

Ebselen Treatment Prevents Islet Apoptosis, Maintains Intranuclear Pdx-1 and MafA Levels, and Preserves β -Cell Mass and Function in ZDF Rats

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We reported earlier that β -cell-specific overexpression of glutathione peroxidase (GPx)-1 significantly ameliorated hyperglycemia in diabetic *db/db* mice and prevented glucotoxicity-induced deterioration of β -cell mass and function. We have now ascertained whether early treatment of Zucker diabetic fatty (ZDF) rats with ebselen, an oral GPx mimetic, will prevent β -cell deterioration. No other antihyperglycemic treatment was given. Ebselen ameliorated fasting hyperglycemia, sustained nonfasting insulin levels, lowered nonfasting glucose levels, and lowered HbA_{1c} levels with no effects on body weight. Ebselen doubled β -cell mass, prevented apoptosis, prevented expression of oxidative stress markers, and enhanced intranuclear localization of pancreatic and duodenal homeobox (Pdx)-1 and v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (MafA), two critical insulin transcription factors. Minimal β -cell replication was observed in both groups. These findings indicate that prevention of oxidative stress is the mechanism whereby ebselen prevents apoptosis and preserves intranuclear Pdx-1 and MafA, which, in turn, is a likely explanation for the beneficial effects of ebselen on β -cell mass and function. Since ebselen is an oral antioxidant currently used in clinical trials, it is a novel therapeutic candidate to ameliorate fasting hyperglycemia and further deterioration of β -cell mass and function in humans undergoing the onset of type 2 diabetes. *Diabetes* 62:3582–3588, 2013

Exposure of β -cells to supraphysiologic glucose levels causes loss of insulin gene transcription factors, insulin mRNA, and glucose-induced insulin secretion (1–3). These adverse changes can be partially ameliorated by antioxidants (4–6), suggesting prolonged hyperglycemia leads to accumulation of excessive levels of reactive oxygen species, which, in turn, cause chronic oxidative stress (7). Oxidants such as H₂O₂, ribose, and streptozotocin have been reported to decrease levels of essential insulin transcription factors, such as pancreatic and duodenal homeobox (Pdx)-1 and v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (MafA) (4,8).

Compared with other cells, the β -cell has very diminished expression and activity of glutathione peroxidase (GPx) (9,10). We demonstrated that β -cell-specific overexpression of GPx-1 in the *db/db* mouse (8) preserved

β -cell mass and reversed the development of hyperglycemia. It is unknown whether this degree of resolution of the diabetic state can be achieved in vivo with an orally administered antioxidant.

Ebselen (2-phenyl-1, 2-benziselenazol-3(2H)-one), a nontoxic seleno-organic drug, is a lipid soluble, orally bioavailable small molecule classified as a GPx mimetic (11–13) and is currently in use in human trials for hearing loss and neurovascular disease (14–17). Zucker diabetic fatty (ZDF) rats are obese, are leptin receptor negative, and develop glucose intolerance, insulin resistance, and fasting hyperglycemia (18,19). Effects of ebselen on β -cell mass and islet function have not been reported. Ebselen was used for 8 weeks to determine whether it would prevent oxidative stress and apoptosis and protect against diminution of β -cell mass and function, as well as loss of intranuclear Pdx-1 and MafA. Study end points included 1) fasting and fed glucose, insulin, and HbA_{1c} levels; 2) insulin sensitivity; 3) morphometric analysis of islets, β -cell apoptosis, and replication; 4) oxidative stress markers; and 5) intranuclear expression of MafA and Pdx-1.

