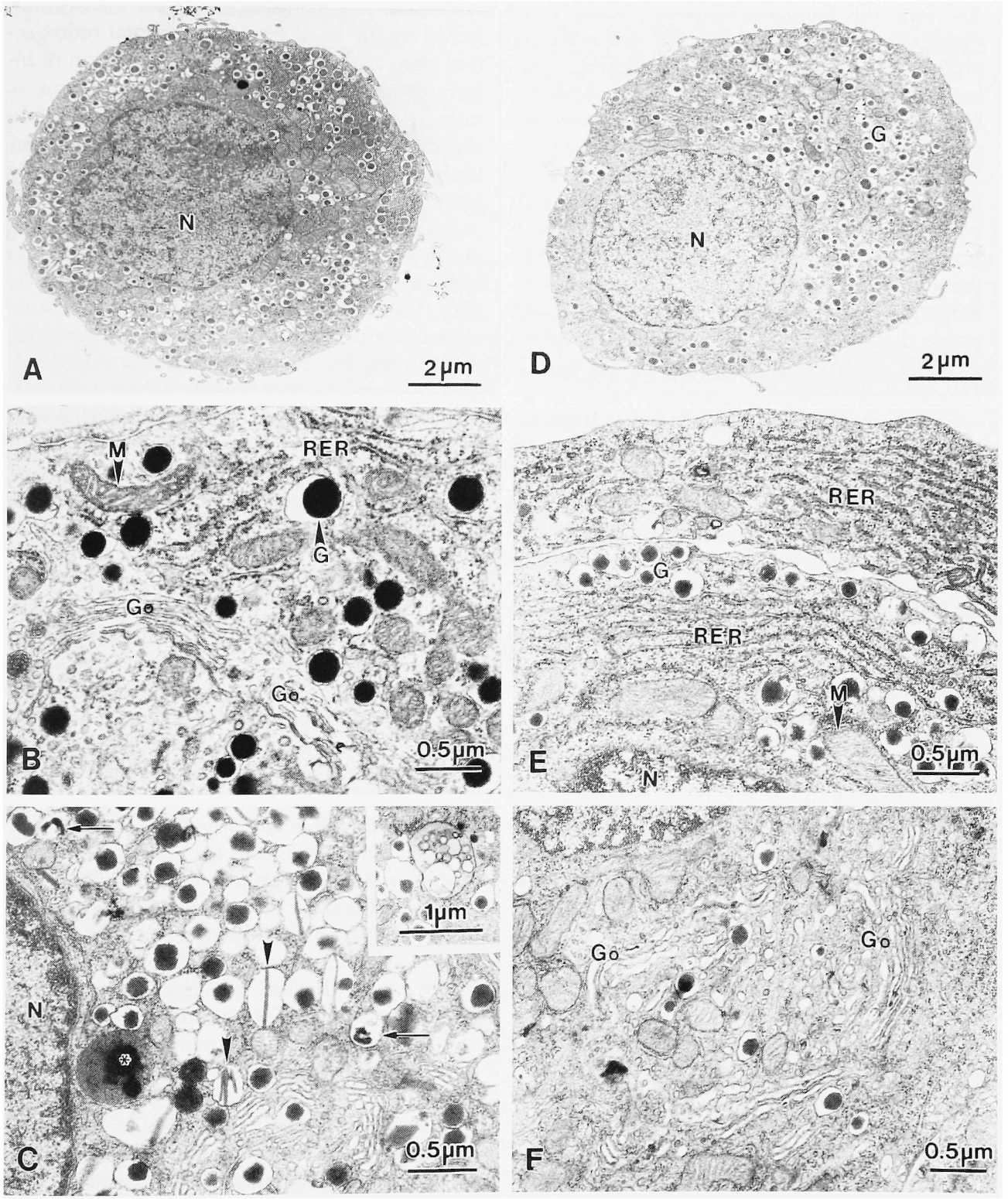

FIG. 4. Electron micrographs of β-cells incubated for 20 hr in cell culture media containing 1.6 m*M* or 28 m*M* glucose, respectively. At the low glucose concentration **(A–C)**, the cells showed abundant secretory granules and moderately developed stacks of rough endoplasmic reticulum (RER) and Golgi complexes (Go). Secondary lysosomes containing secretory granula (asterisks) or multiple vesicles (inset) strongly indicate crinophagic activity. Note also vacuoles (arrows) with fragmented cores or crystalline content (arrowheads). At the high glucose concentration **(D–F)**, the content of secretory granules was much reduced, while the synthetic apparatus (RER, Go) was well developed.

