Hydrogen sulfide: a novel gaseous signaling molecule and intracellular Ca^{2+} regulator in rat parotid acinar cells

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ABSTRACT

In addition to nitric oxide (NO), hydrogen sulfide (H\textsubscript{2}S) is recognized as a crucial gaseous messenger that exerts many biological actions in various tissues. An attempt was made to assess the roles and underlying mechanisms of both gases in isolated rat parotid acinar cells. Ductal cells and some acinar cells were found to express NO and H\textsubscript{2}S synthases. Cevimeline, a muscarinic receptor agonist upregulated endothelial NO synthase (eNOS) in parotid tissue. NO and H\textsubscript{2}S donors increased the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]). This was not affected by inhibitors of phospholipase C and inositol 1,4,5-trisphosphate receptors (InsP\textsubscript{3}Rs) but was decreased by blockers of ryanodine receptors (RyRs), soluble guanylyl cyclase (sGC) and protein kinase G (PKG). The H\textsubscript{2}S donor evoked NO production, which was decreased by blockade of NO synthases or phosphoinositide-3-kinase (PI3K) or by hypotaurine, an H\textsubscript{2}S scavenger. The H\textsubscript{2}S donor-induced [Ca\textsuperscript{2+}] increase was diminished by an NO scavenger or the NO synthases blocker. These results suggest that NO and H\textsubscript{2}S play important roles in regulating [Ca\textsuperscript{2+}] via sGC-cGMP-PKG-RyRs but not via InsP\textsubscript{3}Rs. The effect of H\textsubscript{2}S may be partially through NO produced via PI3K-Akt-eNOS. It was concluded that both gases regulate [Ca\textsuperscript{2+}] in a synergistic way mainly via RyRs in rat parotid acinar cells.

Key words: hydrogen sulfide, intracellular Ca\textsuperscript{2+}, ryanodine receptors, nitric oxide, parotid acinar cells
INTRODUCTION

The parotid gland releases both electrolytes and amylase in an intracellular Ca\(^{2+}\)-dependent manner in response to physiological secretagogues. Gaseous molecules that are produced endogenously as biological messengers have attracted great interest due to their diverse physiological and pathophysiological functions. We have previously demonstrated their roles in regulating intracellular Ca\(^{2+}\) homeostasis in pancreatic acinar cells (28-30). Nitric oxide (NO) is a bioactive gas molecule and is synthesized mostly from L-arginine by NO synthases (NOSs). To date three isoforms of NOS have been identified, including two isoforms that are constitutively expressed in various tissues and are Ca\(^{2+}\)-dependent neuronal NOS (nNOS) and endothelial NOS (eNOS) and a third isoform that is Ca\(^{2+}\)-independent inducible NOS (iNOS).

In the rat and ferret salivary glands, nNOS has been found to be expressed in nerve terminals located in the periphery of glandular tissues, both in acinar and ductal cells (2). This isoform was described to be predominant in rat parotid acinar cells and may be activated prior to amylase secretion in response to methacholine (14). The expression of nNOS was identified in the cytosol of rabbit parotid acinar cells but was barely expressed in ductal cells (38). In addition, nNOS-positive fibers were shown to distribute around acini in the submandibular gland but were scarce around the acini in the sublingual gland (40). Therefore, NOS expression in the salivary glands, including the parotid gland, varies depending on the isoforms, glands, cell types and animals. NO exerts most of its effects by activating soluble guanylyl cyclase (sGC), resulting in the generation of guanosine 3’,5’-cyclic monophosphate (cGMP). This cyclic nucleotide activates cGMP-dependent protein kinase G (PKG), which is assumed to evoke salivary secretion in the rat parotid gland (36). It has also been shown that NO promotes saliva secretion in a wide variety of species (21, 35).

Hydrogen sulfide (H\(_2\)S) is synthesized endogenously and modulates various physiological functions including anti-inflammation (47), neuromodulation (1),
vasoregulation (48), protection from myocardial ischemia-reperfusion injury (7) and
reduction of oxidative stress (46), most of which overlap with the actions of NO. H$_2$S is
generated mainly from cysteine and homocysteine by the enzymes cystathionine β-synthase
(CBS) and cystathionine γ-lyase (CSE). The third synthase, 3-mercaptopropionate
sulfurtransferase, also contributes to H$_2$S production in the presence of cysteine
aminotransferase. In parotid acinar cells, neither the expression of H$_2$S-producing enzymes
nor the roles of H$_2$S have been investigated.

In the last few years, interest has been directed toward a possible cross-talk between
H$_2$S and NO because H$_2$S evokes NO production in some cells, as reported previously (10, 3,
4), and inhibits NO production in other cells (32, 16). In recent reports, we demonstrated that
NO increases the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) via sGC-cGMP and inositol 1,4,5-
trisphosphate (IP$_3$) in pancreatic acinar cells (30). We further examined potential intracellular
cross reactions between H$_2$S and NO and proposed the presence of a novel cross-talk in
which H$_2$S stimulates NO production by activating eNOS, probably via a phosphoinositide-3-
kinase (PI3K) and serine/threonine kinase (PKB/Akt)-dependent mechanism. Therefore, it is
highly possible that some of the H$_2$S actions are through the production of NO in a
synergistic manner. This is especially true for the regulation of intracellular Ca$^{2+}$ homeostasis
in pancreatic acinar cells (28). However, in the parotid gland, an analogous serous exocrine
tissue, no such investigation has been conducted. The aims of the present study are, first, to
determine the expression of the H$_2$S and NO producing enzymes in parotid glandular cells;
second, to investigate the effects of both gases on intracellular Ca$^{2+}$ homeostasis; and third, to
define the possible signaling pathways through which the two gaseous molecules may
interact.

