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The role of CHOP messenger RNA expression in the link between oxidative stress and apoptosis

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Abstract

Low expression of antioxidant enzymes makes pancreatic β-cells susceptible to cell damage by oxidative stress. Pancreatic β-cell loss caused by endoplasmic reticulum stress is associated with the onset of diabetes mellitus. The present studies were undertaken to investigate a possible involvement of proapoptotic gene CHOP in pancreatic β-cells damage by oxidative stress. The induction of CHOP messenger RNA and apoptosis were investigated in βHC-9 cells after the oxidative stress by hydrogen peroxide and ribose. Latter was examined after the suppression of CHOP by small interfering RNA. For in vivo study, the pancreatic β-cells were examined in CHOP-knockout (KO) mice after multiple low-dose streptozotocin (MLDS) administration. In βHC-9 cells, both hydrogen peroxide and ribose obviously increased apoptotic cells, accompanied with enhanced CHOP messenger RNA expression. However, the number of apoptotic cells by those stimulations was significantly reduced by the addition of small interfering RNA against CHOP. In vivo study also showed that CHOP-KO mice were less susceptible to diabetes after MLDS administration. Although the oxidative stress marker level was similar to that of MLDS-treated wild type, the pancreatic β-cell area was maintained in CHOP-KO mice. The present studies showed that CHOP should be important in pancreatic β-cell injury by oxidative stress and indicate that CHOP may play a role in the development of pancreatic β-cell damage on the onset of diabetes mellitus.

1. Introduction

Type 2 diabetes mellitus is a metabolic disorder characterized by impaired glucose homeostasis resulting from defects in insulin action and/or insulin secretion, with underlying genetic and environmental causes. Pancreatic β-cells are known to play an important role in glucose homeostasis by secreting insulin. It has become clear that both the number and volume of pancreatic β-cells change dramatically under various physiologic and pathologic conditions. It is considered that excessive loss of β-cell number will lead to the loss of insulin secretion and cause diabetes. One of the potential mechanisms of the reduction of β-cell number is considered to be apoptosis triggered by oxidative stress that is caused by chronic hyperglycemia [1-6].

Pancreatic β-cells are subject to injury from oxidative stress because the expression of antioxidant enzymes is low compared with cells in other tissues [7]. Both in vitro and in vivo experiments show that hyperglycemia induces pancreatic β-cell dysfunction and that antioxidants are effective in preventing this dysfunction [8-14]. These data indicate the linkage between hyperglycemia, oxidative stress, and β-cell dysfunction in diabetes. However, the precise mechanisms that mediate these 3 factors remain unclear.

It has been reported that one of the pathways to apoptosis that is triggered by various stress is the transcriptional induction of the gene for CHOP (C/EBP

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homologous protein)/GADD 153 (growth arrest and DNA damage) [15-18]. CHOP-10/GADD153 is a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors. It acts as a dominant negative regulator of C/EBPs by forming heterodimers with other C/EBPs [19]. CHOP expression is barely detected under physiologic conditions but strongly induced by cellular stress like DNA damage, growth arrest, and endoplasmic reticulum (ER) stress [20,21]. It is also known that the expression of CHOP increases before apoptosis, and suppression of CHOP expression prevents apoptosis [15-17]. So far, several genes that respond to CHOP have been identified; but none was shown to be directly involved in the process of apoptosis, and the role of CHOP in pancreatic β-cell is not established [22,23].

In this article, we explore the mechanism that links oxidative stress and apoptosis in pancreatic β-cell. Our findings support the idea that expression of CHOP mediates the oxidative stress and apoptosis of pancreatic β-cell in generation of diabetes. We also show that reduction of CHOP expression suppresses apoptosis caused by oxidative stress in pancreatic β-cell. These results indicate that CHOP may be a potential target for the treatment of diabetes.

2. Material and methods

2.1. Cell culture

βHC-9 cells were kindly provided by Dr Douglas Hanahan (University of California at San Francisco). The βHC-9 cell line, derived from pancreatic islets with β-cell hyperplasia, is characterized to have a normal concentration-dependency curve for glucose-stimulated insulin release [24]. Cells were grown in 5% CO_2/95% O_2 at 37°C and maintained in Dulbecco modified Eagles medium containing 25 mmol/L glucose, 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin, and 20 mmol/L glutamine.

2.2. Measurements of intracellular peroxides

As described before [6], oxidative activity was detected by using the fluorescein-labeled dye 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (Invitrogen, Carlsbad, CA). The acetoxymethyl ester derivative readily permeates cell membranes and is trapped within the cell after cleavage by esterases. Oxidation by reactive oxygen species (ROS) converts the dye from its nonfluorescent to fluorescent form. Cells were cultured with 10 mmol/L 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate for 30 minutes at 37°C. After incubation with the dye, cells and islets were washed with phosphate-buffered saline (PBS) to prevent measurement of any extracellular reaction between the dye and H_2O_2 released by the cells into the medium. Cells were dispersed by using trypsin, and intracellular peroxide levels were measured with the flow cytometer FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

2.3. Measurements of CHOP messenger RNA

Total RNA was extracted from βHC-9 cells by using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). One-step reverse transcription–polymerase chain reaction was carried out by using the Assays-on-Demand Gene Expression Products and Taqman One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA) with ABI prism 7700 Sequence detector. All data were standardized by expression of β-actin. The CHOP messenger RNA (mRNA) was analyzed by relative expression to β-actin mRNA expression.

