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Stomach gastrin is regulated by sodium via PPAR-α and dopamine D_1 receptor

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Abstract

Gastrin, secreted by stomach G cells in response to ingested sodium, stimulates the renal cholecystokinin B receptor (CCKBR) to increase renal sodium excretion. It is not known how dietary sodium, independent of food, can increase gastrin secretion in human G cells. However, fenofibrate (FFB), a peroxisome proliferator-activated receptor-α (PPAR-α) agonist, increases gastrin secretion in rodents and several human gastrin-secreting cells, via a gastrin transcriptional promoter. We tested the following hypotheses: (1.) the sodium sensor in G cells plays a critical role in the sodium-mediated increase in gastrin expression/secretion, and (2.) dopamine, via the D_1R and PPAR-α, is involved. Intact human stomach antrum and G cells were compared with human gastrin-secreting gastric and ovarian adenocarcinoma cells. When extra- or intracellular sodium was increased in human antrum, human G cells, and adenocarcinoma cells, gastrin mRNA and protein expression/secretion were increased. In human G cells, the PPAR-α agonist FFB increased gastrin protein expression that was blocked by GW6471, a PPAR-α antagonist, and LE300, a D_1-like receptor antagonist. LE300 prevented the ability of FFB to increase gastrin protein expression in human G cells via the D_1R, because the D_5R, the other D_1-like receptor, is not expressed in human G cells. Human G cells also express tyrosine hydroxylase and DOPA decarboxylase, enzymes needed to synthesize dopamine. G cells in the stomach may be the sodium sensor that stimulates gastrin secretion, which enables the kidney to eliminate acutely an oral sodium load. Dopamine, via the D_1R, by interacting with PPAR-α, is involved in this process.

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Author contribution statement

P X, J J G, P A J, and R A F conceived and supervised the research and wrote the manuscript. P X, J J G, C Z, P K, D B W, and H T T conducted the experiments and analyzed the data. P A J and R A F provided the reagents. All authors read and approved the submission of this manuscript.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Supplementary materials

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Keywords

gastrin; dopamine D_1 receptor; PPAR-α; sodium; fenofibrate; SW626

Introduction

Hypertension develops when renal, neural, and hormonal mechanisms fail to enable the body to excrete the ingested sodium, and the sodium that accumulates in the body can no longer be sequestered (for example, in interstitium/skin); the extracellular fluid volume becomes expanded in salt-sensitive states (Feng *et al.* 2017, Wiig *et al.* 2018). However, an increase in sodium intake in normotensive individuals may not always increase blood pressure or total body water but causes a shift of the interstitial fluid into the intravascular (Heer *et al.* 2000) or non-extracellular compartment (Palacios *et al.* 2004). The sodium load has been shown to accumulate in the skin (Nikpey *et al.* 2017, Selvarajah *et al.* 2017). The ability of the kidney to increase sodium excretion following an oral salt load is enhanced by the increase in blood pressure associated with the increase in sodium intake, a phenomenon called pressure natriuresis (Granger *et al.* 2002, Hall *et al.* 2012). However, orally ingested sodium can be excreted acutely without an increase in blood pressure (Andersen *et al.* 2000, Preston *et al.* 2012). The mechanism(s) by which orally ingested sodium is acutely excreted is not well understood, and the presence of a gastrorenal axis is disputed (Preston *et al.* 2012). We have reported that a gastrorenal pathway exists in which gastrin secreted into the blood from the stomach, in response to ingested sodium, stimulates the gastrin receptor, also known as cholecystokinin B receptor (CCKBR), in the renal proximal tubule; CCKBR inhibits renal sodium transport (Chen *et al.* 2013, Jiang *et al.* 2016, 2017, Jose *et al.* 2016). The gastrorenal axis is abetted by the renal dopaminergic pathway because both the dopamine D_1-like receptors, D_1R and D_5R, physically interact with the renal CCKBR to inhibit sodium transport in renal proximal tubule (RPT) cells (Chen *et al.* 2013, Jiang *et al.* 2016). The natriuresis caused by gastrin or fenoldopam (agonist for both D_1R and D_5R) can be blocked by a CCKBR antagonist or a D_1R/D_5R antagonist in mice and rats (Chen *et al.* 2013, Jiang *et al.* 2016). Thus, gastrin, via the CCKBR, and D_1-like receptors (D_1R and D_5R) synergize to increase urinary sodium by inhibiting renal sodium transport (von Schrenck *et al.* 2000, Chen *et al.* 2013, Liu & Jose 2013, Jiang *et al.* 2016, 2017, Jose *et al.* 2016, Liu *et al.* 2016). The stomach also synthesizes dopamine (Vieira-Coelho & Soares-da-Silva 1993, Eisenhofer *et al.* 1997, Eldrup & Richter 2000) where the D_1R is also expressed (Vaughan *et al.* 2000, Wang *et al.* 2012, 2016), but whether dopamine receptors are expressed in G cells is not known.

Ciprofibrate, a peroxisome proliferator-activated receptor α (PPAR-α) agonist, given by oral gavage, increases gastrin secretion, associated with a doubling of G cell density in the stomach antrum of Sprague–Dawley rats (Martinsen *et al.* 2005). This did not occur in gastric-bypassed rats and PPAR-$\alpha^{-/-}$ mice (Martinsen *et al.* 2005). PPAR-α agonists have been reported also to increase progastrin production in human colorectal carcinoma cells (Lachal *et al.* 2004), but this mechanism has not been studied in normal human stomach G cells. The involvement of the PPAR-α pathway in gastrin secretion is supported by the association of PPAR-α with the gastrin transcriptional promoter by CHIP-SEQ (ENCODE

database). PPAR-α may also positively regulate the number of dopaminergic neurons in the substantia nigra (Gonzalez-Aparicio *et al.* 2011). However, it is not known if PPAR-α is involved in gastrin expression or secretion in humans. Therefore, the current study tested the novel hypothesis that D_1-like receptors, via PPAR-α, increase gastrin production in gastrin-producing cells.

In order to investigate the role of the dopaminergic system and PPAR-α in gastrin expression/secretion in humans, we generated stable cell lines of normal stomach antrum gastrin-secreting cells, that is, G cells. For concurrent controls, we compared these new human G cell lines with established human gastrin-secreting cell lines, that is, human gastric adenocarcinoma (AGS) (Ofori-Darko *et al.* 2000, Datta De *et al.* 2011) and ovarian adenocarcinoma cells (SW626) (Kim *et al.* 1998).

Materials and methods

Cell culture of human gastrin-secreting cells

Human gastrin-secreting carcinoma cell lines (gastric adenocarcinoma AGS (Ofori-Darko *et al.* 2000, Datta De *et al.* 2011) and ovarian adenocarcinoma SW626 (Kim *et al.* 1998) were purchased from ATCC. They were cultured in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 4.5 g/L glucose, 10% FBS, and 1× penicillin/streptomycin (Invitrogen). Human G cells were isolated from human stomach in our laboratory. All cells were grown in humidified conditions at 37°C, 95% air, and 5% CO_2. The cells were grown in standard T flasks, split when 80% confluent, and passaged into T flasks or directly into microplates.

Preparation of human stomach antrum for immunohistochemistry

Human stomach tissue was obtained, after institutional review board-approved informed consent, from patients undergoing gastrectomy. The tissue was immediately placed on ice and transported to the laboratory. Some tissues were snap-frozen and sectioned (10 μm) on a cryostat for immunohistochemistry.

