

# Carbonic anhydrase isozyme-II-deficient mice lack the duodenal bicarbonate secretory response to prostaglandin E<sub>2</sub>

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Duodenal bicarbonate secretion (DBS) is accepted as the primary mucosal defense against acid discharged from the stomach and is impaired in patients with duodenal ulcer disease. The secretory response to luminal acid is the main physiological stimulus for DBS and involves mediation by PGE<sub>2</sub> produced by mucosal cells. The aim of this investigation is to elucidate the role of carbonic anhydrases (CAs) II and IX in PGE<sub>2</sub>-mediated bicarbonate secretion in the murine duodenum. CA II- and IX-deficient mice and different combinations of their heterozygous and WT counterparts were studied. A 10-mm segment of the proximal duodenum with intact blood supply was isolated, and DBS was titrated by pH-stat (TitroLine-easy, Schott, Mainz, Germany). Mean arterial blood pressure (MAP) was continuously recorded, and blood acid/base balance and gastrointestinal morphology were analyzed. The duodenal segment spontaneously secreted HCO<sub>3</sub><sup>-</sup> at a steady basal rate of  $5.3 \pm 0.6 \mu\text{mol}\cdot\text{cm}^{-1}\cdot\text{h}^{-1}$ . Perfusing the duodenal lumen for 20 min with  $47 \mu\text{M}$  PGE<sub>2</sub> caused a significant increase in DBS to  $13.0 \pm 2.9 \mu\text{mol}\cdot\text{cm}^{-1}\cdot\text{h}^{-1}$ ,  $P < 0.0001$ . The DBS response to PGE<sub>2</sub> was completely absent in *Car2*<sup>-/-</sup> mice, whereas basal DBS was normal. The CA IX-deficient mice with normal *Car2* alleles showed a slight increase in DBS. Histological abnormalities were observed in the gastroduodenal epithelium in both CA II- and IX-deficient mice. Our data demonstrate a gastrointestinal phenotypic abnormality associated with CA II deficiency. The results show that the stimulatory effect of the duodenal secretagogue PGE<sub>2</sub> completely depends on CA II.

duodenal mucosal protection | gastroduodenal histological abnormalities | gastrointestinal phenotype

Hydrochloric acid at high concentration intermittently expelled from the stomach constitutes a threat to cause damage to the duodenal mucosa. The proximal duodenum secretes bicarbonate in all species tested, including humans, and luminal acid always increases the secretion (1). A deficiency in basal and acid-stimulated mucosal bicarbonate secretion is observed in patients with duodenal ulcer disease (2). Thus, bicarbonate secretion by the duodenal mucosa is currently accepted as an important duodenal defense mechanism (1, 3).

The duodenal enterocytes transport HCO<sub>3</sub><sup>-</sup> into the continuous layer of viscoelastic mucus gel on top of the epithelial surface, and HCO<sub>3</sub><sup>-</sup> is secreted at higher rates in the duodenum than in more distal segments of the intestine. The alkaline secretion maintains pH in the cell-facing portion of the mucus at neutrality, despite high acidities (pH  $\leq 2.0$ ) in the duodenal luminal bulk solution (1, 3, 4). The major physiological stimulant of the HCO<sub>3</sub><sup>-</sup> secretion is the presence of acid in the duodenal lumen, and the acid-induced HCO<sub>3</sub><sup>-</sup> response is mediated by enteric nervous pathways, involving release of vasoactive intestinal polypeptide and acetylcholine (5) and by E-type prostaglandins released from the mucosal cells (6). The duodenal enterocytes possess several mechanisms for acid/base transport,