Recently, it was demonstrated that pancreatic β-cells in nonobese diabetic (NOD) transgenic mice, overexpressing the antioxidative protein thioredoxin, were significantly protected from oxidative stress-induced apoptosis following both streptozotocin exposure and spontaneous autoimmune insulitis (Hotta *et al.*, 1998). These findings strongly suggest that oxidative stress, indeed, plays an essential role in such β-cell destruction that is caused by infiltrating inflammatory cells during the insulitis that foregoes manifest IDDM. It is now well established that autoimmune insulitis is the first phase during development of IDDM, whereas massive destruction of β-cells is the second (Andre *et al.*, 1996).

Secretory granules of β-cells are well known to contain zinc in high concentrations (Howell *et al.*, 1969, 1978; Orci *et al.*, 1986). Probably they also contain iron (Juntti-Berggren *et al.*, 1987) and, because these two elements are related, it may be difficult for the cells to discriminate completely between them. Furthermore, iron-loaded rats, as well as humans with hemochromatosis accumulate iron within their β-cells. Such an accumulation results in cellular damage and, finally, in diabetes, which indicates the preference of β-cells for iron (Rahier *et al.*, 1987; Lu *et al.*, 1991; Lu and Hayashi, 1994). We hypothesized that in case β-cells secretory granules do contain significant amounts of iron, pronounced crinophagy would lead to a lysosomal enrichment with redox active iron. Such an increment should increase even further the sensitivity to oxidative stress of these cells that are already normally very susceptible to such influence. As expected, following crinophagy induced by cultivation at 1.6 m*M* D-glucose, the cells became more susceptible to oxidative stress and showed lysosomes that were more vulnerable compared to cells that had undergone stimulated exocytosis following culture at 28 m*M* D-glucose. Finally, endocytotic uptake of Des (Hellquist *et al.*, 1997; Olejnicka *et al.*, 1997; Brunk and Svensson, 1999) largely prevented not only lysosomal rupture following oxidative stress but also apoptosis and necrosis. We conclude (i) that β-cells are unusually sensitive to oxidative stress, (ii) that such stress induces lysosomal burst and consequent apoptosis of necrosis depending on the magnitude of the lysosomal burst, (iii) that lysosomal stability and apoptosis are both affected by the level of intralysosomal redox active iron, and (iv) that the concentration of intralysosomal iron in redox active form is influenced by the presence (at low glucose levels) or absence (at high glucose levels) of autophagic/crinophagic degradation of secretory granules.

An outflux of H_2O_2 from activated inflammatory cells that infiltrate the islets during the type of insulitis that usually precedes IDDM may give rise to intralysosomal, iron-catalyzed oxidative reactions in neighboring cells, unless the H_2O_2 is efficiently degraded, *e.g.*, by catalase and/or glutathione peroxidase and, thus, prevented from diffusing into the acidic vacuolar apparatus of the target cell. β-Cells have low levels of catalase (Grankvist *et al.*, 1981; Lenzen *et al.*, 1996), hence they are not well equipped for a rapid and efficient degradation of H_2O_2 that may occur in their vicinity.

It has been shown that β-cells contain high amounts of the natural iron-chelator ferritin. The mRNA of the H-ferritin chain, being a ferroxidase and responsible for keeping iron in a ferric and non-redox-active form, was found to be four- to eight-fold more concentrated in islets kept at 20 m*M* glucose than in islets kept at 1 m*M* glucose. In contrast, the mRNA of L-ferritin behaved in the opposite way and was decreased by 75–90% in islets kept at 20 m*M* glucose when compared to those kept at 1 m*M* (MacDonalds *et al.*, 1994). These pronounced alterations in the relation between the H- and L-chains of ferritin during conditions of exocytosis (20 m*M* glucose) or crinophagy (1 m*M* glucose) may reflect an adaptation to changes in the cellular pool of low-molecular-weight iron. We have previously shown that starvation of NIT insulinoma cells, with subsequent increase of lysosomal redox-active iron, results in a rapidly increased amount of both cytosolic and lysosomal ferritin, with enhanced increased resistance against oxidative stress following lysosomal uptake of ferritin by autophagocytosis (Olejnicka *et al.*, 1998). We hypothesize that modulation of the cytosolic and lysosomal ferritin contents may be of importance for the cellular resistance against ox-

idative stress through influence on the amount of redox-active intralysosomal iron.

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ABBREVIATIONS

AO, acridine orange; Des, desferrioxamine mesylate; FBS, fetal bovine serum; Ga, glutaraldehyde; H_2O_2, hydrogen peroxidase; Ho342, Hoechst dye 33342; HBSS, Hanks' balanced salt solution; $HO^{\bullet}$, hydroxyl radical; IDDM, insulin-dependent (Type I) diabetes mellitus; IFN-γ, interferon-γ; IL-1β, interleukin-1β; NO, nitric oxide; NOD, nonobese diabetic; O_5O_4, osmium tetroxide; PI, propidium iodide; ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide radical; TEM, transmission electron microscopy; TNF-α, tumor necrosis factor-α.

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