Culture of human stomach G cells

Human G cells were isolated using the protocol described by Lambrecht *et al.* (2005, 2006) and Kidd *et al.* (2009) with modifications. Fresh human stomach antrum was obtained from the Biorepository and Tissue Research Facility, The University of Virginia. The outside layer of the stomach wall (serosa) was removed. The remaining stomach wall was purse-string-sutured to make a bag, inside-out with the gastric rugae (mucosa) facing outside. The stomach bag was digested using a series of solutions, as reported by Lambrecht *et al.* (2005, 2006) and Kidd *et al.* (2009). Then, the cells were resuspended in 4 mL DMEM-F12, containing 10% FBS, 1× sodium pyruvate, 1× MEM NEAA, 10 μM SCH28080 to inhibit H^+/K^+ ATPase activity, and 100 mg/mL DTT and transferred into a 6-well dish with 2 mL/well. The medium was changed every other day by removing 1 mL of the medium and adding 1 mL of fresh medium. After 1 week, the cells started to attach to the bottom of the growth dish. The cells were then immortalized by human Tert for 18 h (Gildea *et al.* 2013).

The immortalized G cells were cultured in the same medium with insulin, transferrin, and selenium (1×, ThermoFisher 41400045), in order to maintain normal growth rates.

Immunofluorescence

(a) Human stomach antrum: The serosa of the stomach antrum was removed. The stomach antrum without the serosa was cut into small pieces and treated under different conditions, as indicated below. The pieces of tissue were then rinsed by PBS, fixed, and sectioned following a previously published method (Gildea *et al.* 2015).

(b) Human G, SW626, and AGS cells: The cells were passaged onto a collagen-coated 96-well glass-bottom dish and cultured overnight. After different treatments, as indicated below, the cells were rinsed with PBS, fixed in 4% paraformaldehyde, and then treated with 0.2% Triton ×100 for 5 min. The cells were rinsed twice in PBS and incubated for 10 min with 100 mM Tris pH 7.4, followed by blocking with Odyssey Blocking buffer (Li-Cor, 927–40000) for 30 min. The cells were then stained with anti-goat gastrin antibody (Santa Cruz) or anti-rabbit antibodies to various targets (gastrin, DAKO 1:200), complex type carbohydrate residues marker *Phaseolus vulgaris*-leucoagglutinin (PHA-L, Vector Laboratories), D_1R (generated in our laboratory) (Sanada *et al.* 1999), D_5R (Santa Cruz, 1:100), tyrosine hydroxylase (TH, Cell Signaling, 1:200), and DOPA decarboxylase (DDC, Millipore, 1:200) for 2 h at room temperature. After incubation with the primary antibody, the cells were washed three times in PBST (PBS with Tween-20) and 30 min in TBST (TRIS-buffered saline with Tween-20) and then incubated for 1 h in secondary antibody (Alexa anti-rabbit 647). The cells were washed again in PBST and incubated in TBST containing Hoechst 33342 for 30 min. Finally, the cells were rinsed in PBS before imaging. The images were obtained using an Olympus IX81 automated multi-well spinning disk confocal microscope. D_1R and D_5R antibodies were also used in AGS (Ofori-Darko *et al.* 2000, Datta De *et al.* 2011) and SW626 (Kim *et al.* 1998) cells.

BMG PHERAstar FS was used to quantify (relative fluorescence units (RFU)) the immunofluorescent proteins in the cells. Gastrin RFU was normalized by DAPI RFU for each well. Six confocal images were taken for each intervention (different NaCl concentration) and normalized to control (no fluorescence). Then, the gastrin signal was measured by ImageJ and divided by the number of cells on each image. Finally, the fold-change of each condition, relative to 90 mM NaCl or VEH, was calculated.

Reverse transcription – polymerase chain reaction

mRNA was quantified using real-time quantitative reverse transcription-PCR (qRT-PCR) and the delta/delta method. The gene expression levels were normalized to the expression of α-actin and expressed as fold-change, relative to the expression in 90 mM NaCl- or VEH-treated cells. All PCR reactions were performed in 20 μL volume, using the iCycler CFX Connect (Biorad Laboratories, Inc, Berkeley, CA, USA). Each reaction contained 10 μL SYBR RT-PCR buffer, 10 μM of each primer, 40 μM iScript, and 200 nM cDNA. The amplification was initiated by 10-min incubation at 50°C, followed by two-step amplification of 5 min at 95°C and 30 s at 95°C for 40 cycles. RNA was extracted by the

Zymo Quick-RNA mini-prep, and cDNA was prepared using SuperScript® III First Strand Synthesis Kit.

Primers: Actin sense 5′-AGAAAATCTGGCACCACA CC-3′; Actin anti-sense 5′-CTCCTTAATGTCACGCAC GA-3′; Gastrin sense 5′-ATGCAGCGA CTATGTGTG-3′; Gastrin anti-sense 5′-GTTCTCATCCTCAGCACTG-3′; PPAR-α sense 5′-CTATCATTTGCTGTGGAGATCG-3′; and PPAR-α sense 5′-AAGATATCGTCCGGGTGGTT-3′ (Chandran *et al.* 2016).

The human stomach antrum, isolated human G cells, AGS (Ofori-Darko *et al.* 2000, Datta De *et al.* 2011), and SW626 (Kim *et al.* 1998) were treated with the media containing different sodium concentrations for 4 h and then fixed for immunoblotting and gastrin immunofluorescence staining. For the qRT-PCR studies of the isolated human G cells cultured in the 96-well plates, the duration of the treatment with the different sodium concentrations was for 30 min.

Effect of increased extracellular sodium concentration on gastrin expression and secretion

Preparation of incubation media with different sodium concentrations—Since ingestion of a meal with high NaCl concentration increases sodium excretion that is related to gastrin (Tarasova *et al.* 1996, Wang *et al.* 2017), we determined if increasing the sodium concentration in the incubation medium of human G cells would increase gastrin expression. Low and high sodium solutions were prepared according to Quadri's method (Quadri & Siragy 2016), with some modification. We added 35 mM NaCl to DMEM/F12 (Invitrogen, 135 mM, sodium) to increase the sodium concentration to 170 mM, and 8 or 10 mM NaCl was added to DMEM/F12 to make a 143 or 145 mM sodium buffer and the osmolality was adjusted to be the same as the 170 mM sodium buffer by adding 0.5 M mannitol. The osmolality of the low salt buffer (90 mM Na^+) was also adjusted with mannitol to the same osmolality as the 170 mM sodium buffer. The G cells were plated on 96-well plates and incubated for 4 h or 30 min, as indicated, with media containing varying sodium concentrations.

Measurement of the effect of by the PPAR-α agonist FFB on gastrin expression or secretion in AGS, SW626, and human G cells and stomach antrum—AGS (Ofori-Darko *et al.* 2000, Datta De *et al.* 2011) and SW626 (Kim *et al.* 1998) cells secrete progastrin/gastrin, but it is not known if their ability to express or secrete gastrin can be regulated by PPAR-α. However, PPAR-α has been reported to regulate the secretion of gastrin in colorectal carcinoma cells (Lachal *et al.* 2004) and mouse and rat stomach (Martinsen *et al.* 2005). Therefore, in SW626 (Kim *et al.* 1998), stomach antrum, and isolated human G cells, we studied the effect of the PPAR-α agonist FFB (5 μM/30 min, Sigma-Aldrich) (Lachal *et al.* 2004, Martinsen *et al.* 2005, Gonzalez-Aparicio *et al.* 2011), and the PPAR-α antagonist GW6471 (GW) (5 μM/30 min, Sigma-Aldrich) (Florio *et al.* 2017) alone, or in combination. All cells were washed twice with PBS prior to stimulation. Some of these cells were used to measure the level of gastrin transcription by RT-PCR. Some cells were grown in 96-well glass bottom plates to measure gastrin protein levels.

