ORIGINAL RESEARCH COMMUNICATION

Hydrogen Sulfide Regulates Ca^{2+} Homeostasis Mediated by Concomitantly Produced Nitric Oxide *via* a Novel Synergistic Pathway in Exocrine Pancreas

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Abstract

Aim: The present study was designed to explore the effects of hydrogen sulfide (H_2S) on Ca^{2+} homeostasis in rat pancreatic acini. **Results:** Sodium hydrosulfide (NaHS; an H_2S donor) induced a biphasic increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in a dose-dependent manner. The NaHS-induced $[\text{Ca}^{2+}]_i$ elevation persisted with an EC_{50} of $73.3 \mu\text{M}$ in the absence of extracellular Ca^{2+} but was abolished by thapsigargin, indicating that both Ca^{2+} entry and Ca^{2+} release contributed to the increase. The $[\text{Ca}^{2+}]_i$ increase was markedly inhibited in the presence of NG-monomethyl L-arginine or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), and diaminofluorescein-2/diaminofluorescein-2 triazole (DAF-2/DAF-2T) fluorometry demonstrated that nitric oxide (NO) was also produced by H_2S in a dose-dependent manner with an EC_{50} of $64.8 \mu\text{M}$, indicating that NO was involved in the H_2S effect. The H_2S -induced $[\text{Ca}^{2+}]_i$ increase was inhibited by pretreatment with U73122, xestospongin C, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, KT5823, and GP2A, indicating that phospholipase C (PLC), the inositol 1,4,5-trisphosphate (IP_3) receptor, soluble guanylate cyclase (sGC), protein kinase G (PKG), and G_q -protein play roles as intermediate components in the H_2S -triggered intracellular signaling. **Innovation:** To our knowledge, our study is the first one highlighting the effect of H_2S on intracellular Ca^{2+} dynamics in pancreatic acinar cells. Moreover, a novel cascade was presumed to function *via* the synergistic interaction between H_2S and NO. **Conclusion:** We conclude that H_2S affects $[\text{Ca}^{2+}]_i$ homeostasis that is mediated by H_2S -evoked NO production *via* an endothelial nitric oxide synthase (eNOS)-NO-sGC-cyclic guanosine monophosphate-PKG- G_q -protein-PLC- IP_3 pathway to induce Ca^{2+} release, and this pathway is identical to the one we recently proposed for a sole effect of NO and the two gaseous molecules synergistically function to regulate Ca^{2+} homeostasis. *Antioxid. Redox Signal.* 20, 747–758.

Introduction

THOUGH HYDROGEN SULFIDE (H_2S) is a toxic gas with a repulsive odor, it has recently been identified as a powerful gaseous molecule that exerts diverse biological effects, such as nitric oxide (NO) and carbon monoxide. Two pyridoxal-5'-phosphate-dependent enzymes, cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) are responsible for the majority of the endogenous production of H_2S from L-cysteine as the main substrate (59). These enzymes are expressed in various mammalian cells, indicating biosynthesis of H_2S in those cells. The enzyme CBS is mainly expressed in

the brain, peripheral nervous system, liver, and pancreas (4,45), whereas CSE mRNA is mostly found in the aorta, mesenteric artery, portal vein, and other vascular tissues (16,70). Moreover, the small intestine and stomach express low amounts of CSE (58). In the mouse pancreas, CBS is ubiquitously distributed in both endocrine and exocrine cells, and CSE is found mostly in the exocrine tissue, but in very small amounts in islets (19). In some tissues, both CSE and CBS are required for H_2S synthesis; however, in others, only one of these enzymes is necessary (60). The third enzyme, 3-mercaptopyruvate sulfurtransferase in conjunction with cysteine (aspartate) aminotransferase, was reported to

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Innovation

Roles of hydrogen sulfide (H_2S) on intracellular Ca^{2+} homeostasis and its underlying mechanism are ambiguous in exocrine pancreas. We demonstrated, for the first time, the Ca^{2+} -releasing effect of H_2S *via* the production of nitric oxide. We also demonstrated that this effect is induced *via* the pathway in which activation of each soluble guanylate cyclase, protein kinase G, G_q -protein, and phospholipase C is involved. This hypothesis may provide a useful key to clarify the physiological and/or pathological mechanisms of action of H_2S and eventually may yield clues for potential therapeutic exploitation.

be a possible H_2S generator from L-cysteine in the presence of α -ketoglutarate in the brain and in the vascular endothelium of thoracic aorta (30,49). Another less important endogenous source of H_2S is the non-enzymatic reduction of elemental sulfur to H_2S using reducing equivalents obtained from the oxidation of glucose (47). By the methylene blue method, the endogenous concentration of H_2S has been found to be 50–160 μM in brain tissue and 50–100 μM in human and rat sera (61). By amperometry or gas chromatography, a similar range of values (50–80 μM) has been demonstrated, although lower concentrations were also suggested (27,48).

Recent studies have revealed several physiological and pathophysiological functions of H_2S . It has been shown to relax vascular smooth muscle, induce vasodilation of isolated blood vessels, and reduce blood pressure (43), indicating that it is a cardinal regulator of blood pressure, whereas some contradictory result was reported (18). H_2S has been identified as a potent anti-inflammatory (66) and antioxidant molecule (22). It regulates expression of chemokines, cytokines, and adhesion molecules and has a biphasic effect in acute pancreatitis and associated lung injury (50,53). The physiological functions of H_2S in the brain have been suggested to include Ca^{2+} homeostasis, suppression of oxidative stress, modulation of neurotransmission (44), and enhancement of N-methyl-D-aspartate (NMDA) receptor-mediated responses and they facilitate the induction of hippocampal long-term potentiation (1). Among its presumptive molecular targets, H_2S is known to act on a number of other ion channels such as those of Ca^{2+} and K^+ (12,69,70). H_2S activates K_{ATP} and transient receptor potential (TRP) channels (51,71), whereas it inhibits the big conductance Ca^{2+} -sensitive K^+ channels (BK_{Ca}) (57) and T- and L-type Ca^{2+} channels (31,52). Other targets may be active sites inside the cell such as proteins, enzymes, and transcription factors (27).