2.4. Measurements of apoptosis

Apoptosis was detected by flow-cytometric analysis using Annexin V-FITC kit (Bender MedSystems, Burlingame, CA). The cells were dispersed by using trypsin and washed twice with ice-cold PBS. The cells were resuspended in binding buffer and incubated with Annexin V-FITC for 10 minutes. They were centrifuged and resuspended, and 1 μg/mL propidium iodide (PI) was added. Flow-cytometric analysis was performed; and the percentages of annexin-positive, PI-negative cells (apoptotic cells) of all cells counted (10 000 of βHC-9 cells or 5000 of islet cells) were detected by FACSCalibur (Becton Dickinson).

2.5. Electroporation of βHC-9 cells and transfection of small interfering RNA for CHOP

The small interfering RNA (siRNA) against CHOP, the stealth RNA interference (RNAi), was obtained from Invitrogen. The siRNA against CHOP was transfected by electroporation as described before [25]. The sequences were UUUGGGAUGUGCGUGUGACCUCUGU (sense) for CHOP RNAi and UUUCUGUAGGCGUGUGUGCCAGUGU (sense) for control RNAi.

2.6. Animals

All animals were housed in a temperature-controlled room with a 12-hour light/dark cycle (illumination from 6:00 AM to 6:00 PM). Standard chow pellets and drinking water were available ad libitum. CHOP-knockout (KO) mice were obtained by the method described previously [16,18]. Briefly, the targeting vector, designed to replace a 1.2-kilobase genomic fragment containing parts of exon 3 and 4 of CHOP genome with pMC1-neo, was electroporated into embryonic stem cells; and its clones were microinjected into blastocysts of C57BL/6 mice to generate chimeric mice. The chimeric male mice were mated with C57BL/6 female mice. Isolated islets and oral glucose tolerance test (OGTT) after streptozotocin (STZ) injection of the age-matched C57BL/J male mice (Japan SLC, Shizuoka, Japan) were used as a control. In the histologic

analysis study, control mice were wild male mice that were littermates of KO mice. Similar to what we have reported previously [26], the CHOP-KO male mice had no difference in metabolic state (body weight and circulating glucose, insulin, free fatty acid, triglyceride, and total cholesterol levels) compared with the wild mice. All experimental protocols were approved by the Animal Use and Care Committee of the Gunma University Graduate School of Medicine.

2.7. Pancreatic islet isolation

The pancreata of male mice were infused with 1.5 mg/mL collagenase type XI (Sigma-Aldrich, St Louis, MO)/1% fetal bovine serum/2 U/mL RQ1 DNase (Promega, Madison, WI) solution in Medium 199 (Invitrogen). After surgical dissection, the pancreas was incubated in the collagenase solution at 37°C. Digested tissue was washed twice with ice-cold Hanks balanced salt solution containing 0.1% bovine serum albumin followed by centrifugation at 250*g* for 4 minutes. Islets were separated by using a Histopaque-1083 gradient (Sigma-Aldrich) and then hand-picked and cultured overnight in Pancreatic Islet Media (hCell technology, Reno, NV).

2.8. Measurement of insulin secretion from isolated islets and insulin content

Isolated islets were preincubated for 1 hour with Krebs-Ringer buffer (118.5 mmol/L NaCl, 2.54 mmol/L $CaCl_2$-H_2O, 1.19 mmol/L KHP_2O_4, 4.74 mmol/L KCl, 25 mmol/L $NaHCO_3$, 1.19 mmol/L $MgSO_4$, 10 mmol/L HEPES, 0.1% bovine serum albumin, pH 7.4) containing 1 mmol/L glucose. Five islets per well were incubated for 1 hour with Krebs-Ringer buffer containing 1 or 20 mmol/L glucose after centrifugation, and the supernatant was stored at −20°C until assay. Islets were collected to acidified ethanol and incubated overnight at −20°C, and insulin content was measured. Insulin concentrations were assayed by using Mouse Ultrasensitive Insulin ELISA Kit (Mercodia AB, Uppsala, Sweden).

2.9. Induction of diabetes by multiple low-dose STZ

Streptozotocin (Sigma-Aldrich) was dissolved in citrate buffer (pH 4.5) and intraperitoneally injected into wild-type (WT) or CHOP-KO mice by 50 mg/kg body weight for consecutive 5 days [27].

2.10. Oral glucose tolerance test

At the start of STZ administration and 3 weeks after the treatment, OGTT was performed. After fasting for 16 hours, mice were orally administered glucose solution (1.5 g/kg weight); and blood glucose levels were chronologically measured at 10, 20, 30, 60, 90, and 120 minutes after glucose loading by using glucose oxidase method.

2.11. Histologic analysis

Two weeks after the start of STZ administration, the mice pancreata were removed and fixed in formalin or Bouin solution (for 8-hydroxy-2-deoxyguanosine [8-OHdG] staining; Muto Purechemicals, Tokyo, Japan). Sections, cut from paraffin-embedded tissues, were deparaffinized and then stained by hematoxylin-eosin, anti-insulin and anti-8-OHdG antibody, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method.

For immunohistochemistry with insulin antibody, the sections were blocked for endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 30 minutes, incubated with 10% normal pig serum for 30 minutes to block nonspecific staining, and incubated with anti–human insulin (Oriental Yeast, Tokyo, Japan). After being washed with PBS, the sections were incubated with biotinylated goat anti–rabbit immunoglobulins (DAKO, Glostrup, Denmark) and peroxidase-conjugated streptavidin (DAKO), and then incubated with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin.

