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Endogenous hydrogen sulfide protects pancreatic beta-cells from a high-fat diet-induced glucotoxicity and prevents the development of type 2 diabetes

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ABSTRACT

Chronic exposure to high glucose induces the expression of cystathionine gamma-lyase (CSE), a hydrogen sulfide-producing enzyme, in pancreatic beta-cells, thereby suppressing apoptosis. The aim of this study was to examine the effects of hydrogen sulfide on the onset and development of type 2 diabetes. Middle-aged (6-month-old) wild-type (WT) and CSE knockout (CSE-KO) mice were fed a high-fat diet (HFD) for 8 weeks. We determined the effects of CSE knockout on beta-cell function and mass in islets from these mice. We also analyzed changes in gene expression in the islets. After 8 weeks of HFD, blood glucose levels were markedly increased in middle-aged CSE-KO mice, insulin responses were significantly reduced, and DNA fragmentation of the islet cells was increased. Moreover, expression of thioredoxin binding protein-2 (TBP-2, also known as Txnip) was increased. Administration of NaHS, a hydrogen sulfide donor, reduced TBP-2 gene levels in isolated islets from CSE-KO mice. Gene levels were elevated when islets were treated with the CSE inhibitor DL-propargylglycine (PPG). These results provide evidence that CSE-produced hydrogen sulfide protects beta-cells from glucotoxicity via regulation of TBP-2 expression levels and thus prevents the onset/development of type 2 diabetes.

1. Introduction

Hydrogen sulfide is a toxic gas present in crude oil. The gas is also produced in mammalian tissues and functions as a gaseous signal molecule. Like nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide is able to penetrate plasma membranes and transduces both intracellular and intercellular signals. Studies involving the physiological effects of hydrogen sulfide revealed that this gaseous signal molecule causes various cellular responses, including smooth muscle tone, hormone secretion, neurotransmission, and cell death/survival [1,2].

Hydrogen sulfide is enzymatically synthesized from D/L-cysteine or their metabolites in several distinct ways [1–3]. At least three key enzymes are involved. Cystathionine beta-synthase (CBS) is strongly expressed in neural cells and participates in the modification of long-term synaptic potentiation. 3-Mercaptopyruvate sulfurtransferase (3-MST) is expressed in most mammalian tissues, and produces hydrogen sulfide from 3-mercaptopyruvate, a cysteine metabolite generated by cysteine aminotransferase (CAT) and D-amino acid oxidase (DAO) [4,5]. Cystathionine gamma-lyase (CSE) was identified as an enzyme responsible for producing hydrogen sulfide in smooth muscle cells. Expression of CSE has been identified in several mammalian tissues, including brain, kidney, liver, intestine and pancreas.

The effects of hydrogen sulfide on pancreatic beta-cells have been examined using various insulin-secreting beta-cell lines and isolated islets from rats or mice. Although there is convincing evidence that hydrogen sulfide inhibits insulin secretion from pancreatic beta-cells [6–8], we have a very limited understanding of its physiological roles in these cells. We previously demonstrated that chronic exposure to high glucose increases expression levels of CSE in both mouse islets and the mouse insulin-secreting cell line MIN6 [9]. We also found that the hydrogen sulfide produced by CSE protects beta-cells from oxidative stress. In contrast, there have been some reports that glucose stimulation decreases hydrogen

Abbreviations: CAT, cysteine aminotransferase; CBS, cystathionine beta-synthase; ChREBP, carbohydrate response element-binding protein; CO, carbon monoxide; CSE, cystathionine gamma-lyase; DAO, D-amino acid oxidase; HFD, high-fat diet; K_{ATP}, ATP-sensitive K^+; NO, nitric oxide; STZ, streptozotocin; PPG, DL-propargylglycine; TBP-2, thioredoxin binding protein-2.

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sulfide-producing activity in rat islets and in the rat insulin-secreting cell line INS-1E [8,10]. Moreover, this decrease promotes insulin secretion via the inactivation of ATP-sensitive K^+ (K_{ATP}) channels and protects pancreatic beta-cells from cytotoxic damage [6,8,11,12]. The observed differences are partially due to the species specificity of the cells used and their sensitivity to hydrogen sulfide [10]. It was recently found that streptozotocin (STZ)-treated CSE knockout (CSE-KO) mice at age 10 weeks exhibit a delayed onset of diabetic status [11]. STZ is widely used to generate animal models mimicking type 1 diabetes. In the present study, we examined the effects of CSE deletion on the development of type 2 diabetes in mice.