Role of D_1-like receptors in the regulation of gastrin expression/secretion—To determine the role of D_1-like receptors on gastrin secretion, we studied the effect of the D_1-like receptor antagonist LE300 (Sigma-Aldrich) (Kassack *et al.* 2002) on FFB-stimulated gastrin secretion in SW626 and isolated human G cells. The cells were treated with FFB (5 μM) alone, LE300 (10 μM) alone, or in combination for 30 min. The interaction between FFB and LE300 was also tested in isolated human gastrin antrum but with the incubation period increased to 1 h. The cells and tissues were then fixed, 10 μm-frozen sections were cut, and staining was performed, as described earlier.

In additional studies, the role of D_1-like receptors in the sodium-mediated increase in gastrin mRNA was studied in SW626 cells. The cells were treated with the D_1-like receptor agonist, SKF38393 (10 μM/24 h, Sigma-Aldrich) (Glavin & Hall 1995, Kassack *et al.* 2002), monensin (1 μM/24 h, Sigma-Aldrich) (Aowicki & Huczyński 2013), and the combination of monensin and the D_1-like receptor antagonist LE300 (10 μM/24 h) (Kassack *et al.* 2002). Gastrin mRNA was measured by qRT-PCR, as described previously.

Statistical analysis

The data are expressed as mean ± S.E. Comparisons were made by Student's *t*-test for a two-group comparison or one-way ANOVA for multiple comparisons (groups >2), followed by Tukey post-hoc test. $P < 0.05$ was considered significant.

Results

Human stomach antrum and G cell immunochemistry for gastrin and PHA-L

Gastrin (red) and phytohemagglutinin-leucoagglutinin (PHA-L) (Lueth *et al.* 2005) (specific for complex-type carbohydrate residues which are expressed in gastric parietal cells, green) are observed in fresh human stomach (antrum section) (Fig. 1A). Gastrin and PHA-L colocalize in some cells, indicating that antrum parietal cells produce gastrin as expected (Martinsen *et al.* 2005). However, the green and red fluorescence do not completely overlap (yellow) in most cells but appear polarized on opposite sides of the G cells. The merged image shows that there are cells within the antrum crypts that do not stain for either PHA-L or gastrin.

Human G cell and ovarian adenocarcinoma SW626 cell immunochemistry for gastrin and PHA-L

Immunohistochemistry for gastrin (red) and PHA-L (green) was performed in a G cell line derived from human stomach, as well as a cell line that is known to secrete gastrin (ovarian adenocarcinoma SW626) (Kim *et al.* 1998) (Fig. 1B). In stomach G cells, both gastrin and PHA-L show a punctate staining pattern. Merging the gastrin and PHA-L images shows partial colocalization of gastrin and PHA-L (yellow punctate pattern). The SW626 cells also show positive gastrin staining (red). The negative control, using non-specific rabbit IgG antibody, shows almost no staining in G cells and SW626 cells. Cell nuclei are stained blue with DAPI, except in the PHA-L images.

Gastrin mRNA in G cells and ovarian adenocarcinoma SW626 cells

RT-PCR demonstrated the presence of gastrin mRNA (actin served as a control) in human stomach G cells and gastrin-secreting ovarian adenocarcinoma SW626 cells (Kim *et al.* 1998). Human G and SW626 cells express the appropriate molecular size of gastrin mRNA, ~300 base pairs expected from the primers used (Supplementary Fig. 1, see section on supplementary materials given at the end of this article).

Effect of NaCl on gastrin protein expression in gastric carcinoma AGS and ovarian adenocarcinoma SW626 cells

In addition to SW626 cells (Kim *et al.* 1998), there are other commercially available cell lines of intestinal origin that secrete gastrin, for example, gastric carcinoma (AGS) and human colon cancer cells (Carroll *et al.* 2000, Ofori-Darko *et al.* 2000, Datta De *et al.* 2011). The effect of two different extracellular concentrations of sodium as NaCl (135 mM and 170 mM/4 h, osmolality kept at 340 mosolm/L with mannitol as needed) on gastrin immunofluorescence was studied in AGS and SW626 cells (Supplementary Fig. 2). Gastrin protein expression is increased in both AGS and SW626 cells incubated with 170 mM sodium, relative to 135 mM sodium (as NaCl) ($P < 0.01$, $n = 6$, one-way ANOVA, Tukey test).

Effect of NaCl on gastrin expression in isolated human G cells

We also examined the effect of increasing extracellular sodium concentration on gastrin expression in normal human stomach G cells (Fig. 2). The G cells were plated on 96-well plates and incubated for 4 h with media containing varying sodium concentrations, with the osmolality kept at 340 mosmol/L, using mannitol as needed. The highest sodium concentration (170 mM) increases gastrin protein expression in G cells (Fig. 2A). The individual data points from four independent G cell lines, each studied in triplicate, are shown in Fig. 2B. An increase in extracellular sodium also increases gastrin mRNA, but the increase occurs in both 145 mM and 170 mM NaCl (Fig. 2C). The highest sodium concentration, 170 mM, also increases PPAR-α mRNA expression (Fig. 2C). Similar to the increase in gastrin protein expression in G cells caused by 170 mM sodium, this sodium concentration also increases the secretion of gastrin into the incubation medium (Fig. 2D). G cells were also grown on alginate microcarrier beads, which enabled them to grow as an ion-transporting monolayer, in the polarized state (Global Cell Solutions, Inc.) (Justice *et al.* 2009, Gildea *et al.* 2015). The G cells in 3D culture (Supplementary Fig. 3A) have an increase in gastrin immunofluorescence when exposed for 4 h to 170 mM sodium (as NaCl), relative to 143 mM sodium (as NaCl) (Supplementary Fig. 3B and C), similar to G cells cultured in plates. We also found that gastrin mRNA is increased ($P < 0.05$, $n = 4$) in G cells incubated in media with 143 mM sodium (as NaCl, 286 mosmol/L), containing the ionophore monensin (MON, 1 μM/24 h) (Fig. 2E) (Efendiev *et al.* 2003, Gildea *et al.* 2015).

Gastrin and D_1R expression in human stomach antrum tissue and G cells

Dopamine, via D_1-like but not D_2-like receptors, can stimulate gastric acid secretion in the stomach (Tsai & Cheng 1995). The stomach and other segments of the gastrointestinal tract synthesize dopamine (Vieira-Coelho & Soares-da-Silva 1993, Eisenhofer *et al.* 1997, Mezey

et al. 1999, Eldrup & Richter 2000). The D_1R and D_5R are expressed in the stomach and other segments of the gastrointestinal tract (Vaughan *et al.* 2000, Wang *et al.* 2012). We now show that D_1R mRNA (Fig. 2C) is expressed in G cells which is increased by 145 and 170 mM NaCl, relative to 90 mM NaCl, similar to the effect on gastrin mRNA. D_1R protein (Fig. 3) is also expressed in human G cells in human stomach antrum. There is no D_5R expressed in human G cells or any cell in the human stomach antrum (Supplementary Fig. 4). Therefore, we determined if D_1R colocalizes with gastrin in human stomach antrum (Fig. 3A, B and C). As shown previously in Fig. 1A and B and in this other set of studies (Fig. 3A, red), gastrin is expressed in many, but not all, cells within the crypt of the stomach antrum. By contrast, D_1R (Fig. 3B, green) is expressed in all cells lining the lumen of the crypt. The merged image shows no overlap between gastrin and D_1R staining (Fig. 3C). D_1R is at the apical membrane (Fig. 3B and C) while gastrin is in the cytoplasm of G cells in the stomach antrum (Fig. 3A and C). The isolated human G cells demonstrate similar gastrin and D_1R staining patterns as in fresh stomach antrum, that is, punctate gastrin staining (Fig. 3D) in the cytoplasm and D_1R staining throughout the membrane (Fig. 3E). There is minimal colocalization of gastrin and D_1R staining (Fig. 3F). This could be taken to indicate that a cellular messenger produced in response to D_1R stimulation is needed to enable the D_1R to induce gastrin production. In contrast to the absence of D_5R expression in human G cells (Supplementary Fig. 4), both D_1R and D_5R are expressed in both AGS and SW626 cells (Supplementary Fig. 5). These data indicate the importance of using normal human G cells to study human G cell function – gastrin secretion in this instance.