Ca^{2+} plays essential roles in various cellular functions, including muscle contraction, control of cell growth, activation of platelets, control of secretion, and apoptosis. In pancreatic acinar cells, Ca^{2+} has a central role in the secretory process. It is a trigger, promoter, and modulator in different events leading to digestive enzyme secretion (13,14). Most of the studies on H_2S in the exocrine pancreas have aimed at interpreting mechanism(s) of H_2S by which pancreatitis, nociception, and apoptosis are induced. However, the available data are controversial. H_2S has been shown to reduce caerulein-induced inflammation in pancreatic acini through a phos-

phoinositide-3 kinase signaling pathway (54). In contrast, H_2S has both pro- and anti-inflammatory properties (3). Excessive production may contribute to the pathogenesis of inflammatory diseases, and reduction of its production may be of potential therapeutic value in inflammatory diseases (67,68). Hui *et al.* (17) demonstrated that endogenous vascular H_2S increased in rats with septic shock and endotoxemic shock which suggested that endogenous H_2S was involved in physiological and pathophysiological process during shock. As possible target molecules, sodium hydrosulfide (NaHS)/ H_2S most probably exerts its influence on T-type Ca^{2+} channels, leading to nociception and endogenous H_2S produced by CSE, and T-type Ca^{2+} channels may be involved in pancreatitis-related pain (35).

To elucidate the pathophysiological roles of H_2S , it is of utmost importance to investigate its possible effects on intracellular Ca^{2+} homeostasis in pancreatic acinar cells, as intracellular Ca^{2+} is a double-edged sword and its regulatory disturbance is thought to be related to some types of functional damage (14). In the present study, therefore, an attempt was made to evaluate the possible roles of H_2S in the physiology and pathology of pancreatic exocrine tissue with special reference to its participation in regulating intracellular Ca^{2+} dynamics. Recently, we have reported the presence of cross-talk between Ca^{2+} and NO in pancreatic acini and this cascade may, at least partially, participate in physiological secretagogue-evoked Ca^{2+} dynamics in pancreatic acinar cells (32,33). Although both NO- and H_2S -producing enzymes are known to be expressed in pancreatic acinar cells, and the fact that H_2S can interact with NO has been indicated in smooth muscles, vascular tissue, and the brain (16,25), no studies have examined such possible interactions in pancreatic acinar cells. In the present study, therefore, we attempted to define whether cross-talk between H_2S and NO was present and, if so, how intensively such cross-talk correlated with regulation of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) dynamics in pancreatic acinar cells.

Results

Effect of H_2S on $[\text{Ca}^{2+}]_i$ in pancreatic acinar cells

To investigate the effect of H_2S on $[\text{Ca}^{2+}]_i$ in pancreatic acinar cells, the H_2S donor NaHS was used. Figure 1A shows a typical example of a $[\text{Ca}^{2+}]_i$ increase induced by 0.5 mM NaHS in the cell indicated by the white oval region of interest (ROI) in translucent and pseudocolor images of the acinus shown in Figure 1B. The H_2S -induced $[\text{Ca}^{2+}]_i$ increase occurred rapidly after application of NaHS and the response seemed to be biphasic, with a transient initial increase reaching a plateau followed by a long-lasting gradual increase. Figure 1C shows that NaHS dose dependently (1–1000 μM) increased $[\text{Ca}^{2+}]_i$ with an approximately four-fold increase at 1000 μM NaHS ($p < 0.001$ by analysis of variance [ANOVA]). NaHS at a concentration of 1–10 μM caused faint but significant increases in $[\text{Ca}^{2+}]_i$.

Source of Ca^{2+} in H_2S -induced $[\text{Ca}^{2+}]_i$ increase

Elevation of intracellular $[\text{Ca}^{2+}]_i$ can be due to extracellular Ca^{2+} influx or release of Ca^{2+} from intracellular stores or both. To examine the contribution of Ca^{2+} influx, $[\text{Ca}^{2+}]_i$ changes were evaluated in acini perfused with ethylene glycol

FIG. 1. NaHS-induced dose-dependent increase in [Ca²⁺]_i. (A) A typical [Ca²⁺]_i increase induced by 0.5 mM NaHS. The fluo-3-loaded acini were perfused with normal Ringer's solution for 2 min before the application of NaHS, and the perfusate was changed to a solution containing 0.5 mM NaHS. (B) Pseudocolor images of changes in [Ca²⁺]_i in which the regions of interest analyzed for temporal changes of [Ca²⁺]_i are indicated by white ovals. Images except for the translucent one (a of B) were obtained from the time points shown in (A) by black dots with respective letters (b–f). As shown by the color bar, warm colors indicate higher [Ca²⁺]_i and cold colors indicate low [Ca²⁺]_i. The change in fluorescence intensity is shown by the percent of the prestimulation level as described in the "Materials and Methods" section. (C) Dose-response relationship of [Ca²⁺]_i increases induced by different concentrations of NaHS (1–1000 μ M). Columns represent the SFC $\pm$ standard error ($\pm$ SE) ($n=19$ –36). $p<0.001$ by analysis of variance, ANOVA. NaHS, sodium hydrosulfide; SFC, summed area of fluorescence changes.