8-Hydroxy-2-deoxyguanosine staining was used as an oxidative stress marker. The sections—deparaffinized, hydrated, and blocked for the endogenous peroxidase—were incubated at 4°C with anti–8-OHdG monoclonal antibody (Japan Institute for the Control of Aging, Shizuoka, Japan) overnight and, after washing, incubated with second antibody; and then color development was achieved using DAB substrate. These nuclei were stained with Mayer hematoxylin solution.

Apoptotic nucleus was detected by using the TUNEL method. Deparaffinized, hydrated sections were incubated with 0.3% hydrogen peroxide for 30 minutes to block endogenous peroxidase, incubated with terminal transferase and biotin-16-2′-deoxyuridine,5′-triphosphate (dUTP) (Roche, Basel, Switzerland), and then incubated with peroxidase-conjugated streptavidin. Finally, color development was achieved by using DAB substrate; and the sections were counterstained with methyl green.

For quantitative analysis, we used the method previously reported by Burns and Gold [28], with some modifications. Three nonconsecutive sections randomly selected were examined for each of the 3 mice from the wild or KO group. Up to 10 islets from each section were examined for the evaluation. The area of islet and the TUNEL or 8-OHdG staining positive cells in each islet were counted by using image analysis system (Lumina Vision; Mitani, Fukui, Japan). The means of positive cell count per islet area (count per square millimeter) were calculated for each mice, and the means of each group (wild or KO) were analyzed with Duncan multiple range test.

2.12. Statistics

All data represent mean ± SEM. The statistical analysis was performed by analysis of variance, followed by Duncan multiple range test for the individual difference of the means. Statistical significance was considered at $P < .05$.

3. Results

3.1. Accumulation of intracellular peroxide and induction of CHOP mRNA after exposure to oxidative stress

To investigate the interaction between oxidative stress and CHOP expression in β-cell apoptosis in vitro, we used hydrogen peroxide and ribose as an oxidative stress model. At 50 μmol/L, both hydrogen peroxide and 50 mmol/L ribose similarly induced chronological accumulation of intracellular peroxide in βHC-9 cells (Fig. 1A, B). The CHOP mRNA expression was induced by hydrogen peroxide (50 μmol/L) and ribose (50 mmol/L) (Fig. 1C). The CHOP mRNA induction was significantly higher than control at 4 hours after stimulation with 50 μmol/L hydrogen peroxide ($P < .005$ vs control) and at 72 hours with 50 mmol/L ribose ($P < .05$ vs control).

3.2. Induction of apoptosis

Next, we confirmed that oxidative stress induced apoptosis by using flow cytometry with Annexin V-FITC