**MATERIALS AND METHODS**
Chemicals

Fluo-3/AM, EGTA, HEPES, NG-monomethyl L-arginine (L-NMMA) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were obtained from Dojindo (Kumamoto, Japan). Diaminofluorescein-2 diacetate (DAF-2/DA) was purchased from Daiichikagaku (Tokyo, Japan). Chromatographically purified collagenase (CLSPA) was obtained from Worthington Biochemical (Lakewood, NJ, USA). Cell-Tak was procured from BD Biosciences (San Jose, CA, USA). LY294002 was purchased from Calbiochem (La Jolla, CA, USA). Eagle’s essential amino acid (MEM) was purchased from Invitrogen (Carlsbad, CA, USA). U73122, BSA, soybean trypsin inhibitor, hyaluronidase, ruthenium red (RR), KT5823 cevimeline hydrochloride and hypotaurine (HT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). DETA NONOate was from Enzo Life Science, Inc. Sodium nitroprusside (SNP), sodium hydrosulfide (NaHS), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), Xestospongin c and other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). Nembutal was purchased from Dainippon Sumitomo Pharma, (Osaka, Japan).

Animals and Solutions

Male SPF Wistar rats (70 rats, 8 weeks old) were obtained from Clea Japan (Tokyo, Japan). They were maintained under a controlled environment at an ambient temperature of 22°C and a 12:12-h light-dark cycle. The animals were deprived of food overnight prior to the experiment but were allowed free access to water. 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.
The HEPES-buffered standard solution used throughout the acinar isolation and experimentation contained the following (in mM): NaCl, 138.0; KCl, 4.7; CaCl$_2$, 1.3; MgCl$_2$, 1.13; Na$_2$HPO$_4$, 1.0; d-glucose, 5.5; HEPES, 10.0 supplemented with MEM plus 2 mM L-glutamine and 1 mg/ml BSA. The pH was adjusted to 7.4 with NaOH. A nominally Ca$^{2+}$-free solution was prepared without the addition of CaCl$_2$ but with the addition of 1 mM EGTA.

**Preparation of parotid acini**

Isolated parotid acini were prepared according to a method described by Looms et al. (23) with some modifications originally adopted for isolation of pancreatic acinar cells (28, 29). Briefly, rats were anesthetized by CO$_2$ inhalation and euthanized by exsanguination. The parotid glands were excised and cut into small pieces and incubated with HEPES-buffered standard solution supplemented with 0.1 mg/ml soybean trypsin inhibitor, 75 U/ml collagenase and 153 U/ml hyaluronidase for a total of 60 min at 37°C under vigorous shaking. The tissue was dispersed by gentle suction through pipettes with decreasing orificial size. The tissue suspension was then filtered through 150 μm nylon mesh, washed three times, pelleted (×60 g) and resuspended in a suitable amount of the standard solution.

**Ca$^{2+}$ and NO measurements**

Intracellular changes in Ca$^{2+}$ and NO were measured as described previously (28, 29). Briefly, isolated parotid acini were resuspended in the sonicated standard solution containing 10 μM fluo-3/AM or 10 μM DAF-2/DA and incubated for 60 min at 37°C with mild shaking under dark. Following incubation, the acini were washed with the standard solution, pelleted, resuspended and transferred to a recording chamber, to the bottom of which a Cell-Tak-coated cover glass was attached. The chamber was placed on the stage of an inverted microscope (IX, Olympus, Tokyo, Japan) of a laser scanning confocal imaging system (LSM
FV500, Olympus, Tokyo, Japan). The probe-loaded acini in the recording chamber were perfused with the standard solution at a flow rate of 1 ml/min prior to and throughout the experiments. The 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. Photodamage was minimized by attenuating the laser intensity with a neutral density filter interposed into the illumination path (1% transmission was sufficient to obtain fluorescence). Confocal images of Fluo-3 or triazolofluorescein (DAF-2T) fluorescence were recorded at 10-s intervals. The time courses of changes in fluorescence intensity (F.I.) at regions of interest (ROI) set on several single cells within an acinus were analyzed using bundled software (Fluoview 5.0 with Tiempo, Olympus, Tokyo, Japan). The change in F.I. was calculated as the percent of basal fluorescence intensity obtained by setting the pre-stimulated fluorescence prior to the application of drugs at 100% (baseline) (F/F₀ x 100). The changes in [Ca²⁺]ᵢ and NO production were estimated by calculating the summed area of fluorescence changes (SFC) above the baseline. SFC was calculated in multiple single cells within an acinus and the mean (± SE) was obtained from the accumulated data obtained from multiple cells examined in several experiments for each specific experimental condition. All experiments for the measurement of [Ca²⁺]ᵢ and NO were performed at room temperature, and isolated acini were used for experiments within 4 h after isolation.