tetraacetic acid (EGTA)-containing, Ca²⁺-free buffer. Figure 2A shows a typical example of [Ca²⁺]_i changes in an acinar cell perfused for 5 min with the Ca²⁺-free buffer, then treated with 0.3 mM NaHS in the absence of extracellular Ca²⁺. Different concentrations of NaHS (1–1000 μ M) could still induce [Ca²⁺]_i elevation in the absence of extracellular Ca²⁺ with an appreciable increase at 1000 μ M NaHS with an EC₅₀ of 73.3 μ M (Fig. 2B). However, the NaHS-induced Ca²⁺ elevation was significantly attenuated in the absence of extracellular Ca²⁺, especially at higher concentrations of NaHS (500 and 1000 μ M), indicating that both extracellular and intracellular Ca²⁺ were involved in H₂S-induced Ca²⁺ increase (Fig. 2B). Endoplasmic reticulum (ER) is one of the intracellular Ca²⁺ storage sites and Ca²⁺-ATPase transports calcium from the cytosol to ER, so it restores low Ca²⁺ in cytosol and high Ca²⁺ in ER. To examine the involvement of Ca²⁺ ATPase, thapsigargin (Tg), a Ca²⁺-ATPase inhibitor was employed.

Acini pretreated with 2 μ M Tg for 10 min in the Ca²⁺-free buffer before the application of 0.3 mM NaHS showed complete inhibition of [Ca²⁺]_i elevation (Fig. 2C). These data confirmed that intracellular Ca²⁺, in combination with extracellular Ca²⁺, was responsible for [Ca²⁺]_i elevation in response to H₂S.

Endogenous production of H₂S

The relevance of endogenous H₂S to [Ca²⁺]_i homeostasis was examined using L-cysteine, the substrate of the H₂S-producing enzymes CBS and CSE. Addition of L-cysteine caused a concentration-dependent (10–1000 μ M) increase in [Ca²⁺]_i (Fig. 3A). In addition, effects of 2 mM aminooxyacetic acid (AOAA), a CBS inhibitor, and 2 mM DL-propargylglycine (PAG), a CSE inhibitor, on basal [Ca²⁺]_i were examined. Two millimolar AOAA significantly decreased basal [Ca²⁺]_i (Fig. 3B, C), but PAG was without effect (Fig. 3C).

FIG. 2. Effect of removal of extracellular Ca^{2+} on NaHS-induced $[\text{Ca}^{2+}]_i$. **(A)** A typical time course of Fluo-3 fluorescence changes in an acinar cell treated with 0.3 mM NaHS in the absence of extracellular Ca^{2+} . Acini were perfused with ethylene glycol tetraacetic acid-containing Ca^{2+} -free buffer for 3 min before starting image acquisition. Two minutes after starting image acquisition, acini were treated with 0.3 mM NaHS for a total of 18 min. **(B)** Dose-response relationships between various concentrations of NaHS in the absence of extracellular Ca^{2+} ($n=11-20$). **(C)** A typical result of the effect of Tg on the NaHS-induced Ca^{2+} increase in the absence of extracellular $[\text{Ca}^{2+}]_i$. Two micromolar Tg was applied for 10 min before the treatment with NaHS, and the acini were treated with 0.3 mM NaHS in Ca^{2+} -free buffer containing 2 μM Tg ($n=14$). $p<0.001$ by ANOVA. Tg, thapsigargin.

FIG. 3. Role of effect of endogenous hydrogen sulfide on intracellular Ca^{2+} . **(A)** Dose-response relationship of $[\text{Ca}^{2+}]_i$ increases induced by different concentrations of L-cysteine (0–1 mM) in normal buffer. Columns represent the mean SFC with vertical lines $\pm$ standard error ($\pm$ SE) ($n=20-30$). $p<0.001$ by ANOVA and $*p<0.05$, $***p<0.001$ by Student's t -test (*vs.* unstimulated control). **(B)** An example of the fluorescence change in an acinar cell treated with 2 mM AOAA. **(C)** SFC ($\pm$ SE) of the $[\text{Ca}^{2+}]_i$ in the presence ($n=18$) and absence of 2 mM AOAA ($n=19$) and SFC ($\pm$ SE) of the $[\text{Ca}^{2+}]_i$ in the presence ($n=14$) and absence of 2 mM PAG ($n=15$). $*p<0.05$. AOAA, aminooxyacetic acid; PAG, DL-propargylglycine.

H₂S-induced $[\text{Ca}^{2+}]_i$ increase is largely dependent on NO production

An additive effect has been demonstrated between H_2S and NO in relaxation of smooth muscle, indicating a strong interaction between the two gases (16). To assess the possible involvement of NO in the NaHS-induced $[\text{Ca}^{2+}]_i$ increase in pancreatic acinar cells as well, effects of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), an NO scavenger, and NG-monomethyl L-arginine (L-NMMA),

FIG. 4. Effects of NO scavenger and NOS inhibitor on NaHS-induced $[Ca^{2+}]_i$ increase. (A) Acini were treated with 0.3 mM NaHS in the presence or absence of the NO scavenger cPTIO (0.5 mM) or NOS inhibitor L-NMMA (100 μ M). (B) SFC ($\pm$ SE) in the presence ($n=23$ –42) and absence ($n=39$) of L-NMMA and cPTIO, respectively. *** $p < 0.001$. NO, nitric oxide; NOS, nitric oxide synthase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; L-NMMA, NG-monomethyl L-arginine.

