A novel role for carbon monoxide as a potent regulator of intracellular Ca<sup>2+</sup> and nitric oxide in rat pancreatic acinar cells.

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ABSTRACT

Carbon monoxide (CO) is known as an essential gaseous messenger that regulates a wide array of physiological and pathological processes, similar to nitric oxide (NO) and hydrogen sulfide (H₂S). The aim of the present study was to elucidate the potential role of CO in Ca²⁺ homeostasis and to explore the underlying mechanisms in pancreatic acinar cells. The exogenous application of a CO-releasing molecule dose-dependently increased the intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ). A heme oxygenase (HO) inducer increased [Ca²⁺]ᵢ in a concentration-dependent manner, and the increase was diminished by a HO inhibitor. The CO-induced [Ca²⁺]ᵢ increase persisted in the absence of extracellular Ca²⁺, indicating that Ca²⁺ release is the initial source for the increase. The inhibition of G-protein, PLC and IP₃ receptor diminished the CO-induced [Ca²⁺]ᵢ increase. CO upregulated eNOS expression and stimulated NO production, and NOS inhibitor, CaM inhibitor, or the absence of extracellular Ca²⁺ eliminated the latter response. Blocking the phosphatidylinositol 3-kinase (PI3K)-Akt/PKB pathway abolished the CO-induced NO production. Pretreatment with a NOS inhibitor, NO scavenger or soluble guanylate cyclase inhibitor did not affect the CO-induced [Ca²⁺]ᵢ increase, indicating that NO, sGC and cGMP are not involved in the CO-induced [Ca²⁺]ᵢ increase. CO inhibited the secretory responses to CCK-octapeptide or carbachol. We conclude that CO acts as a regulator not only for [Ca²⁺]ᵢ homeostasis via a PLC-IP₃-IP₃R cascade but also for NO production via the CaM and PI3K-Akt/PKB pathway and both CO and NO interact. Moreover, CO may provide potential therapy to ameliorate acute pancreatitis by inhibiting amylase secretion.
ABBREVIATIONS:
AKT, serine/threonine kinase; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channel; CO, Carbon monoxide; CORM-2, Carbon monoxide releasing molecule-2; CYP450, Cytochrome P450; DAF-2T, Triazolofluorescein; F.I, Fluorescence intensity; HO, Heme oxygenase; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol trisphosphate receptor; NO, Nitric oxide; NOS, Nitric oxide synthase; PI3K, Phosphatidylinositol-3-Kinase; PKB, protein kinase B; ROI, Region of interest; SFC, Summed fluorescence change; sGC, Soluble guanylate cyclase.

Key words: Carbon monoxide; Ca^{2+}, nitric oxide, pancreatic acinar cells
INTRODUCTION

CARBON MONOXIDE (CO) is gaining general acceptance as a potential modulatory molecule in a broad spectrum of biological phenomena including vasodilation, neuromodulation, anti-inflammation, anti-apoptosis and anti-proliferation. CO is generated by three hitherto identified isoforms of heme oxygenase (HO-1, HO-2 and HO-3) in the presence of O₂ and NADPH via the degradation of heme, which releases biliverdin/bilirubin, CO and free iron/ferritin. Like an inducible nitric oxide synthase, various stimuli such as hormones, oxidative stressors, growth factors, and others (30, 39), induce the expression of HO-1, also known as a heat shock protein 32. Its expression is also induced in various disease states (9). HO-2 is a constitutive enzyme expressed in the brain, testes, cardiovascular system, retina, liver and gastrointestinal tract (39). The inducer of HO-2 is limited; adrenal glucocorticoids (61), opiates (36) and estrogen (57) can up-regulate HO-2 expression and the enzyme is activated by Ca²⁺/CaM (3). HO-3 is also a constitutive isoform expressed in the liver, spleen, brain and kidney of rats but its function is thought to be restricted to heme sensing or heme binding rather than heme degradation (11).

In the pancreas, previous studies demonstrated that HO-1 expression was not detected in normal islets isolated from rats (67) and mice but identified in ob/ob mice (38). HO-1 was found to be induced in β cells (53) or whole islets (24). Conversely, HO-2 was found to be expressed in islet endocrine cells (25, 38). In the exocrine pancreas, neither HO-1 nor HO-2 was detected in normal rats (25, 50), but caerulein-induced acute inflammatory pancreatitis in rats (50) or mice (13) was found to be accompanied by a significant increase in the expression of HO-1 in pancreatic tissue.

CO can affect multitudinous sites of cell signaling pathways and preferentially targets heme-containing enzymes. For instance, soluble guanylate cyclase (sGC) is activated to enhance cGMP production, which plays essential roles as an intermediate in various
biological phenomena, such as vascular tone regulation, platelet aggregation, the reduction of ischemia-reperfusion injury in the lung and protection of pancreatic β cells from apoptosis (5, 15, 20, 58). However, the efficacy of CO to activate sGC is reportedly lower than that of nitric oxide (NO) (52), casting doubt on its physiological roles (30). The activation of sGC by CO becomes evident when the intracellular levels of NO are low (47) or in the presence of an enhancer, such as YC-1 (51). Other molecular targets for CO have been demonstrated, including a Ca^{2+}-activated large conductance K^+ (BK_{Ca})-channel in vascular smooth muscle cells (60, 63), p38MAPK in response to stress and inflammation (30) and a serine-threonine kinase (Akt/PKB) in rat endothelial cells (68), isolated rat or mouse cardiomyocytes (14, 54) and rat hepatocytes (31). CO shows a dual effect on L-type Ca^{2+} channels in human intestinal smooth muscle, i.e.; the inhibition or potentiation of the channel activity (37). CO has been reported to reduce the intracellular Ca^{2+} signal in human platelets, partially via the inhibition of capacitative Ca^{2+} entry (17). Like CO, Ca^{2+} is a pleiotropic molecule involved in a wide variety of cellular functions from physiology to pathology. Although Ca^{2+} plays important roles in the physiology and pathology of pancreatic acinar cells, neither the role of exogenous CO nor the role of endogenous CO has been studied with regard to intracellular Ca^{2+} homeostasis.