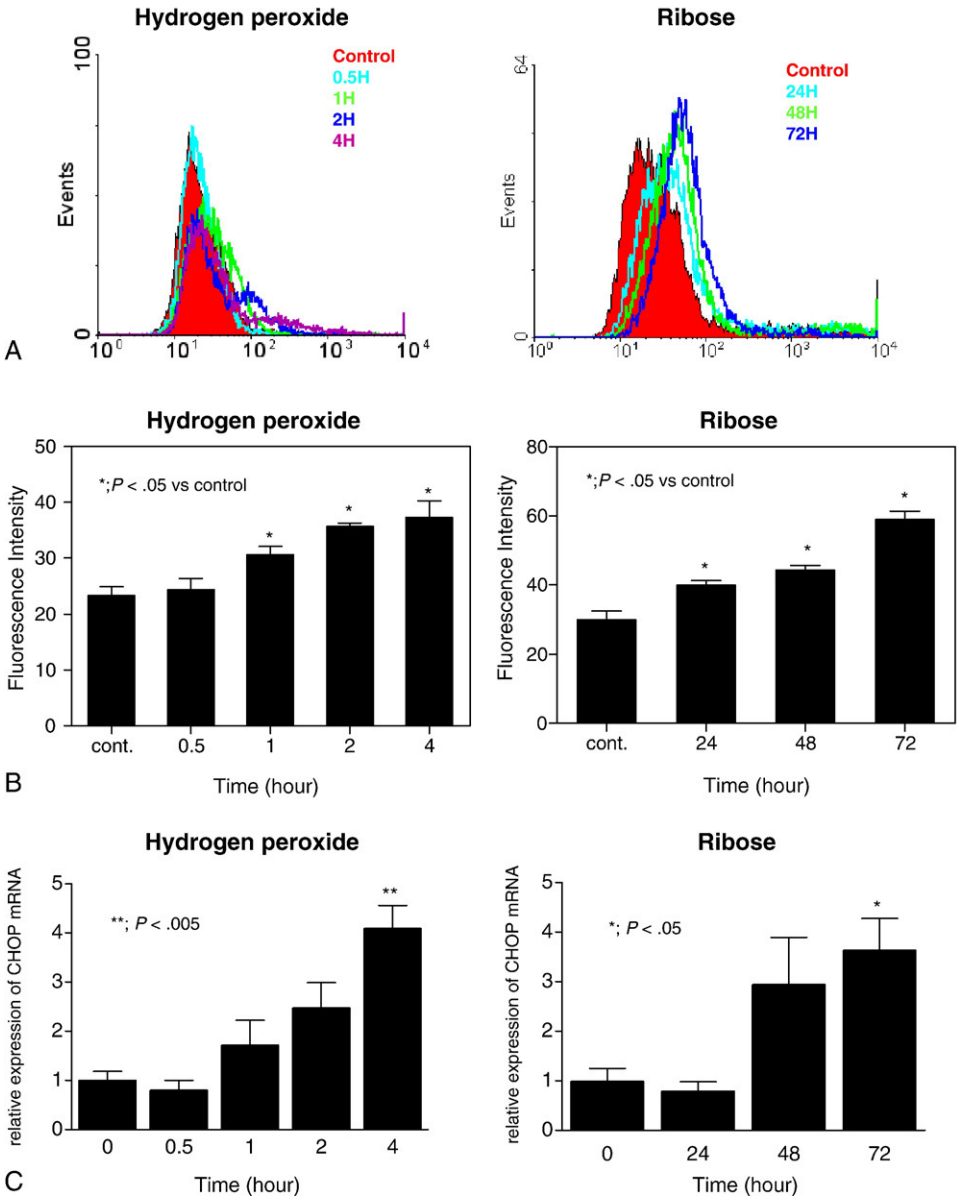

Fig. 1. Intracellular peroxide levels induced by oxidative stress determined by flow cytometry. βHC-9 cells were incubated with a peroxide-sensitive probe, dichlorohydrofluorescein diacetate (using 4 and 10 mmol/L, respectively), during the final 30 minutes of each treatment. Data are expressed as mean ± SEM (n = 3). (A) Actual event in cytometry using dye. Cells were exposed to 50 μmol/L hydrogen peroxide for 0 (red), 0.5 (magenta),1 (green), 2 (blue), and 4 hours (purple) or 50 mmol/L ribose for 24 (blue), 48 (green), and 72 hours (purple). The right shift of peak in x-axis indicates the increase in positive cells. (B) Fluorescence intensity after exposure to H_2O_2 and ribose. (C) Time-dependent relative expression of oxidative stress–induced CHOP mRNA by 50 μmol/L hydrogen peroxide (left panel) for 4 hours or 50 mmol/L ribose (right panel) for 72 hours compared with 0 hour after application.

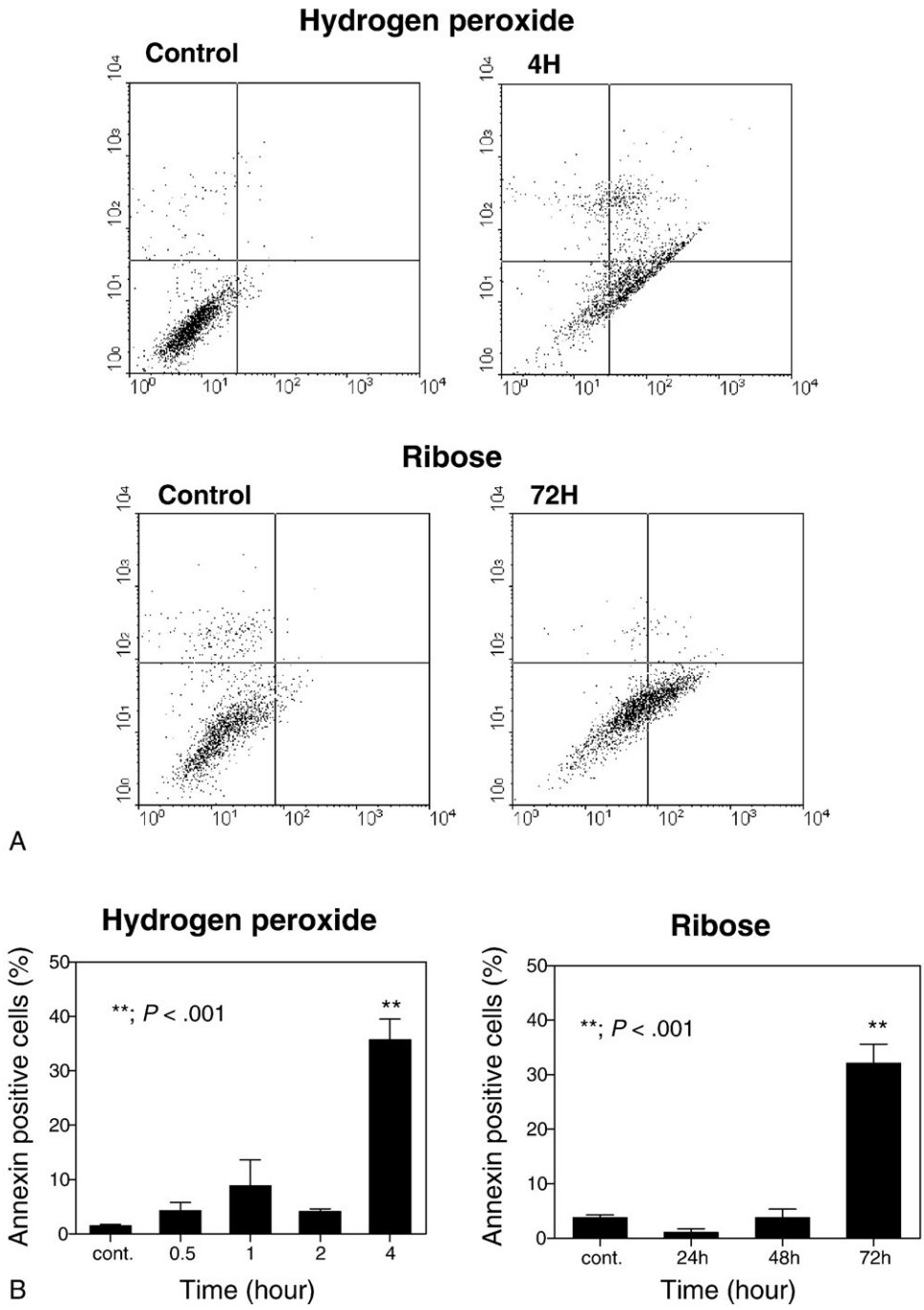

Fig. 2. Induction of apoptosis by oxidative stress. Fifty micromoles per liter of hydrogen peroxide for 4 hours or 50 mmol/L ribose for 72 hours. (A) Actual event in cytometry using dye. (B) The percentage of annexin-positive cells was increased significantly ($P < .001$) compared with control. Data are expressed as mean ± SEM (n = 3).