a non-specific inhibitor of all nitric oxide synthase (NOS) isoforms, were examined. Pretreatment of acini with 100 μ M L-NMMA or 0.5 mM cPTIO significantly attenuated the NaHS-induced $[Ca^{2+}]_i$ increase (Fig. 4A, B). The summed area of fluorescence changes (SFC) was decreased by 37% in the presence of each chemical (the increment from the unstimulated value was decreased by 74%). We next examined whether H₂S itself could stimulate NO production by using a fluorescent NO probe, diaminofluorescein-2/diaminofluorescein-2 triazole (DAF-2/DAF-2T). As shown in Figure 5A, different concentrations of NaHS (0.1–1 mM) induced gradual and continuous NO production in the presence of extracellular Ca²⁺, and NaHS dose dependently increased NO production (Fig. 5B) ($p < 0.001$ by ANOVA). The EC₅₀ was calculated to be 64.8 μ M. The NaHS-induced NO production was partially abolished in the absence of extracellular Ca²⁺ (Fig. 5C). The SFC was decreased by 11% (the increment from the unstimulated value was decreased to 41%). These results indicated that NaHS induced a $[Ca^{2+}]_i$ increase *via* NOS activation and subsequent NO production. Moreover, pretreatment with 100 μ M L-NMMA significantly reduced the extent of NaHS-induced DAF-2/DAF-2T fluorescence increase by 40% compared with that without L-NMMA (data not shown).

FIG. 5. Effect of NaHS on NO production. (A) Temporal changes of DAF-2T fluorescence in acinar cells treated with different concentrations of NaHS (0.1–1 mM). (B) Dose relationship of SFC ($\pm$ SE) in NaHS-induced NO production ($n=16$ –34). $p < 0.001$ by ANOVA followed by Student's *t*-test. (C) SFC ($\pm$ SE) of DAF-2T fluorescence obtained by treatment with 0.3 mM NaHS in the presence ($n=19$) and absence ($n=39$) of extracellular Ca²⁺. *** $p < 0.001$. DAF-2T, diaminofluorescein-2 triazole.

Effect of phospholipase C inhibitor U73122 on H₂S-induced $[Ca^{2+}]_i$ increase

We next conducted a series of experiments to investigate the intracellular signaling mechanism(s) involved in H₂S-induced intracellular Ca²⁺ release. First, we examined whether the phospholipase C (PLC) pathway was involved in the $[Ca^{2+}]_i$ increase in response to H₂S treatment. A specific inhibitor of phospholipase C, U73122, was utilized in the absence of extracellular Ca²⁺. Pretreatment with 2 μ M U73122 for 10 min significantly suppressed the 0.3 mM NaHS-induced $[Ca^{2+}]_i$ increase (Fig. 6A) and caused a significant 28% reduction of the SFC (the increment from the unstimulated value was decreased by 81%) (Fig. 6B), suggesting that H₂S activated PLC, which eventually caused $[Ca^{2+}]_i$ elevation.

FIG. 6. Effect of PLC inhibitor on NaHS-induced $[Ca^{2+}]_i$ increase. **(A)** An example of the fluorescence change in an acinar cell pretreated with the PLC inhibitor U73122 ($2 \mu M$) in Ca^{2+} -free buffer for 10 min, followed by treatment with 0.3 mM NaHS, also perfused with the Ca^{2+} -free buffer containing U73122. **(B)** SFC ($\pm$ SE) of the NaHS-induced $[Ca^{2+}]_i$ increase in the presence ($n=42$) and absence of $2 \mu M$ U73122 ($n=20$). *** $p < 0.001$. PLC, phospholipase C.

Effect of H_2S involves G-protein-inositol 1,4,5-trisphosphate and cyclic guanosine monophosphate/protein kinase G pathway

Second, we examined the involvement of inositol 1,4,5-trisphosphate (IP_3) in the NaHS-induced $[Ca^{2+}]_i$ increase by using xestospongion C, a potent, cell-permeable inhibitor of the IP_3 receptor (IP_3R). Figure 7A and B shows that $3 \mu M$ xestospongion C almost completely inhibited the $[Ca^{2+}]_i$ increase induced by 0.3 mM NaHS. The SFC was reduced by 23% (the increment from the unstimulated value was decreased by 75%), which indicated that the H_2S -induced $[Ca^{2+}]_i$ increase was partly triggered *via* the PLC/ IP_3 signaling pathway.

To further investigate the intracellular signals involved in the H_2S -induced $[Ca^{2+}]_i$ increase in pancreatic acinar cells, we investigated the contribution of soluble guanylate cyclase (sGC) by using 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a potent and selective inhibitor of sGC. Pretreatment with ODQ at $100 \mu M$ significantly abolished the $[Ca^{2+}]_i$ increase elicited by 0.3 mM NaHS, and the SFC decreased by 17% (the increment from the unstimulated value was decreased by 56%) (Fig. 7). Since the downstream product of sGC is cyclic guanosine monophosphate (cGMP), these data suggested that the effect of H_2S on $[Ca^{2+}]_i$ involved production of cGMP. It has been reported that the intracellular action of cGMP is primarily mediated by cGMP-dependent protein kinase G (PKG).