2. Methods

2.1. Animals

CSE-KO mice have previously been generated from C57BL/6J background by our group [13]. Wild-type (WT), CSE-heterozygous and CSE-KO mice were fed standard rodent chow and maintained in an air-conditioned room kept on a 12 h light/dark cycle (8:00 AM–8:00 PM) at the Oita University institutional animal facility. In some experiments, mice were fed a high-fat diet (HFD) that contains 60% fat (Research Diets, New Brunswick, NJ). All animal experiments conformed to the guidelines for the care and use of animals at Oita University and were approved by the institutional committee. Body weight (weekly) and daily food intakes were measured for 8 weeks. Blood glucose levels were measured weekly for 8 weeks in tail bloods using a G-checker (Gunze, Kyoto, Japan). Body weights and glucose levels were measured between 9:30 AM and 10:30 AM.

2.2. Western blot analysis

Isolated pancreases and livers were homogenized by sonication in ice-cold buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 250 mM sucrose, 2 mM 2-mercaptoethanol, 50 μg/ml PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Protein samples were subjected to SDS–PAGE and western blot analysis using several antibodies. Anti-CSE and anti-CBS polyclonal antibodies were established as described previously [9]. Anti-insulin (Seikagaku Corp, Tokyo, Japan), anti-glucagon (CST Japan, Tokyo, Japan), anti-3MST (Abcam, Cambridge, UK) and anti-beta-actin (St. Louis, MO, USA) antibodies were used.

2.3. Glucose tolerance test

Oral glucose tolerance tests were performed after 12 h fasting. Glucose (2 g/kg body weight) was administered orally. Blood samples were taken from the tail vein and blood glucose levels were measured using a G-checker (GUNZE).

2.4. Insulin tolerance test

Mice were fasted for 4 h and then injected intraperitoneally with insulin at 0.75 U/kg body weight. Blood glucose levels were measured before and after injection.

2.5. Quantitative real-time PCR

Total RNA was isolated from islets of each individual animal using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), and was purified using the SV Total RNA Isolation System (Promega, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Gene expression primers and probes used for *Cse*, *Tbp-2* and *beta-actin* detection, were Mm00461247_m1, Mm00452393_m1 and Mm00607939_s1, respectively (Applied Biosystems). Relative expression levels were analyzed using the ddCt method with beta-actin as an internal control.

2.6. Immunohistochemistry of mouse pancreases

Pancreases from mice were fixed with 3% paraformaldehyde and cryosectioned at 5 μm intervals. Cryosections were incubated with anti-insulin and anti-glucagon antibodies, followed by incubation with secondary antibodies.

2.7. Pancreatic and serum insulin content

Isolated pancreases were suspended in cold acid ethanol (1.5% HCl in 75% ethanol), minced with scissors and homogenized. Insulin contents in the supernatant or serum were determined with a Mouse Insulin ELISA kit (Shibayagi, Gunma, Japan) according to the manufacturer's instructions.

2.8. Apoptosis detection

Cytoplasmic histone-associated DNA fragments in islets were quantified using a Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany) as described previously [14].

2.9. Microarray analysis

Quantity and quality of total RNA were examined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Experion System (Bio-Rad, Hercules, CA, USA), respectively. cRNA was amplified, labeled with Cy3 and hybridized with a SurePrint G3 Mouse GE 8×60 K Microarray according to the manufacturer's instructions. Slides were scanned with an Agilent scanner, and relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (v9.5.1.1). Raw signal intensities and flags for each probe were calculated from hybridization intensities (gProcessedSignal) and spot information (gIsSaturated, etc.) according to procedures recommended by Agilent. Flag criteria on GeneSpring Software are: Absent (A): "Feature is not positive and significant" and "Feature is not above background"; Marginal (M): "Feature is not uniform", "Feature is saturated", and "Feature is a population outlier"; and Present (P): others. Raw signal intensities of two samples were $\log_2$-transformed and normalized using the quantile algorithm of the 'preprocessCore' library package [15] in Bioconductor software [16]. We selected probes that called 'P' flags in both samples. To identify up- and down-regulated genes, we calculated *Z*-scores [17] and ratios (non-log scaled fold-change) from the normalized signal intensity of each probe. We then established criteria for regulated genes: up-regulated genes (Z-score $\geqslant 2.0$ and ratio $\geqslant 1.5$-fold) and down-regulated genes (Z-score $\leqslant -2.0$ and ratio $\leqslant 0.66$). The microarray dataset has been deposited in NCBI's Gene Expression Omnibus and is accessible through the GEO Series accession number GSE50458.