Thus, the present study was designed to examine the effects of CO on intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) and to define potential action sites of HO/CO in the Ca^{2+}-associated cell signaling pathway and to define the putative interplay between the CO and NO pathways in pancreatic acinar cells.

MATERIALS AND METHODS

Solutions. 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; Na₂HPO₄, 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 Ca²⁺-free solution was prepared without the addition of CaCl₂ but with the addition of 1 mM EGTA.

**Experimental animals, isolation and perfusion of pancreatic acini.** 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. Male SPF Wistar rats (200-250 g) were obtained from Clea Japan (Tokyo, Japan) and were housed in 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.

The animals were anesthetized by CO₂ inhalation and euthanized by exsanguination. Freshly isolated pancreatic acini were prepared according to a previously reported method (42, 43). In brief, the pancreata were excised, freed from fat and lymph nodes and injected with 5 ml of HEPES-buffered standard solution containing 60–75 U/ml collagenase supplemented with 0.1 mg/ml soybean trypsin inhibitor. The tissue was then incubated in a conical flask at 37°C under vigorous shaking for a total of 30 min. The collagenase was replaced with 5 ml of new collagenase solution and incubated for additional 30 min followed by mechanical disruption 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. The acinar cell viability was assessed by the trypan blue exclusion test and was nearly 100%. For immunohistochemistry, rats were injected with hemin (50 mg/kg, i.p.) and the excised pancreata were employed for immunohistochemical observation as described below.
Confocal imaging of Ca\textsuperscript{2+} and NO. To detect temporal changes in the intracellular Ca\textsuperscript{2+} and NO levels, an imaging system was used as previously reported (42, 43). Briefly, isolated acini were loaded with final concentrations of 10 μM Fluo-3/AM or 10 μM DAF-2/DA in the standard solution with mild shaking in the dark for 60 min at 37°C. The acini were then rinsed 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) were analyzed using bundled software (Fluoview 5.0 with Tiempo, Olympus, Tokyo, Japan). Analyses were conducted by averaging the F.I. changes in all the cells constructing an acinus. The change in the fluorescence intensity was calculated as the percent of basal fluorescence intensity by setting the pre-stimulated fluorescence prior to the application of drugs at 100% (baseline) (F/F₀ x 100). The changes in [Ca\textsuperscript{2+}]\textsubscript{i} and NO production were estimated by calculating the summed area of fluorescence changes (SFC) above the baseline during treatment or stimulation (42, 43). All experiments for the measurement of [Ca\textsuperscript{2+}]\textsubscript{i} and NO were performed at room temperature and isolated acini were used for experiments within 4 h of isolation.
**Immunohistochemical analysis of eNOS.** Immunohistochemical detection of eNOS was conducted as reported previously (48). In brief, paraffin sections (3 µM) from the pancreatic tissues originally fixed in buffered formalin were deparaffinized and rehydrated, and heated in citrate buffer (pH 6.0) for 20 min. After the blockade of endogenous peroxidase activity, rinse, and incubation with 5 % normal goat serum, the sections were incubated overnight at 4°C with a primary antibody against eNOS (rabbit polyclonal, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then treated with a biotinylated secondary antibody, goat anti-rabbit IgG (Sigma, St. Louis, MO, USA), for 30 min at 37°C followed by incubation with streptavidin peroxidase (1:100) for 1 h. Finally, all the sections were treated with DAB reagent for color development and counterstained with hematoxylin. The number of immunoreactive cells was counted under a light microscope and the percentage was calculated. In addition, the immunofluorescence analysis for eNOS expression was also conducted with Alexa Fluor 488 anti-rabbit IgG (1:200; Invitrogen, Waltham, MA, USA), followed by Hoechst 33342 (Dojindo, Kumamoto, Japan). Intracellular localization of eNOS was examined by a fluorescence microscopy (BZ-9000, BIOREVO). As a negative control, the sections were incubated without the primary antibody.

**Amylase measurement.** To assess the pancreatic secretory responses, the amount of amylase released into the incubation medium was measured using a method previously reported (21) with some modifications. Briefly, isolated acini suspensions in a group of conical flasks were stimulated with increasing concentrations of CORM-2 alone, CCK-octapeptide (CCK-8) or carbachol (CCh) in the absence and presence of 0.5 mM CORM-2 for 30 min at 37°C with gentle shaking. At the end of a 30 min incubation period, 1 ml aliquots of acini suspension were pelleted and the supernatants were removed and held on ice. The amylase activity was determined in duplicate for each supernatant using colorimetric assay kits, AMYL-P (Fuji Film, Tokyo, Japan) with an automatic analyzer (Dri-Chem 7000;
Fuji Film Medical, Tokyo, Japan). The amylase released during the 30-min incubation period was expressed as a percentage of the total amount of enzyme initially present in the acinar cells.