FIG. 7. Effects of sGC, PKG, IP_3R , and G-protein inhibitors on the NaHS-induced $[Ca^{2+}]_i$ increase. **(A)** Time courses of the $[Ca^{2+}]_i$ increases induced by 0.3 mM NaHS in the absence and presence of $2 \mu M$ KT5823, $100 \mu M$ ODQ, $3 \mu M$ xestospongion C, and $10 \mu M$ GP2A. Acini were perfused with Ca^{2+} -free buffer containing each inhibitor for 5 min before the treatment with 0.3 mM NaHS. **(B)** SFC ($\pm$ SE) of 0.3 mM NaHS-induced $[Ca^{2+}]_i$ increases in the absence ($n=52$) and presence of KT5823 ($n=39$), ODQ ($n=37$), xestospongion C ($n=26$), and GP2A ($n=40$). *** $p < 0.001$. sGC, soluble guanylate cyclase; PKG, protein kinase G; IP_3R , inositol 1,4,5-trisphosphate receptor; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

Whether PKG mediated the NaHS-induced $[Ca^{2+}]_i$ increase was examined by using a highly cell-permeable and selective inhibitor of PKG, KT5823. Pretreatment with $2 \mu M$ KT5823 significantly suppressed the NaHS-induced $[Ca^{2+}]_i$ increase and the SFC decreased by 15% (the increment from the unstimulated value was decreased by 49%) (Fig. 7). These results indicated that the H_2S -induced $[Ca^{2+}]_i$ increase was caused *via* a cGMP/PKG-dependent pathway.

Several sites of action have been proposed to account for cGMP-dependent regulation of cytosolic Ca^{2+} , including inhibition of receptor/G-protein coupling in smooth muscle (28), PKG activation of the BK channel either by acting as the direct catalyst for phosphorylation (10) or by activating a protein phosphatase that dephosphorylates the BK channel (72), and cGMP/PKG regulation of IP_3R (15). With the data mentioned earlier, the question now is how cGMP activates PLC. We focused on G-protein, as PLC is activated by an α -subunit cleaved from trimeric G-protein. Acini pretreated with the G_q-protein antagonist peptide GP2A

(10 μ M) showed marked inhibition of the NaHS-induced [Ca²⁺]_i increase compared with the control and the SFC decreased by 25% (the increment from the unstimulated value was decreased by 81%) (Fig. 7). These results indicated that H₂S-induced [Ca²⁺]_i elevation occurred through a pathway including G-protein.

Discussion

The current study showed that the superfusion of isolated pancreatic acini with NaHS increased [Ca²⁺]_i in a dose-dependent manner. The increase was biphasic with an initial rapid rise followed by a long-lasting elevation (Fig. 1). L-cysteine-induced dose-dependent increase in [Ca²⁺]_i and the decrease in the basal [Ca²⁺]_i level by the addition of a CBS inhibitor indicates that low concentrations of endogenous H₂S act to keep the basal [Ca²⁺]_i level within a narrow range. These findings suggest that H₂S regulates [Ca²⁺]_i homeostasis in pancreatic acinar cells. The NaHS/H₂S-induced [Ca²⁺]_i rise has been widely reported in various tissues such as astrocytes, microglial cells, neuronal cells, and distal colonic epithelium (24,34,42,63). In contrast, inhibition of the [Ca²⁺]_i increase has been demonstrated in vascular, gastrointestinal, urogenital, atrial, and ventricular smooth muscle, and pancreatic islet cells, causing vasodilation, cardiac muscle relaxation, and inhibition of insulin release (20,27,40,56,62). A protective effect against ischemia-reperfusion injury is also reported (64).

In the exocrine pancreas, H₂S was initially demonstrated to be a pro-inflammatory factor involved in caerulein-induced pancreatitis (5,53). Later, however, anti-inflammatory effects were also reported (50,54). H₂S has also been suggested to be related to pancreatitis-originated nociception (11,35), probably *via* T-type Ca²⁺ channels and TRPV1, leading to extracellular signal-regulated kinase (ERK) phosphorylation that causes pain. Since Ca²⁺ plays crucial roles in both physiology and pathophysiology in a variety of tissues, it is conceivable that, depending on the magnitude of the increase in [Ca²⁺]_i, the final results induced by H₂S may be divergent. In the exocrine pancreas, since Ca²⁺ would function as a double-edged sword (14), it is conceivable that excessive production of H₂S may be related to the pathogenesis of inflammatory diseases and reduction of its formation could be of potential therapeutic value in inflammation (67,68).

The sources of Ca²⁺ utilized for [Ca²⁺]_i elevation were both extracellular and intracellular, as the H₂S-induced [Ca²⁺]_i increase was attenuated but persisted even in the absence of extracellular Ca²⁺. The extent of the dependence on extracellular Ca²⁺ appeared to be large when the acini were treated with higher NaHS concentrations, indicating that Ca²⁺ entry could be a major component causing [Ca²⁺]_i elevation at these concentrations. The H₂S-induced [Ca²⁺]_i increase was completely abolished by pretreatment with Tg (Fig. 2C), indicating that intracellular Ca²⁺ sequestering organella which possessed Ca²⁺ ATPase were involved in Ca²⁺ mobilization. This is consistent with previous studies showing that the H₂S-induced Ca²⁺ waves in astrocytes and [Ca²⁺]_i increase in microglial and neuronal cells were also brought about by both Ca²⁺ influx and the release of intracellularly stored Ca²⁺ (24,34,63).