RESEARCH DESIGN AND METHODS

Male ZDF rats (*ZDF-Lepr^{fa/fa}*) were obtained from Charles River (Wilmington, MA) at 5 weeks of age and fed Purina 5008 chow. At 6 weeks of age, the rats were divided into two experimental groups ($n = 12$ per group) and treated with 128 mg/kg/day ebselen (Cayman Chemicals, Ann Arbor, MI) by oral gavage (64 mg/kg/day twice daily) for 8 weeks. Carboxymethyl cellulose was chosen instead of dimethyl sulfoxide, a frequently used alternative, because the latter has antioxidant properties. Earlier, we had performed ebselen-dose oral glucose tolerance test (OGTT)-response work over 6 weeks using ZDF animals and ebselen doses of 0, 4, 16, and 64 mg/kg b.i.d., which were associated with fasting glucose levels of 293 ± 21 , 257 ± 12 , 199 ± 11 , and 120 ± 10 mg/dL, respectively (20). The 64 mg/kg b.i.d. dose was the optimal dose for reducing the glucose area under the curve during OGTTs. This is consistent with the work of Brodsky et al. who found no effect on hyperglycemia of gavaging 5 mg/kg twice daily in ZDF rats (12). The control group was treated with 0.5% carboxymethyl cellulose in ddH₂O. Ebselen was prepared by making a 20 mg/mL suspension in 0.5% carboxymethyl cellulose fresh daily with constant mixing during dosing. Nonfasting blood glucose and insulin levels, OGTT, insulin tolerance test (ITT), insulin sensitivity in cultured adipocytes, and morphometric analysis were studied previously (4,8) and described in figure legends. Morphometry of islets and immunohistochemistry studies of markers of oxidative stress, Ki67, Pdx-1, and MafA were performed as previously described (8). Results are expressed as mean \pm SEM. Statistical analysis was carried out with InStat Biostatistics 3.0. Comparisons between groups were done by Student *t* test and ANOVA, where appropriate.

RESULTS

Nonfasting glucose and insulin levels. By the end of the study, ebselen treatment had significantly lowered the nonfasting blood glucose level compared with control rats (268 ± 33 vs. 374 ± 33 mg/dL; $n = 10$ each; $P < 0.05$) (Fig. 1A). Insulin levels in both groups were maximally increased

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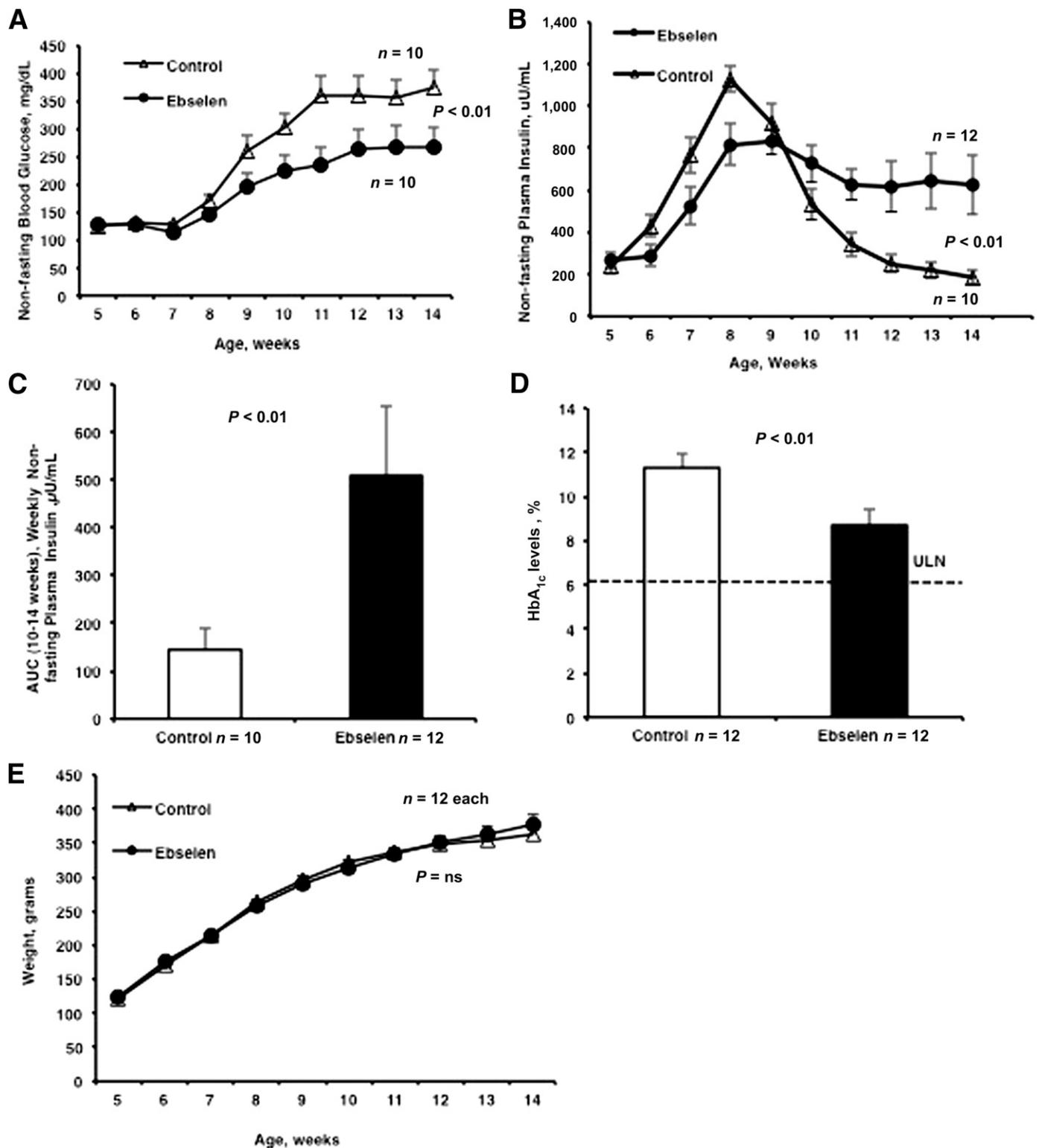