2.10. Statistical analysis

Data are expressed as means ± S.E. Statistical analyses were performed by one-way ANOVA followed by a Tukey's post-test. $P < 0.05$ denotes a significant difference.

3. Results

3.1. Normal glucose tolerance in 8-week-old CSE-KO mice

Western blot analysis revealed the expression of CSE and CBS, but not 3-MST, in pancreases from 8-week-old WT mice (Supplemental Fig. 1A). Levels of CSE expression were reduced by half in pancreases from CSE-heterozygous mice. CSE protein was absent in pancreases from CSE-KO mice, whereas CBS protein levels were indistinguishable between CSE genotypes (Supplemental Fig. 1A).

Previous reports have demonstrated that CSE deletion in mice significantly lowers hydrogen sulfide production in pancreas [11], and we reported that high glucose treatment increases CSE expression and produced hydrogen sulfide protects pancreatic beta-cells from oxidative injury [9,14]. Therefore, we examined the effects of chronic high glucose on CSE-KO mice. Eight-week-old WT and CSE-KO male mice were fed a HFD for 8 or 13 weeks (Supplemental Fig. 1B for experimental scheme), and mice were examined by glucose tolerance test; 8 weeks of HFD induced *Cse* gene expression in pancreatic islets from WT mice (Table S1). After 8 weeks of HFD, blood glucose levels were elevated within 30 min after oral glucose administration and then gradually decreased similarly in WT and CSE-KO mice (Supplemental Fig. 1C). The same results were obtained with WT and CSE-KO mice fed HFD for 13 weeks (Supplemental Fig. 1D). Taken together, the results show that chronic high glucose by HFD had little effect on 8-week-old CSE-KO mice.

3.2. Impaired glucose tolerance in 6-month-old CSE-KO mice

It is known that glucose intolerance increases with age, and therefore, we next examined 6-month-old mice. Middle-aged WT and CSE-KO male mice were fed HFD for 8 weeks (Fig. 1A for scheme), and were examined by glucose tolerance tests. During 8 weeks on HFD, WT and CSE-KO mice displayed similar body weight increases and daily food intakes (Fig. 1B and C, respectively); however, casual blood glucose levels were much higher in CSE-KO mice than WT mice (Fig. 1D).

Although there were no significant differences in glucose tolerance between 6-month-old WT and CSE-KO mice before initiating HFD (Fig. 2A), 8 weeks of HFD significantly impaired glucose tolerance in CSE-KO mice; blood glucose levels after oral glucose administration were consistently higher in CSE-KO mice (Fig. 2B). To examine whether the changes in glucose tolerance could be attributed to altered insulin resistance, we performed insulin tolerance tests; however, CSE-KO mice did not show altered responses to insulin (Fig. 2C). Next, serum insulin levels were monitored during glucose tolerance tests. Serum insulin levels in CSE-KO mice were significantly lower than in WT mice both before and after (30 min) oral glucose administration (Fig. 2D). These results indicate that glucose intolerance in middle-aged CSE-KO mice is caused by impaired insulin secretion rather than insulin intolerance.

3.3. Beta-cell apoptosis and reduced insulin contents in islets of 6-month-old CSE-KO mice

Next, we analyzed islet morphology by immunohistochemistry; pancreatic sections were immunostained with anti-insulin and anti-glucagon antibodies. Although general architecture of islets was maintained and alpha/beta cell ratios were normal, the insulin-positive areas were 40% smaller in 6-month-old CSE-KO mice (Fig. 3A–C). Moreover, insulin contents in the pancreases of 6-month-old CSE-KO mice were 25% lower than those in WT mice (Fig. 3D). Our previous study demonstrated that CSE-produced hydrogen sulfide protects beta-cells against oxidative injury [9,14], and thus, we examined apoptosis in islet cells. DNA fragmentation levels in islet cells of 6-month-old CSE-KO mice were 43% higher than those of WT mice (Fig. 3E).

