

# L-Cysteine Inhibits Insulin Release From the Pancreatic $\beta$ -Cell

## Possible Involvement of Metabolic Production of Hydrogen Sulfide, a Novel Gasotransmitter

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Hydrogen sulfide ( $H_2S$ ) was historically recognized as a toxic gas generated by natural resources. However, its enzymatic production from L-cysteine has recently been demonstrated in mammals. Cystathionine  $\beta$ -synthase and cystathionine  $\gamma$ -lyase, both of which can produce  $H_2S$ , were expressed in mouse pancreatic islet cells and the  $\beta$ -cell line, MIN6. L-Cysteine and the  $H_2S$  donor NaHS inhibited glucose-induced insulin release from islets and MIN6 cells. These inhibitory effects were reproduced when insulin release was stimulated by  $\alpha$ -ketoisocaproate, tolbutamide, or high  $K^+$ . L-Cysteine and NaHS inhibited glucose-potentiated insulin release in the copresence of diazoxide and high  $K^+$ . Real-time imaging of intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) demonstrated that both L-cysteine and NaHS reversibly suppressed glucose-induced  $[Ca^{2+}]_i$  oscillation in a single  $\beta$ -cell without obvious changes in the mean value. These substances inhibited  $Ca^{2+}$ - or guanosine 5'-0-3-thiotriphosphate-induced insulin release from islets permeabilized with streptolysin-O. L-Cysteine and NaHS reduced ATP production and attenuated glucose-induced hyperpolarization of the mitochondrial membrane potential. Finally, L-cysteine increased  $H_2S$  content in MIN6 cells. We suggest here that L-cysteine inhibits insulin release via multiple actions on the insulin secretory process through  $H_2S$  production. Because the activities of  $H_2S$ -producing enzymes and the tissue  $H_2S$  contents are known to increase under diabetic conditions, the inhibition may participate in the deterioration of insulin release in this disease. *Diabetes* 55:1391-1397, 2006

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$\alpha$ -KIC,  $\alpha$ -ketoisocaproate;  $[Ca^{2+}]_i$ , intracellular  $Ca^{2+}$  concentration; CBS, cystathionine  $\beta$ -synthase; CSE, cystathionine  $\gamma$ -lyase; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenyl hydrazone; GTP $\gamma$ S, guanosine 5'-0-3-thiotriphosphate;  $K_{ATP}$  channel, ATP-sensitive  $K^+$  channel; SLO, streptolysin-O; TBST, Tris-buffered saline with Tween.

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**D**iabetes is a chronic metabolic disorder that affects the metabolism of carbohydrates and other nutrients as a result of impaired insulin release and/or insulin resistance. There is a line of evidence that diabetic conditions affect metabolism of the sulfur-containing amino acids, i.e., methionine, homocysteine, and L-cysteine, in human subjects (1) and also in diabetic model animals, such as streptozotocin-treated rats (2,3) and Zucker fatty rats (4). Plasma L-cysteine levels were elevated in diabetic patients with diabetic nephropathy renal complications (1) and in the streptozotocin-treated rats (2). Moreover, the cysteine contents were reported to be increased in the diabetic liver and kidney in the streptozotocin-treated rats (5).

Recent studies show that L-cysteine may be a metabolic source of hydrogen sulfide ( $H_2S$ ), a novel gasotransmitter in various types of cells, including neural cells and smooth muscle cells (6). Cystathionine  $\beta$ -synthase (CBS) and cystathionine  $\gamma$ -lyase (CSE), the key enzymes of the transsulfuration pathway, are reported to be able to produce  $H_2S$  from L-cysteine (7). Both of the enzymes distribute in a wide variety of tissues in a tissue-specific manner and have the potential to catalyze the desulfuration of L-cysteine (8,9). The activities of these enzymes were reported to become higher in diabetic animals (10,11). Nevertheless, the physiological or pathological roles of  $H_2S$  have been studied only in a few types of cells (6,12), and those in the secretory cells have not been reported previously.

In this study, we examined the effects of L-cysteine and the  $H_2S$  donor NaHS on insulin secretion using isolated mouse islets and the mouse  $\beta$ -cell line MIN6. We also examined the expression and distribution of the  $H_2S$ -producing enzymes in the pancreatic islets. We found that L-cysteine potently inhibited insulin secretion partly via production of  $H_2S$ , the action sites of which seem to be versatile.

This is the first report showing that  $H_2S$  may regulate insulin secretion from pancreatic islets. These findings suggest to us that elevation of the  $H_2S$  levels as a result of L-cysteine metabolism may be involved in a vicious cycle of impaired insulin secretion under diabetic conditions.

## RESEARCH DESIGN AND METHODS

**Isolation of mouse pancreatic islets.** Pancreatic islets were isolated from male ICR mice (SLC, Hamamatsu, Japan) aged 8–12 weeks by the collagenase digestion method. The solution used for the isolation was Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 119 mmol/l NaCl, 4.75 mmol/l KCl, 5 mmol/l NaHCO<sub>3</sub>, 2.54 mmol/l CaCl<sub>2</sub>, 1.2 mmol/l MgSO<sub>4</sub>, 1.2 mmol/l KH<sub>2</sub>PO<sub>4</sub>, and 20 mmol/l HEPES (pH 7.4 adjusted with NaOH) supplemented with 3 mmol/l glucose. All experiments using laboratory mice in this study were approved by the University Committee on Animal Experiments in accordance with the Guideline for Animal Experimentation in Oita University.

**RT-PCR.** Total RNA was extracted from various tissues of mice and the  $\beta$ -cell line MIN6 using an RNeasy Total RNA kit (Qiagen, Hilden, Germany). Reverse transcription and PCR were performed using a One-Step PCR kit (Qiagen) with the gene-specific primers (CSE forward, 5'-ATGGATGAAGTGTATGGA GG-3'; CSE reverse, 5'-ACGAAGCCGACTATTGAGGT-3'; CBS forward, 5'-ACTA CGATGACACCGCCGAG-3'; and CBS reverse, 5'-AGTCCTTCCTGTGC GATGAG-3') and primers for glyceraldehyde-3-phosphate dehydrogenase as an internal control (forward, 5'-AACGACCCCTTCATTGAC-3'; and reverse, 5'-TCCACGACATACTCAGCAC-3'). mRNA samples obtained from mouse liver and kidney were used as positive expression controls for both CSE and CBS. The PCR products were separated by 1.5% agarose gel electrophoresis followed by staining with ethidium-bromide, and the intensity of each band was analyzed with the NIH image software.