Effect of PPAR-α agonist, fenofibrate (FFB), D_1-like receptor antagonist, LE300, and high NaCl concentration on gastrin expression in human stomach antrum

As aforementioned, PPAR-α associates with the gastrin promoter (CHIP-SEQ) and increases progastrin production in gastrin-secreting carcinoma cells (Lachal *et al.* 2004) and gastric antrum (Martinsen *et al.* 2005). In isolated human G cells, when PPAR-α expression is decreased by about 70% using PPAR-α-specific siRNA (Supplementary Fig. 6A), there is about a 40% decrease in gastrin mRNA expression ($P < 0.05$, $n = 4$) (Supplementary Fig. 6B). In the human stomach antrum, the D_1R-mediated gastrin synthesis may occur through PPAR-α, because the stimulatory effects of FFB (PPAR-α agonist, 5 μM/1 h) ($P < 0.05$, $n = 6$) (Fig. 4A) and high extracellular sodium concentration (170 mM/4 h) ($P < 0.05$, $n = 4$) (Fig. 4B) on gastrin protein tend to be decreased by the addition of LE300 (D_1R/D_5R receptor antagonist) (Kassack *et al.* 2002) (Fig. 4A and B). The immunohistochemistry (Fig. 4C), corresponding to the numerical data in Fig. 4A and B, shows that LE300 (10 μM/4 h) tends to block the gastrin immunofluorescence induced by FFB (5 μM/1 h) and 170 mM NaCl (4 h). Both FFB and 170 mM sodium also increase gastrin mRNA measured by real time RT-PCR. LE300 (10 μM/4 h) impairs the ability of 170 mM sodium (4 h) to increase gastrin mRNA in stomach antrum (Fig. 4D and E). However, as in the immunofluorescence data (Fig. 4A and B), LE300 (10 μM/4 h) (Fig. 4D and E), tends to, but does not, significantly impair the ability of the PPAR-α agonist FFB to increase gastrin mRNA. It must be noted that PPARα gene silencing decreases PPARα mRNA expression to a much greater extent than the decrease in gastrin mRNA expression (Supplementary Fig. 6). The D_1-like receptor antagonist LE300 only partially blocks the 170 mM sodium-induced gastrin mRNA (Fig. 4D and E) and only tends to block gastrin protein expression (Fig. 4B). These

findings indicate that sodium increases gastrin secretion by pathways in addition to the D_1R and PPARα. Preliminary studies suggest the involvement of D_1R-stimulated cAMP production in the D_1R-mediated stimulation of gastrin expression (Xu *et al.* 2019). However, it has to be kept in mind that these studies were performed in the stomach antrum in which counter regulatory pathways in cells other than G cells could have occurred (Tsai & Cheng 1995, Li *et al.* 2006, Eliassi *et al.* 2008). For example, the D_2R, in gastric myenteric neurons, can inhibit gastrin secretion (Li *et al.* 2006, Eliassi *et al.* 2008), thus the need to study D_1R and PPARα in isolated normal human G cells (see below).

Gastrin synthesis, D_1R, and the PPAR-α pathway in ovarian adenocarcinoma SW626 cells

Gastrin expression in SW626 cells was studied in the presence of a D_1-like receptor agonist (SKF-38393 (SKF)) (Kassack *et al.* 2002). SKF (10 μM/24 h) markedly increases the transcription of gastrin, relative to vehicle (VEH) treatment (Supplementary Fig. 7). Monensin (1 μM/24 h) (Efendiev *et al.* 2003, Gildea *et al.* 2015), an ionophore that increases intracellular sodium, also increases gastrin mRNA expression in SW626 cells that is completely blocked by LE300 (10 μM/24 h), a D_1-like receptor antagonist (Supplementary Fig. 7). It should be noted that monensin also increases gastrin RNA in isolated human G cells (Fig. 2E). We also determined if the monensin-induced increase in gastrin expression in SW626 cells could be related to PPAR-α. GW6471, a PPAR-α antagonist, by itself, reduces the expression of gastrin suggesting that PPAR-α constitutively stimulates basal gastrin synthesis in SW626 cells (Fig. 5A). The PPAR-α agonist FFB-mediated increase in gastrin expression is also blocked by the PPAR-α antagonist GW6741 (Fig. 5A). Monensin also slightly increases gastrin protein but is not blocked by GW6741 (Fig. 5A), which could be taken to indicate that intracellular sodium can increase gastrin secretion independent of PPAR-α, in gastrin tumor-secreting cells. As aforementioned, preliminary studies suggest the involvement of D_1R-stimulated cAMP production in the D_1R-mediated stimulation of gastrin expression (Xu *et al.* 2019).

Gastrin synthesis and the PPAR-α pathway in human G cells

We next determined the role of the PPAR-α pathway in the synthesis of gastrin in isolated normal human G cells by treating them with the PPAR-α agonist FFB, PPAR-α antagonist GW6471, or D_1-like receptor antagonist LE300 for 30 min (Fig. 5B). FFB (5 μM/30 min) increases gastrin protein ($P < 0.01$, $n = 6$) in isolated human G cells, similar to that observed in human stomach antrum (Fig. 4A) and ovarian adenocarcinoma SW626 cells (Fig. 5A). The PPAR-α antagonist GW6471 (5 μM/30 min) by itself has no effect, unlike that observed in ovarian adenocarcinoma SW626 cells. However, similar to those observed in ovarian adenocarcinoma SW626 cells, in isolated human G cells, GW6471 also completely blocks the increase in gastrin protein expression stimulated by FFB ($P < 0.05$, $n = 6$) (Fig. 5B). We also tested the effect of blocking the D_1R (D_5R is not expressed in G cells) with LE300 and found that LE300 (10 μM/30 min) by itself has no effect but completely blocks the FFB-induced stimulation of gastrin protein expression ($P < 0.05$, $n = 6$). These studies show some minor differences in gastrin secretion among intact G cells in human antrum, isolated human G cells, and gastrin-secreting carcinoma SW626 cells, bearing in mind that SW626 cells constitutively secrete gastrin while G cells need to be stimulated to secrete gastrin. There is also a minor difference between isolated G cells and intact G cells in the human stomach

antrum; in the human stomach antrum, LE300, the D_1-like receptor antagonist tends to block the stimulatory effect of FFB, the PPAR-α agonist, on gastrin protein expression but in isolated human G cells, LE300 completely blocks the stimulatory effect of FFB on gastrin secretion. As aforementioned, this could be related to counter regulatory mechanisms in intact human stomach antrum not found in isolated G cells; the D_2R, expressed in gastric myenteric neurons, can inhibit gastrin secretion (Li *et al.* 2006, Eliassi *et al.* 2008).