kit. As Fig. 2A shows, the annexin-positive cells (ie, preapoptotic cells) locate to the right direction; and PI-positive cells (ie, necrotic cells) locate to the upper direction in the panel. We counted the percentage of cells in the right lower quarter area as the percentage of apoptotic cells. As shown in Fig. 2B, apoptosis was significantly induced at 4 hours after the start of stimulation with 50 μmol/L hydrogen peroxide ($P < .001$ vs control) and at 72 hours with 50 mmol/L ribose ($P < .001$ vs control).

Although the level of peroxide and CHOP mRNA had a continuous time-dependent increase (as shown in Fig. 1), the induction of apoptosis occurred only in 4 hours (hydrogen peroxide) and 72 hours (ribose) after the application. This result indicates that accumulation of peroxide or CHOP

mRNA to a certain threshold level is required for the actual induction of apoptosis.

3.3. Induction of apoptosis after suppression of CHOP by siRNA

To examine whether CHOP induction is directly associated with apoptosis by oxidative stress, we transfected the siRNA against CHOP gene (CHOP siRNA) and checked the induction of apoptosis by oxidative stress induced by hydrogen peroxide and ribose using βHC-9 cells. By transfection with siRNA, CHOP mRNA was reduced by 50% of controls at both 48 and 72 hours. Application of 20 μmol/L hydrogen peroxide for 4 hours induced apoptosis about 3 times higher in the cells transfected with control siRNA than in nonstimulated cells. In contrast, the apoptotic cell rate was significantly reduced by transfection of CHOP siRNA ($P < .005$ vs control; Fig. 3A, C). In addition, application of 50 mmol/L ribose for 48 hours also induced significantly less apoptosis in the cells transfected with CHOP siRNA than control (nonsense) siRNA ($P < .005$ vs control; Fig. 3B, D).

3.4. Glucose-stimulated insulin secretion in CHOP-KO mice treated with multiple low-dose STZ

We investigated the hypothesis that CHOP may play an important role in apoptosis of β-cells by oxidative stress in vivo. We used CHOP-KO mice and examined glucose-stimulated insulin secretion in the islets of WT and CHOP-KO mice. As shown in Fig. 4A, insulin secretions in response to high glucose were not significantly different between WT and KO mice by nature. Next, we treated these mice with STZ as an oxidative stress model for diabetes. Fig. 4B shows change of blood glucose concentration after oral glucose load. Before STZ treatment, there was no difference between wild and CHOP-KO mice in blood glucose excursion after the load. Three weeks after treatment with multiple low-dose streptozotocin (MLDS), blood glucose levels were significantly higher than before the treatment in WT mice. However, although the glucose levels were elevated in KO mice, they were smaller than those observed in STZ-treated WT mice.

3.5. Histologic analysis

Fig. 5A, B shows hematoxylin-eosin staining of pancreas from WT or CHOP-KO mouse treated with vehicle or STZ. Whereas WT mice's islet was seriously damaged by MLDS, islet area was not obviously changed in CHOP-KO mice. The number of insulin-positive cells was decreased in WT but preserved in KO mouse after MLDS treatment (Fig. 5C).