**Anti-CBS antibody and immunoblot analysis.** The anti-CBS antibody used in this study was raised as described by Enokido et al. (13). Isolated islets were sonicated in an ice-cold homogenization buffer consisting of 20 mmol/l Tris-HCl (pH 7.4), 2 mmol/l EDTA, 2 mmol/l 2-mercaptoethanol, 50  $\mu$ g/ml phenylmethylsulfonylfluoride, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, and 250 mmol/l sucrose. Protein samples were boiled in a sample buffer containing 62.5 mmol/l Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue. They were then separated on a 10% polyacrylamide gel and transferred to polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk in a buffer containing 10 mmol/l Trizma base, 137 mmol/l NaCl, and 0.1% Tween 20 (Tris-buffered saline with Tween [TBST]) and incubated overnight with an anti-CBS antibody (diluted  $\times$ 1,000 in TBST). After washings in TBST, the membrane was further incubated with a horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:25,000; Amersham Biosciences, Piscataway, NJ), and immunopositive bands were visualized by a commercialized kit (ECL Plus Western Blotting Reagent Pack; Amersham Biosciences).

**Cell culture of MIN6 cells.** MIN6 cells (donated by Prof. J.-I. Miyazaki, Osaka University, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 66 mg/l kanamycin sulfate (Sigma) and 15% fetal bovine serum (JRH Biosciences, Lenexa, KA) at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub>. They were passaged and harvested using trypsin/EDTA. The culture medium was replaced every other day.

**Static incubation of isolated pancreatic islets and MIN6 cells.** Groups of five size-matched islets were preincubated in 1 ml KRBH with 0.1% BSA (fraction V; Sigma) and 3 mmol/l glucose for 1 h at 37°C. These islets were then incubated for 1 h in various conditions. The solutions containing NaHS (Sigma) were prepared by diluting 1 mol/l NaHS stock solution just before its application. For MIN6 cells, the cells were seeded onto a 24-multiwell plate (Nalge-Nunc, Rochester, NY) at a density of  $1 \times 10^5$  cells per well. After 2 days of culturing in the culture medium described above, the secretion experiments were carried out as for isolated islets. At the end of the incubation, incubation media were collected. For MIN6 cells, the aliquots were briefly centrifuged to avoid any detached cells. Samples were kept at  $-20^\circ\text{C}$  for later assay, and the amount of insulin in the media was measured by a radioimmunoassay kit (Shionogi, Osaka, Japan) using bovine insulin as a standard.

**Membrane permeabilization of the pancreatic islets.** Experiments of insulin secretion from permeabilized islets were carried out as previously described (14). Groups of five size-matched islets were preincubated as described above. After preincubation, these islets were washed twice with 1 ml glutamate buffer containing 100 mmol/l potassium glutamate, 42 mmol/l sodium glutamate, 16 mmol/l HEPES (pH 7), 5 mmol/l BSA, 1 mmol/l EGTA, and 3 mmol/l MgATP (Sigma). The islets were then permeabilized by coinubation with 0.125 IU/ml streptolysin-O (SLO) (Nissui Pharmaceutical, Tokyo, Japan) in the same glutamate buffer, under various conditions. At the end of the 1-h incubation, incubation media were retained, and the amount of insulin in the media was measured by radioimmunoassay.

**Primary culture of mouse islet cells and measurement of the intracellular Ca<sup>2+</sup> concentration.** Experiments for the measurement of the intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) using dispersed islet cells were carried out as described previously (15). Isolated islets were dispersed to single islet cells by dispase (Godo Shusei, Tokyo, Japan). Dispersed islet cells were plated

onto coverslips and cultured overnight in RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum, 100  $\mu$ g/ml streptomycin (Meiji Seika, Tokyo, Japan), and 100 units/ml penicillin (Meiji Seika). The cells were loaded with 4  $\mu$ mol/l fura PE3-AM (Sigma) for 2 h at 37°C. They were then mounted in a chamber on the stage of an inverted microscope (Diaphot TMD 300; Nikon, Tokyo, Japan) and were continuously superfused with KRBH with 3 mmol/l glucose and 0.1% BSA for 30 min at 37°C using a peristaltic pump at a flow rate of 1 ml/min. [Ca<sup>2+</sup>]<sub>i</sub> was measured with an Argus-50/CA system (Hamamatsu Photonics, Hamamatsu, Japan), with alternating excitation of cells at 340 and 380 nm and monitoring of the resultant emission at 510 nm. Pairs of 340- and 380-nm fluorescence images were captured every 10 s and converted to 340:380 ratio images after subtraction of the background fluorescence. At the end of these experiments, tolbutamide at 300  $\mu$ mol/l was applied to verify that the recording was obtained from a  $\beta$ -cell.

**ATP assay.** MIN6 cells ( $2 \times 10^4$  cells/well) were seeded and cultured for 2 days before experimentation. After preincubation in KRBH containing 3 mmol/l glucose and 0.1% BSA for 1 h, they were incubated for 60 min in the presence of 20 mmol/l glucose and various concentrations of L-cysteine or NaHS. Cellular ATP was extracted and measured using a commercialized kit (ATPlite; PerkinElmer Life and Analytical Sciences, Groningen, the Netherlands) according to the manufacturer's instructions.