H₂S-induced NOS activation and NO production

A previous study demonstrated that an NO donor, sodium nitroprusside (SNP), up-regulated H₂S production and an-

other NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP), increased the transcriptional level of CSE in cultured aortic smooth muscle cells (70). Subsequent studies conducted by other groups revealed the functioning of a retrograde pathway, that is, H₂S exerts its effect, at least in part, *via* the activation of endothelial nitric oxide synthase (eNOS) by phosphorylation (31) *via* serine/threonine kinase/protein kinase B (Akt/PKB) activation (7,64). NO production by H₂S mediated by an increase in [Ca²⁺]_i was also indicated (39). Moreover, H₂S reacts with S-nitrosothiols to form thionitrous acid (HSNO). At the cellular level, HSNO can be metabolized to afford NO⁺, NO, and NO⁻ species (9). It has been proposed that H₂S functions as a paracrine signaling molecule (8). Conversely, an inhibitory effect by H₂S on lipopolysaccharide-induced NO production was reported in macrophages, in which up-regulation of heme oxygenase-1 was involved (36). Further, inhibition by NaHS of all three NOS isoforms was demonstrated (23). These findings suggested that H₂S and NO could be symbiotic or interdependent in eliciting some physiological and/or pathological effects (26) and suggested the existence of cross-talk between NO and H₂S (6).

Based on the idea mentioned earlier, we scrutinized potential increases of [NO]_i by H₂S and found that NOS activation and NO production were indeed involved in the H₂S-induced [Ca²⁺]_i increase in pancreatic acini, as the NaHS-evoked [Ca²⁺]_i rise was largely decreased by the NOS inhibitor L-NMMA and NO scavenger cPTIO (Fig. 4). More directly, [NO]_i measurement with DAF-2/DAF-2T clearly showed that NO was actually formed by the treatment of acini with NaHS, whose effect was gradual and long lasting (Fig. 5A, B). What we would like to especially stress is the temporal change in [NO]_i; the [NO]_i started increasing with some delay after NaHS addition and this time course appeared to be synchronized with the second phase of the [Ca²⁺]_i increase (Fig. 5A *vs.* Fig. 4A solid line). This most likely indicated that the second phase of the NaHS-induced [Ca²⁺]_i could be largely attributed to the NaHS-induced [NO]_i increase. A possible underlying mechanism will be discussed next. In support of this hypothesis, pharmacokinetic analyses of the dose-response relationship for the NaHS-induced NO production (Fig. 5B) and the [Ca²⁺]_i increase in the absence of extracellular Ca²⁺ (Fig. 2B) demonstrated that these EC₅₀ values were in close proximity, being 73.3 and 64.8 μ M, respectively. All these lines of evidence strongly suggested that NOS activation and the resultant NO production were largely involved in the downstream cascade of H₂S action in pancreatic acinar cells, indicative of the multifaceted and complicated nature of the biological actions of H₂S. Altogether, it is likely that the first H₂S-induced increase in [Ca²⁺]_i was due to a solo effect by H₂S and the secondary long-lasting increase was caused by synergism between H₂S and H₂S-produced NO.

Intracellular mechanism of H₂S-induced [Ca²⁺]_i elevation

Presumptive targets of H₂S proposed so far include the K_{ATP} channel and myosin-light-chain phosphatase, which cause vasodilation and atrial and ventricular relaxation. Other sites of action may be BK_{Ca}, L-type and T-type Ca²⁺ channels, Cl⁻ channels and TRPV channels, and some kinases such as mitogen-activated protein kinase, ERK, and PKC are also molecular targets of H₂S (27). In the endocrine pancreas, H₂S has been reported to open the K_{ATP} channel and reduce

exocytosis of insulin (20,40,62). It is well understood that hampering intracellular Ca^{2+} homeostasis results in distortion of normal cellular functions. In a recent report, we proposed the presence of a novel pathway underlying regulation of NO-induced intracellular Ca^{2+} dynamics in rat pancreatic acinar cells (32,33), and here we show evidence that such a pathway synergistically operates in the H_2S -triggered $[\text{Ca}^{2+}]_i$ increase. In the present study, similar characteristics were found in the NaHS-induced $[\text{Ca}^{2+}]_i$ elevation. First, the NaHS-induced $[\text{Ca}^{2+}]_i$ was significantly attenuated by the PLC inhibitor U73122 (Fig. 6). This provided evidence that H_2S might directly or indirectly activate PLC and the resultant Ca^{2+} response. This is contradictory to the result reported in microglial cells, in which U73122 failed to affect the action of H_2S on the intracellular Ca^{2+} store (24), but a result similar to the present one has been reported for neuronal SH-SY5Y cells, in which U73122 significantly suppressed the H_2S -induced $[\text{Ca}^{2+}]_i$ elevation (63). Second, the NaHS-induced $[\text{Ca}^{2+}]_i$ elevation was found to be greatly reduced in the presence of the IP_3R blocker xestospongine C (Fig. 7), which indicated that $\text{IP}_3\text{-IP}_3\text{R}$ interaction was present in the downstream of PLC activation to cause Ca^{2+} release from the IP_3 -sensitive intracellular Ca^{2+} store. Third, the increase of $[\text{Ca}^{2+}]_i$ by NaHS was markedly depressed by pretreatment with the G-protein antagonist GP2A (Fig. 7). This provided evidence that G-protein, possibly G_q -protein, was involved in PLC- $\text{IP}_3\text{-IP}_3\text{R}$ pathway. Fourth, the NaHS-induced $[\text{Ca}^{2+}]_i$ increase was inhibited by the presence of the sGC inhibitor ODQ and the PKG inhibitor KT5823 (Fig. 7). This suggested that the sGC-cGMP-PKG cascade was functioning even in the NaHS-caused $[\text{Ca}^{2+}]_i$ increase in pancreatic acinar cells. These four findings are analogous to what we previously observed in the SNP-induced $[\text{Ca}^{2+}]_i$ increase in pancreatic acinar cells (32,33), strongly indicating that the NO-triggered process was also included in the NaHS-induced increase in $[\text{Ca}^{2+}]_i$. Additional evidence that supports this hypothesis would be the significant production of NO by NaHS

