H₂S, Endoplasmic Reticulum Stress, and Apoptosis of Insulin-secreting Beta Cells*

Guangdong Yang †1, Wei Yang ‡2, Lingyun Wu §3, and Rui Wang ¶‡4,5*

From the Departments of †Physiology and ‡Pharmacology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5 and the §Department of Biology, Faculty of Science and Environmental Studies, Lakehead University, Thunder Bay, Ontario P7B 5E1, Canada

Cystathionine γ-lyase (CSE) is a key enzyme in the trans-sulfuration pathway, which uses l-cysteine to produce hydrogen sulfide (H₂S). Functional changes of pancreatic beta cells induced by endogenous H₂S have been reported, but the effect of the CSE/H₂S system on pancreatic beta cell survival has not been known. In this study, we demonstrate that H₂S at physiologically relevant concentrations induced apoptosis of INS-1E cells, an insulin-secreting beta cell line. Transfection of INS-1E cells with a recombinant defective adenovirus containing the CSE gene (Ad-CSE) resulted in a significant increase in CSE expression and H₂S production. Ad-CSE transfection also stimulated apoptosis. The other two end products of CSE-catalyzed enzymatic reaction, ammonium and pyruvate, had no effects on INS-1E cell apoptosis, indicating that overexpression of CSE may stimulate INS-1E cell apoptosis via increased endogenous production of H₂S. Both exogenous H₂S (100 μM) and Ad-CSE transfection inhibited ERK1/2 but activated p38 MAPK. Interestingly, BiP and CHOP, two indicators of endoplasmic reticulum (ER) stress, were up-regulated in H₂S- and CSE-mediated apoptosis. Inhibition of p38 MAPK, but not of ERK1/2, inhibited the expression of BiP and CHOP and decreased H₂S-stimulated apoptosis, suggesting that p38 MAPK activation functions upstream of ER stress to initiate H₂S-induced apoptosis. It is concluded that H₂S induces apoptosis of insulin-secreting beta cells by enhancing ER stress via p38 MAPK activation. Our findings may help unmask a novel role of the CSE/H₂S system in regulating pancreatic functions under physiological condition and in diabetes.

Cystathionine γ-lyase (CSE), EC 4.4.1.1, is a pyridoxal 5’-phosphate-dependent enzyme in the trans-sulfuration pathway, which uses l-cysteine to produce hydrogen sulfide (H₂S), a novel and important gasotransmitter (1–3). Endogenous productions of H₂S in different organs and tissues as well as the circulatory concentration of H₂S have been elucidated, and the physiological importance of H₂S has gained increasing recognition (2–5).

Diabetes is a spectrum of clinical conditions arising from relative or absolute insulin deficiency with decreased functional beta cell mass (6). Any change in beta cell mass must reflect an imbalance between proliferation (neogenesis or replication) and cell death (necrosis or apoptosis) (7). Excessive loss of beta cells constitutes one of the causes of diabetes, and apoptosis is considered to be the main mode of beta cell death in type I and type II diabetes (8). In recent years, pathophysiological implications of the CSE/H₂S system in diabetes have been reported (9, 10). Endogenous production of H₂S together with the expression of CSE and cystathionine β-synthase (CBS), another H₂S-producing enzyme, was identified in rat pancreatic tissues (9, 10). CSE mRNA expression and H₂S formation in the rat pancreas were significantly increased after diabetes induction by streptozotocin injection (10). Pancreatic H₂S level in Zucker diabetic fatty rats was significantly higher than that in nondiabetic Zucker fatty rats (9). Inhibition of CSE activity by N-propargylglycine (PPG) significantly decreased production of H₂S and increased plasma insulin levels in Zucker diabetic fatty rats (9). Increased CSE and CBS activity or decreased homocysteine levels in diabetic animals and human have also been observed in different laboratories (10–13). In vitro studies showed that incubation of INS-1E cells (an insulin-secreting beta cell line) with H₂S drastically decreased insulin secretion because of the activation of plasma ATP-sensitive potassium (K₅ ATP) channels (14). Given the importance of pancreatic β cell mass for the pathogenesis of diabetes and altered endogenous pancreatic production of H₂S in diabetes, it becomes impera-

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4 To whom correspondence should be addressed: Office of Vice President (Research), Lakehead University, 955 Oliver Rd., Thunder Bay, Ontario P7B 5E1, Canada. Tel.: 807-343-8180; Fax: 807-346-7749; E-mail: rwang@lakeheadu.ca.