### Fluorometrical measurement of mitochondrial membrane potential.

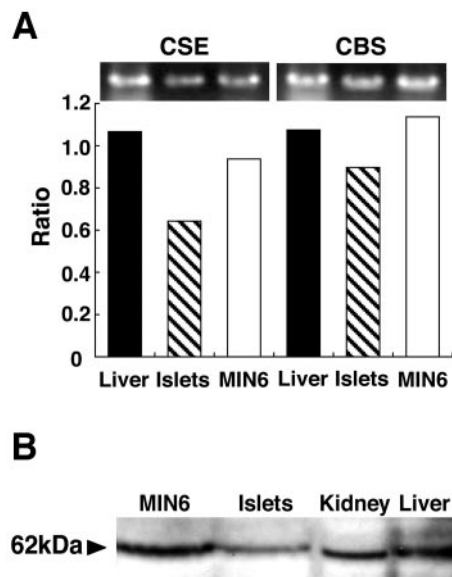
Mitochondrial membrane potential ( $\Delta\psi_m$ ) was monitored with rhodamine 123 (Sigma), a fluorescent indicator of  $\Delta\psi_m$  (16,17). Cells were loaded by incubation with 10  $\mu$ g/ml rhodamine 123 for 10 min in KRBH containing 0.1% BSA and 3 mmol/l glucose at 37°C. After washing, they were transferred to an incubation chamber mounted on the stage of a laser scanning confocal microscope (LSM510; Carl Zeiss; Oberkochen, Germany), and cells were perfused with the BSA-containing KRBH at a flow rate of 1 ml/min using a peristaltic pump. Rhodamine 123 fluorescence was excited with a 488-nm argon laser, and its resultant fluorescence was acquired through a 505- to 550-nm band-pass filter every 15 s. Calbonyl cyanide *p*-(trifluoromethoxy) phenyl hydrazone (FCCP; Sigma), a protonophore, was applied at the end of the experiment as an indicator of the maximal  $\Delta\psi_m$  depolarization. When necessary, obtained data were expressed as a percentage of the maximal value obtained by the protonophore in each experiment.

**H<sub>2</sub>S assay.** The levels of H<sub>2</sub>S were measured by the modified method previously described by Tang and Santschi (18). After 1 h of preincubation with 3 mmol/l glucose, MIN6 cells ( $1.85 \times 10^7$  cells) were incubated for 1 h with 20 mmol/l glucose in the presence or absence of 10 mmol/l L-cysteine. They were then washed twice with PBS and centrifuged three times at 1,000 rpm for 10 min. The pellet of cells was resuspended in 0.8 ml 0.01 mol/l NaOH, followed by the addition of 80  $\mu$ l 20 mmol/l *N,N*-dimethyl-*p*-phenylenediamine sulfate (Sigma) in 7.2 mol/l HCl and 80  $\mu$ l 30 mmol/l FeCl<sub>3</sub> in 1.2 mol/l. After 20 min at room temperature, the mixed solution was centrifuged at 5,000 rpm for 10 min, and the supernatant was applied to a Waters Sep-Pak Light tC18 cartridge, which was conditioned by passing 1 ml methanol and 1 ml milli-Q water. After the cartridge was rinsed with 5 ml milli-Q water, 0.5 ml methanol was passed through the cartridge in the reverse direction to elute methylene blue. The eluent was dried with speedvac and dissolved in 50  $\mu$ l milli-Q water. The levels of methylene blue were measured by a high-performance liquid chromatographer (Waters 2695; Waters, Milford, MA). An acetonitrile gradient from 30 to 50% with complementary 70 to 50% of 50 mmol/l ammonium acetate and 5 mmol/l pentanesulfonic acid sodium salt (Wako, Osaka, Japan) was performed on a C18 column (Waters Symmetry C18; 5  $\mu$ m 4.6  $\times$  250 mm) using a flow rate of 1.0 ml/min in 12 min. Absorbance was monitored at a wavelength of 668 nm with UV/Vis detector (Waters 2487).

**Statistics.** Results were expressed as means  $\pm$  SE. Statistical evaluations were made by one-way ANOVA followed by the Tukey's method. For Fig. 8, statistical significance was assessed by the unpaired Student's *t* test. A probability of  $P < 0.05$  was accepted as the level of statistical significance.

## RESULTS

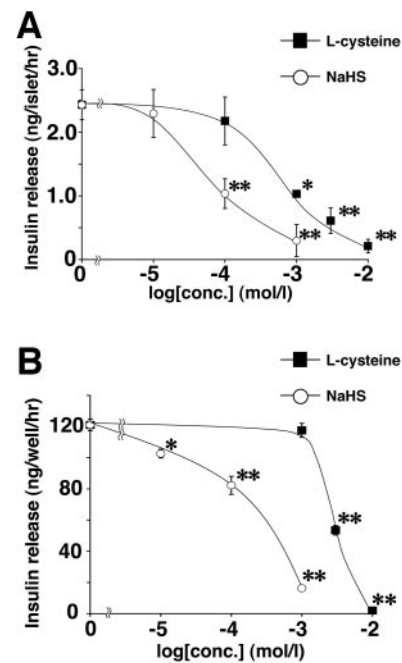
**Expression of CSE and CBS in the mouse pancreatic islets and MIN6 cells.** We assessed mRNA expression levels of CSE and CBS in mouse pancreatic islets and MIN6 cells by RT-PCR. A PCR-amplified 384-bp fragment of CSE and a 337-bp fragment of CBS were detected in mouse islets, kidney, liver, and MIN6 cells (Fig. 1). Western blot analysis using the polyclonal anti-CBS antibody revealed an immunopositive band with an apparent molecular weight of 62 kDa in mouse islets, kidney, and liver (Fig. 1).



**FIG. 1.** Expression of CSE and CBS in mouse pancreatic islets. **A:** Expression of CSE and CBS mRNA was assessed in mouse pancreatic islets, liver, and MIN6 cells by RT-PCR. Each column shows the level standardized by that of glyceraldehyde-3-phosphate dehydrogenase expression. **B:** Expression of the CBS protein was detected with an anti-CBS antibody in mouse pancreatic islets, kidney, liver, and MIN6 cells by Western blotting analysis.

