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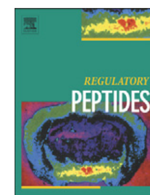

Gene expression profiling of gastric mucosa in mice lacking CCK and gastrin receptors

Chun-Mei Zhao [a,*], Yosuke Kodama [a], Arnar Flatberg [a], Vidar Beisvag [a], Bård Kulseng [a], Arne K. Sandvik [a,b], Jens F. Rehfeld [c], Duan Chen [a]

[a] Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, 7006 Trondheim, Norway
[b] Department of Gastrointestinal and Liver Diseases, St. Olav's University Hospital, 7006 Trondheim, Norway
[c] Department of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, 2100 København Ø, Denmark

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ABSTRACT

The stomach produces acid, which may play an important role in the regulation of bone homeostasis. The aim of this study was to reveal signaling pathways in the gastric mucosa that involve the acid secretion and possibly the bone metabolism in CCK_1 and/or CCK_2 receptor knockout (KO) mice. Gastric acid secretion was impaired and the ECL cell signaling pathway was inhibited in CCK_2 receptor KO mice but not in CCK_1 receptor KO mice. However, in CCK_{1+2} receptor double KO mice the acid secretion in response to pylorus ligation-induced vagal stimulation and the ECL cell pathway were partially normalized, which was associated with an up-regulated pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 receptor (PAC1). The basal part of the gastric mucosa expressed parathyroid hormone-like hormone (PTHLH) in a subpopulation of likely ECL cells (and possibly other cells) and vitamin D3 1α hydroxylase probably in trefoil peptide2-immunoreactive cells. In conclusion, mice lacking CCK receptors exhibited a functional shift from the gastrin-CCK pathways to the neuronal pathway in control of the ECL cells and eventually the acid secretion. Taking the present data together with previous findings, we suggest a possible link between gastric PTHLH and vitamin D and bone metabolism.
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1. Introduction

The stomach produces acid and pepsin, facilitating the digestion of food and the absorption of calcium, iron and vitamin B_{12}. The stomach is also an endocrine organ with distinct endocrine cell types that are known to produce gastrin, ghrelin, pancreastatin and somatostatin. These peptide hormones are involved in the control of gastric acid secretion, hunger and perhaps calcium homeostasis and/or bone metabolism [1–3].

There is crosstalk between hormonal and neuronal pathways in the regulation of gastric acid secretion, revealed by phenotyping of various gene knockout (KO) mouse models [4]. For instance, gene expression profiling and functional analysis have shown that genes in the parietal cells were down-regulated and the acid secretion was impaired in gastrin KO mice but normalized by cholinergic vagal stimulation in gastrin-CCK double KO mice, suggesting that the control of gastric acid secretion was shifted from both gastrin and CCK hormonal pathways to neuronal pathway in the gastrin and CCK double KO mice [5–7]. The action of gastrin is known to be mediated via the CCK_2 receptor, while CCK acts both on the CCK_1 and CCK_2 receptors [8]. Thus, the first aim of the present study was to examine whether crosstalk also exists in CCK_{1+2} receptor double KO mice in comparisons with CCK_1 receptor KO mice or CCK_2 receptor KO mice.

Impaired gastric acid secretion is believed to negatively affect calcium homeostasis and bone mass; which was recently claimed by phenotyping of the stomach and bone of CCK_2 receptor KO mice [9]. However, histidine decarboxylase (HDC) KO mice also suffered from impaired gastric acid secretion, but exhibited a very different bone phenotype [10]. This argues against the view that impaired gastric acid causes bone loss [11]. Thus, the second aim of the present study was to explore new gastric factors that may be involved in the control of bone metabolism.