FIG. 1. Ebselen effects on weekly nonfasting blood glucose level, nonfasting plasma insulin level, HbA_{1c}, and body weight. (A) The nonfasting blood glucose levels demonstrated a significant difference at 14 weeks when comparing control rats with ebselen-treated animals ($n = 10$ each; $P < 0.01$). (B) Ebselen-treated rats ($n = 12$) sustained nonfasting plasma insulin levels whereas the control rats ($n = 10$) had significantly lower insulin levels from 11 through 14 weeks of age; $P < 0.01$. (C) The area under the curve insulin levels at 10 through 14 weeks was significantly lower in the controls versus the ebselen-treated rats ($P < 0.01$). (D) HbA_{1c} levels were greater in controls compared with ebselen-treated rats at the end of 14 weeks (11.3 vs. 8.3%; $n = 12$ each; $P < 0.01$). The dashed line represents the upper limit of normal. (E) Body weights measured weekly showed no significant difference between control rats and ebselen-treated rats ($n = 12$ each; $P = ns$). AUC, area under the curve; ULN, upper limit of normal.

by 8–9 weeks of age. The control rats thereafter had decreased insulin levels, whereas the ebselen-treated rats maintained higher, relatively constant insulin levels. There was a significant difference in the insulin levels between control and ebselen-treated rats from 10 to 15 weeks of age ($P < 0.01$) (Fig. 1B). The area under the curve for insulin levels at 10–15 weeks was significantly less in the controls versus the ebselen-treated rats ($P < 0.01$) (Fig. 1C). HbA_{1c} levels were greater in controls compared with ebselen-treated rats (11.3% or 100 mmol/mol vs. 8.3% or 67 mmol/mol; $P < 0.01$) (Fig. 1D). There were no significant differences in body weight between controls and ebselen-treated rats (363 ± 10 vs. 378 ± 12 g at 14 weeks; $P = ns$) (Fig. 1E).

OGTTs and ITTs and glucose uptake in cultured adipocytes. Fasting blood glucose levels were significantly less in ebselen-treated versus untreated animals (155 ± 14 vs. 259 ± 16 mg/dL). Control rats exhibited higher glucose levels during OGTT (Fig. 2A). Data from OGTT, expressed as change above fasting glucose levels, demonstrated a significant difference when comparing control rats to ebselen-treated rats (149 ± 13 vs. 92 ± 10 mg/dL; delta glucose level at 2 h; $P < 0.01$) (Fig. 2B).

Blood glucose levels during insulin tolerance tests (ITTs) demonstrated no significant difference in rate of fall in glucose in the first 60 min nor was there a significant difference in the blood glucose nadirs (Fig. 2C). Cultured 3T3L1 adipocytes were used as an independent model to test the effect of ebselen on insulin sensitivity. Only a small tendency toward increased insulin sensitivity with ebselen treatment was observed (Fig. 2D).