**Dose-dependent inhibition of glucose-induced insulin secretion by L-cysteine and NaHS.** Expression of  $H_2S$ -generating enzymes in pancreatic islets and MIN6 cells led us to examine the effects of the enzyme substrate L-cysteine and the  $H_2S$  donor NaHS on insulin release from pancreatic islets and MIN6 cells. L-Cysteine within a millimolar range inhibited glucose (10 mmol/l)-induced insulin release from mouse pancreatic islets in a dose-dependent manner, and NaHS more potently inhibited glucose-induced insulin release (Fig. 2A). L-Cysteine and NaHS did not decrease insulin release in the copresence of a non-stimulatory concentration (3 mmol/l) of glucose (data not shown). Inhibition of glucose (20 mmol/l)-induced insulin release by these substances was also observed in MIN6 cells at similar ranges (Fig. 2B). These inhibitory effects of L-cysteine and NaHS disappeared immediately after the removal in the perfusion system (data not shown).

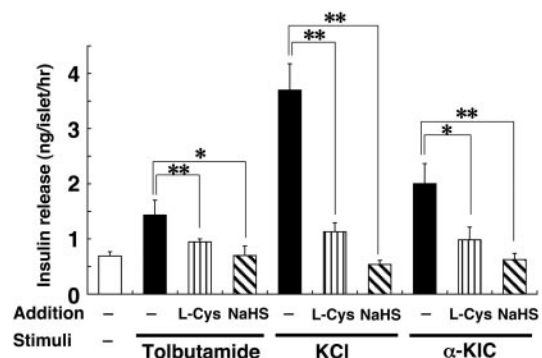
**Consistent inhibition by L-cysteine and NaHS of insulin release evoked by various secretagogues.** In smooth muscle cells,  $H_2S$ -induced relaxation has been reported to be due to its activating effect on the ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channel (19). In the  $\beta$ -cell, closure of this channel by intracellular ATP plays a crucial role in glucose-induced depolarization of the plasma membrane, which causes  $Ca^{2+}$  influx through the voltage-dependent  $Ca^{2+}$  channel (20). Exposure of isolated islets to tolbutamide (100  $\mu$ mol/l), a  $K_{ATP}$  blocker, or  $\alpha$ -ketoisocaproate ( $\alpha$ -KIC; 10 mmol/l), a mitochondrial fuel, induces insulin release via the  $K_{ATP}$  closure. Both L-cysteine (3 mmol/l) and NaHS (100  $\mu$ mol/l) strongly inhibited insulin release by these substances in the concomitant presence of 3 mmol/l glucose (Fig. 3). However, L-cysteine and NaHS also inhibited insulin release when the voltage-dependent  $Ca^{2+}$  channel was directly opened by high  $K^+$  (30 mmol/l) depolarization (Fig. 3). To ascertain that the inhibition involves  $K_{ATP}$ -independent mechanism, we further examined the effects of L-cysteine and NaHS on high  $K^+$  (30



**FIG. 2.** Dose-dependent inhibition of glucose-induced insulin release by L-cysteine and NaHS. **A:** Five mouse islets were incubated with 10 mmol/l glucose in the copresence of L-cysteine (0.1–10 mmol/l) and NaHS (10  $\mu$ mol/l to 1 mmol/l). **B:** MIN6 cells seeded at a density of  $1 \times 10^5$  cells/well were incubated with 20 mmol/l glucose and various concentrations of L-cysteine and NaHS. The amount of insulin released into the media during a 1-h incubation was measured by radioimmunoassay. Each value is the mean  $\pm$  SE for four to seven observations from two independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  compared with the value with glucose alone.

mmol/l)-induced insulin release (Fig. 4). L-Cysteine (3 mmol/l) inhibited insulin release in the copresence of the  $K_{ATP}$  opener diazoxide (200  $\mu$ mol/l). Such inhibition was also observed with NaHS. These results suggest that the inhibition of insulin release by L-cysteine and NaHS involves a mechanism independent of their effects on the  $K_{ATP}$  activity.

**Effects of L-cysteine and NaHS on glucose-induced  $[Ca^{2+}]_i$  oscillation.** We examined the effect of L-cysteine and NaHS on glucose-induced  $[Ca^{2+}]_i$  oscillation. In single  $\beta$ -cells,  $[Ca^{2+}]_i$  was low and stable at 3 mmol/l glucose. When the extracellular glucose concentration was in-



**FIG. 3.** Effects of L-cysteine and NaHS on insulin release evoked by tolbutamide, KCl, and  $\alpha$ -KIC. Five size-matched islets were incubated for 1 h in the presence of tolbutamide (100  $\mu$ mol/l), KCl (30 mmol/l), and  $\alpha$ -KIC (10 mmol/l) with or without L-cysteine (3 mmol/l) and NaHS (100  $\mu$ mol/l) as indicated. All of these experiments were carried out in the presence of 3 mmol/l glucose. Each value is the mean  $\pm$  SE for 4–12 observations. \* $P < 0.05$ , \*\* $P < 0.01$ .

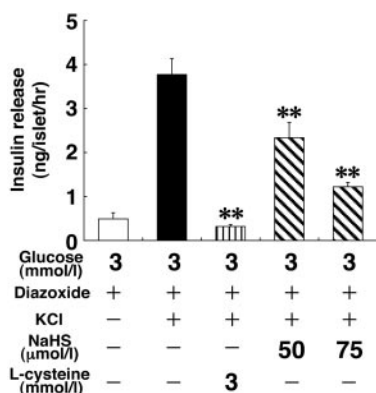


FIG. 4. Inhibition of high K<sup>+</sup>-induced insulin release by L-cysteine and NaHS in the presence of diazoxide. Pancreatic islets were incubated for 1 h in the consistent presence of diazoxide (200 μmol/l) and glucose (3 mmol/l) with 30 mmol/l KCl L-cysteine or NaHS as described. Each column represents the mean ± SE for five observations. \*\**P* < 0.01 vs. the value with 3 mmol/l glucose, 200 μmol/l diazoxide, and 30 mmol/l KCl.

creased to 10 mmol/l, most β-cells showed [Ca<sup>2+</sup>]<sub>i</sub> oscillation (Fig. 5). Exposure to L-cysteine (3 mmol/l) and NaHS (100 μmol/l) promptly suppressed glucose-induced [Ca<sup>2+</sup>]<sub>i</sub> oscillation without obvious changes in the mean [Ca<sup>2+</sup>]<sub>i</sub> value (Fig. 5). The oscillatory changes in [Ca<sup>2+</sup>]<sub>i</sub> reappeared at 9.5 min after the removal of these substances. These results suggest that the inhibition of insulin secretion by L-cysteine and NaHS was accompanied by suppression of [Ca<sup>2+</sup>]<sub>i</sub> oscillation in mouse pancreatic β-cells.