Expression of dopamine biosynthetic enzymes in G cells

As aforementioned, the stomach and other segments of the gastrointestinal tract can synthesize dopamine (Vieira-Coelho & Soares-da-Silva 1993, Eisenhofer *et al.* 1997, Mezey *et al.* 1999, Eldrup & Richter 2000). However, it is not known if G cells, per se, can synthesize dopamine. Therefore, we studied the expression of dopamine biosynthetic enzymes in human stomach antrum (Supplementary Fig. 8A) and cultured human G cells (Supplementary Fig. 8B). There is widespread tyrosine hydroxylase (TH) red fluorescent staining (Supplementary Fig. 8A) throughout the crypt cells in the stomach antrum. Gastrin and TH colocalize in G cells but not always in the same intracellular domain (Supplementary Fig. 8A, merge). These data prove the expression of the first enzyme, TH, in the synthesis of dopamine in human G cells. The gastrin and TH distribution in isolated G cells in culture (Supplementary Fig. 8B) is similar to that found in the stomach antrum (Supplementary Fig. 8A), that is, only in some cells do gastrin and TH colocalize.

We next examined the location of the enzyme responsible for the catalysis of dopamine from L-DOPA (aromatic L-amino acid decarboxylase (DOPA decarboxylase, DDC)) in human stomach antrum (Supplementary Fig. 9A) and isolated human G cells in culture (Supplementary Fig. 9B). As with TH, there is widespread DDC red fluorescent staining (Supplementary Fig. 9A) in most cells in the stomach antrum. Gastrin green fluorescent staining is similar to that in Supplementary Figs 8A and 9A. The merged image (Supplementary Fig. 9A) demonstrates that there is colocalization of gastrin and DDC in human stomach G cells but not always in the same intracellular domain, as is the case for TH (Supplementary Fig. 8A). Similar to those observed regarding the colocalization of gastrin and TH in G cells (Supplementary Fig. 8B), gastrin and DDC colocalization is also seen in some but not all G cells (Supplementary Fig. 9B).

Discussion

Sensing the amount of ingested sodium by the stomach and other segments of the gastrointestinal tract may be an important mechanism by which sodium balance is regulated (Michell *et al.* 2008, Haid *et al.* 2012, Furness *et al.* 2013, Jose *et al.* 2016, Yang *et al.* 2017). However, previous reports on gastrorenal communication have not dealt with the sodium sensor in the stomach. Therefore, we isolated G cells from the antrum of the human stomach (Kasacka & Majewski 2007, Takaishi *et al.* 2011, Haid *et al.* 2012) in order to determine the effect of sodium, independent of food, on gastrin secretion. Similar to rodents, we found that gastrin secretion, by human G cells, can be increased by sodium, independent of food (Survé & Håkanson 1998). Moreover, we found that increasing the intracellular sodium concentration, using the non-selective ionophore monensin, increases gastrin mRNA and

protein in isolated human G cells. We also found that an increase in extracellular or intracellular sodium concentration increases gastrin expression in human G cells and other gastrin-secreting cells, such as AGS (Ofori-Darko *et al.* 2000, Datta De *et al.* 2011) and SW626 (Kim *et al.* 1998) cells.

PPAR-α activation increases gastrin secretion, independent of gastric pH (Lachal *et al.* 2004, Martinsen *et al.* 2005). The incubation of human stomach antrum, isolated human G cells, and gastrin-secreting SW626 adenocarcinoma cells (Kim *et al.* 1998) with the PPAR-α agonist FFB increases gastrin expression. This effect can be blocked by the PPAR-α antagonist GW6471. Because the D_1R is expressed in G cells and its plasma membrane expression can be increased by an increase in intracellular sodium, at least in renal epithelial cells (Efendiev *et al.* 2003), we examined the interaction between the gastrin and dopaminergic systems. We found that SKF38393 (a D_1R/D_5R agonist) (Glavin & Hall 1995, Kassack *et al.* 2002) increases gastrin mRNA expression in human SW626 gastrin-producing carcinoma cells. The increase in gastrin mRNA expression caused by monensin, presumably due to an increase in intracellular sodium, in SW626 cells can be blocked by the D_1R/D_5R antagonist LE300 (Kassack *et al.* 2002). LE300 also completely blocks the ability of FFB to stimulate gastrin protein expression in isolated normal human G cells. Which D_1-like receptor, D_1R or D_5R, is involved in the dopaminergic stimulation of gastrin secretion was not determined in SW626 cells, but these cells express both D_1R and D_5R. By contrast, D_5R is not expressed in human G cells. Therefore, the dopaminergic stimulation of gastrin expression/secretion in human G cells is mediated by the D_1R, not the D_5R. Preliminary studies suggest that cAMP produced by D_1R may stimulate PPARα expression (Xu *et al.* 2019).

In the normal human stomach antrum, the D_1-like receptor antagonist LE300 tends to, but does not, significantly block the ability of high NaCl concentration or the PPAR-α agonist FFB to increase gastrin protein expression. This is in contrast to the ability of LE300 to block the stimulatory effect of high intracellular NaCl, induced by monensin in SW626 cells, or the PPAR-α agonist FFB in isolated human G cells. As aforementioned, in the stomach antrum, counter regulatory pathways in cells other than G cells could have occurred (Tsai & Cheng 1995, Li *et al.* 2006, Eliassi *et al.* 2008). For example, the D_2R, in gastric myenteric neurons, can inhibit gastrin secretion (Li *et al.* 2006, Eliassi *et al.* 2008).

Our present study suggests that dopamine, via D_1-like receptors, can acutely (30 min) stimulate gastrin secretion from gastrin-secreting cells. Dopamine can be synthesized by neuronal and non-neuronal cells in the gastrointestinal tract, including the stomach (Vieira-Coelho & Soaresda-Silva 1993, Eisenhofer *et al.* 1997, Mezey *et al.* 1999, Eldrup & Richter 2000, Zheng *et al.* 2014). However, the enzymes that are involved in dopamine synthesis have not been studied in G cells. We now report that human G cells express tyrosine hydroxylase, which converts tyrosine to L-DOPA and DOPA decarboxylase which converts L-DOPA to dopamine. An increase in salt intake increases jejunal dopamine (Lucas-Teixeira *et al.* 2000). However, it remains to be determined if an increase in ingested sodium increases dopamine synthesis in G cells.

In summary, exposure of human G cells to increased extracellular sodium concentration or monensin that presumably leads to an increase in intracellular sodium concentration stimulates gastrin expression/secretion. The presence of dopamine-synthesizing enzymes in G cells suggest that G cells can increase dopamine synthesis. Dopamine, via the D_5R, stimulates gastrin expression, in part, by interacting with PPAR-α (Fig. 6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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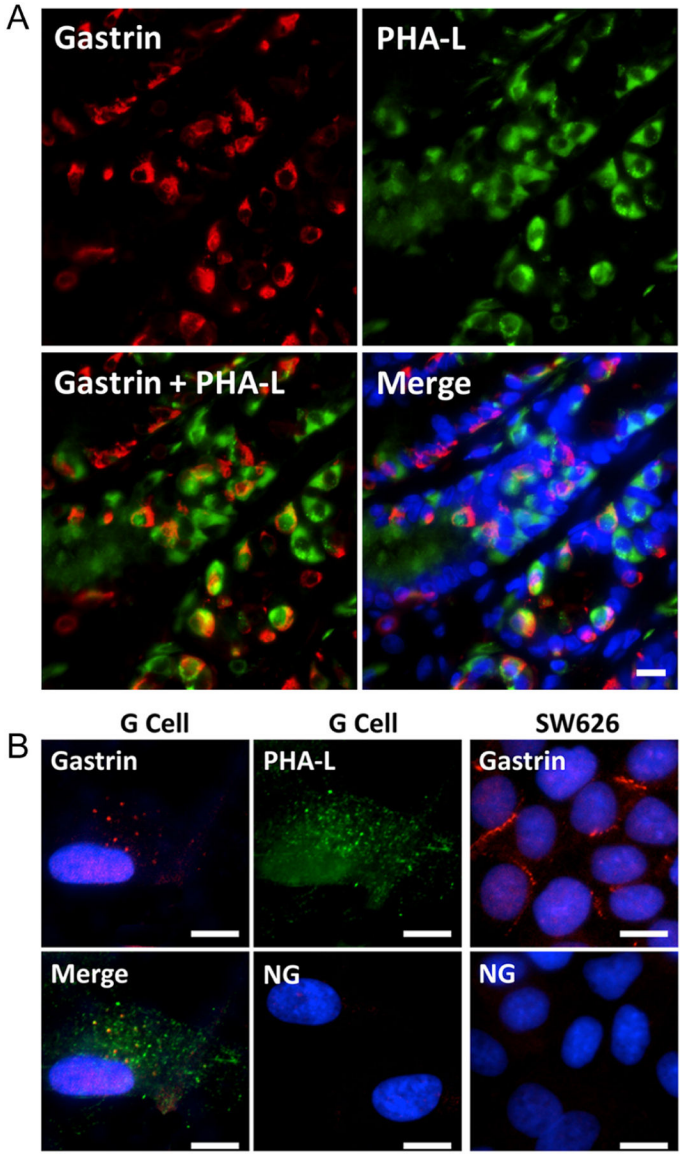