5 The abbreviations used are: CSE, cystathionine γ-lyase; Ad-CSE, adenovirus containing CSE gene; Ad-lacZ, adenovirus containing β-galactosidase gene; CBS, cystathionine β-synthase; DTT, dithiothreitol; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; HASMC, human aorta smooth muscle cell; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; m.o.i., multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid); PPG, N-propargylglycine; siRNA, short-interfering RNA; SREBP-1c, sterol regulatory element-binding protein-1c; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.
to understand whether the apototic status of pancreatic β cells is regulated by H2S and the underlying signaling cascade.

In this study, we overexpressed the CSE gene in INS-1E cells using a highly effective replication-deficient adenovirus expression system. The successful adenovirus-mediated overexpression of CSE was confirmed by measuring CSE protein levels and endogenous H2S production. Cell survival was examined and compared after the increased CSE expression. Whether CSE overexpression-induced changes in cell survival were because of overproduced H2S was determined and compared with the effect of exogenously applied H2S. Finally, the involvement of the mitogen-activated protein kinase (MAPK) pathway and endoplasmic reticulum (ER) stress on the effect of the CSE/H2S system on INS-1E cell apoptosis was examined. Our findings suggest the hypothesis that H2S plays a fundamental role in regulating pancreatic β cell apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—INS1-E cells derived from a rat insulinoma (kindly provided by Dr. C. B. Wollheim, Geneva, Switzerland) were cultured with RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin, as described previously (14). The experiments were performed when the cells reached 70–80% confluence between passages 56 and 70. In all studies, cells were first incubated in the serum-free medium for 12 h and then 10% serum was added together with different treatments.

**Construction of a Recombinant CSE Adenovirus and Gene Transfer**—Recombinant adenovirus containing the CSE gene (Ad-CSE) was prepared as described previously (14). The recombinant adenovirus encoding bacterial β-galactosidase (Ad-lacZ) derived from the same vector was used as control. For adenoviral transfection, subconfluent INS-1E cells were incubated with Ad-CSE or Ad-lacZ at 50 multiplicities of infection (m.o.i.) in serum-free media (14). After 4 h of incubation, media were removed, and cells were incubated in appropriate media for another 44 h.

**Cellular Viability Assays**—Cell viabilities were assessed based on conversion of trazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, according to manufacturer's instruction (CellTiter96®, Promega, Madison, WI). Briefly, cells at equal number were plated onto each well of 96-well plates for 24 h. At different time points after treatment with different concentrations of H2S or transfection with adenovirus, 0.5 mg/ml MTT was added to each well. The cells were then cultured at 37 °C for 4 h, and absorbance of formazan products at 570 nm was measured in a Multiskan spectrum microplate spectrophotometer (Thermo Labsystems, Franklin, MA). The cells incubated with control medium were considered 100% viable.

**Apoptosis Assays**—Apoptosis was monitored by measuring nuclear condensation by Hoechst 33258 staining and DNA fragmentation by the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL), and DNA electrophoresis was performed on 1.8% agarose gel containing 0.5 μg/ml ethidium bromide as described previously (15).

**CSE Activity Measurement**—CSE activity was determined by a sensitive method using a colorimetric assay for the determination of pyruvate formation (18). Briefly, after different treatments the cells were collected and homogenized in 20 mM ice-cold potassium phosphate buffer (pH 7.8). The flasks containing reaction mixture (100 mM potassium phosphate buffer, 10 mM L-cysteine, 2 mM pyridoxal 5’-phosphate, and 10% (w/v) cell homogenates) and center wells containing 0.5 ml 1% zinc acetate and a piece of filter paper (2 × 2 cm) were flushed with N2 and incubated at 37 °C for 90 min. The reaction was stopped by adding 0.5 ml of 50% trichloroacetic acid, and the flasks were incubated at 37 °C for another 60 min. The contents of the center wells were transferred to test tubes each containing 3.5 ml of water. Then 0.5 ml of 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl and 0.5 ml 30 mM FeCl3 in 1.2 M HCl were added. The absorbance of the resulting solution at 670 nm was measured 20 min later with a Multiskan spectrum microplate spectrophotometer.

To measure H2S concentration, 200 μl of culture media from each treatment were collected and added to microcentrifuge tubes containing zinc acetate (1% w/v, 600 μl) to trap H2S. After 5 min, the reaction was terminated by adding 400 μl of N,N-dimethyl-p-phenylenediamine sulfate (20 μM in 7.2 M HCl) and 400 μl of FeCl3 (30 mM in 1.2 M HCl). After the mixture was kept in the dark for 20 min, 300 μl of trichloroacetic acid (10% w/v) was added to precipitate any protein that might be present in the culture media. Subsequently, the mixture was centrifuged at 10,000 × g for 10 min. H2S in the sampled culture media interacts with N,N-dimethyl-p-phenylenediamine sulfate to form methylene blue, and the absorbance of the resulting solution was determined at 670 nm (17). H2S concentration in the culture media was calculated against the calibration curve of standard H2S solutions.