**Effect of L-cysteine and NaHS on insulin release from the SLO-treated pancreatic islets.** Treatment of living cells with SLO forms small pores in the plasma membrane and enables cell-impermeant molecules to access the intracellular space (21). Insulin release from SLO-treated islets was stimulated by Ca<sup>2+</sup> at 10 μmol/l (Fig. 6). Thus Ca<sup>2+</sup>-stimulated insulin release from permeabilized islets was suppressed by L-cysteine (3 mmol/l; Fig. 6A) and NaHS (100 μmol/l; Fig. 6B). Insulin release from SLO-treated islets was also stimulated by 100 μmol/l guanosine 5'-0-3-thiotriphosphate (GTPγS) in the copresence of 100 nmol/l Ca<sup>2+</sup> (0.230 ± 0.035 and 1.043 ± 0.060 ng · islet<sup>-1</sup> · h<sup>-1</sup> for control and GTPγS, respectively; *n* = 4). NaHS (100 μmol/l) inhibited GTPγS-induced insulin release from SLO-treated islets (0.615 ± 0.116 ng · islet<sup>-1</sup> · h<sup>-1</sup>; *n* = 4,

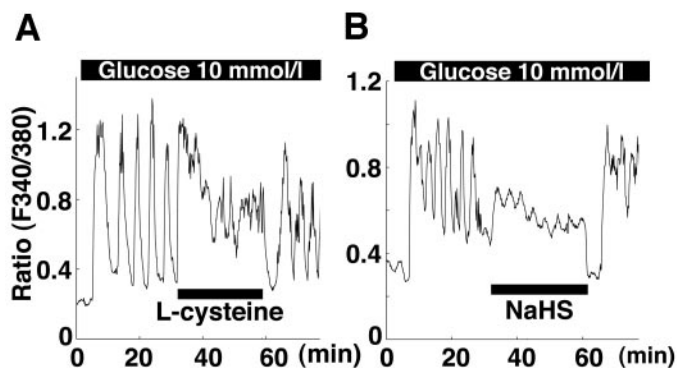


FIG. 5. Inhibitory effects of L-cysteine and NaHS on glucose-induced [Ca<sup>2+</sup>]<sub>i</sub> oscillations in isolated mouse pancreatic β-cells. L-Cysteine (3 mmol/l; A) and NaHS (100 μmol/l; B) were applied in the superfusing solution containing 10 mmol/l glucose. Horizontal bars indicate the periods when the cells were exposed to these agents. Tracings in A and B are the representatives of 238 and 278 cells from three independent experiments, respectively.

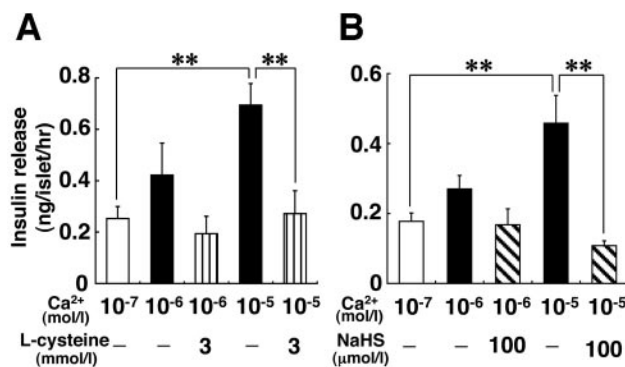


FIG. 6. Effects of L-cysteine and NaHS on Ca<sup>2+</sup>-induced insulin release from permeabilized pancreatic islets. Groups of five pancreatic islets were incubated for 1 h in SLO-supplemented glutamate buffer under various concentrations of free Ca<sup>2+</sup> and L-cysteine (3 mmol/l; A) or NaHS (100 μmol/l; B). Each value is the mean ± SE for four to five observations. \*\**P* < 0.01.

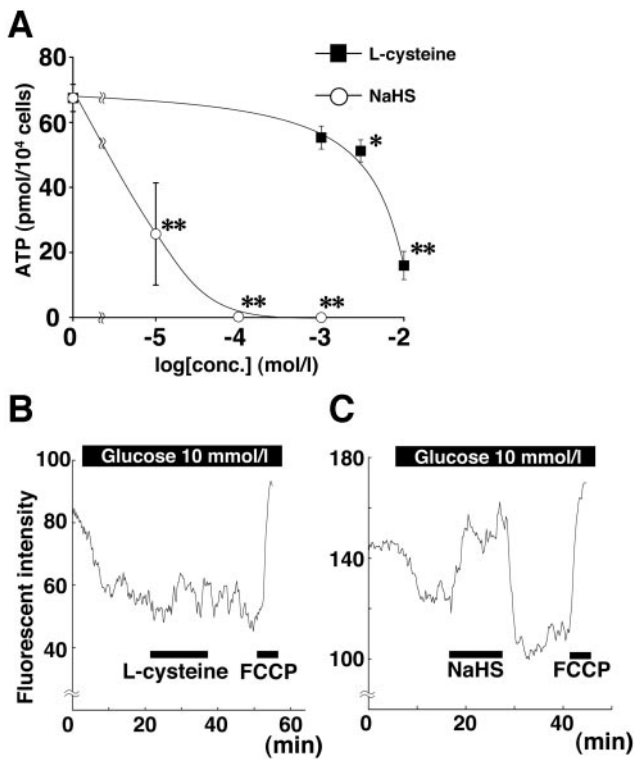
*P* < 0.01). Under this condition, [Ca<sup>2+</sup>]<sub>i</sub> is not altered by either Ca<sup>2+</sup> influx or Ca<sup>2+</sup> mobilization because of its chelation by EGTA. Therefore, the inhibition of insulin release by L-cysteine and NaHS may, at least in part, be independent of the changes in [Ca<sup>2+</sup>]<sub>i</sub>. This inhibition may not merely result from their suppression of ATP production, because metabolic inhibitors such as carbonylcyanide *m*-chlorophenylhydrazone and oligomycin failed to decrease Ca<sup>2+</sup>-induced insulin release from permeabilized islets (data not shown).