β -cell mass, apoptosis, and replication. Immunoperoxidase staining revealed islets in the control rats that had irregular shape with poor insulin staining [Fig. 3A(a)]. β -Cell mass was doubled in ebselen-treated compared with control rats (6.8 ± 1.25 vs. 13.6 ± 1.53 mg; $n = 6$; $P < 0.05$). Islets from the ebselen group exhibited regular shape with intense insulin staining [Fig. 3A(b)]. TUNEL-positive cells were easily detectable in untreated rats [Fig. 3B(a)], whereas β -cell apoptosis was rarely positive in ebselen-treated rats [Fig. 3B(b)]. Quantification of apoptotic cells revealed a statistically significant difference between the groups (control = $4.56\% \pm 0.31\%$; ebselen treated = $0.03\% \pm 0.18\%$; $n = 4$; $P < 0.001$). Ki67 immunostaining to estimate β -cell replication was minimal in both groups (Fig. 3C). The number of cells that costained

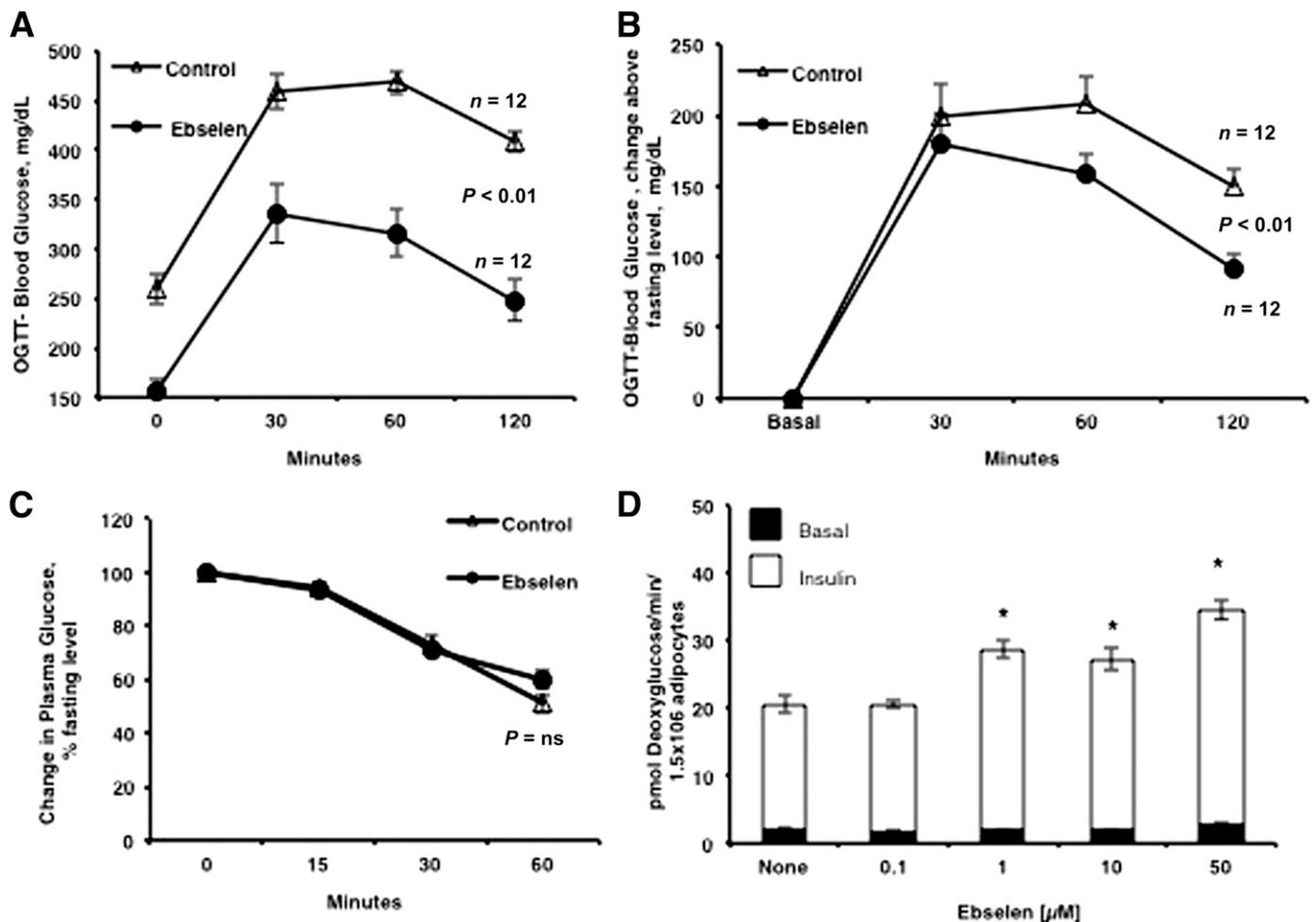


FIG. 2. Effect of ebselen on OGTTs and ITTs. (A) Ebselen normalized fasting hyperglycemia and lowered blood glucose levels during OGTT compared with controls ($n = 12$ each; $P < 0.01$). (B) Change above fasting glucose levels during OGTT demonstrated a significant difference when comparing control rats with ebselen-treated rats ($n = 12$ each; $P < 0.01$). (C) Insulin sensitivity was measured by ITT. There were no significant differences in the initial rate of fall of blood glucose or the nadirs in control rats versus ebselen-treated rats. (D) Glucose uptake was measured in differentiated 3T3L1 adipocytes. Ebselen treatment resulted in no significant differences in basal glucose uptake but caused a small increase in insulin-stimulated glucose uptake ($*P < 0.05$).