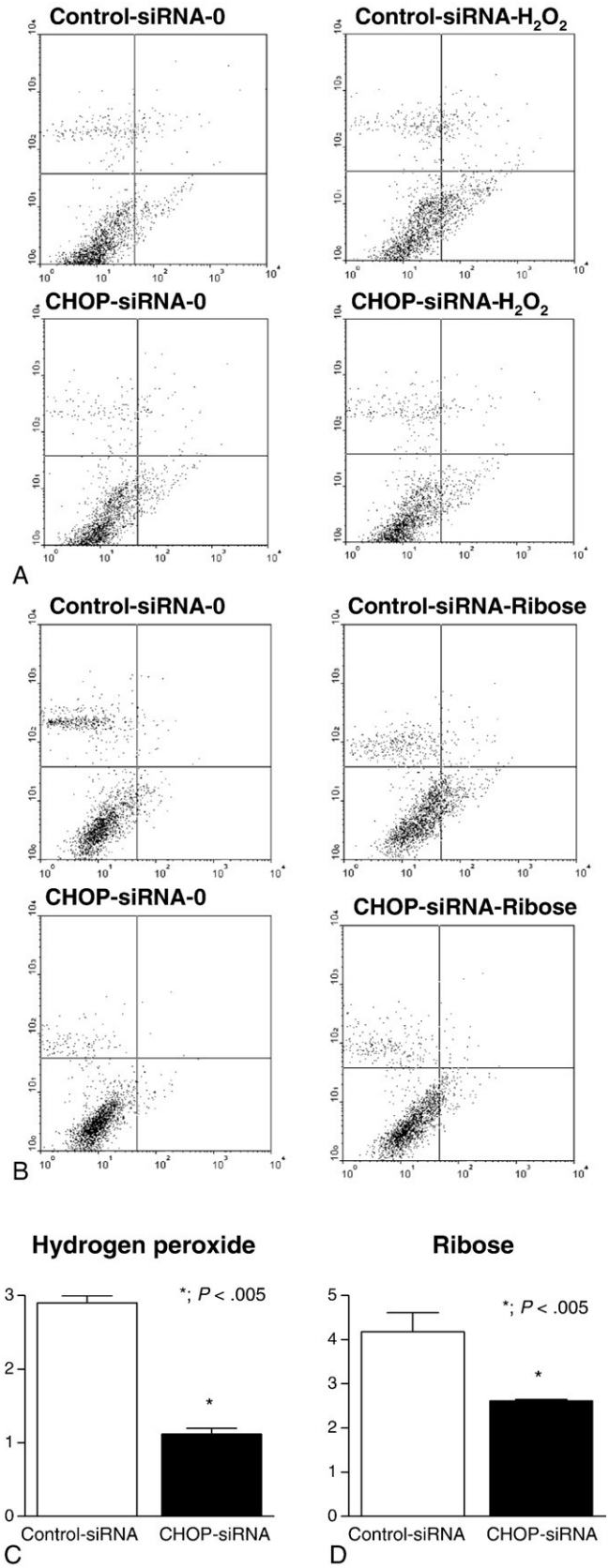

Fig. 3. Change of oxidative stress–induced apoptosis by interfering CHOP expression by siRNA. The number of apoptotic cells was significantly less after transduction with CHOP siRNA when stimulated with 20 μmol/L hydrogen peroxide for 2 hours (A and C) or 50 mmol/L ribose for 48 hours (B and D) (n = 3).

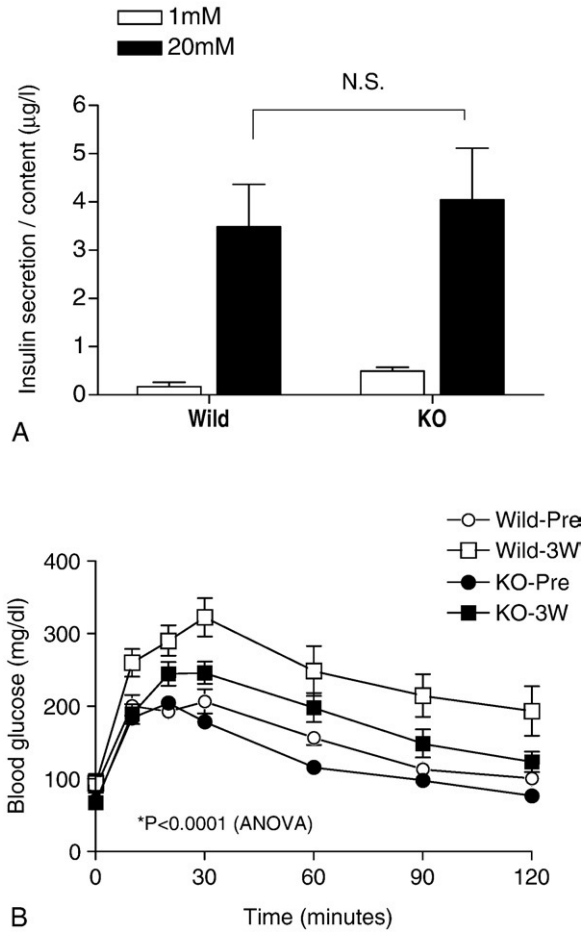

Fig. 4. (A) Glucose-stimulated insulin secretion from the islet of WT and CHOP-KO mice. The isolated islets were collected from the mice at the age of 10 weeks and stimulated with 1 or 20 mmol/L glucose for 1 hour (n = 4 from each group). No significant differences were observed between WT and KO mouse. (B) Results of OGTT in WT and KO mice before or 3 weeks after STZ injection (n = 7). Blood glucose levels after glucose injection were not significantly different between WT (open circle) and KO (closed circle) mice before STZ injection.

The control (treated with vehicle) mice islets were not stained with 8-OHdG, and the islets of WT or KO mice treated with STZ were stained well with 8-OHdG (Fig. 5D). The number of oxidative stress–positive cells (detected by oxidative stress marker 8-OHdG) per islet area was not different between WT and KO mice (Fig. 5D; wild, 330 ± 78/mm^2; KO, 610 ± 110/mm^2; not significant). On the other hand, the number of TUNEL-positive cells per islet area was lower in KO mouse than in WT mouse, although the difference was not significant (Fig. 5E; wild, 420 ± 90/mm^2; KO, 49 ± 6/mm^2; P = .06).

4. Discussion

Oxidative stress may play an important role in the development of diabetes. However, its precise mechanism is still unclear. In the present study, we investigated how oxidative stress induces apoptosis of pancreatic β-cells and the induction of CHOP is related to the process in vitro and in vivo.

First, we have shown that chronologically increased oxidative stress either by hydrogen peroxide or ribose induced CHOP mRNA and the apoptosis in βHC-9 cells. Although the intracellular peroxide levels increased chronically, the induction of CHOP mRNA and the apoptosis were not confirmed until 4 hours after the application of hydrogen peroxide and 72 hours after the application of ribose. This difference of required time between hydrogen peroxide and ribose can be explained by the different mechanisms of oxidative stress induction by these compounds. Hydrogen peroxide is considered as a rapid oxidative stress model that includes mechanisms such as N(+)/H(+) exchanger (NHE) inhibition [29-33], poly (adenosine diphosphate ribose) polymerase activation [34], adenosine triphosphate hydrolysis [29], and H^+ redistribution [32]. On the other hand, D-ribose increases oxidative stress through autoxidation and/or protein glycosylation in cells [35]; so intracellular oxidation occurs slowly compared with hydrogen peroxide.

