Intracellular Calcium Release and Protein Kinase C Activation Stimulate Sonic Hedgehog Gene Expression During Gastric Acid Secretion

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BACKGROUND & AIMS: Hypochlorhydria during *Helicobacter pylori* infection inhibits gastric Sonic Hedgehog (Shh) expression. We investigated whether acid-secretory mechanisms regulate Shh gene expression through intracellular calcium ($Ca2^{+}_{i}$)-dependent protein kinase C (PKC) or cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) activation. **METHODS:** We blocked Hedgehog signaling by transgenically overexpressing a secreted form of the Hedgehog interacting protein-1, a natural inhibitor of hedgehog ligands, which induced hypochlorhydria. Gadolinium, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) + 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), PKC-overexpressing adenoviruses, and PKC inhibitors were used to modulate Ca^{2+}_{i}-release, PKC activity, and Shh gene expression in primary gastric cell, organ, and AGS cell line cultures. PKA hyperactivity was induced in the H^{+}/K^{+}-β-cholera-toxin–overexpressing mice. **RESULTS:** Mice that expressed secreted hedgehog-interacting protein-1 had lower levels of gastric acid (hypochlorhydria), reduced production of somatostatin, and increased gastrin gene expression. Hypochlorhydria in these mice repressed Shh gene expression, similar to the levels obtained with omeprazole treatment of wild-type mice. However, Shh expression also was repressed in the hyperchlorhydric H^{+}/K^{+}-β-cholera-toxin model with increased cAMP, suggesting that the regulation of Shh was not solely acid-dependent, but pertained to specific acid-stimulatory signaling pathways. Based on previous reports that Ca^{2+}_{i} release also stimulates acid secretion in parietal cells, we showed that gadolinium-, thapsigargin-, and carbachol-mediated release of Ca^{2+}_{i} induced Shh expression. Ca^{2+}-chelation with BAPTA + EGTA reduced Shh expression. Overexpression of PKC-α, -β, and -δ (but not PKC-ε) induced an Shh gene expression. In addition, phorbol esters induced a Shh-regulated reporter gene. **CONCLUSIONS: Secretagogues that stimulate gastric acid secretion induce Shh gene expression through increased Ca^{2+}_{i}-release and PKC activation. Shh might be the ligand transducing changes in gastric acidity to the regulation of G-cell secretion of gastrin.**

Keywords: Somatostatin; Gastrin; Hedgehog Interacting Protein; Chelation; Stomach.

Sonic Hedgehog (Shh) is expressed by gastric parietal cells,[1–3] and correlates with several mechanisms related to parietal cell acid secretion. First, Shh stimulates H^{+}/K^{+}-adenosine triphosphatase (H^{+}/K^{+}-ATPase) gene expression and subsequently enhances histamine-stimulated acid secretion by parietal cells.[4] Second, Shh protein co-localizes with H^{+}/K^{+}-ATPase.[3] Third, gastric acid induces processing of the Shh 45-kilodalton precursor form to the 19-kilodalton biologically active form.[5] Fourth, blocking gastric acid secretion with a proton pump inhibitor inhibits Shh gene expression.[2,6] The latter observation is relevant to the observation that *Helicobacter pylori* infection down-regulates Shh gene expression apparently through its ability to induce proinflammatory cytokines, which inhibit acid secretion.[6] Parietal cell Shh production is important because tissue-specific deletion of Shh using the Cre-Lox system induces foveolar hyperplasia,[7] which is reminiscent of the premalignant changes induced by *H pylori*. Nevertheless, the mechanism by which gastric acid regulates Shh is not well understood.

In gastric cells, the process of acid secretion is associated with protein kinase A (PKA) and protein kinase C (PKC) signaling.[8] Although the increase in cyclic adenosine monophosphate (cAMP) levels and activation of PKA pertain to histaminic stimulation,[8] both cholinergic (acetylcholine) and hormonal (gastrin) stimulation of acid

Abbreviations used in this paper: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; $Ca^{2+}_{(i)}$, intracellular calcium; Ctox, H^{+}/K^{+}-β-cholera-toxin–overexpressing; DAG, diacylglycerol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Gd^{3+}, gadolinium; Gli-1, glioma-associated oncogene-1; Hh, hedgehog; PCR, polymerase chain reaction; PKC, protein kinase C; Ptch-1, Patched-1; Shh, Sonic Hedgehog; sHip-1, secreted hedgehog-interacting protein 1; TPA, phorbol-12-myristate-13-acetate.

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secretion increase intracellular calcium ($Ca^{2+}{}_i$) in parietal cells.[9,10] The release of $Ca^{2+}{}_i$ activates several signal transduction pathways including Ca^{2+}-dependent PKC isoforms, α and β.[11] Although the effects of $Ca^{2+}{}_i$ on Shh expression have not been investigated previously, Ca^{2+} chelation inhibits Indian hedgehog gene expression in chick chondrocytes.[12] Furthermore, in the chick wing bud, PKC sustains Shh gene expression.[13] Collectively, these studies suggest that calcium-dependent PKCs, when activated, transduce increased $Ca^{2+}{}_i$ levels to downstream targets.