**Measurement of H2S Production and Concentration**—H2S production rate was measured as described previously (4, 17). Briefly, after different treatments, the cells were collected and homogenized in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The flasks containing reaction mixture (100 mM potassium phosphate buffer, 10 mM L-cysteine, 2 mM pyridoxal 5’-phosphate, and 10% (w/v) cell homogenates) and center wells containing 0.5 ml 1% zinc acetate and a piece of filter paper (2 × 2 cm) were flushed with N2 and incubated at 37 °C for 90 min. The reaction was stopped by adding 0.5 ml of 50% trichloroacetic acid, and the flasks were incubated at 37 °C for another 60 min. The contents of the center wells were transferred to test tubes each containing 3.5 ml of water. Then 0.5 ml of 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl and 0.5 ml 30 mM FeCl3 in 1.2 M HCl were added. The absorbance of the resulting solution at 670 nm was measured 20 min later with a Multiskan spectrum microplate spectrophotometer.

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**GSH Level Measurement**—GSH levels were determined by using the high pressure liquid chromatography method described as before (16). Protein content was determined by using the bicinchoninic acid procedure with bovine serum albumin as reference.

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tometer. The CSE-specific activity was expressed as the optical density unit of absorbance at 727 nm per mg of protein.

Western Blot Analysis—Cultured cells were harvested and lysed. Equal amounts of proteins were boiled and separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane as described previously (14). The primary antibody dilutions were 1:500 for anti-CSE antibody or BiP, 1:1000 for phosphorylated or total extracellular signal-regulated kinase (ERK), p38 MAPK, or c-Jun N-terminal kinase (JNK), and 1:5000 for /H9252-actin. Horseradish peroxidase-conjugated secondary antibody was used at 1:5000. The immunoreactions were visualized by ECL and exposed to x-ray film (Kodak Scientific Imaging film).

Short Interfering RNA (siRNA) Transfection—Pre-designed CHOP-targeted siRNA (CHOP-siRNA) was purchased from Ambion (Austin, TX). Negative siRNA (Neg-siRNA), a 21-nucleotide RNA duplex with no known sequence homology with all the genes, was also from Ambion. Transfection of siRNA into INS-1E cells was achieved using the siPORT™ lipid transfection agent from Ambion (14). Briefly, the cells were plated overnight to form 60–70% confluent monolayers. CHOP-siRNA or Neg-siRNA at different concentrations and transfection reagent complex were added to the cells in serum-free medium for 4 h. Fresh normal growth medium was then added, and the cells were incubated for another 20 h. As control, Neg-siRNA was used to transfect INS-1E cells.

Determination of mRNA Level by Real-time PCR—After treatment with H2S or transfection with adenovirus or siRNA, the cells were harvested from 100-mm culture dishes. Total RNA was prepared using TriReagent (Molecular Research Center, Cincinnati) and DNA-free kit (Ambion), and then 2 μg of total RNA was reverse-transcribed into cDNA with avian myeloblastosis virus reverse transcriptase using random hexamer primers according to manufacturer’s protocol (Roche Applied Science). Controls containing no reverse transcriptase were used to safeguard for genomic DNA contamination in each sample. Real-time PCR was performed in an iCycler iQ apparatus (Bio-Rad) associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Master Mix, as described previously (19). Briefly, all PCRs were performed in a volume of 20 μl, using 96-well optical-grade PCR plates and an optical sealing tape. The cycling was conducted at 95 °C for 90 s followed by 38 cycles of 95 °C for 10 s and at 60 °C for 20 s. The primers of CSE (GenBank™ accession number AY032875) were 5'-AGCGATCACACCAGACCAAG-3' (sense, position 432–453) and 5'-ATCCACCCAGAGCCAAAGG-3' (antisense, position 589–609). These primers produced a product of 178 bp. The primers of BiP (GenBank™ accession number M14050) were 5'-TCCGGCGTGAGGTAGAAAAG-3' (sense, position 422–441) and 5'-GAGTAGATCCGCCAACCAGAACAA-3' (antisense, position 635–658) with a product of 236 bp. The primers of CHOP (GenBank™ accession number BC100664) were 5'-CAGAGGTCAAGACCT-3' (sense, position 338–355) and 5'-TCCCTGGTCAGGCGCTC-3' (antisense, position 562–578) with a product of 240 bp. The primers of SREBP-1c (GenBank™ accession number XM213329) were 5'-GGAGCCATGGGATTGCACATT-3' (sense, position 95–115) and 5'-AGGGAGGCTTCCAGAGAGGA-3' (antisense, position 267–286) with a product of 191 bp. The primers of β-actin were purchased from Ambion, which produce a product of 295 bp. A
standard curve was constructed using a series of dilution of total RNA (Ambion) transcribed to cDNA using the same protocol outlined above to confirm the same amplifying efficiency in the PCR. A standard melting curve analysis was performed using the following thermal cycling profile: 95 °C for 10 s, 55 °C for 15 s, and ramping to 95 °C at 1 °C increments to confirm the absence of primer dimers. Relative mRNA quantification was calculated by using the arithmetic formula "2^ΔΔCT" (18), where ΔCT is the difference between the threshold cycle of a given target cDNA and an endogenous reference β-actin cDNA. Based on the calculated ΔCT value, the target mRNA level in the treated carotid arteries was subsequently expressed as the percentage of that in the untreated controls.