**Chemicals.** Chromatographically purified collagenase (CLSPA) was obtained from Worthington Biochemical (Lakewood, NJ, USA). Fluo-3/AM, EGTA, NG-monomethyl L-arginine (L-NMMA), HEPES and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were obtained from Dojindo (Kumamoto, Japan). MEM was purchased from Invitrogen (Carlsbad, CA, USA). DAF-2/DA was purchased from Daiichikagaku (Tokyo, Japan). Fluphenazine-N-2-chloroethane (SKF-7171A), Zinc (II) Protoporphyrin IX (ZnPPIX) and D-3-Deoxy-2-O-methyl-myo-inositol 1-[(R)-2-methoxy-3-(octadecyloxy) propyl hydrogen phosphate] (SH-5) were obtained from Enzo Life Sciences (Farmingdale, NY, USA). CCK-8 was obtained from Peptide Institute (Minoh, Osaka, Japan). GP2A, LY29004 and manganese tetrakis (4-benzoic acid) porphyrin chloride (Mn-TBAP) were purchased from Calbiochem (La Jolla, CA, USA). Cell-Tak was procured from BD Biosciences (San Jose, CA, USA). 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and xestospongic were obtained from Wako Pure Chemicals (Osaka, Japan). Other drugs, including tricarboxyldichlororuthenium (II) dimer (CORM-2), were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Statistical analysis.** The reported values are means ± S.E. Statistical significances were determined using one-way ANOVA and unpaired Student’s *t*-tests depending on the number of groups for comparison (http://www.physics.csbsju.edu/stats/); *p* < 0.05 was considered significant.
RESULTS

Effect of CO on [Ca\(^{2+}\)]\(_{i}\) in pancreatic acinar cells. The effects of CO on [Ca\(^{2+}\)]\(_{i}\) were examined using a CO donor, CORM-2 from which CO is released spontaneously, mimicking the biological actions of CO. Fig. 1 shows typical time courses of [Ca\(^{2+}\)]\(_{i}\) changes induced by CORM-2 at concentrations ranging from 0.1 to 1 mM. The [Ca\(^{2+}\)]\(_{i}\) changes were found to be dose-dependent; the [Ca\(^{2+}\)]\(_{i}\) increase was gradual at the lowest concentration, oscillatory at medium concentrations, and monophasic at the highest concentration (Fig. 1, A-E) (\(p < 0.001\) by ANOVA). The dose-dependent manner of CORM-2-induced [Ca\(^{2+}\)]\(_{i}\) changes was analogous to changes recorded in acini when stimulated with physiological secretagogues, such as CCK-8 or ACh (22). The maximal [Ca\(^{2+}\)]\(_{i}\) response was obtained at 0.5 mM (186%, Fig. 1E). The EC\(_{50}\) of the increasing phase was calculated to be 287 µM. Fig. 1F shows 0.5 mM CORM-2-induced temporal and spatial [Ca\(^{2+}\)]\(_{i}\) dynamics with pseudo-colors, the time course of which is described in Fig. 1C.

To investigate the potential involvement of intrinsic HO, hemin, a substrate of HO-1, was applied. Hemin at concentrations ranging from 0.01 to 0.5 mM caused a dose-dependent increase in [Ca\(^{2+}\)]\(_{i}\) (Fig. 2, A and B, \(P < 0.001\) by ANOVA) without an oscillatory [Ca\(^{2+}\)]\(_{i}\) response. When compared with the response induced by CORM-2, the hemin-induced [Ca\(^{2+}\)]\(_{i}\) increase was small; the response to 0.5 mM hemin (~30% increment) appeared to be approximately equal to that of 0.1 mM CORM-2, which caused a non-oscillatory, gradual increase in [Ca\(^{2+}\)]\(_{i}\). The 0.1 mM hemin-induced increase was partially but significantly inhibited by the HO inhibitor, ZnPPiX (\(P < 0.05\) by Student’s \(t\)-test). The increment from the unstimulated value was diminished by 33% (Fig. 2C). The results suggested that pancreatic acinar cells possess an innate HO-1/CO signaling system which triggers the [Ca\(^{2+}\)]\(_{i}\) response.

Next, sources of Ca\(^{2+}\) were investigated. The isolated acini were perfused with nominally Ca\(^{2+}\)-free buffer containing EGTA, and CO was applied by perfusing cells with CORM-2 at
0.4 mM. The $[Ca^{2+}]_i$ response persisted even in the absence of extracellular $Ca^{2+}$ (Fig. 3A). The dose-response relationship of the $[Ca^{2+}]_i$ increase in the presence and absence of extracellular $Ca^{2+}$ revealed that the $[Ca^{2+}]_i$ increase was unaffected in the absence of extracellular $Ca^{2+}$, except for 0.5 mM CORM-2 (Fig. 3B, $P < 0.05$), indicating that the source of $Ca^{2+}$ for the CO-induced $[Ca^{2+}]_i$ increase is intracellularly stored $Ca^{2+}$. Nevertheless, the CORM-2-induced $[Ca^{2+}]_i$ increase was partially reduced in the absence of extracellular $Ca^{2+}$ at 0.5 mM CORM-2 which elicited maximal $[Ca^{2+}]_i$ response.

Effect of inhibition of $G_q$, PLC, or IP$_3$R on $[Ca^{2+}]_i$ increase initiated by CO. Our recent studies indicated that the NO and hydrogen sulfide (H$_2$S) signaling pathways cross-react (42, 43). In the current study, we attempted to define the intracellular signals that are triggered by CO, resulting in $[Ca^{2+}]_i$ increase. First, we focused on the potential contribution of the inositol 1,4,5-trisphosphate (IP$_3$)-producing enzyme, PLC. In the absence of extracellular $Ca^{2+}$, the treatment of acinar cells with 2 µM U73122, a PLC inhibitor, 5 min before and during the addition of 0.1 mM CORM-2 significantly attenuated the $[Ca^{2+}]_i$ increase induced by CORM-2 (Fig. 4A). The increment was significantly reduced by 33% (Fig. 4B, $P < 0.05$), suggesting the involvement of PLC in the CORM-2-induced $[Ca^{2+}]_i$ increase. Second, we examined the involvement of IP$_3$ and IP$_3$ receptor (IP$_3$R) using a potent, cell-permeable IP$_3$R inhibitor, xestospongin C, and found that 3 µM xestospongin C appreciably inhibited the CORM-2-induced $[Ca^{2+}]_i$ increase (Fig. 4A) by 30%. (Fig. 4B, $P < 0.05$). The results indicated that the CORM-2-induced $[Ca^{2+}]_i$ increase was partly triggered by the PLC/IP$_3$ pathway. Third, to further test the possible involvement of the $G_q$ in PLC/IP$_3$ pathway initiated by CO, the effect of GP2A, a $G_q$ protein inhibitor, was examined. Fig. 4C shows the inhibitory effect of 10 µM of GP2A in the absence of extracellular $Ca^{2+}$. The SFC was decreased by 30% (Fig. 4D, $P < 0.05$), indicating that $G_q$-protein contributed to the CO-
induced [Ca\(^{2+}\)]\(_i\) increase. Collectively, all of these results suggest that G\(_q\)-protein, PLC, IP\(_3\) and IP\(_3\)R, at least in part, mediate the CO-triggered intracellular Ca\(^{2+}\) mobilization.