Previously, we reported that Shh enhances acid secretion in gastric parietal cells by increasing H^+/K^+-ATPase gene expression thereby increasing enzyme content.[4] In this report, regulation of Shh gene expression was examined in vivo and in vitro. Transgenic overexpression of an inhibitor of Hedgehog (Hh) ligands called hedgehog-interacting protein-1 (Hip-1) from parietal cells resulted in hypochlorhydric mice. This permitted us to examine the effect of low gastric acidity on the Shh gene locus (Shh gene expression). Because Shh gene expression was reduced, we hypothesized that the change occurred in response to the hypochlorhydria. Knowing that PKC regulates Shh gene expression during development, we tested the hypothesis that hypochlorhydria reduces $Ca^{2+}{}_i$ and PKC activation, ultimately inhibiting Shh gene expression.

BASIC–ALIMENTARY TRACT

Materials and Methods

Generation of Transgenic Mice

The Hip-1 complementary DNA (cDNA) lacking the transmembrane domain (sHip-1)[14] was subcloned downstream of the H^+/K^+-ATPase β-subunit promoter.[15] The H^+/K^+-ATPase–Hip transgene was injected into fertilized eggs obtained by mating (C57BL/6 X SJL)F1 (UM Transgenic Core, Ann Arbor, MI). Transgenic founders were bred to C57BL/6 mice. H^+/K^+-β-cholera-toxin–overexpressing (Ctox) mice (line 7) were generated as described previously.[15]

Omeprazole Treatment

Omeprazole (Sigma-Aldrich, St Louis, MO) stock (80 μmol/mL) was prepared in a 9:1 solution of dimethyl sulfoxide:polyethylene glycol 400 (Fluka, Sigma-Aldrich). Intraperitoneal injections of omeprazole (400 μmol/kg) were administered once daily for 3 consecutive days.

Gastric Acidity

After opening the stomach along the greater curvature, the gastric contents were collected in 1.5 mL of 0.9% NaCl. The samples were centrifuged to collect a clear supernatant. The hydrogen ion concentration was determined by titrating with 0.005 N sodium hydroxide using the PHM290 pH-Stat Controller titration system (Radiometer, Cleveland, OH).

Western Blot Analysis

Western blotting was performed according to previously published conditions[5] using a 1:200 dilution of goat polyclonal anti-Shh (sc-1194; Santa Cruz Biotechnology, Santa Cruz, CA), 1:500 GAPDH antibody (Molecular Probes, Invitrogen, Carlsbad, CA), and 1:1000 phospho-PKCα/β_{II} (Thr638/641) antibody (9375; Cell Signaling, Boston, MA).

Results

Hypochlorhydria in sHip-1–Expressing Mice

Prior studies of primary parietal cell cultures showed a role for Shh in H^+/K^+-ATPase gene expression, implicating an indirect effect of Shh on gastric acid production.[4] Indeed, we recently showed that a proinflammatory cytokine inhibits both gastric acid and Shh gene expression.[6] Therefore, to understand the relationship between gastric acidity and Shh expression in vivo, we generated a transgenic mouse expressing the natural inhibitor of Shh, called *Hip-1*. The secreted form of Hip-1 (sHip-1), which lacks the transmembrane domain, inhibits Hh signaling in the intestine by binding Hh ligands.[14] We therefore expressed sHip-1 from the H^+/K^+-ATPase-β subunit promoter to block Hh signaling in the stomach (Figure 1*A*). To confirm expression of the sHip-1 transgene in the stomachs of 2-month-old mice, Hip-1 messenger RNA (mRNA) expression in nontransgenic mice was compared with transgenic littermates by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Hip-1 protein was verified by Western blotting. sHip-1 mice showed a 40-fold increase in Hip-1 mRNA (Figure 1*B*), and a marked increase in Hip-1 protein (Figure 1*C*). There was only a slight reduction in size (2.4 kilodaltons) of the sHip-1 transgene compared with endogenous Hip-1 because the deleted transmembrane domain is only 22 amino acids.[14] The increase in Hip-1 corresponded to a significant reduction in Hh-target gene expression (glioma-associated oncogene-1 [Gli-1] and Patched-1 [Ptch-1]), showing efficient suppression of hedgehog signaling (Figure 1*D*), and was consistent among 3 different founder lines (Supplementary Figure 1*A–C*).

Previous reports have shown that Hh signaling induces H^+/K^+-ATPase gene expression and enhances histamine-stimulated acid secretion in primary canine parietal cell cultures.[4] Therefore, we examined whether H^+/K^+-ATPase gene expression was affected by the overproduction of sHip-1. Indeed, H^+/K^+-ATPase-β subunit mRNA and protein were reduced significantly in 3 founder lines (Figure 2*A* and *C*, and Supplementary Figure 2), despite the normal morphologic distribution of parietal cells observed with H&E staining (Figure 2*B*, *insert*). This correlated with reduced levels of gastric acid in 3 founder lines (Figure 2*B*, and Supplementary Figure 1*D*). In contrast, H^+/K^+-ATPase-α subunit ex-