Reagents and Chemicals—H2S stock solution was freshly prepared by directly bubbling distilled water with pure H2S gas (Praxair) to make the saturated H2S solution (0.09 M at 30 °C) (4). H2S stock solution was diluted to different concentrations into cell culture medium, and the pH of medium was adjusted to 7.4. The anti-CSE antibody was homemade. Briefly, synthesized and conjugated targeted peptide VGLEDEQDILEDLD (GenBank™ accession number AAL99218.1, position 377–390) was used to immunize male New Zealand rabbits. After the titer of anti-CSE antisera reached 1:4,000, the antisera were purified by protein G affinity. Purified antibody was eluted with 0.1 M glycine buffer (pH 2.5) and stored at −80°C for later use. The anti-MAPK antibodies and U0126 were obtained from Sigma or New England Biolabs. Anti-BiP antibody was purified by protein G affinity. Purified antibody was eluted with 0.1M glycine buffer (pH 2.5) and stored at 80°C for later use.

Statistical Analysis—All data are expressed as means ± S.E. and represent at least three independent experiments. Statistical comparisons were made using Student’s t test or one-way analysis of variance followed by a post hoc analysis (Tukey test) where applicable. Significance level was set at p < 0.05.

RESULTS

Overexpression of CSE in INS-1E Cells—Transfection efficiency was first determined using the control adenovirus, Ad-lacZ. INS-1E cells were transfected with Ad-lacZ at an m.o.i. ranging from 10 to 200 for 48 h. At an m.o.i. of 50, >90% cells showed nuclear staining for β-galactosidase (data not shown). All subsequent experiments were performed at an m.o.i. of 50. The Ad-CSE-transfected cells or nontransfected control cells did not exhibit any intrinsic β-galactosidase activity or false-positive staining.

After 48 h of transfection of INS-1E cells with Ad-CSE, the incubation medium was removed, and cell lysates were prepared. As shown in Fig. 1A, CSE mRNA expression level in Ad-CSE-transfected cells was 1.87 ± 0.21 times higher than that in Ad-lacZ-transfected cells or control cells (p < 0.05). Significant increase in CSE proteins was also detected after transfection with Ad-CSE (Fig. 1B). As expected, the cells transfected with Ad-CSE exhibited marked increases in CSE activity and H2S production rate, 4.2 ± 0.4 and 2.4 ± 0.5 times that in Ad-lacZ-transfected cell (p < 0.05) (Fig. 1, C and D). The concentrations of H2S of culture media, in which different types of INS-1E cells were incubated, were further tested. It was found Ad-CSE-transfected cells produced and released much higher concentration of H2S in their culture media than that of control cells or Ad-LacZ-transfected cells (p < 0.05). H2S concentration of the culture medium of control cells is 17.4 ± 1.6 μM, although it is 31.0 ± 1.3 μM for CSE overexpressed cells (Fig. 1E).

Effects of Exogenous Applied H2S and CSE Overexpression on Cell Viability and Apoptosis—As shown in Fig. 2A, exogenously applied H2S at 50–200 μM reduced cell viability in a dose-dependent manner, and the cell viability with 100 μM H2S treatment was only 78.9 ± 1.9% that in the absence of H2S (p < 0.05). The cells transfected with Ad-CSE also had reduced cell viability in comparison with that of Ad-lacZ-transfected or control cells. At 48 h after Ad-CSE transfection, cell viability was 80.9 ± 2.6% that observed in Ad-lacZ-transfected cells (p < 0.05) (Fig. 2B).