In order to exclude the possibility that the abnormalities are attributed to the developmental dysregulation, we analyzed islet

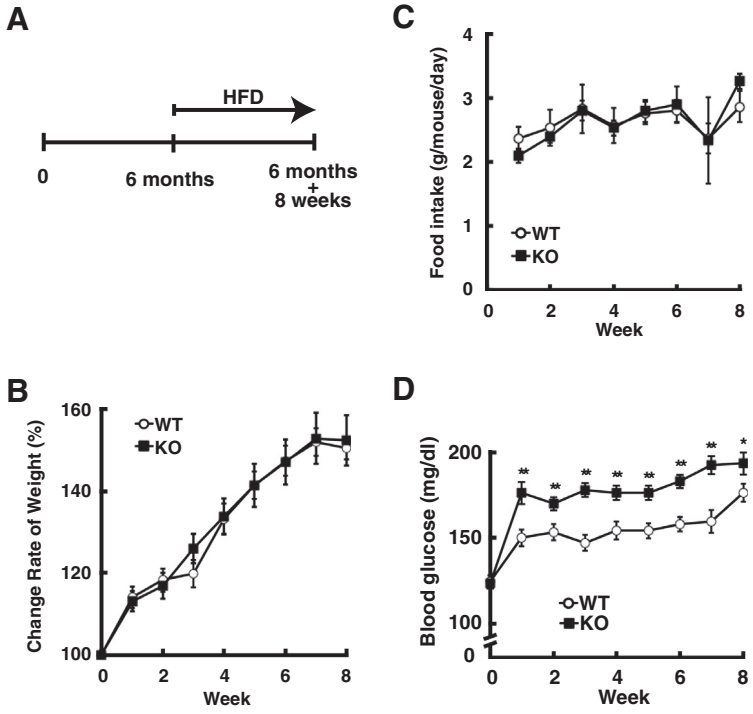

Fig. 1. Impaired glucose tolerance in 6-month-old CSE-KO mice. (A) Experimental scheme. (B–D) Body weight changes (B, $n \geqslant 9$), daily food intake (C, $n \geqslant 9$) and casual blood glucose levels (D, $n \geqslant 23$) of 6-month-old mice *ad lib* fed HFD for 8 weeks were assessed. Data represent means ± S.E. Significant differences were observed between WT and CSE-KO mice; $^{*}P < 0.05$ and $^{**}P < 0.01$.

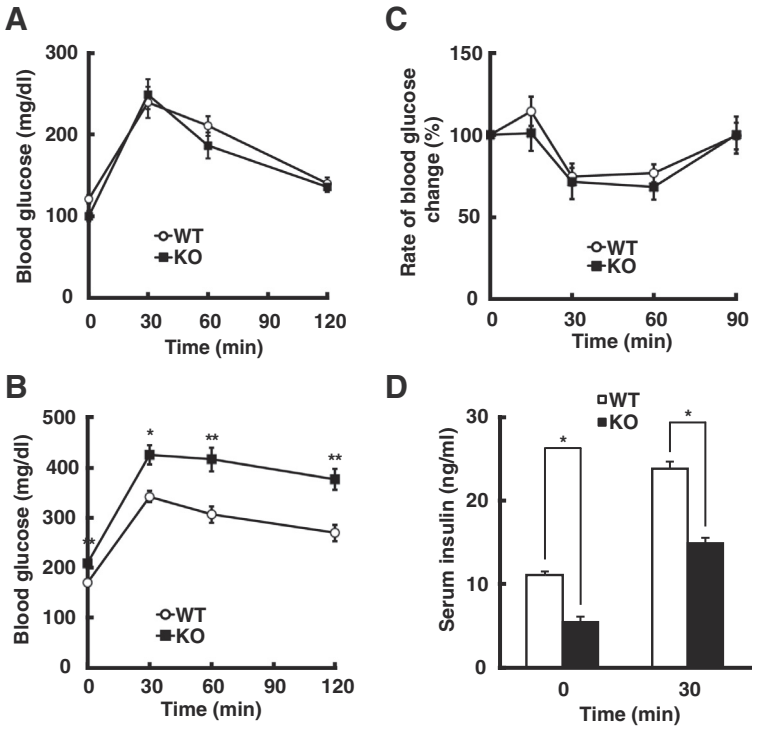

Fig. 2. Oral glucose tolerance test and insulin tolerance test in HFD-fed 6-month-old mice. (A and B) Oral glucose tolerance test in 6-month-old mice before (A, $n \geqslant 9$) and after (B, $n \geqslant 22$) the 8 weeks feeding with HFD. (C) Insulin tolerance test in HFD-fed mice ($n \geqslant 5$). (D) Serum insulin levels during oral glucose tolerance test on HFD-fed mice ($n = 3$ each). Data represent means ± S.E. $^{*}P < 0.05$ and $^{**}P < 0.01$.