By genome-wide gene expression profiling and validation analysis of the gastric mucosa, we have found that impaired acid secretion was associated with inactivated ECL cell signaling pathway in CCK_2 receptor KO mice. These phenotypes were partially "normalized" in CCK_{1+2} receptor double KO mice, probably by the switch from the hormonal pathways to the neuronal pathway including both vagus nerve and pituitary adenylate cyclase-activating polypeptide (PACAP). Furthermore, the stomach expressed parathyroid hormone-like hormone (Pthlh) [12], which was down-regulated in both CCK_2 receptor KO mice and CCK_{1+2} receptor double KO mice. The stomach also expressed vitamin D3 1α hydroxylase, which was unchanged in both CCK_2 receptor KO mice and CCK_{1+2} receptor double KO mice. Thus, there is a

* Corresponding author at: Erling Skjalgssons Gate 1, Laboratory Centre of St. Olav's Hospital, 7006 Trondheim, Norway. Tel.: +47 725 73080; fax: +47 725 76612.
E-mail address: chun-mei.zhao@ntnu.no (C.-M. Zhao).

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possible link between stomach and bone metabolism, the so-called "stomach–bone axis" [11].

2. Materials and methods

2.1. Animals

Male and female KO mice, originally developed at Dr. Alan Kopin's laboratory, Tufts University (Boston, MA), and age- and sex-matched C57BL6/J wild-type (WT) mice were purchased from the Jackson Laboratory (Boston). The KO mice had been backcrossed for more than 10 generations onto C57BL6/J background. All the mice (WT and KOs) used were bred in-house. The present study includes 4 strains of mice (WT, CCK_1 receptor KO, CCK_2 receptor KO, and CCK_{1+2} receptor double KO) with 26 mice at 3–4 months of age (14 males and 12 females) in each KO group compared with 36 WT mice. All mice were housed under standard conditions with free access to tap water and standard pellet food (RM1 801002, Scanbur BK AS, Sweden) under specific pathogen free conditions. In addition, adult Sprague–Dawley rats were purchased from Taconic (Ejby, Denmark). Immediately after sample collection, the anesthetized animals (isoflurane) were killed. The animal experiments were performed after approval by the Norwegian National Animal Research Authority.

2.2. Blood sampling and stomach samplings and analyses

Plasma was collected from the inferior vena cava for measurement of α-amidated gastrins (including gastrin-34, gastrin-17 and gastrin-14), glycine-extended gastrins, progastrin and CCK concentrations by epitope-defined radioimmunoassays (RIA) [13–17].

2.3. Histology and transmission electron microscopy

Small whole wall specimens (3 × 5 mm) were collected from the greater curvature of the stomach for routine histology. The thickness of the oxyntic mucosa was measured. Immunohistochemistry was performed using a DAKO AutoStainer (Universal Staining System with DAKO EnVision System, Dako, Glostrup, Denmark). Immuno double-staining was performed with a combination of two individual antigen visualization methods, i.e. DAB showing brown and TBM showing blue according to the protocol provided by KPL Technical Manual ML-168 (www.kpl.com/docs/datasheet/547800.pdf). Antibodies used included H^+,K^+-ATPase (1:100, mouse monoclonal, from Dr. L. Friis-Hansen at Rigshospitalet, Copenhagen, Denmark), histamine (8431: 1:1000, rabbit polyclonal, from Dr. P. Panula at University of Helsinki, Helsinki, Finland), somatostatin (A0566, 1:75, rabbit polyclonal, Dako, Denmark), ghrelin (H-031-31, 1:2000, rabbit polyclonal, Phoenix Europe GmbH, Karlsruhe, Germany), Pthlh (ARP33885_T100, 8 μg/mL, rabbit polyclonal, Aviva System Biology, San Diego, CA, USA), VD3 1-alpha-hydroxylase (ABIN249303, 1:500, sheep polyclonal, Antibodies-online Gmbh, Aachen, Germany), TFF2 (orb13709, 1:600, rabbit polyclonal, Biorbyt, Cambridgeshire, UK), and CGA (M9517, 1:400, guinea pig polyclonal, Dr. L. Person, Lund University, Lund, Sweden). Immunostained cells were counted and expressed as number of cells per gland (for parietal cells) or millimeter visual field (for others).

Minute tissue specimens (<1 mm^3) were collected from gastric oxyntic gland area for routine transmission electron microscopy. Parietal, ECL, D and A-like cells were identified on the basis of their characteristic ultrastructural appearance and photographed [18].