The nuclei of untreated control cells with normal morphology (Fig. 3A, panel a) were uniformly stained by Hoechst 33258 (Fig. 3A, panel d). When incubated with 100 μM H2S, the cells exhibited increased condensed apoptotic nuclei and the morphological changes typical of apoptosis (Fig. 3A, panels b and e). Similar apoptotic changes occurred in Ad-CSE-transfected cells (Fig. 3A, panels c and f). Pro-apoptotic effect of H2S was further evidenced by TUNEL assay. TUNEL-positive staining increased in H2S-treated cells (Fig. 3A, panel h) and Ad-CSE-
transfected cells (Fig. 3A, panel i) compared with the control cells (Fig. 3A, panel g). INS-1E cell apoptosis induced by H₂S and Ad-CSE was also confirmed by internucleosomal DNA fragmentation. Oligonucleosomal DNA fragmentation was observed in the presence of 100 μM H₂S or transfection of Ad-CSE for 48 h but not in control cells or Ad-lacZ-transfected cells (Fig. 3B). Ammonium and pyruvate, another two products of CSE-catalyzed cysteine degradation, had no effect on cell viability and apoptosis at 100 μM (data not shown).

Effects of Exogenously Applied H₂S or CSE Overexpression on the MAPK Pathway—The MAPK pathway is an important signal mechanism for cell growth regulation. Whether MAPKs were phosphorylated and activated by exogenously applied H₂S or CSE, overexpression in INS-1E cells was examined using anti-phospho-MAPK antibodies. The same blots were later stripped and re-probed with an antibody that recognized both phosphorylated and unphosphorylated forms of MAPK. As shown in Fig. 4A, ERK1/2 activation was decreased during the first 30 min of H₂S treatment and continued to decline for 4 h. Conversely, p38 MAPK activation was induced by H₂S during the first 30 min, peaked at 2 h, and later subsided (Fig. 4A). Reduced ERK1/2 activation and increased p38 MAPK activation were also observed in Ad-CSE-transfected cells compared with Ad-lacZ-transfected cells and the control cells (Fig. 4B). JNK activity, however, was quiescent during the period of H₂S treatment or Ad-CSE transfection (Fig. 4, A and B). The total amounts of ERK1/2, p38 MAPK, and JNK proteins remained unchanged with H₂S stimulation or Ad-CSE transfection. U0126 (an inhibitor of MEK/ERK) and SB203580 (a p38 MAPK inhibitor) were used in the following experiments. As shown in

**FIGURE 3.** Exogenously applied H₂S or CSE overexpression induced apoptosis of INS-1E cells. A, after incubation with 100 μM H₂S for 12 h or transfection at 50 m.o.i. with Ad-CSE for 48 h, the cells were fixed and processed for Hoechst 33258 staining or TUNEL assay. Cell morphologies are shown in panels a–c observed under light microscope. Stained nuclear chromatin with Hoechst 33258 are shown in panels d–f. TUNEL staining results are presented in panels g–i. Arrows indicate representative apoptotic cells. Scale bar represents 20 μm. B, oligonucleosomal DNA fragmentation induced by H₂S (100 μM) treatment for 12 h or Ad-CSE transfection for 48 h. MW, molecular weight. The results are representative of three independent experiments.

**FIGURE 4.** Activation of MAPK pathway by H₂S in INS-1E cells. A, exogenously applied H₂S (100 μM) decreased ERK but increased p38 MAPK phosphorylation, detected with Western blot using specific antibodies. B, CSE overexpression decreased ERK but increased p38 MAPK phosphorylation. After transfection at 50 m.o.i. with Ad-lacZ or Ad-CSE for 48 h, the cells were collected and subjected to Western blot using specific antibodies. C, pharmacologic interference of the interaction of H₂S with ERK and p38 MAPK. INS-1E cells were pretreated with or without the MEK/ERK inhibitor U0126 or p38 MAPK inhibitor SB302580 at indicated concentrations for 1 h prior to the addition of 100 μM H₂S. Two hours later, ERK and p38 MAPK activations were examined. The data in A–C are typical experiment from three independent experiments.
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Fig. 4C, 10 μM U0126 augmented the inhibitory effect of H₂S on ERK1/2 activity but had no effect on H₂S-induced phosphorylation of p38 MAPK. SB203580 at 20 μM completely inhibited H₂S-induced phosphorylation of p38 MAPK but had no effect on the phosphorylation of ERK1/2 (Fig. 4C).

**ER Stress Induced by H₂S or CSE Overexpression**—The expression of BiP and CHOP, two indicators of ER stress (20), was up-regulated in INS-1E cells by exogenously applied H₂S or Ad-CSE transfection. BiP mRNA levels in H₂S-treated or Ad-CSE-transfected cells were 1.8 ± 0.1 or 4.0 ± 0.5 times higher than that in the control cells, respectively (*p < 0.05*) (Fig. 5A). These transcriptional changes in BiP expression were consistent with the corresponding protein expression levels (Fig. 5B). Similarly, CHOP mRNA levels in H₂S-treated or Ad-CSE-transfected cells were 3.4 ± 0.5 and 4.8 ± 0.4 times, respectively, that in the control cells (*p < 0.05*) (Fig. 5C). Ad-lacZ transfection did not change the expression level of BiP and CHOP. Sterol regulatory element-binding protein (SREBP)-1c, a member of the SREBP family (21), was also up-regulated by exogenously applied H₂S or CSE overexpression (Fig. 5D).