**Immunohistochemistry**

Immunohistochemical detection of NOS isoforms (eNOS and nNOS), CBS and CSE was conducted as reported previously (29). In brief, parotid glands were excised, fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated in an alcohol series and embedded in paraffin. The paraffin block was cut into 3-µm sections and placed on poly-L-Lysine-coated slides. The sections were deparaffinized, rehydrated and washed twice with
distilled water for 5 min each time. Antigen retrieval was conducted by heating in citrate buffer (pH 6.0) for 20 min. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide in absolute methanol for 30 min at 4°C. Then, the nonspecific antibody binding was blocked by 10% normal goat serum. Sections were incubated overnight with specific primary antibodies (polyclonal rabbit anti eNOS, nNOS, CSE or CBS antibodies; 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were subsequently incubated for 1 h with the biotinylated secondary antibody and then for 30 min with an avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories. Burlingame, CA). 3, 3′-diaminobenzidine tetrahydrochloride (DAB) was used as a chromogenic substance. Finally, the slides were washed and counterstained with hematoxylin. Negative control samples were treated in the same way, but the primary antibody was replaced with PBS. Effects of cholinergic stimulation on eNOS expression in parotid gland were examine in in vivo. Cevimeline (5.0 mg/kg) was intravenously injected in the tail vein of anesthetized rats with pentobarbital sodium (Nembutal, 40 mg/kg) and parotid glands were removed at 0, 5, 10 and 30 min after the injection, fixed with 4% paraformaldehyde and embedded in paraffin. Percentage of area fraction (percentage of immunopositive area against whole area) was estimated using ImageJ (ver. 1.46r).

Statistical analysis

The results are means ± SE. Statistical significance was determined using one-way ANOVA or unpaired Student’s t-tests. \( P < 0.05 \) was considered significant.

RESULTS

Expression and localization of NOSs, CBS and CSE in rat parotid gland
First, we examined expression and localization of constitutive NOS isoforms and two H$_2$S synthases in the rat parotid gland. Immunohistochemical analyses showed cytoplasmic expression of nNOS in intralobular and interlobular ducts, in addition to nuclear expression in intercalated ductal cells. This isoform was also present in some nuclei of the acinar cells (Fig. 1A, upper left panel). Expression of eNOS was found in the nuclei of all ductal cells and in the endothelial cells (Fig. 1A, upper right panel). Second, we investigated the H$_2$S synthases and found that both CBS and CSE were expressed in the nuclei of all ductal cells and in some nuclei of acinar cells as shown in Fig. 1A (lower panels). Therefore, in parotid acinar cells, all four synthases examined were found to be present with some variations in localization. Third, the expression and localization of NO- and H$_2$S-producing synthases after the stimulation with 5 mg/kg cevimeline, a cholinomimetic muscarinic agonist, were examined. The restricted eNOS expression as described above was changed to scattered distribution at 10 min after cevimeline injection (Fig. 1B). This alteration was found at 5 and 30 min also as indicated by percentage of area fraction (Fig. 1C). Cevimeline treatment had no appreciable effects on nNOS, CBS and CSE expression in the parotid gland (data not shown).

**Effect of NO and H$_2$S on [Ca$^{2+}$]$_i$**

To assess the roles of NO and H$_2$S in [Ca$^{2+}$]$_i$ regulation, SNP and NaHS were used as NO and H$_2$S donors, respectively. Fig. 2A shows typical pseudo-color images of [Ca$^{2+}$]$_i$ changes recorded before and after treatment with 100 µM SNP (left panel) or 50 µM NaHS (right panel). Fig. 2B and C depict the time courses of [Ca$^{2+}$]$_i$ changes calculated in ROIs, indicated with red ovals in the Fig. 2A left and right panels before and after treatment with 100 µM SNP and 50 µM NaHS, respectively. The [Ca$^{2+}$]$_i$ increases were gradual for both donors, though some oscillatory changes were also found only in a small population of acinar cells treated with SNP (data not shown). With these concentrations, the extent of the donor-
induced [Ca\textsuperscript{2+}]\textsubscript{i} increase was much higher for NaHS than SNP. The donor-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases were dose-dependent, as described in Fig. 3A and B (P < 0.001 for both donors by ANOVA). Like SNP, DETA NONOate another NO donor (5-100 µM) induced [Ca\textsuperscript{2+}]\textsubscript{i} increases as well (data not shown). Next, whether intracellular and/or extracellular Ca\textsuperscript{2+} is mandatory for the NO- and H\textsubscript{2}S-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase was evaluated. Perfusion of isolated acini with EGTA-containing Ca\textsuperscript{2+}-free buffer prior to treatment with SNP (50-500 µM) or NaHS (5-300 µM) tended to decrease the donor-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases, but the effects were not significant (Fig. 3A and B), suggesting that the source of Ca\textsuperscript{2+} for the gaseous molecule-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases is most likely to be intracellular stores, at least for the duration of treatment in this experiment.

**Characterization of NO- and H\textsubscript{2}S-induced Ca\textsuperscript{2+} release**

Ca\textsuperscript{2+} release from intracellular stores can be initiated through either or both inositol trisphosphate receptors (InsP\textsubscript{3}Rs) or ryanodine receptors (RyRs). We attempted to define internal stores that are involved in the SNP- and/or the NaHS-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase. First, possible involvement of PLC-IP\textsubscript{3} pathway in the SNP- and the NaHS-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase was evaluated. Pretreatment of acini with a PLC inhibitor, U73122 (2 µM), in the absence of extracellular Ca\textsuperscript{2+} had no effect on the [Ca\textsuperscript{2+}]\textsubscript{i} increases elicited by SNP or NaHS (Fig. 4A, C). Blockade of InsP\textsubscript{3}Rs with its potent inhibitor xestospongin c (3 µM) in Ca\textsuperscript{2+}-free medium did not significantly decrease the SNP- or the NaHS-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase (Fig. 4B, D). However, the SNP-induced [Ca\textsuperscript{2+}]\textsubscript{i} was markedly elevated by xestospongin c (Fig. 4B, P < 0.001). These results indicate that the PLC-InsP\textsubscript{3}Rs pathway is not significantly attributable to the SNP- or the NaHS-induced Ca\textsuperscript{2+} release. Second, we investigated whether RyRs are involved in the [Ca\textsuperscript{2+}]\textsubscript{i} increase induced by both donors using ruthenium red a RyRs blocker. Pretreatment of acini with RR at 30 µM in the absence of extracellular Ca\textsuperscript{2+} significantly
attenuated the SNP- and the NaHS-induced $[Ca^{2+}]_i$ increase (Fig. 5). The change from the unstimulated value was attenuated by 48 and 57%, respectively (Fig. 5B, $P < 0.05$ and D, $P < 0.001$). These findings suggest that ryanodine-sensitive $Ca^{2+}$ stores are responsible for the $[Ca^{2+}]_i$ increase generated by SNP and NaHS in rat parotid acinar cells.