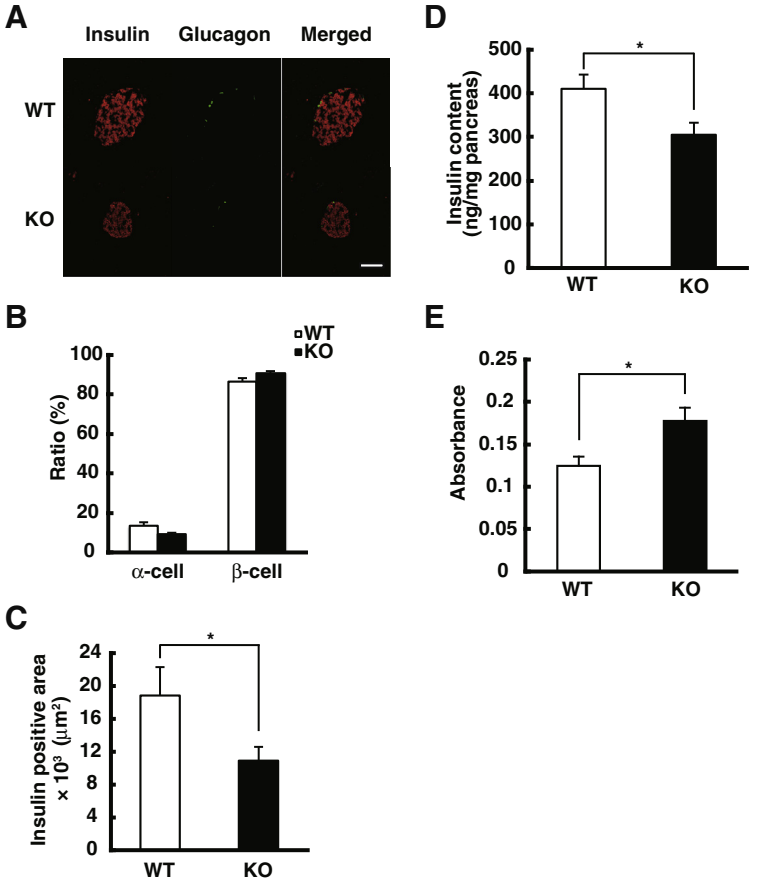

Fig. 3. Morphology and function of pancreatic beta-cells in HFD-fed 6-month-old mice. (A) Pancreatic sections from HFD-fed 6-month-old WT and CSE-KO mice were double-stained with anti-insulin and anti-glucagon antibodies. The scale is 100 μm. (B and C) Pancreatic alpha/beta-cell ratios (B, $n \geqslant 12$) and beta-cell areas (C, $n \geqslant 4$) were measured. (D) Insulin contents in whole pancreases were measured ($n \geqslant 4$). (E) Histone-associated DNA fragments were quantified by ELISA to evaluate apoptotic cell death ($n = 3$ each). Data represent means ± S.E. $^{*}P < 0.05$.

morphology of CSE-KO mice receiving normal diet. Although alpha/beta cell ratios were slightly lower in CSE-KO mice (Supplemental Fig. 2A and B), there were no significant differences in the insulin-positive areas and DNA fragmentation levels between WT and CSE-KO mice fed normal diet (Supplemental Fig. 2C and D). These results suggest that apoptotic beta-cell loss was promoted in 6-month-old CSE-KO mice fed HFD.