**Correlation of p38 MAPK and ER Stress in H₂S-induced Apoptosis**—To explore the correlation of H₂S-induced MAPK activation and ER stress, we tested the effects of U0126 and SB203580 on the expression of BiP and CHOP. After incubation of INS-1E cells with 20 μM SB203580, but not with U0126, H₂S-stimulated BiP and CHOP mRNA expressions were decreased to near basal levels (Fig. 6, A and B). These results indicate that p38 MAPK kinase activation functions upstream of ER stress induced by H₂S.

We also examined effects of U0126 and SB203580 on INS-1E cell apoptosis induced by exogenous H₂S. SB203580 alone (20 μM) had no effect on cell apoptosis. This treatment, however, significantly reduced H₂S-induced apoptosis of INS-1E cells by 33.3 ± 2.9% (*p < 0.05*) (Fig. 6C). Inhibiting ERK1/2 activation with U0126 (10 μM), on the other hand, did not alter the apoptotic effect of H₂S. To further explore the role of H₂S-induced ER stress in INS-1E cell apoptosis, we employed the RNA interference approach to knock down CHOP mRNA expression. CHOP-siRNA at 10, 30, and 50 nM inhibited H₂S-mediated CHOP gene expression by 43.8 ± 4.8% (*p < 0.05*), 78.8 ± 4.3% (*p < 0.05*), and 79.8 ± 8.1% (*p < 0.05*), respectively (Fig. 7A). Neg-siRNA transfection had no effect on H₂S-stimulated CHOP gene expression. Because there was no significant difference in the inhibitory effects of CHOP-siRNA on CHOP gene expression between 30 and 50 nM, we used only 30 nM CHOP-siRNA to perform the next study. After transfection of INS-1E cells with 30 nM CHOP-siRNA, cell apoptosis induced by exogenous H₂S was reduced to 3.9 ± 0.7%, whereas it was 8.7 ± 1.2% (*p < 0.05*) in native untransfected cells (Fig. 7B). Transfection of INS-1E cells with 30 nM Neg-siRNA had no effect on H₂S-stimulated apoptosis.

**Comparison of the Effects of H₂S and DTT**—DTT is a known reducing agent and ER stress inducer. Similar to the effects of H₂S, DTT treatment decreased ERK1/2 activation during the first 30 min, and this declination continued for 2 h with a slight increase at 4 h (Fig. 8A). The activities of p38 MAPK and JNK, however, were quiescent during the period of DTT treatment (data not shown). DTT at 1 mM increased the expression of CHOP mRNA but not that of BiP mRNA. Incubation of the cells with 10 μM U0126 or 20 μM SB203580 did not alter the effects of DTT on CHOP or BiP expression (Fig. 8, B and C). DTT also significantly induced apoptosis of INS-1E cells (Fig. 8D). DTT treatment, but not H₂S treatment, significantly changed cellular redox status, as evidenced by increased intracellular GSH levels (Fig. 8E). Cysteine at 1 mM mimicked the effect of H₂S on INS-1E cell apoptosis but had no effect on intracellular GSH level (Fig. 8, D and E).
DISCUSSION

H₂S, a colorless and flammable gas with the characteristic smell of rotten eggs, has long been regarded as a toxic environmental agent of little physiological significance. This conventional thought has gradually lost ground. Recent studies show that H₂S is actually endogenously generated and has profound biological and physiological effects (2–5, 22). The endogenous concentration of circulating H₂S is 50–160 μM in rat, bovine, and human (2, 3). Tissue level of H₂S is known to be higher than its circulating level. H₂S at physiologically relevant concentrations hyperpolarizes cell membranes, relaxes smooth muscle cells, modulates neuronal excitability, and regulates cell proliferation or apoptosis (2–5, 15, 18).