Cyclic GMP is an intracellular signal that mediates the actions of NO on various physiological functions. Therefore, whether the cGMP-linked pathway is involved in the NO- and H$_2$S-induced $[Ca^{2+}]_i$ release was investigated. Pretreatment of acini with 100 or 300 µM ODQ, a potent and selective inhibitor of sGC, attenuated the $[Ca^{2+}]_i$ increase elicited by 100 µM SNP (Fig. 6A, B) by 54 and 59%, respectively ($P < 0.01$). However, 300 µM ($P < 0.05$) but not 100 µM ODQ significantly inhibited the 50 µM NaHS-induced $[Ca^{2+}]_i$ increase by 50% (Fig. 6C, D). Because one of the main cellular targets of cGMP is PKG, we next assessed its involvement in the $[Ca^{2+}]_i$ increase. Pretreatment of acini with 2 µM KT5823, a highly cell permeable and selective inhibitor of PKG, in Ca$^{2+}$-free buffer significantly reduced the $[Ca^{2+}]_i$ increase elicited by 100 µM SNP or 50 µM NaHS (Fig. 7). The increase from the unstimulated value was attenuated by 53 and 46%, respectively (Fig. 7B, $P < 0.05$ and D, $P < 0.01$). Together, these results indicate that SNP and NaHS induce the $[Ca^{2+}]_i$ increase via a cGMP/PKG-dependent pathway.

H$_2$S-induced NO production in parotid acini

An interaction between H$_2$S and NO at different levels of the signaling cascade has been proposed. Here, we examined the effects of NaHS on NO production in parotid acinar cells using the fluorescent NO probe DAF-2. As shown in Fig. 8A, various concentrations of NaHS (5-300 µM) induced NO production ($P < 0.001$ by ANOVA) with a maximal effect at 50 µM. Intracellular signaling mechanisms that may be involved in the NaHS-induced NO production were then investigated. Pretreatment of acini with 100 µM L-NMMA, a
nonselective NOS inhibitor, significantly decreased the 50 µM NaHS-induced NO production (Fig. 8B and C). The increase from the unstimulated value was attenuated by 42%, \( P < 0.01 \). Moreover, blockade of the PI3K-PKB/Akt signaling pathway by LY294002 (20 µM), a potent PI3K inhibitor, also markedly attenuated the NaHS-induced NO production (Fig. 8B). The increase from the unstimulated value was attenuated by 73% (Fig. 8C, \( P < 0.001 \)). These results suggest that the NaHS-induced NO production is via the PI3K-PKB/Akt pathway. Moreover, HT, an \( \text{H}_2\text{S} \) scavenger, was used to further examine whether endogenously produced NO is \( \text{H}_2\text{S} \) dependent or not. The NaHS-induced NO production was significantly inhibited in the presence of 100 µM HT (\( P < 0.01 \), the increment from the unstimulated value was decreased by 63%), indicating that NO production is in large part \( \text{H}_2\text{S} \)-dependent. To that end, we further examined the potential involvement of NO in the \( \text{H}_2\text{S} \)-induced \([\text{Ca}^{2+}]_i\) increase. Both a NO scavenger, cPTIO, 300 µM, and L-NMMA (100 µM) decreased the \([\text{Ca}^{2+}]_i\) increase in response to 50 µM NaHS. The increase from the unstimulated value was attenuated by 25 and 47%, respectively, Fig. 9). These results support a model where \( \text{H}_2\text{S} \)-induced NO production is partially involved in the NaHS-induced \([\text{Ca}^{2+}]_i\) increase. Additionally, potential effect of cholinomimetic agonist, carbachol (CCh) on NO production was examined. Carbachol at the maximal concentration (\( 3 \times 10^{-6} \) M) increased NO production and \([\text{Ca}^{2+}]_i\) (data not shown).