**Effect of CO on NO production.** CO is a heme-binding gaseous molecule that is, known to modify the activities of intracellular enzymes, such as sGC, nitric oxide synthases (NOS) and cytochrome C oxidase, which contain a heme domain in their molecular structures. The possible production of intracellular NO by CO was investigated using a fluorescent NO probe, DAF-2/DAF-2T. We found that increasing the concentrations of CORM-2 (0.1-1 mM) induced gradual and continual NO production in the presence of extracellular Ca\(^{2+}\) (Fig. 5A). The CO-induced NO production was dose-dependent (Fig. 5B, \(P < 0.001\) by ANOVA). The EC\(_{50}\) was calculated to be 277 µM, which is nearly equal to that of the CO-induced [Ca\(^{2+}\)]\(_i\) increase (287 µM as stated before). Moreover, increasing concentrations of an HO-1 inducer, hemin (10-500 µM) could stimulate the NO production, though some decrease in the increment was found at a higher concentration (Fig. 5C, \(P < 0.001\) by ANOVA). The immunohistochemical and immunofluorescence analyses showed the baseline expression of eNOS in the nuclei of acinar, centroacinar and intercalated ductal cells, in addition to the vascular endothelial cells (Fig. 5E). Interestingly, the percentage of immunopositive nuclei was increased by hemin treatment (54.2 ±1.06%) as compared with the control without hemin (34.6 ±3.3%) (Fig. 5D, \(P < 0.001\)). Taken together, these findings suggest that CO stimulates the NO production via eNOS localized in the acinar cells.

An attempt was made to explore the mechanism of NO production by CO. The 0.1 mM CORM-2-induced NO production was significantly diminished by the removal of extracellular Ca\(^{2+}\), a NOS inhibitor, L-NMMA (100 µM), or by a CaM antagonist, SKF-7171A (10 µM). The increment of SFC from the unstimulated value was diminished by 43%, 37% and 61%, respectively, (Fig. 6, \(P < 0.001\)), suggesting the involvement of intracellular Ca\(^{2+}\) and CaM in the CO-induced NOS activation, which leads to NO production. Next, we
attempted to assess whether the phosphoinositide 3-kinase (PI3K)-Akt/PKB signaling pathway is involved in NOS activation and the resultant NO production by CORM-2. Both a PI3K inhibitor, LY29004 (20 µM), and an Akt/PKB inhibitor, SH-5 (10 µM), significantly decreased the NO production induced by 0.1 mM CORM-2 (Fig. 6A). The increment of SFC was attenuated by 62% and 34%, respectively (Fig. 6B, \( P < 0.001 \)). The results indicated that CO activates PI3K, which in turn activates Akt/PKB and enhances NO production, likely via eNOS activation. To assess the possible involvement of reactive oxygen species (ROS) in the CO-induced NO production, a cell permeable super oxide dismutase mimetic and peroxynitrite scavenger, Mn-TBAP (50 µM), was applied. As shown in Fig. 6, the pretreatment with the ROS scavenger did not affect the CORM-2-induced NO production. However, the hemin-induced NO production was partially attenuated in the presence of Mn-TBAP (Fig. 6C, \( P < 0.01 \)).

**Effect of NOS inhibitor, NO scavenger and sGC inhibitor on CO-induced \([Ca^{2+}]_i\) increase.**

The heme-HO and L-arginine-NOS pathways have been demonstrated to interact at multiple sites and mutually influence each other at the levels of activity and function (12, 39). To investigate whether the CORM-2-induced \([Ca^{2+}]_i\) increase is, at least in part, attributed to the CO-induced NO production, the effects of a non-specific inhibitor of all NOS isoforms, L-NMMA, and an NO scavenger, cPTIO, were examined. The CORM-2-induced \([Ca^{2+}]_i\) increase was found to be unaffected by treatment with 100 µM L-NMMA or 300 µM cPTIO (Fig. 7, A and B). Moreover, 100 µM and 300 µM ODQ, a specific sGC inhibitor, also did not affect the \([Ca^{2+}]_i\) increase induced by 0.1 mM CORM-2 (Fig. 8, A and B). Taken together, these findings imply that NO and the resultant sGC activation are not attributable to the CORM-2-induced \([Ca^{2+}]_i\) increase in pancreatic acinar cells.

**Effect of CO on secretagogue-induced amylase release.** The effects of CO alone or those on the CCK-8- or CCh-induced amylase release from pancreatic acini were studied. The dose-
response curve for amylase release by CORM-2 was found to be bell-shaped; significant increases in amylase release (53% increment from the basal) at a medium concentration (0.5 mM) followed by a significant decrease (71% reduction from the basal) at a higher concentration (1 mM) were observed (Fig. 9A, \( P < 0.01 \)). When acini were stimulated with increasing concentrations of CCK-8 or CCh in the presence of 0.5 mM CORM-2, the dose-response curve obtained for each secretagogue alone was shifted downward, indicating that CO significantly inhibited the secretagogue-induced secretory response (Fig. 9B and C).