Decreased homocysteine level was observed in type 1 diabetic patients (11). In streptozotocin-induced diabetic animals or Zucker diabetic fatty rats, hepatic or pancreatic activities of CBS and CSE were significantly up-regulated and were associated with decreased plasma homocysteine levels (12, 13). The above studies involving CBS or CSE in diabetes had concentrated on altered homocysteine metabolism with altered H₂S metabolism largely neglected. Significantly increased H₂S formation was recently reported in Zucker diabetic fatty rats and streptozotocin-induced diabetic rats (9, 10). Inhibition of endogenous H₂S production by PPG significantly decreased glucose levels in Zucker diabetic fatty rats (9), suggesting that H₂S derived from CSE participates in the etiology or development of diabetes in these animals. Yusuf et al. (10) reported that insulin treatment of streptozotocin-injected rats reversed the rise in pancreatic H₂S synthesizing activity. No mechanistic data to interpret these observations, however, were provided. It is possible that insulin treatment of streptozotocin-injected rats reversed the rise in H₂S synthesizing activity by a direct effect of insulin on CSE expression or activity (13). Functional correlations of H₂S and insulin secretion in beta cells also have been realized as insulin secretion from INS-1E cells was significantly decreased by exogenously applied H₂S or CSE overexpression (9, 14). Cysteine and the H₂S donor NaHS also inhibited insulin release from isolated mouse islets and the mouse beta cell line MIN6 in a dose-dependent manner (23).
By activating K\textsubscript{ATP} channels in pancreatic beta cells, H\textsubscript{2}S may inhibit pancreatic insulin release, leading to insulin deficiency in circulation. By inducing cell apoptosis, H\textsubscript{2}S may reduce pancreatic beta cell mass, inhibit secretion of insulin, and deteriorate the status of diabetes. These pathogenic events would partially contribute to diabetes development. The former hypothesis has been confirmed (14), but the latter had only been deduced from the reported effects of H\textsubscript{2}S on proliferation or apoptosis of other types of cells. H\textsubscript{2}S possesses an antiproliferative effect on T-lymphocytes and induces apoptotic death of polymorphonuclear cells (24, 25). Our laboratory also has shown that overproduction of H\textsubscript{2}S via up-regulation of the expression of CSE inhibits HEK-293 cell proliferation and induces human aorta smooth muscle cell (HASMC) apoptosis, and that exogenously applied H\textsubscript{2}S at physiologically relevant concentrations induces apoptosis of HASMCs (15, 18, 19). To date, the effect of exogenous and endogenous H\textsubscript{2}S on pancreatic beta cell growth has not been determined. In this study, we used adenovirus-mediated gene transfer technique to overexpress CSE in INS-1E cells. The successful overexpression was demonstrated by a near 2-fold increase in CSE mRNA expression and 4-fold increase in CSE activity in transfected INS-1E cells (Fig. 1) with significantly increased endogenous H\textsubscript{2}S production. The overexpression of CSE in INS-1E cells significantly decreases cell viability, manifested as condensed nuclei, positive TUNEL staining, and DNA fragmentation (Figs. 2 and Fig. 3). Exogenously applied H\textsubscript{2}S at physiologically relevant concentrations also reduces cell viability and induces apoptosis of INS-1E cells. The other CSE products, ammonium and pyruvate, had little effect on cell survival, further indicating that the pro-apoptotic effect of CSE overexpression is mainly due to endogenously produced H\textsubscript{2}S. H\textsubscript{2}S-induced apoptosis of INS-1E cells is not likely linked to altered redox status because the cellular GSH level was not changed by H\textsubscript{2}S treatment (Fig. 8E). Elevated cellular GSH level has been used as an indicator of increased reduction of oxidized glutathione (26). Our previous studies have also shown that the excitatory effect of H\textsubscript{2}S on K\textsubscript{ATP} channels (14) and H\textsubscript{2}S-induced vasorelaxation (4) were not related to altered redox status.

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One of the characteristic features of pancreatic beta cells is a highly developed ER, apparently because of heavy engagement in insulin secretion. The proper function of ER is essential to cell survival. Disruption of ER function may lead to the accumulation of misfolded or unfolded proteins, triggering ER stress (27). The first ER stress response involves up-regulation of the genes encoding ER chaperone proteins including BiP, which increases protein-folding activity and prevents protein aggregation. When severe and prolonged ER stress extensively impairs ER functions, CHOP (encoding the C/EBP homologous protein-10, also known as GADD153) gene expression is upregulated and apoptosis occurs, which is necessary not only for removing the cells that threaten the integrity of the whole organ but also for proper development and differentiation (27). ER stress plays a key role in pancreatic beta cell apoptosis and dia-
Many types of cells by many research teams. It may be questioned whether H$_2$S-induced apoptosis of pancreatic beta cells merely represents a general cell type-independent cellular response. Several lines of evidence disprove this notion. First, H$_2$S induces apoptosis of HASMCs, polymorphonuclear cells, pancreatic acinar cells, INS-1E cells, and some other cells (15, 18, 25, 36, 37). H$_2$S only inhibits cell proliferation in HEK-293 cells but does not induce apoptosis (19). In contrast, H$_2$S prevents apoptosis of human polymorphonuclear neutrophils and colon cancer cells (38, 39). All these observations suggest that different cell types have different reactions to H$_2$S.