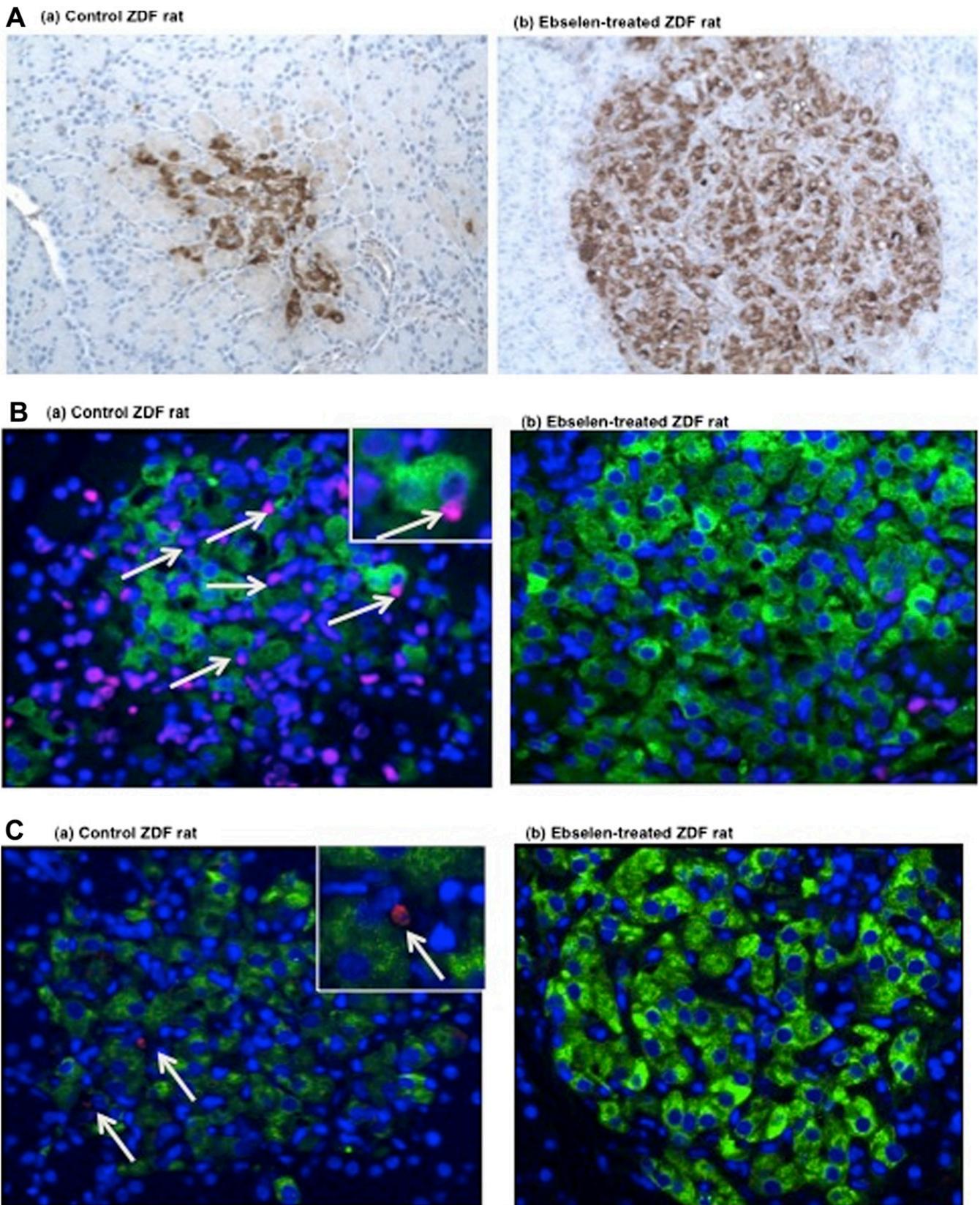


FIG. 3. Morphometric analysis of β -cells and apoptosis. (A) Islet morphology of control rats (a) and ebselen-treated rats (b) as shown by immunoperoxidase staining for insulin. (a) The control rats showed degranulation and irregularity in islet shape. (b) Ebselen-treated rat islets were double the size of islets from control rats, had robust granulation and a normal morphological pattern, and exhibited intense islet insulin staining (hematoxylin counterstained, $n = 4$) in β -cells. (B) The pancreatic tissue sections were stained for apoptosis using TUNEL method. TUNEL-positive nuclei (indicated by arrows) were (a) present in control rats and (b) rarely found in ebselen-treated rats ($n = 4$; magnification is $20\times$). The box represents the magnified area shown in insets. (C) Measurement of β -cell replication. Double immunostaining for Ki67 (red fluorescence indicated by arrows) and insulin (green fluorescence) in islets from rats treated with or without ebselen. (a) Control rats and (b) ebselen-treated rats islets did not stain for Ki67 ($n = 4$ each; magnification is $20\times$).