The result from βHC-9 cells indicates that the mechanism by which oxidative stress induces apoptosis in pancreatic β-cells and its reduction of cell number may lead to reduced amount of insulin secretion and therefore would result in diabetes. Furthermore, the addition of siRNA against CHOP gene significantly reduced the number of apoptotic cells by both stimulations in vitro. These results strongly indicate that the factor that links oxidative stress and apoptosis is the induction of CHOP.

Second, we have used the CHOP-KO mice to clarify the interference of CHOP in the induction of apoptosis in pancreatic β-cells in vivo. In the experiments using CHOP-KO mice, we have shown that CHOP-KO mice are more resistant to pancreatic β-cell injury caused by oxidative stress induced by MLDS than WT mice. This is consistent with results we have obtained from in vitro experiments.

A number of studies have shown that oxidative stress contributes to experimental diabetes induced by single administration of high-dose STZ [36-38]. On the other hand, it has been reported that inhibitory and/or scavenging ROS protect against the development of diabetes by MLDS [39] and that electrolyzed-reduced water, a potent ROS scavenger, has antidiabetic effects in MLDS-treated mice [40]. However, the details about how intracellular signals directly interact with oxidative stress have not been established. In the present study, we have demonstrated that the MLDS causes oxidative stress assessed by oxidative stress marker 8-OHdG and that it reduces the number of pancreatic β-cells, therefore causing diabetes. Although the degree of oxidative stress was not different from that of wild mice, the number of apoptotic β-cells was reduced and pancreatic islet area was preserved even after

MLDS treatment in CHOP-KO mice compared with the WT. These data strongly suggest that CHOP should play an important role in the induction of pancreatic β-cell apoptosis after oxidative stress.

CHOP is known to increase in preapoptotic stage, and it is known that various kinds of stress enhance its gene expression [17,19,41-43]. The most known stress factor is the ER stress [16-18,21,22]. It has been reported that ER stress increases ROS from the ER [22,44,45] or from mitochondria [46]. On the other hand, there is a report about ER stress caused by increase in nitric oxide level, indicating that ER stress may exist downstream of oxidative stress [47]. It is supposed that there may be a cross-stalk between oxidative stress and ER stress pathways, although further studies are needed to clarify this point. Except for CHOP, it was reported that mitogen-activated protein kinase 8 (MAPK8/JNK1) and caspase-12 translation is enhanced when the cell apoptosis is induced by severe ER stress [48-50].

In pancreatic β-cells, oxidative stress is reported to play a role in the development of diabetes [12-14,51-53]. However, the relationship between CHOP, oxidative stress, and apoptosis in pancreatic β-cells remains unclear. From our results, we have shown for the first time that CHOP