**Influence of L-cysteine and NaHS in glucose metabolism in the pancreatic β-cell.** Figure 7A shows dose-dependent effects of L-cysteine and NaHS on ATP content in MIN6 cells. L-Cysteine progressively decreased ATP production from glucose (20 mmol/l). NaHS at lower concentrations also inhibited ATP production. Figure 7B and C depicts the effects of L-cysteine (3 mmol/l) and NaHS (100 μmol/l) on glucose metabolism in dispersed β-cells assessed by a fluorescent dye, rhodamine 123. Activation of the respiratory chain induces H<sup>+</sup> transport through the inner mitochondrial membrane, resulting in Δψ<sub>m</sub> hyperpolarization. Glucose (10 mmol/l) caused Δψ<sub>m</sub> hyperpolarization, indicated by a fall in the intensity of the fluorescence. Application of L-cysteine caused a small but significant inhibition of glucose-induced hyperpolarization (Fig. 7B), and that of NaHS resulted in an immediate and more potent inhibition of glucose metabolism (Fig. 7C). When the peak value of the inhibition was expressed as a percentage of the FCCP-induced depolarization, 24.9 ± 4.2 and 82.0 ± 6.3% were obtained by L-cysteine and NaHS, respectively (*n* = 18 for L-cysteine and *n* = 7 for NaHS).

**Production of H<sub>2</sub>S from L-cysteine in MIN6 cells.** Figure 8 demonstrates the levels of H<sub>2</sub>S in MIN6 cells. In the presence of 20 mmol/l glucose, the content of H<sub>2</sub>S was 0.099 ± 0.006 nmol/10<sup>6</sup> cells (*n* = 6), and when the cells were incubated with 20 mmol/l glucose and 10 mmol/l L-cysteine, the H<sub>2</sub>S level were elevated to 0.20 ± 0.022 nmol/10<sup>6</sup> cells (*n* = 6, *P* < 0.01 compared with the value without L-cysteine). This result and other findings described above indicate that inhibition of insulin release by L-cysteine involves its metabolic production of H<sub>2</sub>S.

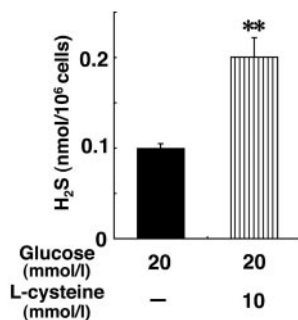
## DISCUSSION

Historically, H<sub>2</sub>S has been recognized as a toxic gas, and it produces serious intoxication in certain occasions such as



**FIG. 7.** Suppression of ATP production and glucose-induced hyperpolarization of  $\Delta\psi_m$  by L-cysteine and NaHS. **A:** MIN6 cells ( $2 \times 10^4$  cells/well) were incubated with 20 mmol/l glucose and various concentrations of L-cysteine and NaHS as indicated. Cellular ATP was extracted and measured by a method based on the luciferin-luciferase reaction. Each symbol represents the mean  $\pm$  SE for six observations.  $*P < 0.05$ ,  $**P < 0.01$  compared with the value with 20 mmol/l glucose alone. **B** and **C:** Primary cultured islet cells were loaded with rhodamine 123, a fluorescent indicator of  $\Delta\psi_m$ . Glucose (10 mmol/l), L-cysteine (3 mmol/l; **B**), and NaHS (100  $\mu$ mol/l; **C**) were applied as indicated. FCCP (1  $\mu$ mol/l) was applied at the end of each experiment to determine the maximal value of the  $\Delta\psi_m$  depolarization. These traces are the representatives of 18 and 7 observations for L-cysteine and NaHS, respectively.

an environmental pollution (22). However, recent studies show that  $H_2S$  may be endogenously produced as a metabolite of L-cysteine and that the gas may be involved in the regulation of various cellular phenomena, i.e., relaxation of vascular or visceral smooth muscle cells and cardiomyocytes (8,23–25), endotoxin-induced inflammation (26), and also some neural functions such as intracellular  $Ca^{2+}$  dynamics and long-term potentiation (27,28).



**FIG. 8.**  $H_2S$  production from L-cysteine by MIN6 cells. MIN6 cells were incubated with 20 mmol/l glucose with or without 10 mmol/l L-cysteine. At the end of the 60-min incubation, the cells were pelleted, and their  $H_2S$  content was measured by high-performance liquid chromatography. Each column represents the mean  $\pm$  SE for six observations from two independent experiments.  $**P < 0.01$ .

$H_2S$  has been suggested to be implicated in the pathogenesis of certain diseases. Changes in the tissue  $H_2S$  contents and activities of  $H_2S$ -producing enzymes have been reported in Down syndrome (29,30) and acute pancreatitis (31).

The present findings indicate that L-cysteine potently inhibited insulin release from both mouse islets and MIN6 cells. The inhibition is likely to result from L-cysteine metabolism and eventual  $H_2S$  production, because 1) the major  $H_2S$ -generating enzymes from L-cysteine, CBS and CSE, were distributed in the pancreatic islets and MIN6 cells; 2) L-cysteine inhibited insulin release in a manner similar to NaHS-induced inhibition; and 3)  $H_2S$  was produced from L-cysteine in MIN6 cells.

As has been suggested in literature,  $H_2S$  exhibits its toxicity via inhibiting cellular metabolism by suppressing the respiratory chain in the mitochondria (32). Suppression of glucose-induced insulin release by L-cysteine and NaHS may result from their inhibition of glucose metabolism as shown by the fluorometrical measurements in the present study. However, we consider that L-cysteine and NaHS may also act on the  $\beta$ -cell secretory machinery in addition to inhibiting glucose metabolism, because these substances also inhibited insulin release from SLO-permeabilized islets under the conditions where ATP was exogenously supplied. Moreover, it was reported that glucose metabolism is nullified in permeabilized islets (33), and metabolic inhibitors examined so far in this study failed to decrease insulin release from SLO-treated islets.