**DISCUSSION**

**Expression of NOSs, CBS and CSE in parotid gland**

Immunohistochemical analyses in the present study showed that nNOS expression was localized in the cytoplasm of intralobular and interlobular ductal cells, the nuclei of intercalated ductal cells and some nuclei of acinar cells. Endothelial NOS was localized in the nuclei of all parotid ductal cells in addition to endothelial cells. This feature was changed
after in vivo stimulation of parotid gland with cevimeline. Expression of eNOS was thoroughly detected in acinar cell nuclei and cytoplasm after the stimulation of the gland. Reports of NOS expression in the salivary glands have varied according to isoforms and localization. NOS activity in the cytoplasmic fraction of the rat parotid gland was reported (27). β-adrenergic receptor-activated NOS activity, as estimated by cGMP production, was detected in rat parotid acinar cells (42). Furthermore, nNOS-positive nerve fibers were identified around acini of rat parotid gland (2). The expression of nNOS in the glandular parenchyma and the excretory ducts of parotid and submandibular glands was reported (6). This isoform was also reported to be expressed in the cytosol of rabbit parotid acinar cells but less in ductal cells (38). NOS activity, coupled with muscarinic receptor activation, was reported (35). Western blot analysis identified nNOS, but not iNOS or eNOS, in rat parotid acinar cells (14). However, parotid gland from duct-ligated rats presents an increase in iNOS activity (26). In the other two major salivary glands, expression of eNOS was detected exclusively in blood vessels and capillaries, whereas nNOS was found at high levels in the cell periphery, associated with the plasma membrane in all cell types of the submandibular gland (44). Neuronal NOS was not found in acinar cells of the submandibular gland but was identified in neuroterminals, as well as excretory and striated ducts (21). In the submandibular gland, an nNOS-positive fiber was found around the acini, but expression was scarce in the sublingual gland (40). Reasons for these discrepancies are unclear, but according to our present findings, it seems most likely that at least nNOS is expressed in rat parotid acinar cells. In major human salivary glands, most duct epithelial cells were found to be immunoreactive for nNOS, suggesting that NO might directly regulate saliva secretion and it is a putative source of nitrates secreted into saliva (37). However, increased iNOS expression was reported in salivary gland of Sjögren's syndrome patients suggesting that NO may contribute to inflammatory damage and acinar cell atrophy in the syndrome (18), and
inhibition of NO synthesis was found to ameliorate the dysfunction of irradiated salivary glands (41). An alternative notable outcome found in the present study was nuclear localization of the H\textsubscript{2}S-producing enzymes, CBS and CSE, in all ductal cells in addition to some nuclei of acinar cells. To the best of our knowledge, this is the first report showing the expression of CBS and CSE in the rat parotid gland, implicating a functional role/s of H\textsubscript{2}S in parotid acinar cells. Together, it is conceivable that NO and H\textsubscript{2}S are endogenously produced in the rat parotid salivary gland.

Gaseous messenger-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase and underlying mechanisms

In our recent studies, carried out in rat pancreatic acinar cells, we found that both NO and H\textsubscript{2}S could increase the [Ca\textsuperscript{2+}]\textsubscript{i}, mainly through release from intracellular stores via InsP\textsubscript{3}Rs (30, 28). In the present study, we assessed whether a similar mechanism is operating in rat parotid acinar cells, which are exocrine cells functionally analogous to pancreatic acinar cells. In isolated parotid acinar cells, SNP elevated the [Ca\textsuperscript{2+}]\textsubscript{i} in a dose-dependent manner, similar to our previous results in isolated pancreatic acinar cells. The characteristics of the [Ca\textsuperscript{2+}]\textsubscript{i} increase induced by a NO donor were similar in pancreatic and parotid acinar cells. The NO-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase has already been reported in rat parotid acinar cells and NO/cGMP signal transduction was reported to play a crucial role in the acetylcholine-stimulated Ca\textsuperscript{2+} homoeostasis in addition to the regulation of aquaporine-5 levels in the apical plasma membrane of rat parotid gland (23, 13). One novel finding obtained in the present study is that the H\textsubscript{2}S donor could also increase the [Ca\textsuperscript{2+}]\textsubscript{i} in a dose-dependent fashion in parotid acinar cells, which was clearly demonstrated in rat pancreatic acinar cells in our recent study (28). The presence of the H\textsubscript{2}S-activated Ca\textsuperscript{2+} signaling pathway has been proposed in many types of cells including astrocytes and cerebral arteriole smooth muscle cells (31, 20). In the present study, the H\textsubscript{2}S-induced [Ca\textsuperscript{2+}]\textsubscript{i} elevation was also presumed to be largely a result of intracellular Ca\textsuperscript{2+} release, at least for the duration of treatment with the donor. These findings
obtained for NO and H2S led us to define whether a very similar machinery is associated with the Ca2+ release initiated by NO and H2S in parotid acinar cells.

In general, Ca2+ release is caused by InsP3Rs and RyRs, which are embedded in the membranes of Ca2+ storing cellular organelles such as sarcoplasmic and endoplasmic reticula. Our previous studies demonstrated that the IP3-InsP3Rs pathway is clearly associated with the NO- and the H2S-induced [Ca2+]i increases in pancreatic acinar cells. In parotid acinar cells, receptors or channels involved in Ca2+ release have not been fully defined. Previous studies have proposed that RyRs, acting after secretagogue-induced Ca2+ release via InsP3Rs, are involved in water secretion (12, 14). Both ryanodine-sensitive/ryanodine-insensitive stores contribute to the Ca2+ release (42, 23), and a Ca2+ release mechanism that is distinct from IP3-sensitive Ca2+ channels is operating (33).

The present results that neither the PLC inhibitor nor the InsP3Rs blocker was effective (Fig. 4) would rule out presumed involvement of the PLC-IP3-InsP3Rs pathway in the gaseous messenger-induced [Ca2+]i increases or suggest that this pathway, if available, plays only a minor role in the Ca2+ release by NO or H2S, highlighting the second possibility that RyRs may be the major component that is related by the gaseous messenger-induced Ca2+ release in parotid acinar cells. The finding that RR, the RyR blocker (8, 19, 24, 45), reduced the NO- and the H2S-induced [Ca2+]i increases supports the second hypothesis that the NO- and the H2S-induced [Ca2+]i increases occur through RyRs.