Second, the role of MAPK pathway in H$_2$S-mediated cellular apoptosis is different in different types of cells. MAPK family represents important signal transduction machinery and occupies a central position in cell growth, differentiation, and programmed cell death (40). Different MAPK are activated by different stimuli, target at different downstream molecules, and perform different functions (41). Our previous studies showed that H$_2$S treatment or CSE overexpression increases ERK and p38 MAPK activities in HASMCs (15, 18). Here we provide evidence that Ad-CSE transfection or H$_2$S application decreased ERK activity and increased p38 MAPK activity in INS-1E cells (Fig. 4). Inactivation of p38 MAPK, but not ERK, inhibited H$_2$S-induced apoptosis of INS-1E cells (Fig. 6), suggesting that p38 MAPK plays a major role in apoptosis induction by H$_2$S in INS-1E cells. In contrast, it is ERK, not p38 MAPK, that plays a key role in CSE/H$_2$S-mediated apoptosis of HASMCs (15, 18). H$_2$S treatments have been shown to increase ERK and p38 MAPK activation in IEC-18 cells (37) and inhibit p38 MAPK activation in polymorphonuclear neutrophils (38). NO and carbon monoxide, another two gasotransmitters (2, 22), also have been reported to differentially regulate the activity of MAPK in different cell types (42–45).

Third, H$_2$S induced different ER stress levels in INS-1E cells from that of other types of cells. Whereas H$_2$S induces expression of BiP, CHOP, and SREBP-1c expression in INS-1E cells, at the same concentration H$_2$S only induced BiP expression, but not that of SREBP-1c and CHOP, in HASMCs. Furthermore, treatment with H$_2$S led to a significant increase in tumor necrosis factor-α expression in human monocytic cell line U937 (46) but a decrease in gastric mucosa (47).

In addition, a difference in substrate specificity of CSE between different species should also be noticed. Although rat CSE and human CSE have higher homologous sequences, rat CSE is able to cleave both C–S and C–S bonds of L-cystathionine, and human CSE appears to selectively act on C–S bonds (48). Rat CSE can use cysteine as substrate to produce H$_2$S (10, 15). Recombinant human CSE also caused concentration-dependent L-cysteine degradation (48). Furthermore, exogenously applied cysteine induced chloride secretion from human submucosa/mucosa preparations, which was diminished by PPG treatment (49). In our own study, HASMC lysate produced significant amounts of H$_2$S using L-cysteine as the substrate (18). These observations suggest that human CSE can also use cysteine as the substrate for H$_2$S production although cysteine only contains one C–S bond. Therefore, our results obtained with the rat CSE cDNA gene would provide clues to altered CSE expression and activity in human diseases. Similarly decreased homocysteine levels in diabetic patients (11) and increased CSE activities in diabetic rats (12, 13) further support our opinion.

In summary, our findings demonstrate that CSE/H$_2$S induces apoptosis of INS-1E cells, an insulin-secreting beta cell line, possibly via the phosphorylation of p38 MAPK and up-regulation of the mRNA level of BiP and CHOP (Fig. 6). This observation supports the notion that H$_2$S-induced BiP and CHOP induction results from activation of the p38 MAPK pathway. The expression of CHOP gene is regulated tightly by stress in a wide variety of cells and closely associated with the level of the ER chaperone BiP (31, 32). At the moment, the mechanism by which p38 MAPK activates BiP and CHOP expression still remains unclear. Previous studies reported that CHOP is phosphorylated on two adjacent serine residues (78 and 81) by p38 MAPK, and SB203580 abolished the stress-induced phosphorylation of CHOP (33). p38 MAPK signaling is also necessary for CHOP induction and apoptosis in several mammalian cells (34, 35).

Apoptotic changes induced by H$_2$S have been reported in many types of cells by many research teams. It may be questioned whether H$_2$S-induced apoptosis of pancreatic beta cells merely represents a general cell type-independent cellular response. Several lines of evidence disprove this notion. First, H$_2$S induces apoptosis of HASMCs, polymorphonuclear cells, pancreatic acinar cells, INS-1E cells, and some other cells (15, 18, 25, 36, 37). H$_2$S only inhibits cell proliferation in HEK-293 cells but does not induce apoptosis (19). In contrast, H$_2$S prevents apoptosis of human polymorphonuclear neutrophils and colon cancer cells (38, 39). All these observations suggest that different cell types have different reactions to H$_2$S.
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luted expression of ER stress-related genes, BiP, CHOP, and SREBP-1c. These novel results may help our understanding of the important role of the CSE/H₂S system in homeostatic control of pancreatic structure and function, and insight may be perceived regarding the pathogenesis and new therapeutic targets of diabetes as well.

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