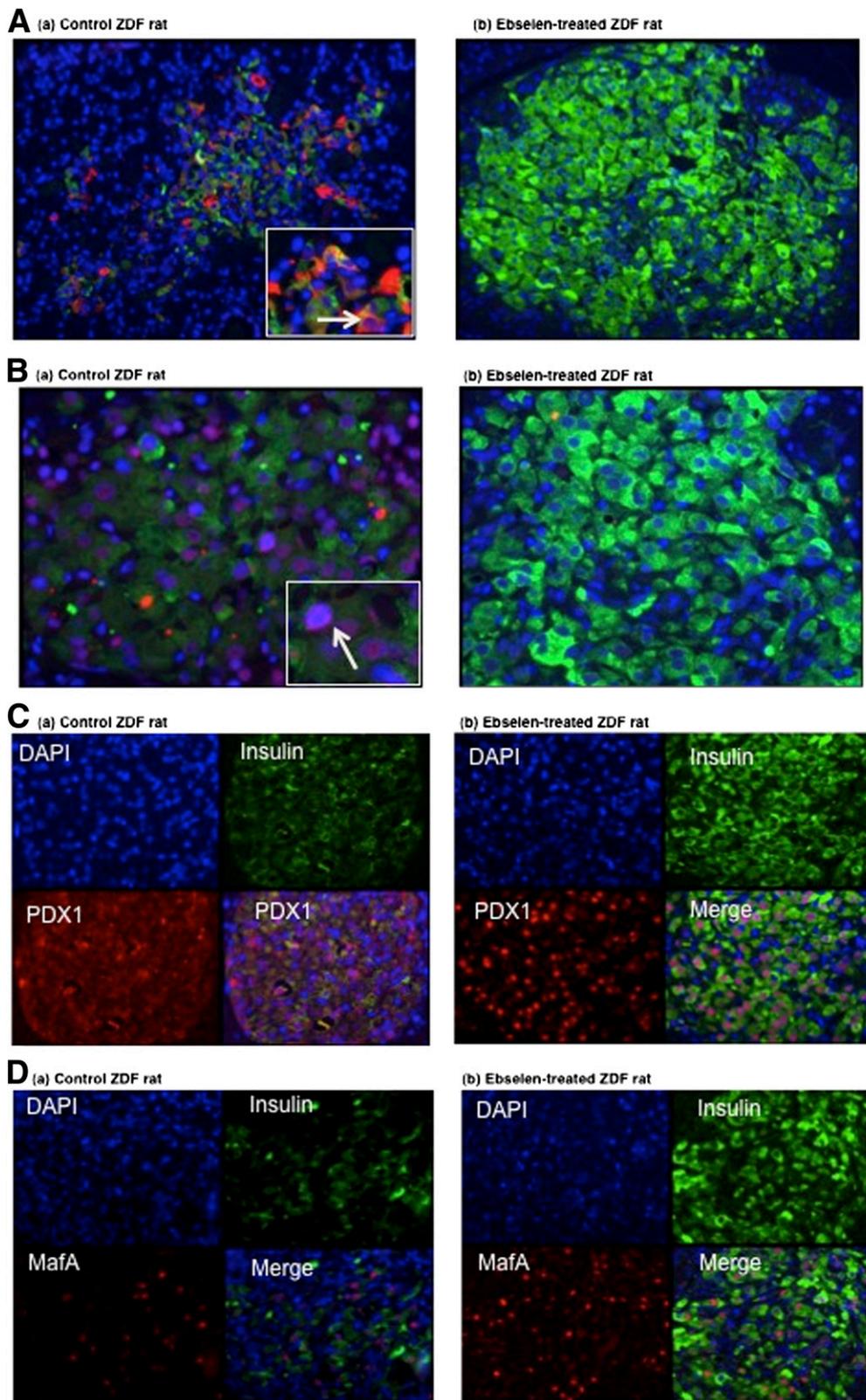


FIG. 4. Effect of ebselen on lipid peroxidation, DNA oxidation, Pdx-1, and MafA. See RESULTS for quantitative data and statistics. (A) Pancreatic sections were double labeled for insulin (green fluorescence) and 4-HNE (red fluorescence) using islets from control rats and ebselen-treated rats. (a) The control rats showed intense expression of 4-HNE in the cytoplasm of β -cells (red fluorescence indicated by arrow). (b) Inset represents a magnified area. Ebselen treatment prevented the expression of 4-HNE in β -cells, as shown in Fig. 2A (magnification is 20 \times). (B) Pancreatic sections were double immunostained for insulin (green fluorescence) and 8-OHdG (red fluorescence). (a) The control rats showed intense expression of 8-OHdG in the nuclei of β -cells (red fluorescence indicated by arrow). (b) β -Cells stained poorly for 8-OHdG in the ebselen-treated rats ($n = 4$). (C) Double-labeling immunofluorescent staining for insulin (green) and Pdx-1 (red) in islets from untreated or ebselen-treated rats. In control rats, the nuclear counts for Pdx-1 were fewer than were observed in the ebselen-treated rats. (D) Double-labeling immunofluorescence staining for insulin (green fluorescence) and MafA (red fluorescence) in islets from untreated or ebselen-treated rats. (a) Very few β -cells were present in islets from control animals, but most stained for nuclear MafA, (b) whereas islets from ebselen-treated rats showed many β -cells and greater intranuclear expression of MafA (magnification 20 \times). DAPI, 4',6-diamidino-2-phenylindole.

for both insulin and Ki67 was divided by the total number of insulin-positive cells per each field to obtain the percentage of actively dividing β -cells (6-week ZDF rats = $1.8 \pm 0.23\%$ vs. 14-week control ZDF rats = $0.56 \pm 0.19\%$; $n = 4$; $P < 0.05$). Ebselen-treated ZDF rat islets did not stain for Ki67 ($n = 4$). We also used 4-week *db/db*-GPx animals (8) as positive controls to be certain that the antibody works well. In addition, we performed negative staining to be certain fluorescein isothiocyanate and CY3 did not stain tissues when omitting the antibody.