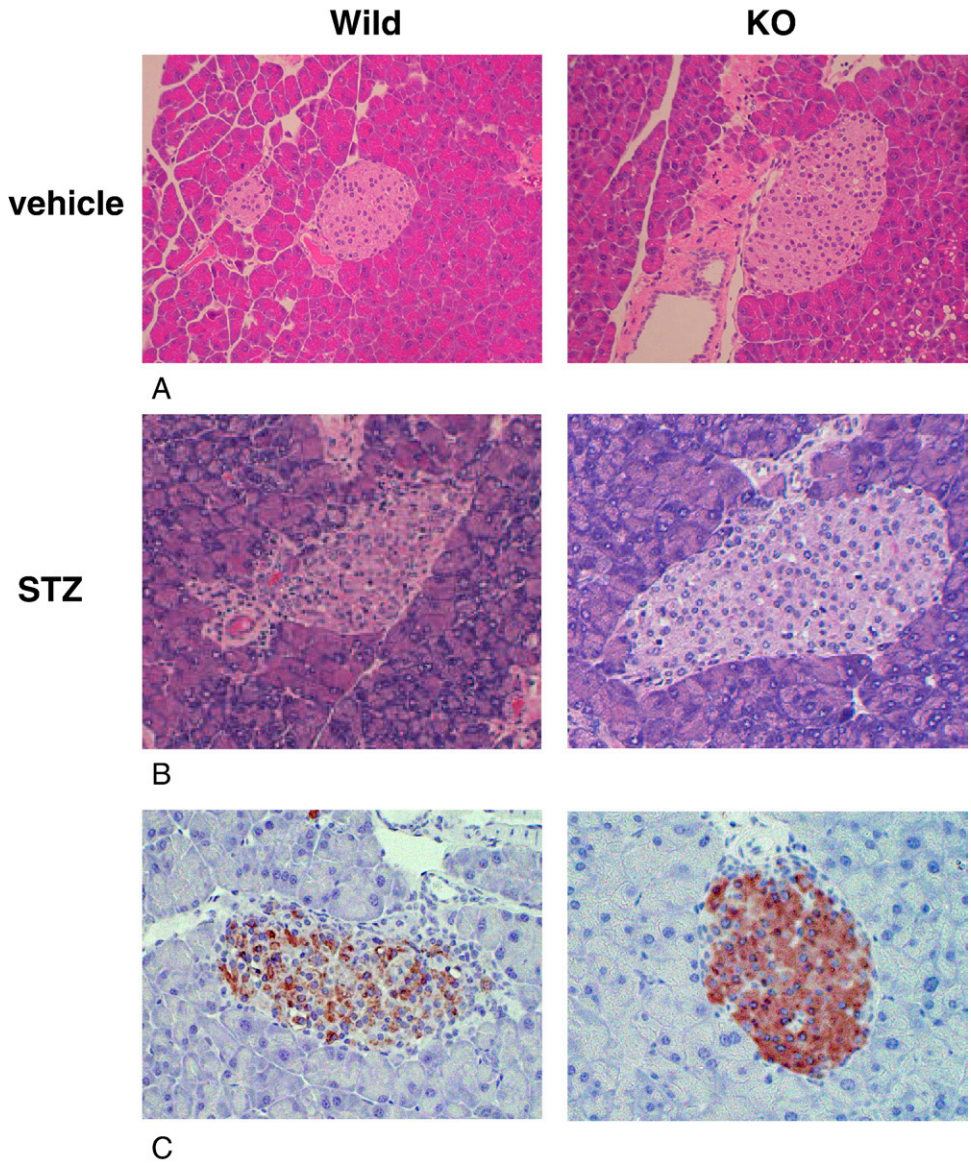

Fig. 5. Histologic analysis of islets from STZ-treated mice. A and B, Inflammatory cells were invaded, and the islet was destroyed in WT mouse (left panels); but the islet was not significantly influenced by MLDS in CHOP-KO mouse (right panels). (C) Insulin-positive cells were lost in the islets of WT mouse, but these were preserved in the islet of CHOP-KO mice. (D) The count of 8-OHdG–positive cells was not different between wild and KO mice. (E) TUNEL staining of islet for detection of apoptosis. Number of TUNEL-positive cells was lower in KO than in wild mice, although it was not significant ($P = .06$). For quantitative analysis, 3 sections from each of the 3 mice were used for the evaluation.

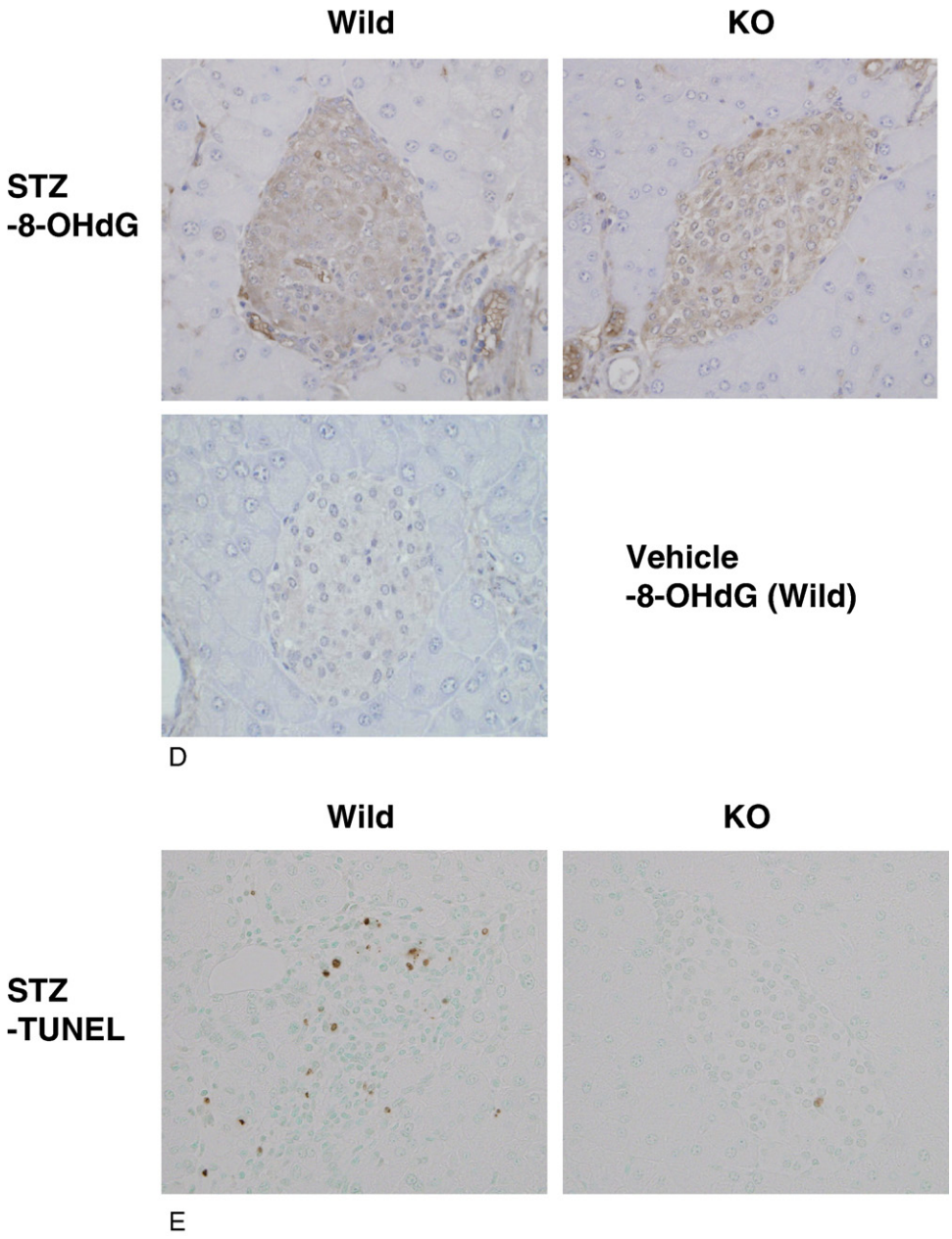

Fig. 5 (*continued*).

mediates the pathway between oxidative stress and apoptosis of pancreatic β-cell. Therefore, CHOP should contribute to the development of diabetes triggered by oxidative stress. Our results indicate the possibility that the reduction of CHOP gene expression might be an effective new strategy for the treatment of diabetes that is caused by the reduction of pancreatic β-cell number.

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