as mentioned earlier. Some contradictory reports have indicated that H_2S inhibits NO-induced intracellular cGMP accumulation in the salamander retina (41) and that it does not affect sGC or the level of cGMP in the course of vascular relaxation (69). Moreover, H_2S does not affect the sGC or the cGMP concentration, but it increases the intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) level, especially in neurons (21). On the other hand, cGMP was reported to be involved in the combined effect of NO and H_2S on pacemaker currents generated by interstitial cells of Cajal (65). A clear-cut interpretation for these controversial findings is not apparent, but they may partly be due to the types of cells utilized. In any case, our present findings implied that, in pancreatic acini, the cGMP-PKG cascade was involved in the H_2S -provoked $[\text{Ca}^{2+}]_i$ increase and the putative overall cascade seems identical to our recently proposed model (32,33).

However, certain missing links in our model remain to be identified. The first missing link is how cGMP activates membranous G_q -protein and the second one is how H_2S activates NOS. To obtain an overall model, the following reports appear to be beneficial, providing us with significant hints. According to Akaike *et al.* (2) and Saito *et al.* (46), NO, in addition to activating sGC, nitrosylates various intracellular substances as post-translational modifications, producing 8-nitro-cGMP in the case of cGMP. The nitrosylated cGMP then binds with $\text{G}\alpha_q$ in the cell membrane forming $\text{G}\alpha_q$ -cGMP, which can activate PLC. Tang *et al.* (55) documented that cGMP-activated PKG could phosphorylate a regulator of G-protein signaling-2 and its translocation from cytosol to the cellular membrane, which can also activate the $\text{G}\alpha_q$ subunit. Thus, it is highly possible that cGMP can activate G_q -protein, leading to PLC activation. Yong *et al.* (64) have demonstrated that endogenous production of H_2S after ischemia-reperfusion phosphorylates Akt, a serine/threonine kinase, followed by eNOS activation, inducing a protective effect of NO against cellular injury. Minamishima *et al.* (31) also reported that H_2S

FIG. 8. Schematic diagram of H_2S -triggered putative cascade that induces Ca^{2+} release in pancreatic acinar cells. H_2S stimulates IP_3 production and resultant intracellular Ca^{2+} release via the NO-cGMP-PLC pathway. Some of the steps are inhibited by the various chemicals used in the current study as indicated by the dotted lines. cGMP, cyclic guanosine monophosphate.

phosphorylates Akt and NOS3. Coletta *et al.* (7) proposed, in their recent report, a model in which H₂S and NO synergistically affect angiogenesis and vasodilation.

In conclusion, the present study suggests the hypothesis that H₂S accelerates NO production *via* NOS activation, which, in turn, activates sGC and elevates the intracellular cGMP level. The latter can activate G-protein followed by PLC activation and mobilize intracellularly stored Ca²⁺ that is triggered by IP₃ bound to IP₃R (Fig. 8). This pathway is identical to that proposed for the NO-induced [Ca²⁺]_i increase in pancreatic acinar cells. To best of our knowledge, this is the first report that highlights a novel pathway by which H₂S modulates intracellular Ca²⁺ dynamics and the resultant physiology and/or pathology of pancreatic acinar cells.

Materials and Methods

Chemicals

Chromatographically purified collagenase (CLSPA) was obtained from Worthington Biochemical (Lakewood, NJ). A G_q-protein antagonist peptide, GP2A, and a PKG inhibitor, KT5823, were purchased from Calbiochem (La Jolla, CA). U-73122, bovine serum albumin (BSA), AOAA, and soybean trypsin inhibitor (type1-S) were purchased from Sigma (St. Louis, MO). Fluo-3/AM, EGTA, L-NMMA, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and cPTIO were from Dojindo (Kumamoto, Japan). Eagle's minimum essential medium (MEM) was purchased from Invitrogen (Carlsbad, CA). DAF-2/DA was from Daiichikagaku (Tokyo, Japan). Cell-Tak was from BD Biosciences (San Jose, CA). DL-propargylglycine (PAG) was purchased from Cayman Chem. (Ann Arbor, MI). Other chemicals, including the H₂S donor NaHS and L-cysteine hydrochloride, were obtained from Wako Pure Chemicals (Osaka, Japan).

Recently, it is claimed that circulating sulfide (HS⁻, H₂S, S²⁻) level is very low, achieving nM level at highest (37,38). Tissue sulfide concentration is not easy to measure or estimate, as multiple factors are related to determining actual concentration. H₂S can easily diffuse from the site of production (8) and readily permeate the cell (29) but, on the other hand, H₂S is rapidly consumed by tissues in the presence of oxygen. Exogenously applied H₂S is quickly and efficiently consumed by tissues at pO₂ > 10 torr (38). It is estimated that sulfide presents ~20% H₂S/80% HS⁻ ration in extracellular fluid (37). Under the present experimental condition, pO₂ of the perfusate in the chamber is estimated to be ~140 torr, equilibrating rapidly with environmental dry air. Thus, based on earlier consideration, actual concentration of H₂S at the tissue level after the administration of 300 μM NaHS could be lower than 60 μM at most or much lower in the current study.