However, we unexpectedly found that the NO- but not the H2S-evoked [Ca2+]i increase was significantly potentiated in the presence of xestospongin c (Fig. 4B). Although the exact reasons are unclear, this phenomenon can be interpreted in light of the available evidence. In particular, two reports give a significant hint that may account for this result. 1) Whereas xestospongin c is a selective InsP3Rs blocker extracted from a marine sponge, its hydroxylated derivatives such as xestospongin b showed a sensitizing effect on RyRs,
inducing potentiating of Ca\(^{2+}\)-induced Ca\(^{2+}\) release by a mechanism mediated by RyR type 1 channels (39). In parotid acinar cells, types 1 and 3 RyRs are reported to be expressed, so there is a possibility that even xestospongin c can sensitize type 1 or 3 RyRs in parotid acinar cells. 2) Alternative possibility may be due to the impurity of the chemical. Xestospongin c we used is approximately 90% pure, as was assumed by Ta et al. (39). The possibility that hydroxylated xestospongins that are coexisted with xestospongin c might cause sensitization of RyRs before treatment with the NO donor cannot be excluded. 3) Although our finding and others (33) did not support a significant involvement of IP\(_3\)Rs in the NO- or H\(_2\)S-induced \([Ca^{2+}]_i\) increase, a minor role for InsP\(_3\)Rs in parotid acinar cells could not be ruled out (42, 22). If so, the potentiating effect of xestospongin c in the SNP-induced \([Ca^{2+}]_i\) may be interpreted by putative interactions between InsP\(_3\)Rs and RyRs, as proposed by McGeown (25). Whatever the reasons, the present finding corroborates a major involvement of RyRs in the NO-induced \([Ca^{2+}]_i\) increase in parotid acinar cells. However, the potentiating effect of xestospongin c was not observed in the case of H\(_2\)S-induced \([Ca^{2+}]_i\) increase. This may be due to a relatively strong effect of the H\(_2\)S donor compared with the NO donor at the concentration used, which may mask the potentiating effect by xestospongin c.

Cyclic ADP-ribose (cADPR) has been first proposed as an endogenous activator of RyRs in the sea urchin egg (8) and in various tissues (49); including RyR type 1 in Purkinje cells (15). In addition, NO has been shown to induce Ca\(^{2+}\) mobilization via a signaling pathway involving cADPR in the sea urchin egg (43). The cADPR-RyRs-Ca\(^{2+}\)-induced Ca\(^{2+}\) release (33) and the NO-induced \([Ca^{2+}]_i\), release via cARPR-RyRs (23) are implicated in parotid acinar cells as well. H\(_2\)S has been demonstrated to increase the \([Ca^{2+}]_i\) due to the activation of RyRs in frog motor nerve endings (9). As mentioned above, our present findings suggested that the NO- and H\(_2\)S-induced \([Ca^{2+}]_i\), increase is through RyRs but not InsP\(_3\)Rs, raising the possibility that cADPR mediates RyR activation. Together, it is likely that there is
a prominent difference in the mechanism(s) of Ca\(^{2+}\) release between pancreatic acinar cells and parotid acinar cells; in the former, IP\(_3\)-InsP\(_3\)Rs-induced Ca\(^{2+}\)-release appears to be major (28, 30), but in the latter, RyR-mediated Ca\(^{2+}\) release would be dominant for both the gaseous messenger- and secretagogue-induced [Ca\(^{2+}\)], increase. This finding supports the intriguing idea that each acinar cell, which is functionally analogous, possesses a unique pathway in terms of Ca\(^{2+}\) releasing receptors/channels.

Next, we investigated the mechanism(s) that leads to the RyR-mediated [Ca\(^{2+}\)]\(_i\) release by NO and H\(_2\)S. The sGC enzyme has been known to regulate the NO/cGMP signaling cascade. ODQ, a selective inhibitor of sGC, significantly inhibited the NO-induced [Ca\(^{2+}\)]\(_i\) increase at 100 and 300 µM. However, only the higher concentration was effective for the H\(_2\)S-induced [Ca\(^{2+}\)]\(_i\) increase (Fig. 6). This may be due to a higher efficacy of 50 µM H\(_2\)S in inducing the [Ca\(^{2+}\)]\(_i\) increase than 100 µM NO, which masked the inhibitory effect of the lower concentration of ODQ. Subsequently, we assessed the possible involvement of PKG, which is downstream of the sGC-cGMP cascade, in the NO- and H\(_2\)S-induced [Ca\(^{2+}\)]\(_i\) increase using the cGMP-dependent PKG inhibitor, KT-5823. Significant inhibition of both gaseous messenger-induced [Ca\(^{2+}\)]\(_i\) was found (Fig. 7). These two findings suggest that the sGC-cGMP-PKG pathway mediates not only the NO-induced (23) but also H\(_2\)S-induced [Ca\(^{2+}\)]\(_i\) release, potentially from ryanodine-sensitive stores, via cADPR in rat parotid acinar cells. Carbachol has been reported to mediate amylase release in rat parotid acinar cells via acceleration of the NO-sGC-cGMP-PKG pathway (36).