Oxidative stress marker expression in β -cells from ZDF rats. To determine the mechanisms by which ebselen preserved β -cell morphology and function, 10 pancreatic sections from each of four control and four ebselen-treated rats were stained for 4-hydroxy-2-nonenal (HNE; an oxidative stress marker for lipid peroxidation) and another oxidative stress marker, 8-hydroxydeoxyguanosine (8-OHdG), which binds to oxidized DNA. The untreated rats exhibited intense cytoplasmic staining of 4-HNE in β -cells [Fig. 4A(a)], which was virtually absent in ebselen-treated rats [Fig. 4A(b)], indicating that ebselen had reduced lipid peroxidation. The untreated rats exhibited 8-OHdG abundantly in nuclei of β -cells [Fig. 4B(a)], whereas the ebselen-treated rats did not ($68 \pm 3.1\%$ vs. $1.7 \pm 0.2\%$; $P > 0.0001$) [Fig. 4B(b)].

Expression of insulin transcription factors Pdx-1 and MafA. To examine the effect of ebselen on Pdx-1 and MafA levels, double-labeling immunofluorescence staining was performed using 10 pancreatic sections from each of four control and four ebselen-treated rats for each nuclear transcription factor. The untreated rats had cytoplasmic localization of Pdx-1 with fewer β -cells showing nuclear staining [Fig. 4C(a)] compared with the ebselen-treated rats whose Pdx-1 was more abundant (718 ± 11 vs. 431 ± 10 ; $P < 0.001$) and localized exclusively in the nucleus [Fig. 4C(b)]. Expression of MafA was present in the control rats, although fewer β -cells were observed [Fig. 4D(a)]. In ebselen-treated rats, MafA was clearly evident in the nuclei of β -cells and more abundant (849 ± 7 vs. 212 ± 6 ; $P < 0.0001$) [Fig. 4D(b)].