It must be noted that the effects of L-cysteine and NaHS on glucose-induced changes of the  $\beta$ -cell  $[Ca^{2+}]_i$  were very distinctive. Exposure of dispersed  $\beta$ -cells to L-cysteine and NaHS resulted in the disappearance of the  $[Ca^{2+}]_i$  oscillation, whereas the average value of the  $[Ca^{2+}]_i$  did not decline significantly. These results may corroborate an idea that oscillatory changes of  $[Ca^{2+}]_i$  are crucial in glucose-induced insulin release (34). In SLO-treated islets,  $[Ca^{2+}]_i$  is clamped by the  $Ca^{2+}$ -EGTA buffer. Therefore, the inhibition of insulin release from permeabilized islets also implies that these effects of L-cysteine and NaHS are at least partly independent of the modulation of the  $[Ca^{2+}]_i$  levels.

Taking the results obtained in this study into our consideration, we suggest here that L-cysteine and NaHS may share multiple sites for their inhibitory actions on insulin release. However, inhibition of glucose metabolism by L-cysteine was much smaller than that by NaHS, whereas these substances at the tested concentrations inhibited insulin release to a similar extent. This may suggest to us that L-cysteine may inhibit insulin release via a mechanism independent of  $H_2S$  production and the resultant inhibition of glucose metabolism. For example, taurine, known to be produced from L-cysteine (35), was reported to inhibit glucose-induced insulin release from mouse islets (36).

Plasma L-cysteine levels in the normal subjects have been reported to be within a submillimolar range (1). However, we consider that insulin release in vivo can be regulated by endogenous  $H_2S$ . It is reported that 10–150  $\mu$ mol/l  $H_2S$  occurs in various normal tissues (8), and the plasma L-cysteine levels are reported to become higher in diabetic patients with renal complications (1,37). The activities of the  $H_2S$ -producing enzymes CBS and CSE are elevated under diabetic conditions (10,11). Moreover, the tissue contents of  $H_2S$  were reported to be increased in the liver and pancreas in streptozotocin-treated diabetic rats (11).  $H_2S$  is known to penetrate cellular membranes freely

as another gasotransmitter nitric oxide (NO) does (38). Therefore, it is likely that insulin release from the pancreatic  $\beta$ -cell may be modulated by H<sub>2</sub>S, which is generated by pancreatic islets or by the surrounding tissues under diabetic conditions.

In the  $\beta$ -cell, NO, generated as an arginine metabolite, modulates insulin release in a bimodal fashion (15). Because it was previously reported that H<sub>2</sub>S may regulate the production of NO in the vascular smooth muscle cells (24), we examined the effects of NaHS on the intracellular NO levels in the pancreatic  $\beta$ -cells using the NO-specific fluorescent dye, DAF-2. However, we could not obtain any evidence for the involvement of H<sub>2</sub>S in the regulation of NO synthesis in the  $\beta$ -cells (data not shown).

Insulin release is sophisticatedly regulated by physiological substances, such as various hormones, autacoids, neurotransmitters, and nutrients. Most of these substances are considered to modulate the levels of their relevant intracellular signal molecules, which increase or enhance insulin release. However, an intracellular signal molecule that directly inhibits insulin release has never been reported. From this aspect, H<sub>2</sub>S is the first found signal molecule that plays an inhibitory role in insulin release from the pancreatic  $\beta$ -cell. Inhibitory mechanisms of insulin release by physiological substances are poorly understood at present (39). Therefore, it is tempting to investigate the involvement of H<sub>2</sub>S production in the hormones or neurotransmitters that decrease insulin release.

Here, we demonstrated that L-cysteine and NaHS, a donor of its metabolite H<sub>2</sub>S, have a potent inhibitory effect on insulin release via multiple actions on the insulin secretory machinery. These findings may participate in the physiological and/or pathological regulation of insulin release in vivo. We speculate that it may explain why the  $\beta$ -cell suffers from progressive functional deterioration in type 2 diabetic patients.