**Cross-talk between NO and H\(_2\)S**

Interaction between NO and H\(_2\)S at various sites of intracellular signaling has been implicated (3, 4). We also proposed the possibility that H\(_2\)S induces Ca\(^{2+}\) release partly via the produced NO in pancreatic acinar cells (28). The present study showed that the H\(_2\)S donor increased NO production, which was significantly inhibited by the NOS inhibitor, the PI3K
inhibitor and the H\textsubscript{2}S scavenger HT (Fig. 8). Furthermore, the H\textsubscript{2}S-induced [Ca\textsuperscript{2+}]\textsubscript{i} release was partially attenuated by either the NOS inhibitor or the NO scavenger (Fig. 9). The constitutive NOSs, namely eNOS and nNOS, are phosphorylated and activated or upregulated by Akt, a downstream target of PI3K (11, 5). These results indicate that in rat parotid acinar cells, the H\textsubscript{2}S-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase is partly mediated by NO production. This action is most likely via the PI3K-Akt-NOS pathway, in addition to a NO-independent mechanism in rat parotid acinar cells. These findings are in agreement with a hypothesis proposed by Predmore et al. (34) and Kondo et al. (17) for endothelial cells. Together, a putative overall cascade was described in Fig. 10.

In summary, we described the expression and localization of CBS, and CSE for the first time in rat parotid tissue. The expression of the various NOS isoforms was also detected. Both H\textsubscript{2}S and NO induced an [Ca\textsuperscript{2+}]\textsubscript{i} increase via the sGC-cGMP-PKG-cADPR-RyRs signaling cascade. H\textsubscript{2}S induces NO production via PI3K/Akt/NOS, and this may be upstream of sGC activation. We herein postulate a new role for the gaseous messengers H\textsubscript{2}S and NO in the [Ca\textsuperscript{2+}]\textsubscript{i} homeostasis that regulates saliva secretion in parotid acinar cells. In parotid and pancreatic acinar cells, this signaling cascade is shared, but the final target after PKG activation appears to be disparate, namely the RyR-mediated Ca\textsuperscript{2+} release predominates in parotid acinar cells but the InsP\textsubscript{3}R-mediated release predominates in pancreatic acinar cells.

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**DISCLOSURES**
The authors declare no conflicts of interest, financial or otherwise.

**AUTHOR CONTRIBUTIONS**

A.M. performed the experiments, prepared the figures and analyzed the data; A.M. and Y.H. interpreted the results of the experiments, and drafted the manuscript; A.M. and Y.H. edited and revised the manuscript, and approved the final version of the manuscript.

**REFERENCES**


**FIGURE LEGENDS**

**Fig. 1. Expression of NO and H$_2$S producing synthases in parotid gland.**

(A) Immunohistochemically detected localization of nNOS, eNOS, CBS and CSE in the parotid gland. Arrows represent ductal cells (intralobular and interlobular), and intercalated ductal cells are represented by arrowheads. (B) Immunohistochemical localization of eNOS at 0, 5, 10 and 30 min after the treatment of the parotid gland with cevimeline. (C) % area fraction of eNOS expression in parotid gland at 0, 5, 10 and 30 min after stimulation with cevimeline ($n = 12-22$). *** $P < 0.001$, significant difference by unpaired Student’s *t*-test. Scale bar in negative control photographs: 50 µm.

**Fig. 2. NO and H$_2$S donors induce [Ca$^{2+}$]$_i$ increase in rat parotid acinar cells.**

(A) Examples of pseudo-color images of [Ca$^{2+}$]$_i$ changes recorded in isolated parotid acini loaded with Fluo-3/AM before (0 min) and after (9 or 13, and 18 min, respectively) challenge with 100 µM SNP or 50 µM NaHS in the presence of extracellular Ca$^{2+}$. Warm colors
indicate higher and cold colors lower [Ca\(^{2+}\)]\(_i\). Scale bar: 20 µm. (B, C) Typical time courses of Fluo-3 F.I. analyzed in areas of region of interest shown with red ovals in translucent images in control with no addition of donors (dashed lines) and in 100 µM SNP- or 50 µM NaHS-treated acini (solid lines) in the presence of extracellular Ca\(^{2+}\). Changes in F.I. are shown as the percent of basal fluorescence intensity as described in the MATERIALS AND METHODS.

**Fig. 3. Effect of removal of extracellular Ca\(^{2+}\) on the SNP- and NaHS-induced [Ca\(^{2+}\)]\(_i\) increase.**

(A) Dose-response relationship of SNP-induced changes in SFC (± SE) in the presence (black bars, \(n = 13-18\)) and absence (gray bars, \(n = 9-25\)) of extracellular Ca\(^{2+}\). \(P < 0.001\) and \(P < 0.01\) by ANOVA in the presence and absence of extracellular Ca\(^{2+}\), respectively. (B) Dose-response relationship of NaHS-induced changes in SFC in the presence (black bars, \(n = 11-25\)) and absence (gray bars, \(n = 11-28\)) of extracellular Ca\(^{2+}\). \(P < 0.001\) and \(P < 0.05\) by ANOVA in the presence and absence of extracellular Ca\(^{2+}\), respectively. Acini were perfused with standard or EGTA-containing Ca\(^{2+}\)-free buffer for 5 min before treatment with donors and throughout the experiments.

**Fig. 4. PLC and InsP\(_3\)Rs inhibitors have no effect on the SNP- or NaHS-induced [Ca\(^{2+}\)]\(_i\) increase.**

(A, B) Summarized SFC (± SE) of 100 µM SNP-induced [Ca\(^{2+}\)]\(_i\) changes in the absence (\(n = 23\) and 37) and presence of 2 µM U73122 (\(n = 26\)) or 3 µM xestospongin C (Xe, \(n = 23\)). (C, D) Summarized SFC (± SE) of 50 µM NaHS-induced [Ca\(^{2+}\)]\(_i\) changes in the absence (\(n = 26\) and 37) and presence of 2 µM U73122 (\(n = 29\)) or 3 µM xestospongin c (\(n = 25\)). Acini were perfused with Ca\(^{2+}\)-free buffer containing U73122 or xestospongin c for 5 min before
treatment with donors and throughout image acquisition. *** $P < 0.001$, significant difference for SNP alone vs. SNP + xestospongin c.