Animals

All experiments conformed to the guidelines on the ethical use of animals set by the U.S. National Institutes of Health and were approved by the institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult SPF male Wistar rats (200–250 g) purchased from Clea Japan (Tokyo, Japan) were housed under a controlled environment at an ambient temperature of 22°C and a 12:12-h

light-dark cycle. Animals were deprived of food overnight before the experiment but had free access to water.

Solutions

Normal Ringer's solution, used throughout acinar isolation and experimentation (Standard HEPES-buffered solution), contained (mM): sodium chloride, 138.0; potassium chloride, 4.7; calcium chloride (CaCl₂), 1.3; magnesium chloride, 1.13; disodium phosphate, 1.0; D-glucose, 5.5; and HEPES, 10.0 supplemented with MEM plus 2 mM L-glutamine and 1 mg/ml BSA. For isolation of acini, soybean trypsin inhibitor was added to the normal Ringer's solution. The pH was adjusted to 7.4 with sodium hydroxide. In some experiments, where indicated, CaCl₂ was omitted from the solution and 1 mM EGTA was added. All experiments except for isolation of acini and dye loading were performed at room temperature and isolated acini were used for experiments within 4 h after isolation.

Preparation of pancreatic acini

The animals were anesthetized by CO₂ inhalation and euthanized by exsanguination. Pancreatic acini were obtained from the rat pancreas by collagenase digestion according to the method previously reported (32,33). Briefly, pancreata were removed, freed from fat and lymph nodes, and 5 ml of standard solution containing 60–75 U/ml collagenase and 0.1 mg/ml soybean trypsin inhibitor was injected into the interstitium of the pancreatic tissue, which was incubated in a conical flask at 37°C under vigorous shaking for total of 60 min. After 30 min of incubation, 5 ml of new collagenase was added to the flask. Mechanical disruption of the tissue was performed by gentle suction through pipettes with decreasing orifice size. The acinar suspension was then filtrated through 150 μm nylon mesh, rinsed thrice, pelleted (×60 g), and resuspended in a suitable amount of the standard solution. Acinar cell viability was virtually 100% when assessed by the trypan blue exclusion test (32,33).

Optical imaging of Ca²⁺ and NO

Imaging was carried out as previously reported (32). Briefly, isolated acini were resuspended in the normal Ringer's solution containing 10 μM Fluo-3/AM or 10 μM DAF-2/DA and incubated at 37°C with constant mild shaking in the dark for 60 min. After incubation, the acini were washed in the standard solution, transferred to a recording chamber, on the bottom of which a cover glass previously coated with Cell-Tak was attached, and left for at least 5 min. The chamber was placed on the stage of an inverted microscope. Imaging was performed using a confocal laser scanning microscope (Fluoview FV500; Olympus, Tokyo, Japan). The acini were continuously perfused with normal Ringer's solution at a flow rate of 1 ml/min before and throughout the experiments. Acini were illuminated at 488 nm with a krypton/argon laser, and the emission light (>505 nm) was guided through a ×40 water immersion objective to a pinhole diaphragm. Photo-damage was minimized by attenuating the laser intensity by interposing a neutral density filter into the illumination path (usually 1% transmission was sufficient to obtain fluorescence). Confocal images of acini were taken at 10 s intervals. The time courses of changes in fluorescence intensity at the ROI were analyzed using Fluoview 5.0 with Tiempo. The

change in the fluorescence intensity was expressed as the percent of basal fluorescence intensity by setting the pre-stimulated fluorescence just before the application of chemicals at 100% (baseline). In addition, the degree of the change of $[Ca^{2+}]_i$ was estimated by calculating the SFC above the baseline during stimulation as previously reported (32).

Statistical analysis

The results are reported as means $\pm$ standard error, with n denoting the number of cells in the acinus analyzed. Statistical significance was determined using Student's t -test and ANOVA, with a value of $p < 0.05$ being considered significant.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ANOVA = analysis of variance
 AOAA = aminooxyacetic acid
 BK_{Ca} = big conductance Ca²⁺-sensitive K⁺ channels
 BSA = bovine serum albumin
 [Ca²⁺]_i = intracellular Ca²⁺ concentration
 CaCl₂ = calcium chloride
 cAMP = adenosine 3', 5'-cyclic monophosphate
 CBS = cystathionine β -synthase
 cGMP = cyclic guanosine monophosphate
 cPTIO = 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
 CSE = cystathionine γ -lyase
 DAF-2 = diaminofluorescein-2
 EGTA = ethylene glycol tetraacetic acid
 eNOS = endothelial nitric oxide synthase
 ER = endoplasmic reticulum
 ERK = extracellular signal-regulated kinase
 GP2A = G_q-protein antagonist peptide
 H₂S = hydrogen sulfide
 HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
 HSNO = thionitrous acid
 IP₃ = inositol 1,4,5-trisphosphate
 IP₃R = inositol 1,4,5-Trisphosphate Receptor
 L-NMMA = NG-monomethyl L-arginine
 MAPK = mitogen-activated protein kinase
 MEM = Eagle's minimum essential medium
 NaHS = sodium hydrosulfide
 NMDA = N-methyl-D-aspartate
 NO = nitric oxide
 NOS = nitric oxide synthase
 ODQ = 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
 PAG = DL-propargylglycine
 PKB = protein kinase B
 PKG = protein kinase G
 PLC = phospholipase C
 ROI = region of interest
 SE = standard error
 SFC = summed area of fluorescence changes
 sGC = soluble guanylate cyclase
 SNAP = s-nitroso-N-acetyl-DL-penicillamine
 SNP = sodium nitroprusside
 Tg = thapsigargin
 TRP = transient receptor potential

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