3.4. Increased gene expression of TBP-2 in 6-month-old CSE-KO mice

In order to assess the effects of HFD on 6-month-old mice, we examined global gene expression in the pancreas by DNA microarray (Table S2). Twenty transcripts were identified to encode known proteins. We next verified gene expression of thioredoxin binding protein-2 (TBP-2, also known as Txnip) by quantitative real-time PCR, because it is a redox protein that promotes beta-cell apoptosis and is involved in diabetes [18,19]. Gene expression levels of TBP-2 in 6-month-old CSE-KO mice were 3.6-fold higher than those in WT mice (Fig. 4A).

In order to examine the involvement of CSE-produced hydrogen sulfide in increased TBP-2 gene expression, islet cells isolated from 6-month-old CSE-KO mice were exposed to 20 mM glucose for 18 h in the presence or absence of 0.1 mM NaHS, a hydrogen sulfide donor. Glucose exposure significantly increased TBP-2 gene expression (3.3-fold), whereas co-treatment with NaHS completely cancelled this response (Fig. 4B). Moreover, the treatment of islet cells from 6-month-old WT mice with DL-propargylglycine (PPG, a CSE inhibitor) promoted TBP-2 gene expression (1.8-fold) (Fig. 4C), suggesting that CSE-produced hydrogen sulfide regulates TBP-2 expression and its absence may lead to increased beta-cell apoptotic loss in 6-month-old CSE-KO mice.

4. Discussion

Hydrogen sulfide is known to inhibit insulin release in MIN6 cells and isolated islets [7]. In addition to such acute effects, long-term exposure to hydrogen sulfide has been shown to inhibit beta-cell death [9,14]. We previously demonstrated that hydrogen sulfide is produced by CSE and that its expression levels are increased by long-term exposure to high glucose levels [9]. The objective of this work was to investigate the involvement of CSE-produced hydrogen sulfide in the onset/development of type 2 diabetes.

When fed HFD for 8 weeks, 6-month-old CSE-KO mice displayed hyperglycemia (Fig. 1D) and glucose intolerance due to impaired insulin secretion rather than insulin intolerance (Fig. 2). The change was accompanied by apoptotic beta-cell loss (Fig. 3) and increased TBP-2 gene expression (Table S2 and Fig. 4A). Because TBP-2 deficiency has been shown to protect against diabetes by inhibiting beta-cell apoptosis [18,19], we propose a model in which CSE-produced hydrogen sulfide protects beta-cells from glucotoxicity-induced apoptosis by suppressing TBP-2 expression levels (Fig. 4D). In this context, CSE in islet cells plays a key role by regulating hydrogen sulfide levels and thereby the development of type 2 diabetes associated with beta-cell loss.