Figure 1.

Human stomach antrum and G cell immunochemistry for gastrin and PHA-L. (A) Gastrin, phytohemagglutinin-leucoagglutinin (PHA-L, marker of complex type carbohydrate residues which are expressed in gastric parietal cells), and nuclei (blue) in the antrum of fresh human stomach. Scale bar = 10 μm. (B) Gastrin and PHA-L staining in G cells isolated from human stomach and gastrin staining in SW626 cells (ovarian adenocarcinoma cells). G cell- and SW626 cell-control using non-specific rabbit IgG antibody show almost no staining. NG = negative control, non-specific rabbit IgG antibody. Scale bar = 10 μm.

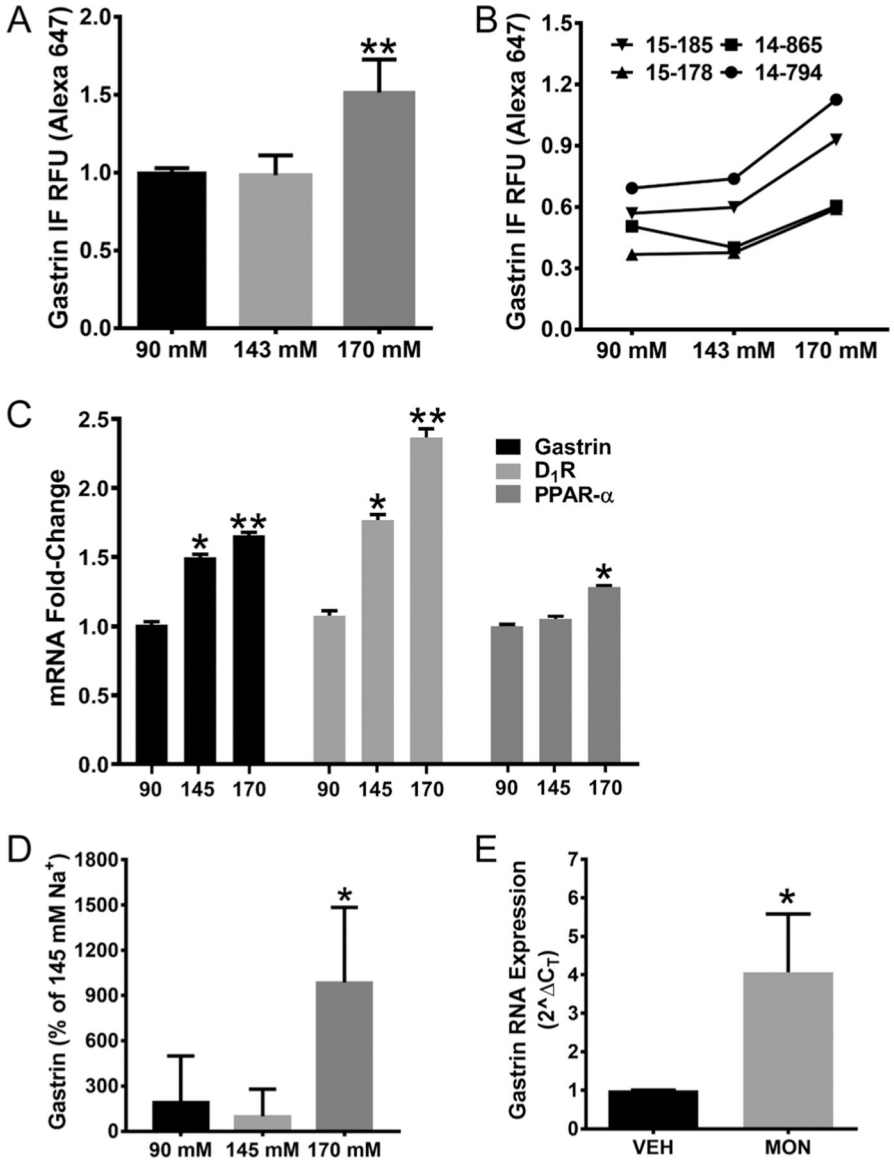

Figure 2.

Effect of NaCl on gastrin, D_1R, and PPAR-α expression in isolated human G cells. Isolated human G cells, cultured in 96-well cells, were incubated in media with sodium concentrations ranging from 90 to 170 mM NaCl/4 h; osmolality was kept at 340 mosmol/L with mannitol. (A and B) Group data and individual data points show an increase in gastrin expression in isolated human G cells incubated with 170 mM NaCl (** $P < 0.01$, vs 143 mM and others, one-way ANOVA, Tukey test, $n = 4$/group). BMG PHERAstar FS was used to quantify the immunofluorescence (IF) in G cells, expressed as relative fluorescence units (RFU). (C) A 30-min incubation of another set of isolated human G cells in varying amounts of sodium concentration also increases Gastrin, D_1R, and PPAR-α mRNA, quantified by qRT-PCR (*, ** $P < 0.01$, vs others in a particular group, one-way ANOVA, Tukey test, $n = 3$/group for Gastrin, $n = 4$/group for D_1R and PPAR-α). (D) Gastrin secreted by human G cells into the incubation medium is increased by the highest concentration of NaCl (170

mM/4 h) (*$P < 0.01$, vs others, one-way ANOVA, Tukey test, $n = 4$/group). (E) Increasing intracellular sodium with the ionophore monensin (MON, 1 μM/24 h) increases gastrin mRNA expression in human G cells (*$P < 0.05$, t-test, $n = 4$/group). All data (except the individual data points) are expressed as M ± S.E.