**DISCUSSION**

*CO induces \([Ca^{2+}]_i\) increase in pancreatic acinar cells.* The current study demonstrated that CO released from CORM-2 dose-dependently (0.1-1 mM) increases \([Ca^{2+}]_i\) in the exocrine pancreas. The increase was gradual at lower concentrations (0.1-0.3 mM), oscillatory at moderate concentrations (0.4-0.5 mM) and monophasic at a higher concentration (1 mM) of CORM-2 (Fig. 1), which appeared to be analogous to what we usually observe in acinar cells stimulated with physiological secretagogues, such as CCK-8 (22). In pancreatic acinar cells, \(Ca^{2+}\) plays essential roles for the secretagogue-induced release of zymogens by exocytosis and the secretion of fluids by \(Ca^{2+}\)-activated Cl- and BK\(_{Ca}\) channels. To date, only a limited number of investigations on the effect of the third gaseous molecule, CO, on \([Ca^{2+}]_i\) have been conducted. Xi *et al.* (63) reported that exogenously applied CO increased the open probability of low-affinity \(K_{Ca}\) channels by increasing the \(Ca^{2+}\) sensitivity of the channel. The same group subsequently showed the same effect of CO and glutamate on BK\(_{Ca}\) channel activity depending on the \(Ca^{2+}\) spark frequency (34), but the global \([Ca^{2+}]_i\) was reduced in cerebral arteriole smooth muscle cells of piglet (64), leading to dilation in smooth muscles. Gende (17) also revealed that multiple agonist-induced \([Ca^{2+}]_i\)
increases in human platelets were attenuated in a solution bubbled with CO, partially via the inhibition of capacitative Ca\(^{2+}\) entry. The promotion of the appearance of Ca\(^{2+}\) transients and synchronization by hemin was described in glucose-treated pancreatic β cells, (38). Thus, the effect of CO is variable depending on tissues.

The current finding that hemin caused a concentration-dependent increase in [Ca\(^{2+}\)]\(_{i}\) and that this increase was diminished by a hemin inhibitor, ZnPPIX (Fig. 2) supports an idea that endogenous CO triggers [Ca\(^{2+}\)]\(_{i}\) elevation in pancreatic acinar cells. In the exocrine pancreas, multiple conditions, such as oxidative stress and an overdose application of caerulein (13, 50), reportedly induce HO-1 and the resultant production of CO is thought to be a key factor that serves to ameliorate inflammatory insult. In conjunction with this mechanism, hemin can offer a therapeutic approach to prevent acute pancreatitis via the upregulation of HO-1 (44).

Collectively, the current findings suggested the involvement of exogenously and endogenously produced CO in the regulation of intracellular [Ca\(^{2+}\)]\(_{i}\) in pancreatic acinar cells. The source of Ca\(^{2+}\) for CO-induced [Ca\(^{2+}\)]\(_{i}\) was found to be mostly intracellular at low and moderate concentrations of CORM-2; extracellular Ca\(^{2+}\) is also attributable at a 0.5 mM concentration (Fig. 3). We examined how CO could induce the release of intracellularly stored Ca\(^{2+}\). Pharmacological scrutiny using various inhibitors indicated that each G\(_{q}\)-protein, PLC, IP\(_{3}\) and IP\(_{3}\)R pathway takes part in the CO-induced [Ca\(^{2+}\)]\(_{i}\) release in pancreatic acinar cells (Fig. 4). This cascade is mostly identical to the Ca\(^{2+}\)-releasing mechanism proposed in our previous papers for two other gaseous messengers, NO and H\(_{2}\)S (42, 43). Inverse results have been reported in other tissues; in an in vivo study, a HO-1 inducer, hemin, suppressed PLC activity, attenuated IP\(_{3}\) and reduced the resting [Ca\(^{2+}\)]\(_{i}\) in the aorta and left ventricle of spontaneously hypertensive rats with high aldosterone levels (45, 46). It seems contradictory but the apparent discrepancy can be interpreted by the fact that aldosterone stimulates PLC (6) and hemin inhibits aldosterone level in vivo (45, 46). Thus, at the cellular level, hemin
can activate PLC-IP$_3$-Ca$^{2+}$ release via the production of CO, as was found in the present study.

*CO stimulates production of NO.* Understanding the cross-talk between CO and NO, or even including H$_2$S, draws attention to the physiological and/or pathophysiological effects of these gases (8, 42, 62). The cellular physiology of CO is complex and CO can modulate NO production in multiple ways. CO may activate NOS and NO production or may induce NO release from a storage pool but suppress NOS (55). It was also revealed that high levels of CO inhibit NOS activity and NO generation, while lower concentrations induce the release of NO, which is most likely a pre-existing intracellular heme-bound pool of NO, rather than a direct stimulation of eNOS (55). Hemin has been reported to inhibit glucose-induced NO production in pancreatic islets, suggesting CO-induced NOS inhibition (24, 25). On the contrary, the activation of eNOS via Akt by CO has been demonstrated, which protected from ischemia-reperfusion injury (14). Moreover, the activation of L-type Ca$^{2+}$ channels by CO via NOS-NO production in HEK-293 cells has been described (37), though the attenuation and quenching of NO production by CORM-2 in aortic smooth muscle cells has been reported, suggesting that CORM-2 inhibits NO-induced signaling and vasorelaxation (40). Thus, the effect of CO on NO production has been regarded as two-sided (47). The dose-dependent NO production by CORM-2 and hemin was demonstrated in the present study (Fig. 5, A-C). Moreover, we found that the eNOS immunoreactivity was present in the cell nucleus of not only acinar but also centroacinar, intercalated ductal cells and endothelial cells, and that eNOS expression was further enhanced by hemin treatment (Fig. 5, D and E), leading us to conclude that NO is produced in the pancreatic acinar cells when eNOS is activated by various factors such as CORM-2 and hemin. Western blot analysis revealed the expression of eNOS in the whole pancreas as well as pancreatic acinar cell lysates (2) and, reportedly, ~30% of whole eNOS protein was expressed in pancreatic acinar cells (10). In addition, the
nucleus-localized eNOS expression was implied in various cell types (16, 18, 19, 33). The hemin-induced eNOS gene expression was reported in endothelial progenitor cells (59). Taken together, it is highly possible that CORM-2 and hemin upregulate the eNOS expression, resulting in the NO production in pancreatic acinar cells.