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#### REFERENCES

- Herrmann W, Schorr H, Obeid R, Makowski J, Fowler B, Kuhlmann MK: Disturbed homocysteine and methionine cycle intermediates S-adenosylhomocysteine and S-adenosylmethionine are related to degree of renal insufficiency in type 2 diabetes. *Clin Chem* 51:891–897, 2005
- Glanville NT, Anderson GH: Altered methionine metabolism in streptozotocin-diabetic rats. *Diabetologia* 27:468–471, 1984
- Gursu MF, Baydas G, Cikim G, Canatan H: Insulin increases homocysteine levels in a dose-dependent manner in diabetic rats. *Arch Med Res* 33:305–307, 2002
- Wijekoon EP, Skinner C, Brosnan ME, Brosnan JT: Amino acid metabolism in the Zucker diabetic fatty rat: effects of insulin resistance and of type 2 diabetes. *Can J Physiol Pharmacol* 82:506–514, 2004
- Brosnan JT, Man KC, Hall DE, Colbourne SA, Brosnan ME: Interorgan metabolic of amino acids in streptozotocin-diabetic ketoacidotic rat. *Am J Physiol* 244:E151–E158, 1983
- Boehning D, Snyder SH: Novel neural modulators. *Annu Rev Neurosci* 26:105–131, 2003
- Maclean KN, Kraus JP: Hydrogen sulfide production and metabolism in mammalian tissues. In *Signal Transduction and the Gasotransmitters*. Wang R, Ed. Clifton, NJ, Humana Press, 2004, p. 275–292
- Moore PK: Hydrogen sulfide and visceral smooth muscle contractility. In *Signal Transduction and the Gasotransmitters*. Wang R, Ed. Clifton, NJ, Humana Press, 2004, p. 333–344
- Kamoun P: Endogenous production of hydrogen sulfide in mammals. *Amino Acids* 26:243–254, 2004
- Jacobs RL, House JD, Brosnan ME, Brosnan JT: Effects of streptozotocin-induced diabetes and of insulin treatment on homocysteine metabolism in the rat. *Diabetes* 47:1967–1970, 1998
- Yusuf M, Kwong Huat BT, Hsu A, Whiteman M, Bhatia M, Moore PK: Streptozotocin-induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis. *Biochem Biophys Res Commun* 333:1146–1152, 2005
- Moore PK, Bhatia M, Moochhala S: Hydrogen sulfide: from the smell of the past to the mediator of the future? *Trends Pharmacol Sci* 24:609–611, 2003
- Enokido Y, Suzuki E, Iwasawa K, Nametaka K, Okazawa H, Kimura H: Cystathionine  $\beta$ -synthase, a key enzyme for homocysteine metabolism, is preferentially expressed in the radial glia/astrocyte lineage of developing mouse CNS. *FASEB J* 19:1854–1856, 2005
- Okazaki K, Niki I, Iino S, Kobayashi S, Hidaka H: A role of calyculin, a Ca<sup>2+</sup>-binding protein, on the Ca<sup>2+</sup>-dependent insulin release from the pancreatic  $\beta$  cell. *J Biol Chem* 269:6149–6152, 1994
- Kaneko Y, Ishikawa T, Amano S, Nakayama K: Dual effect of nitric oxide on cytosolic Ca<sup>2+</sup> concentration and insulin secretion in rat pancreatic  $\beta$ -cells. *Am J Physiol Cell Physiol* 284:C1215–C1222, 2003
- Emaus RK, Grunwald R, Lemasters JJ: Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim Biophys Acta* 850:436–448, 1986
- Duchen MR, Biscoe TJ: Relative mitochondrial membrane potential and [Ca<sup>2+</sup>]<sub>i</sub> in type I cells isolated from the rabbit carotid body. *J Physiol* 450:33–61, 1992
- Tang D, Santschi PH: Sensitive determination of dissolved sulfide in estuarine water by solid-phase extraction and high-performance liquid chromatography of methylene blue. *J Chromatogr A* 883:305–309, 2000
- Wang R: Interaction of hydrogen sulfide and adenosine triphosphate-sensitive potassium channels in vascular smooth muscle cells. In *Signal Transduction and the Gasotransmitters*. Wang R, Ed. Clifton, NJ, Humana Press, 2004, p. 345–355
- Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP IV, Boyd AE III, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA: Cloning of the  $\beta$  cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* 268:423–426, 1995
- Lindau M, Gomperts BD: Techniques and concepts in exocytosis: focus on mast cells. *Biochim Biophys Acta* 1071:429–471, 1991
- Roth SH: Toxicological and environmental impacts of hydrogen sulfide. In *Signal Transduction and the Gasotransmitters*. Wang R, Ed. Clifton, NJ, Humana Press, 2004, p. 293–313
- Cheng Y, Ndisang JF, Tang G, Cao K, Wang R: Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. *Am J Physiol Heart Circ Physiol* 287:H2316–H2323, 2004
- Zhao W, Zhang J, Lu Y, Wang R: The vasorelaxant effect of H<sub>2</sub>S as a novel endogenous gaseous K<sub>ATP</sub> channel opener. *EMBO J* 20:6008–6016, 2001
- Geng B, Yang J, Qi Y, Zhao J, Pang Y, Du J, Tang C: H<sub>2</sub>S generated by heart in rat and its effects on cardiac function. *Biochem Biophys Res Commun* 313:362–368, 2004
- Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ, Anuar FB, Whiteman M, Salto-Tellez M, Moore PK: Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J* 19:1196–1198, 2005
- Abe K, Kimura H: The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* 16:1066–1071, 1996
- Nagai Y, Tsugane M, Oka J, Kimura H: Hydrogen sulfide induces calcium waves in astrocytes. *FASEB J* 18:557–559, 2004
- Kamoun P, Belardinelli MC, Chabli A, Lallouchi K, Chadeaux-Vekemans B: Endogenous hydrogen sulfide overproduction in Down syndrome. *Am J Med Genet A* 116:310–311, 2003
- Taub JW, Huang X, Matherly LH, Stout ML, Buck SA, Massey GV, Becton DL, Chang MN, Weinstein HJ, Ravindranath Y: Expression of chromosome 21-localized genes in acute myeloid leukemia: differences between Down syndrome and non-Down syndrome blast cells and relationship to in vitro sensitivity to cytosine arabinoside and daunorubicin. *Blood* 94:1393–1400, 1999
- Bhatia M, Wong FL, Fu D, Lau HY, Moochhala SM, Moore PK: Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J* 19:623–625, 2005
- Khan AA, Schuler MM, Prior MG, Yong S, Coppock RW, Florence LZ, Lillie LE: Effects of hydrogen sulfide exposure on lung mitochondrial respiratory chain enzymes in rats. *Toxicol Appl Pharmacol* 103:482–490, 1990
- Pace CS, Tarvin JT, Neighbors AS, Pirkle JA, Greider MH: Use of a high

- voltage technique to determine the molecular requirements for exocytosis in islet cells. *Diabetes* 29:911-918, 1980
34. Hellman B, Gylfe E, Grapengiesser E, Lund PE, Berts A: Cytoplasmic Ca<sup>2+</sup> oscillations in pancreatic  $\beta$ -cells. *Biochim Biophys Acta* 1113:295-305, 1992
35. Brand A, Leibfritz D, Hamprecht B, Dringen R: Metabolism of cysteine in astroglial cells: synthesis of hypotaurine and taurine. *J Neurochem* 71:827-832, 1998
36. Tokunaga H, Yoneda Y, Kuriyama K: Streptozotocin-induced elevation of pancreatic taurine content and suppressive effect of taurine on insulin secretion. *Eur J Pharmacol* 87:237-243, 1983
37. Wollesen F, Brattstrom L, Refsum H, Ueland PM, Berglund L, Berne C: Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. *Kidney Int* 55:1028-1035, 1999
38. Wang R: Two's company, three's a crowd: can H<sub>2</sub>S be the third endogenous gaseous transmitter? *FASEB J* 16:1792-1798, 2002
39. Sharp GW: Mechanisms of inhibition of insulin release. *Am J Physiol* 271:C1781-C1799, 1996