2.4. Microarray and quantitative PCR analyses

The gastric mucosa was scraped from both antral and oxyntic parts of each mouse. Total RNA was isolated from the gastric mucosa and purified using an Ultra-Turrax rotating-knife homogenizer and the mirVana miRNA Isolation Kit (AM1560, Ambion, TX). Concentration and purity of total RNA were assessed using a NanoDrop (NanoDrop Technologies, Wilmington, DE) photometer. The A260/280 ratios were 2.1305 ± 0.031 (mean ± SEM). RNA integrity was assessed using a Bioanalyzer (Agilent Technologies, Palo Alto, CA) with RIN values of 9.21 ± 0.31 (mean ± SEM). The microarray gene expression analysis followed standard protocols, analyzing 300 ng total RNA per sample with the Illumina MouseWG-6 (Illumina, San Diego, CA) and the data were deposited in the Gene Expression Omnibus (GEO accession no. E-MTAB-2824).

Quantitative real-time PCR (TaqMan gene expression assay) was used for determining the expression of *Adcyap1r1*, *Cyp27B1* and *Pthlh*. Two and a half micrograms of total RNA from six stomach samples from each of the four groups (WT, CCK_1KO, CCK_2KO and CCK_1KO + CCK_2KO) was reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. qRT-PCR was performed in triplicate with cDNA corresponding to 25 ng of total RNA in a 20 μL reaction, containing the following components: 10 μL 2× TaqMan Gene Expression Master Mix, 1 μL 20× TaqMan Gene Expression Assay and 4 μL of cDNA sample solution. The TaqMan gene expression assay consist of unlabeled PCR primers and TaqMan MGB probes predefined for the selected target genes by Applied Biosystems. qRT-PCR primer/probes were CCGCAGGCTCAAACGCGCTGTGTCT (target exon 3, AB assay ID Mm00436057_m1, NCBI gene reference NM_008970.3) for *Pthlh*, ATCAGATGTTTGCC TTTGCCCAGAG (4, Mm01165918_g1, NM_010009.2) for *Cyp27b1*, AATGGGGAGGT ACAGGCAGAGATTA (16. Mm00431683_m1, NM_001025372.2) for *Adcyap1r1*, and GGTGTGAA CGGATTTGGCCGTATTG (2, Mm99999915_g1, NM_008084.2) for *Gapdh*. AB Assay ID relates to the ID number assigned to each individual predesigned primer/probe mix by Applied Biosystems for product identification. Context sequence is genomic location identifiers. The qRT-PCR was performed on an Applied Biosystems Real-Time PCR System (Applied Biosystems, LifeTechnologies, Foster City, CA). The PCR reaction condition was 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C (annealing temperature). Relative gene expression quantification was performed using the modified ΔΔCT equation [19]. *Gapdh* gene expression levels were not different between the four groups of mice (data not shown) and *Gapdh* was used for normalization.

2.5. Measurement of gastric acid acidity and acid secretion

The animals were fasted but had free access to water for 24 h. Under anesthesia, the abdomen was opened through a midline incision. A cannula was placed in the pylorus via the duodenum, and the stomach rinsed with 1 mL of 37 °C saline through the cannula. The stomach washings were collected for measuring pH, and then pylorus ligation was performed by applying silk thread firmly around the junction between the pylorus and the duodenum, and gastric juice was collected 4 h later and assessed by titration with 0.02 N NaOH. The secretory response in the pylorus-ligation model is known to be mediated by vagal nerve stimulation but not the ECL cells [20,21].

2.6. Statistical analysis

The results are expressed as means ± SEM, and comparisons between WT mice and different strains of KO mice were performed using two-way Dunnett's two-tailed *t* test (otherwise stated) and a *p*-value of <0.05 was considered statistically significant. Gene expression differences between groups were tested using moderated t-tests as implemented in the limma software library {Smyth, 2005 #183} [*]. p-Values were controlled for false discovery rate using the Benjamini–Hochberg approach. Adjusted *p*-values < 0.01 were considered statistically significant. A list of significant genes was tested for functional over-representation using pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) and biological processes from the Gene Ontology (GOBP). Hypergeometric *p*-values < 0.01 were used to define

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