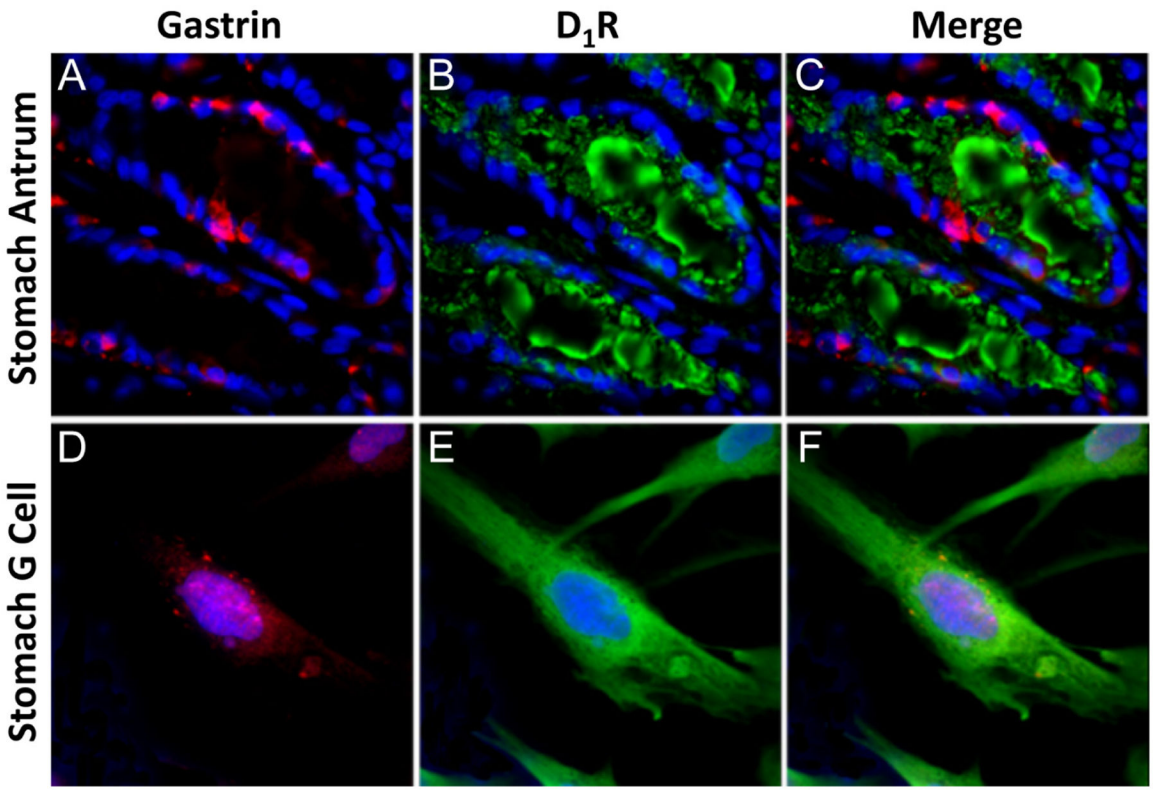

Figure 3.

Gastrin and D_1R expression in human stomach antrum and isolated human G cells. (A) Gastrin (red) is found in the cytoplasm of some cells within the crypts of the stomach antrum. (B) D_1R in green is expressed in all cells lining the lumen of the crypts. (C) Merged images of both gastrin and D_1R in stomach antrum. (D) Gastrin, stained red, is mainly in the cytoplasm of an isolated G cell. (E) D_1R green staining is throughout the cell membrane of isolated G cells. (F) Merged images of gastrin and D_1R in isolated G cells. Nucleus is stained with DAPI (blue). Scale bar = 10 μm.

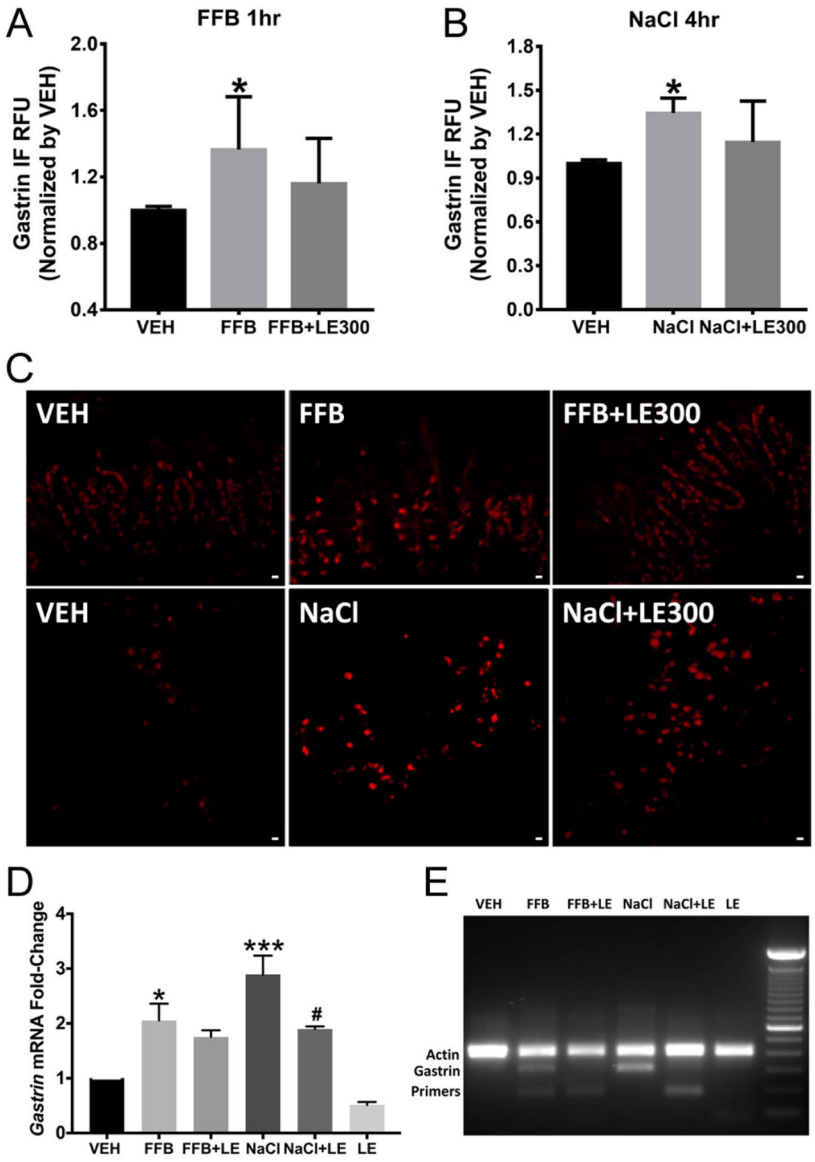

Figure 4.

Effect of the PPAR-α agonist, fenofibrate (FFB), D_1-like receptor antagonist, LE300, and high NaCl concentration on gastrin expression in human stomach antrum. (A) FFB (5 μM/1 h) stimulates gastrin expression in human stomach antrum ($P < 0.05$, vs VEH, one-way ANOVA, $n = 6$, Tukey test). (B) 170 mM (4 h) sodium increases gastrin expression in intact human stomach G cells ($P < 0.05$, vs. VEH, one-way ANOVA, $n = 4$, Tukey test). The stimulatory effects of FFB and 170 mM NaCl tend to be blocked by the D_1-like receptor antagonist LE300 but do not achieve statistical significance. (C) Immunostained human stomach antrum shows an increase in gastrin expression with fenofibrate (FFB, upper center image), relative to vehicle (VEH, upper left image). The D_1R/D_5R antagonist LE300 (10 μM/1 h) tends to block the stimulatory effect of FFB (upper right image). High extracellular NaCl (170 mM) (lower center image) also increases antrum gastrin staining, relative to VEH (lower left image). The D_1R/D_5R antagonist LE300 (10 μM/4 h) tends to block the

stimulatory effect of 170 mM NaCl on gastrin expression (lower right image). (D) RT-PCR of gastrin in stomach antrum treated with FFB (5 μM/1 h) and 170 mM NaCl (4 h); LE300 blocks the stimulatory effect of 170 mM NaCl on gastrin mRNA but not the stimulatory effect of FFB (* and *** $P < 0.05$ vs VEH; #$P < 0.05$, NaCl + LE vs NaCl, one-way ANOVA, Tukey test). (E) Representative DNA gel image of gastrin and actin mRNA. All data are expressed as M ± S.E.