Regarding the mechanisms by which CO stimulates NO production, the interaction between the CO/Akt pathway (29, 68) as well as the activation of the PI3K-Akt/PKB-NOS pathway (35) and Akt/PKB-eNOS pathway (14) by CO have been proposed. Pharmacological experiments conducted in the present study showed that the NO production depended on the dose of the CO donor, the presence of extracellular Ca\(^{2+}\), the activation of NOS and CaM, the production of PI3K, and the upregulation of Akt/PKB. Based on these results and previous reports, it was presumed that, in addition to Ca\(^{2+}/CaM\)-mediated NOS activation and resultant NO production, a PI3K-Akt/PKB signaling pathway is involved in CO-induced NO production in pancreatic acinar cells (Fig. 6).

ROS and NO have been implicated to mediate not only physiological signalling responses but also pathophysiological causal effects. It has been reported that the NO formation and the bioavailability could be impaired by ROS (41) and that Ca\(^{2+}\) may act at both upstream and downstream of ROS and NO production (66). These complicated interactions make the understanding of intracellular cascade trigged by the gaseous molecules difficult. We found that the scavenger of peroxynitrite and superoxide, MnTBAP, had no appreciable effect on the CORM-2-induced NO production but it partially inhibited the hemin-induced NO production (Fig. 6C), indicating that ROS is not involved in the former but is partly attributable to the latter. Although a clear interpretation for the potential involvement of ROS in the hemin-induced NO production waits for further experimentation, it is speculated that hemin degradation products such as free iron but not CO are, at least in part, involved in the hemin-induced NO production due to its ROS-generating catalyzing action (56).
Is NO and sGC activation involved in the CO-induced \( [Ca^{2+}]_i \) increase? We recently hypothesized that NO itself and \( H_2S \) could induce \( [Ca^{2+}]_i \) via an increase in NO production due to the activation of sGC (42, 43). However, despite the significant increase in NO by CO possibly via the activation of NOS (Fig. 5), the present study did not support the positive involvement of NO in the CO-induced \( [Ca^{2+}]_i \), increases as both NOS inhibitor and NO scavenger did not affect the \( [Ca^{2+}]_i \) increases (Fig. 7). The exact reason is unclear, but we presume that the amount of NO produced by CO is not sufficient to trigger \( [Ca^{2+}]_i \) dynamics. Alternatively, CO may rather diminish \( Ca^{2+} \) entry after the depletion of the \( Ca^{2+} \) pool, possibly via the inhibition of cytochrome P450 (CYP450) (1, 4, 17, 49), as discussed below.

Neither sGC nor cGMP was demonstrated to be involved in the CO-induced \( [Ca^{2+}]_i \), increase in the present study (Fig. 7 and 8). Like NO, CO has been reported to have the potential to activate sGC, as sGC is a heme-containing enzymes (62). Compared with NO, CO can reportedly only weekly activate sGC; NO could enhance the sGC activity by a factor of 130, but CO resulted in only a 4.4-fold increase (52). A 4-fold activation has also been reported (28). Christova et al. (7) demonstrated that CO can inhibit platelet aggregation and relax blood vessels by activating sGC and elevating the intracellular level of cGMP. Conversely, significant increase in the cGMP level due to CO have not been reported in other tissues (12, 32). Even attenuation of NO-induced sGC activation by CO has been demonstrated in cerebellar granule cells (26). The final biological effect of CO has been suggested to depends on various factors, such as the amount of CO, the amount of endogenously produced NO and the concentration of CO that can react with NO (40). Moreover, when the local concentration of NO is low, CO could modestly activate sGC (27). These data indicate that, CO likely acts as a partial activator of sGC, while CO alone is much less effective in activating sGC than NO alone, CO rather inhibits NO-induced sGC activation by competitively acting on sGC molecules. Thus, although CO produces a significant amount of NO, the CO released from
CORM-2 would mask sGC activation by the produced NO and CO itself could not significantly activate sGC. Therefore, NO is not related to the CO-induced \([\text{Ca}^{2+}]\), increase in pancreatic acinar cells.