Fig. 5. Blocking RyRs diminishes the SNP- and NaHS-induced $[\text{Ca}^{2+}]_i$ increase.

(A, C) Typical examples of temporal changes in fluo-3 F.I. induced by 100 µM SNP or 50 µM NaHS, alone (solid lines), or in the presence (dashed lines) of 30 µM RR with no added extracellular Ca$^{2+}$. Acini were perfused with Ca$^{2+}$-free buffer containing 30 µM RR for 3 min before starting image acquisition and throughout the experiments. (B, D) Summarized SFC (± SE) induced by 100 µM SNP and 50 µM NaHS, alone ($n = 39$ and $26$), or in combination with RR ($n = 15$ and $17$). * $P < 0.05$ and *** $P < 0.001$, significant difference for SNP alone vs. SNP + RR and NaHS alone vs. NaHS + RR, respectively, by unpaired Student’s $t$-test.

Fig. 6. Effect of sGC inhibitor on the SNP- and NaHS-induced $[\text{Ca}^{2+}]_i$ increase.

(A, C) Typical examples of temporal changes in fluo-3 F.I. induced by 100 µM SNP or 50 µM NaHS in the absence (solid lines) and presence of 100 (dotted lines) or 300 µM (dashed lines) ODQ in the absence of extracellular Ca$^{2+}$. Acini were perfused with Ca$^{2+}$-free buffer containing ODQ for 5 min before the application of SNP or NaHS and throughout the experiments. (B, D) Summarized SFC (± SE) of SNP- and NaHS-induced $[\text{Ca}^{2+}]_i$ changes in the absence ($n = 23$ and $37$) and presence of 100 ($n = 11$ and $14$) or 300 µM ($n = 13$ and $15$) ODQ. * $P < 0.05$ and ** $P < 0.01$, significant difference by unpaired Student’s $t$-test.

Fig. 7. SNP- and NaHS-induced $[\text{Ca}^{2+}]_i$ increases are reduced by PKG inhibition.

(A, C) Typical time courses of $[\text{Ca}^{2+}]_i$ increases induced by 100 µM SNP or 50 µM NaHS in the absence (solid lines) and presence (dashed lines) of 2 µM KT5823, respectively. Acini were perfused with Ca$^{2+}$-free buffer containing KT5823 for 3 min before starting and
throughout image acquisition. (B, D) SFC (± SE) of SNP- and NaHS-induced [Ca$^{2+}$]$_i$ changes in the absence ($n = 19$ and $12$) and presence ($n = 18$ and $37$) of $2 \mu$M KT5823, respectively. * $P < 0.05$ and ** $P < 0.01$, significant difference for SNP alone vs. SNP + KT5823 and NaHS alone vs. NaHS + KT5823 by unpaired Student’s $t$-test, respectively.

**Fig. 8. NaHS elevates NO production**

(A) Summarized dose-response relationship of NO production induced by different concentrations of NaHS (5-300 µM). Each column represents mean SFC (± SE) of DAF-2T F.I. changes ($n = 7$-27). $P < 0.001$ by ANOVA and * $P < 0.05$ by unpaired Student’s $t$-test.

(B) Examples of temporal changes in DAF-2T F.I. changes recorded in parotid acinar cells treated with $50 \mu$M NaHS alone (solid line), or in combination with $100 \mu$M L-NMMA (dotted line) or $20 \mu$M LY29004 (dashed line) or $100 \mu$M HT (gray line). DAF-2-loaded acini were perfused with the standard solution containing each inhibitor for 3 min before starting and throughout image acquisition. (C) Summarized SFC (± SE) obtained by treatment with $50 \mu$M NaHS in the absence ($n = 35$) and presence of $100 \mu$M L-NMMA ($n = 28$) or $20 \mu$M LY29004 ($n = 22$) or $100 \mu$M HT ($n = 17$). ** $P < 0.01$, significant difference for NaHS alone vs. NaHS + L-NMMA and for NaHS alone vs. NaHS + HT. *** $P < 0.001$, significant difference for NaHS alone vs. NaHS + LY29004 by unpaired Student’s $t$-test.

**Fig. 9. Effect of a NO scavenger and NOS inhibitor on the NaHS-induced [Ca$^{2+}$]$_i$ increase.**

(A) Temporal changes in Fluo-3 F.I. induced by $50 \mu$M NaHS in the absence (solid line) and presence of $300 \mu$M cPTIO (dotted line) or $100 \mu$M L-NMMA (dashed line) with no added extracellular Ca$^{2+}$. Acini were perfused with Ca$^{2+}$-free buffer containing each inhibitor for 5 min before the application of $50 \mu$M NaHS and throughout the experiments. (B) Summarized
SFC (± SE) of 50 µM NaHS-induced [Ca²⁺]ᵢ changes in the absence (n = 37) and presence of 300 µM cPTIO (n = 33) or 100 µM L-NMMA (n = 28). * P < 0.05 and *** P < 0.001, significant difference for NaHS-induced SFC increase in the absence vs. presence of cPTIO or L-NMMA by unpaired Student’s t-test, respectively.

**Fig. 10. Schematic diagram of putative cascade triggered by H₂S and NO, and their possible cross-talk in rat parotid acinar cells.**

Blockades by inhibitors used in the present study are described by dotted line.