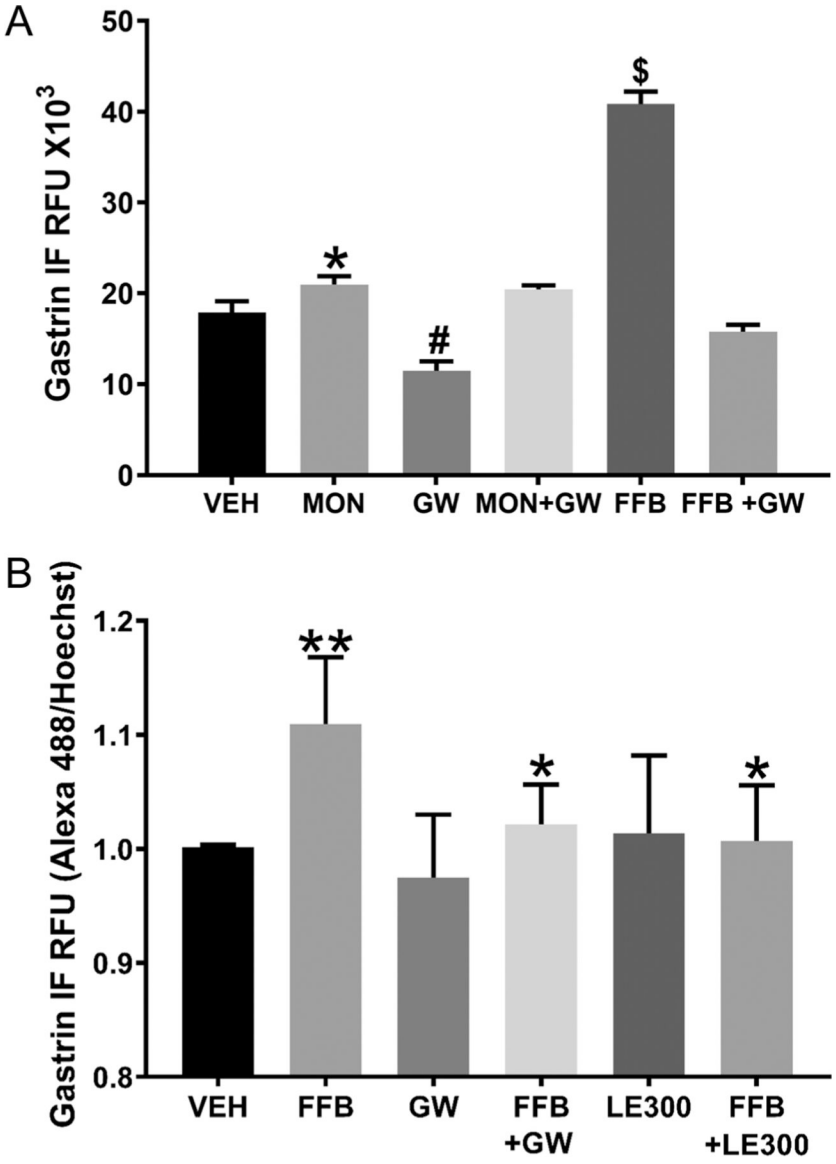

Figure 5.

Gastrin synthesis, D_1R, and PPAR-α in SW626 and human G cells. (A) Monensin (MON, 1 μM/24 h) or fenofibrate (FFB, PPAR-α agonist, 5 μM/30 min) increases gastrin protein in SW626 cells (IF, immunofluorescence; RFU, relative fluorescence units) (*$P < 0.05$ vs vehicle (VEH/24 h), one-way ANOVA, Tukey test). GW6471 (GW, PPAR-α antagonist, 5 μM/30 min) decreases gastrin protein (#$P < 0.05$, vs VEH, FFB, one-way ANOVA, Tukey test) and prevents the stimulatory effect of FFB but not MON on gastrin protein ($$P < 0.001$ vs others, one-way ANOVA, Tukey test). $n = 10$/group. (B) FFB (5 μM/30 min) increases gastrin protein in isolated human G cells (relative fluorescence units, RFU) (**$P < 0.01$, vs others, one-way ANOVA, Tukey test, $n = 6$/group). GW6471 (GW, PPAR-α antagonist, 5 μM/30 min) and LE300 (D_1-like receptor antagonist, 10 μM/30 min), by themselves, do not affect gastrin protein expression but prevent the stimulatory effect of FFB on gastrin protein expression (*$P < 0.05$, vs FFB, one-way ANOVA, Tukey test, $n = 6$/group).

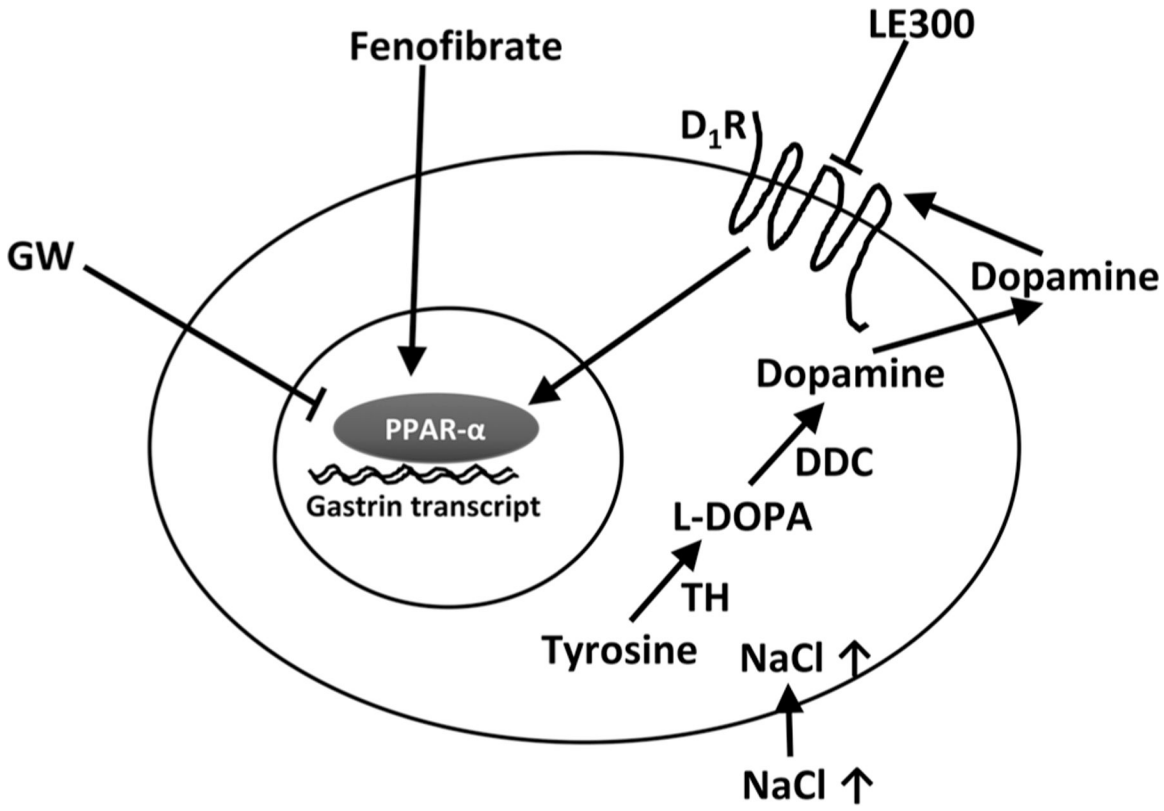

Figure 6.

Putative mechanism by which sodium induces gastrin expression in human stomach G cell. Sodium stimulates the production of dopamine. Dopamine, via the D_1R, increases gastrin secretion that involves the PPAR-α pathway, TH, tyrosine hydroxylase; L-DOPA, L-3,4-dihydroxyphenylalanine; DDC, DOPA decarboxylase.