*CO alone induces faint amylase release but markedly inhibits secretagogue-induced amylase release.* CO was found to induce a faint release of amylase but it unexpectedly markedly inhibited CCK-8- or CCh-induced amylase release (Fig. 9). In general, a secretory response in pancreatic acinar cells is thought to be triggered when \([\text{Ca}^{2+}]\), is increased by mobilization from intracellular stores, entry from the extracellular space or both. The faint increase in amylase release by CO could be due to an increase in \([\text{Ca}^{2+}]\), that is mainly the result of \(\text{Ca}^{2+}\) mobilization. We hypothesized that the postprandial long-lasting exocrine secretion is due to a mandatory continuous increase in \([\text{Ca}^{2+}]\), which can be first accomplished by the release of \(\text{Ca}^{2+}\) from intracellular stores followed by sustained entry from the extracellular space (23). In other words, intracellularly stored \(\text{Ca}^{2+}\) is attributed to the early stage of secretion, and the presence of extracellular \(\text{Ca}^{2+}\) is necessary for the subsequent secretory response. Regarding intracellular \(\text{Ca}^{2+}\) dynamics, CO has been reported to inhibit capacitative \(\text{Ca}^{2+}\) entry by interfering with and inhibiting CYP450, which accelerates plasma membrane \(\text{Ca}^{2+}\) influx triggered after \(\text{Ca}^{2+}\) store depletion (1, 4, 17, 49). Thus, the inhibition of the secretagogue-induced secretory response by CO is presumably due to the inhibition of CYP450 by CO via a mechanism distal to the \(\text{Ca}^{2+}\) mobilizing mechanism but related to the decreased \(\text{Ca}^{2+}\) influx in pancreatic acini. Several lines of evidence have demonstrated the protective and therapeutic role of HO-1 and CO in various inflammatory conditions, especially acute pancreatitis. CORM-2 suppressed the levels of systemic inflammatory cytokines, as well as macrophage TNF-\(\alpha\) secretion, serum amylase and lipase secretion (65). However, the mechanism by which CO physiologically or pathophysiologically regulates pancreatic exocrine secretion in the normal and inflammatory
pancreas has not yet been elucidated. The above-noted hypothesis shed light on the
therapeutic potentials of CO and/or CYP450 in the amelioration of acute pancreatitis.

In conclusion, the current study demonstrated for the first time the role of CO in
intracellular Ca\(^{2+}\) homeostasis in pancreatic acinar cells that possess the HO/CO system. The
system represents a novel regulatory system of physiological and pathophysiological
significance for the regulation of [Ca\(^{2+}\)]. This pathway is independent of sGC-cGMP, but an
unknown signaling factor may act as an intermediate in the CO and G\(_{q}\)-protein-PLC-IP\(_3\)-IP\(_3\)R
pathway. Moreover, a positive interaction between CO and eNOS/NO via the Ca\(^{2+}/\)CaM and
PI3K-Ak/PKB system has been suggested, and both gases play a fundamental role in
regulating [Ca\(^{2+}\)], in pancreatic acinar cells (Fig. 10).

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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; A.M. and Y.H. analyzed the data,
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; Y.H. organized the research; A.M. and Y.H. are equally responsible for conception and design of the research.

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**FIGURE CAPTIONS**

**Fig. 1.** CORM-2 dose-dependently increases \([\text{Ca}^{2+}]_i\) in rat pancreatic acinar cells. (A-D)

Typical examples of temporal changes in fluo-3 F.I. induced by increasing concentrations of CORM-2 (0.1-1 mM) in the presence of extracellular \( \text{Ca}^{2+} \). Changes in F.I. are shown by the pre-stimulation percentage, as described in the MATERIALS AND METHODS. (E) Summarized dose-response relationship of F.I. increase induced by CORM-2. Columns represent SFC (± SE) \((n = 10-29)\). \( P < 0.001 \) by ANOVA. (F) Translucent and pseudo-color images of acini used in the experiment treated with 0.5 mM CORM-2. ROI analyzed for temporal changes of F.I. is indicated in white oval in a. All images except for the translucent image \((a \text{ of } F)\) were obtained from time points shown in \((C)\) by black dots with respective letters \((b-h)\). Warm colors in the look-up-table indicate higher and cold colors indicate lower \([\text{Ca}^{2+}]_i\). Scale bar: 20 \( \mu \text{m} \).
Fig. 2. A heme oxygenase inducer, hemin, also increases \([Ca^{2+}]_i\) in a dose-dependent manner and its effect is blocked by HO inhibitor, ZnPPIX. (A) Temporal changes in F.I. after treatment with increasing concentrations of hemin (0.01-0.5 mM) in the presence of extracellular Ca\(^{2+}\). (B) Summarized dose-response relationship of the hemin-induced increase in F.I. Columns represent SFC (± SE) (\(n = 22-47\)). \(P < 0.001\) by ANOVA. (C) Increases in 0.1 mM hemin-induced SFC (± SE) in the presence (\(n = 38\)) and absence (\(n = 47\)) of 10 \(\mu\)M ZnPPIX. \(* P < 0.05\), significant difference for hemin alone vs. hemin + ZnPPIX by unpaired Student’s \(t\)-test.

Fig. 3. Removal of extracellular Ca\(^{2+}\) does not markedly affect CORM-2-induced \([Ca^{2+}]_i\) increase. (A) A typical oscillatory change in F.I. in an acinar cell treated with 0.4 mM CORM-2 in the absence of extracellular Ca\(^{2+}\). (B) Dose-response relationship of CORM-2-induced changes in SFC (± SE) in the presence (closed squares, \(n = 10-29\)) and absence (opened squares, \(n = 8-25\)) of extracellular Ca\(^{2+}\). \(* P < 0.05\), significant difference for 0.5 mM CORM-2-induced SFC increase in the presence vs. absence of extracellular Ca\(^{2+}\) by unpaired Student’s \(t\)-test.

Fig. 4. CORM-2-induced \([Ca^{2+}]_i\) increase is inhibited by PLC, IP\(_3\)R and G-protein inhibitors. (A) Temporal changes in F.I. induced by 0.1 mM CORM-2 in the absence and presence of 2 \(\mu\)M U73122 (dashed line) or 3 \(\mu\)M xestospongin C (dotted line) in the absence of extracellular Ca\(^{2+}\). (B) Summarized SFC (± SE) of 0.1 mM CORM-2-induced F.I. changes in the absence (\(n = 42\)) and presence of U73122 (\(n = 25\)) or xestospongin C (\(n = 32\)). (C) An example of temporal change in F.I. induced by 0.1 mM CORM-2 in the absence (solid line) and presence (dotted line) of 10 \(\mu\)M GP2A with no added extracellular Ca\(^{2+}\). (D) Summarized SFC (± SE) of 0.1 mM CORM-2-induced F.I. increase in the absence (\(n = 15\)) and presence (\(n = 22\)) of 10 \(\mu\)M GP2A. \(* P < 0.05\), significant difference for 0.1 mM CORM-2-induced
SFC increase in the presence vs. absence of U73122, xestospongin C or GP2A by unpaired Student’s t-test.