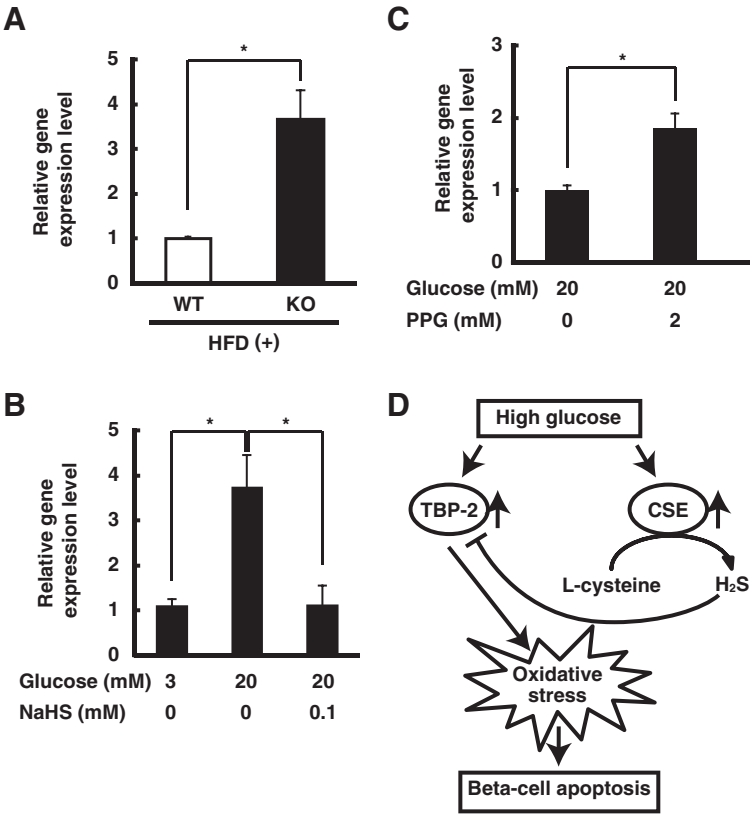

Fig. 4. Regulation of *TBP-2* expression in isolated islets. (A) HFD-induced *Tbp-2* expression in islets from 6-month-old WT mice. Expression of *Tbp-2* was analyzed by quantitative real-time PCR and was normalized against housekeeping *beta-actin*. The average *Tbp-2*/*beta-actin* expression ratio in HFD-fed WT mice was set at 1. Data represent means ± S.E ($n = 3$). $^{*}P < 0.05$. (B) Isolated islets were incubated with/without 0.1 mM NaHS for 18 h and *TBP-2* gene expression was evaluated by quantitative real-time PCR ($n \geqslant 3$). (C) Isolated islets were cultured in medium containing 20 mM glucose with/without 2 mM PPG for 18 h, and *TBP-2* gene expression was evaluated. Data represent means ± S.E. $^{*}P < 0.05$. (D) A proposed model showing hydrogen sulfide-mediated protection against beta-cell apoptosis upon glucotoxicity. High glucose induces TBP-2 expression in pancreatic beta-cells, and TBP-2 binds to and inhibits thioredoxin, thereby promoting oxidative stress-induced beta-cell apoptosis. High glucose also induces CSE expression to enhance hydrogen sulfide production, which counteracts glucotoxicity by suppressing TBP-2 expression. Therefore, the absence of CSE promotes progressive beta-cell failure and the development of type 2 diabetes.

We previously reported that glucose promotes CSE expression via a calmodulin kinase II-dependent MAPK cascade [20]. Glucose also promotes TBP-2 expression via carbohydrate response element-binding protein (ChREBP) in a Ca^{2+}-dependent manner [21,22]. Therefore, glucose-stimulated Ca^{2+} signaling is essential for gene expression of both CSE and TBP-2. Interestingly, *ChREBP* expression was slightly upregulated in CSE-KO mice (*Z*-score: 1.7). Moreover, recent reports have shown that hydrogen sulfide inhibits both K_{ATP} channel activity and L-type Ca^{2+} channels in mouse pancreatic beta-cells [11,23]. These studies raise the possibility that hydrogen sulfide inhibits *ChREBP* expression both directly and indirectly. The inhibition of calcium ion influx also suppresses CSE expression. This may be a homeostatic mechanism by which the cells maintain intracellular concentrations of hydrogen sulfide constant, as the gas has the opposite effect on the death of pancreatic beta-cells [9,12].

In the present study, we found that the lack of CSE activity (by either genetic deletion (Fig. 4A) or pharmacological inhibition (Fig. 4C)) promotes TBP-2 gene expression and hydrogen sulfide donor administration inhibits it (Fig. 4B). TBP-2 is a ubiquitously expressed protein that binds to and inhibits thioredoxin [24], and thioredoxin constitutes a small molecule-based redox buffer system essential for controlling redox homeostasis [25]. Therefore, we believe that hydrogen sulfide activates thioredoxin, and thereby modulates the redox state and protects beta-cells from glucotoxicity. This is consistent with previous data that thioredoxin overexpression slows the progression of diabetes by protecting beta-cells from destruction [26,27].

Both TBP-2 deficiency and hydrogen sulfide production specifically protect beta-cells from oxidative stress, but have very few protective effects against ER stress [14,28]. Together with our previous finding that hydrogen sulfide increases the production of glutathione in MIN6 cells [7], our results indicate that hydrogen sulfide regulates redox status by modulating anti-oxidative mechanisms including glutathione and thioredoxin.

A previous report showed that CSE deficiency protects pancreatic beta-cells and delays the development of STZ-induced diabetes [11]. On the other hand, our results suggest that a lack of CSE induces apoptotic beta-cell death and promotes the development of HFD-induced diabetes. This discrepancy may be related to differences between the two types of diabetes. STZ and HFD are widely used to generate animal models mimicking type 1 and type 2 diabetes, respectively. The observed discrepancy raises the possibility that hydrogen sulfide promotes apoptosis when beta-cells are damaged by severe stimulation and that the gas may also protect beta-cells when the damage is insufficiently severe. Endogenous hydrogen sulfide may regulate beta-cell functions differently depending on age or stage/type of diabetes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.023.

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