DISCUSSION

The current work was designed to document oxidative stress markers within β -cells of hyperglycemic ZDF rats and to determine whether these markers were associated with apoptosis and absence of two critical insulin transcription factors in the nucleus and, if so, whether these abnormalities could be prevented by the drug ebselen, an oral GPx mimetic. In this study, 8-week treatment of ZDF rats with oral ebselen alone and no other drugs significantly ameliorated fasting hyperglycemia, improved glucose tolerance and postprandial levels of glucose and insulin, and maintained lower levels of HbA_{1c} compared with nontreated animals. Ebselen prevented accumulation of 4-HNE and 8-OHdG (markers of oxidative stress) in the β -cell and increased β -cell mass twofold over that observed in age-matched untreated animals. The expansion of β -cell mass in ebselen-treated animals was accompanied by robust insulin immunostaining. Control animals had 50% fewer β -cells that stained poorly for insulin. Islet apoptosis was abundant in the untreated animals but rare in the ebselen-treated animals. Intranuclear Pdx-1 and MafA levels were enhanced in β -cells of ebselen-treated animals compared with the untreated animals. ITTs failed to detect effects of ebselen on insulin sensitivity in ZDF rats. Ebselen treatment of

cultured adipocytes caused a small increase in insulin-stimulated glucose uptake consistent with a mild antioxidant effect (21). There were no differences in β -cell replication or body weight between the two groups. These favorable outcomes of ebselen therapy indicate that the primary mechanism of action of this drug on β -cells is prevention of oxidative stress. This led to prevention of apoptosis and maintenance of sufficient β -cell mass to compensate for the insulin resistance caused by obesity characteristic in ZDF rats (18) with an associated enhancement of intranuclear Pdx-1 and MafA levels.

Several interesting points arise when comparing our ZDF data with those previously published (12,22–26). The fasting glucose levels in the ebselen group, although not normal by human standards, were similar to levels are found in Zucker lean control animals (12). The HbA_{1c} levels reached were not normalized by ebselen treatment but are consistent with the persistence of impaired glucose tolerance in the high-fat-fed state and the fact that chow was available in the cages 24 h/day during these 8 weeks. The low levels of Ki67 in 14-week-old control and ebselen-treated ZDF animals compared with 6-week-old control animals suggests that by 14 weeks ZDF rats have aged sufficiently to enter a period of low islet replication. This, combined with the impressively high rate of islet apoptosis in the control, but not the ebselen-treated, group contributes heavily to the natural history of severe hyperglycemia in ZDF animals fed a high-fat diet.

Type 2 diabetes (T2D) in humans is a disease that is typified by an inexorable decline over time in β -cell function despite optimal therapy with conventional anti-hyperglycemic therapeutic agents. The initial cause of hyperglycemia in T2D is considered to involve polygenic/epigenetic defect(s) in β -cell function, which are amplified by states of insulin resistance. Studies in cell lines and animal models have suggested that this relentless decline in β -cell function is secondary to chronic hyperglycemia, which causes chronic oxidative stress (3–5). This line of thinking is supported by the fact that β -cells have poor intrinsic antioxidant activity (9,10) and are therefore at major risk for oxidative damage. Our current study demonstrates for the first time that the oral antioxidant drug ebselen, approved for use in human trials (14–17), prevents not only oxidative stress and the nucleocytoplasmic dislocation of Pdx-1 and MafA in β -cells, but also β -cell apoptosis associated with hyperglycemia in a robust rodent model of T2D. These findings point to the important opportunity to evaluate the potential use of oral ebselen for prevention of continuing deterioration of β -cell function and mass in humans with impaired glucose tolerance.

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J.M. wrote the manuscript and performed the imaging studies. S.P. and E.O. performed laboratory assays. A.V.H. performed adipocyte studies. D.A.B., S.N.V., and J.S.H. contributed to discussion. C.-q.L. and M.L. performed the animal gavages. R.P.R. wrote the manuscript. R.P.R. is the

guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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AUTHORS' NOTE

There are changes between this version of the manuscript and the version initially posted online on 25 June 2013. Figure 3C here is a new version representing the Ki67 studies. The former was mistakenly chosen. The legend to the figure has been changed, as well as the corresponding text. The changes report that ebselen-treated rats did not stain for Ki67.

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