**Fig. 5.** CORM-2 stimulates NO production in a dose-dependent fashion and immunohistochemical detection of eNOS expression. (A) Temporal changes of DAF-2T F.I. in acinar cells treated with increasing concentrations of CORM-2 (0.1-1 mM) in the presence of extracellular Ca\(^{2+}\). (B) Summarized dose-response relationship of SFC (± SE) of DAF-2T F.I. \((n = 10-43)\). \(P < 0.001\) by ANOVA. (C) Summarized dose-response relationship of SFC (± SE) of hemin-induced DAF-2T F.I. \((n = 20-25)\). \(P < 0.001\) by ANOVA. (D) Summarized results of the percentage of eNOS immunoreactive nuclei in hemin-untreated control and hemin-treated pancreata averaged from eight randomly selected fields. (E) Immunohistochemical detection of eNOS localization in hemin-untreated \((b, e, h, k)\) and hemin-treated \((c, f, l, l)\) pancreata. Photographs \((a, d, g, j)\) are negative controls. Arrows; nuclei of acinar cell, arrowheads; nuclei of centroacinar cell, asterisks; nuclei of intercalated ductal cell, and tailed arrows; nuclei of endothelium of blood vessels. eNOS expression \((e\) and \(f\), green) and nuclei \((h\) and \(i\), originally blue but changed to red for clarity) are merged \((k\) and \(l)\). Scale bar: 50 µm. ** \(P < 0.01\), *** \(P < 0.001\), significant difference for hemin-untreated control vs. hemin-treated pancreata by unpaired Student’s t-test.

**Fig. 6.** Extracellular Ca\(^{2+}\) removal, inhibitors of NOS, CaM, PI3K and Akt/PKB diminish CORM-2-induced NO production but Mn-TBAP does not. (A) Examples of temporal changes in DAF-2T F.I. in acinar cells treated with 0.1 mM CORM-2 alone (solid black line), or in combination with different types of inhibitors. (B) Summarized SFC (± SE) induced by 0.1 mM CORM-2 in the absence of extracellular Ca\(^{2+}\) \((n = 37)\), or the presence of 100 µM L-NMMA \((n = 47)\), 10 µM SKF77171A \((n = 27)\), 20 µM LY29004 \((n = 22)\), 10 µM SH-5 \((n = 43)\) or 50 µM Mn-TBAP \((n= 21)\). (C) Effect of 50 µM Mn-TBAP on 100 µM hemin-induced increase in SFC. ** \(P < 0.01\), *** \(P < 0.001\), significant difference for
CORM-2 alone vs. CORM-2 + Ca\(^{2+}\)-free, CORM-2 + L-NMMA, CORM-2 + SKF7171A, CORM-2 + LY29004 or CORM-2 + SH-5 or hemin vs. hemin + Mn-TBAP by unpaired Student’s \(t\)-test.

**Fig. 7. Lack of an inhibitory effect of NOS inhibitor and NO scavenger on CORM-2-induced \([\text{Ca}^{2+}]_i\) increase.** (A) Typical example of temporal changes in fluo-3 F.I. induced by 0.1 mM CORM-2 alone (solid line), or in the presence of 100 µM L-NMMA (dotted line) or 300 µM cPTIO (dashed line) with no added extracellular Ca\(^{2+}\). (B) Summarized SFC (± SE) induced by 0.1 mM CORM-2 alone (\(n = 13\)), or CROM-2 plus L-NMMA (\(n = 23\)) or cPTIO (\(n = 25\)).

**Fig. 8. Lack of inhibitory effect of sGC inhibitor on CORM-2-induced \([\text{Ca}^{2+}]_i\) increase.** (A) Typical example of temporal changes in fluo-3 F.I. induced by 0.1 mM CORM-2 in the absence and presence of 100 (dotted line) or 300 µM (dashed line) ODQ without added extracellular Ca\(^{2+}\). (B) Summarized SFC (± SE) of CORM-2-induced increase in fluo-3 F.I. in the absence (\(n = 42\)) and presence of 100 (\(n = 17\)) or 300 mM (\(n = 32\)) ODQ.

**Fig. 9. CO inhibits CCK-8- or CCh-induced amylase release.** (A) Dose-response relationship of amylase release from pancreatic acini treated with increasing concentrations of CORM-2 (0.1-1 mM, \(n = 3\), respectively) for 30 min. \(P < 0.001\) by ANOVA. (B) Dose-response relationship of CCK-8-induced amylase release in the absence (filled squares) or presence (open squares) of 0.5 mM CORM-2 (\(n = 3\), respectively). (C) Dose-response relationship of CCh-induced amylase release in the absence (filled circles) or presence (open circles) of 0.5 mM CORM-2 (\(n = 3\), respectively). * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\), significant difference for CCK-8 or CCh alone vs. CCK-8 + CORM-2 or CCh + CORM-2 by unpaired Student’s \(t\)-test.
Fig. 10. Schematic diagram of putative intracellular mechanism triggered by CO with special reference to [Ca$^{2+}$]$_i$ homeostasis and possible cross-talk with NO in pancreatic acinar cells. Blockade by inhibitors used in the current study are described by dotted line.