and induction of transport may possibly reflect the second messenger system activated. HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> enter the epithelium via the blood, and HCO<sub>3</sub><sup>-</sup> is imported across the basolateral membrane by Na<sup>+</sup>(n)-HCO<sub>3</sub><sup>-</sup> cotransport. High levels of CO<sub>2</sub> are also generated from the luminal mixture of gastric acid with duodenal- and pancreatic-secreted HCO<sub>3</sub><sup>-</sup> (7, 8). CO<sub>2</sub> enters the enterocytes by diffusion, and HCO<sub>3</sub><sup>-</sup> is formed intracellularly in a reaction (CO<sub>2</sub> + H<sub>2</sub>O  $\rightarrow$  HCO<sub>3</sub><sup>-</sup> + H<sup>+</sup>), catalyzed by carbonic anhydrase (CA). The enterocytes export HCO<sub>3</sub><sup>-</sup> at the apical membrane by the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger and by an anion-conductive pathway. It has been suggested that the cystic fibrosis transmembrane conductance regulator (CFTR) is a ubiquitous membrane-spanning channel that transports Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> (9–11). The amiloride-sensitive Na<sup>+</sup>/H<sup>+</sup> exchanger extrudes protons at both the apical and basolateral membranes. Suppression of CA activity with acetazolamide decreases the basal and stimulated duodenal mucosal HCO<sub>3</sub><sup>-</sup> secretion in humans (12), rabbits (13), and guinea pigs (14). In rats, acetazolamide has been reported to decrease bicarbonate secretion (4), whereas other authors have found no effect (15). In mice, the basal bicarbonate secretion is slightly reduced after CA inhibition with methazolamide (16, 17).

CA II is the main isozyme of the CA family that has been suggested to be associated with alkaline secretion. CA II is located mainly in the villi and not in the duodenal crypts (18). In the murine duodenum *in vitro*, it was shown that cAMP-stimulated HCO<sub>3</sub><sup>-</sup> transport occurred through the CFTR channel and through the CA II- and CFTR-dependent Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger (9). CA IX is another CA isozyme detected in the gastroduodenal mucosa (19). In duodenum, CA IX is mainly located in the crypt enterocytes. Even though it shows high enzymatic activity and is predominantly expressed in the gastrointestinal tract (20), CA IX's role in physiological ion and pH homeostasis remains unclear.

The aim of this study was to examine the role of CA II and IX in the regulation of duodenal bicarbonate secretion (DBS). We used genetically modified mice lacking either CA II, CA IX, or both to evaluate their role in the DBS response to prostaglandin (PG)E<sub>2</sub>. PGE<sub>2</sub> as a stimulus was chosen for the following reasons: (i) It has been accepted as one of the most important physiological stimulants of DBS; (ii) experimentally, the response to PGE<sub>2</sub> mimics that to luminal acid (17); (iii) it is a well characterized physiological secretagogue; and (iv) the response to PGE<sub>2</sub> is proposed to involve CA activity.

The results show clearly that CA II activity plays an essential role in the PGE<sub>2</sub>-stimulated mucosal bicarbonate secretion in

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Abbreviations: CA, carbonic anhydrase; DBS, duodenal bicarbonate secretion; MAP, mean arterial blood pressure; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

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the murine duodenum. Thus, CA II-deficient mice ( $Car2^{-/-}/Car9^{+/+}$ ,  $Car2^{-/-}/Car9^{+/-}$ , or  $Car2^{-/-}/Car9^{-/-}$ ) did not increase alkaline secretion when stimulated with PGE<sub>2</sub>. In contrast,  $Car2^{+/+}/Car9^{-/-}$  mice showed both a slightly higher basal DBS and an increased secretory response to PGE<sub>2</sub> compared with the WT control mice.

## Materials and Methods

**Mice.** Generation of C57BL/6 strain  $Car2^{-/-}$  mice by treating them by chemical mutagenesis has been described in refs. 21 and 22. Generation of  $Car9^{-/-}$  mice by targeted disruption of the *Car9* gene was recently reported (23). These mice were introduced into the animal facility of Oulu University by embryo transfer, backcrossed 10 generations (F10) to C57BL/6J strain, and heterozygous mice were intercrossed to produce mice homozygous for the targeted gene.  $Car2^{-/-}$  and  $Car9^{-/-}$  mice were intercrossed to produce mice with various genotypic combinations.

One  $Car2^{-/-}/Car9^{-/-}$  mouse, three  $Car2^{-/-}/Car9^{+/+}$  mice, four  $Car2^{+/+}/Car9^{-/-}$  mice, five  $Car2^{-/-}/Car9^{+/-}$  mice, five  $Car2^{+/+}/Car9^{-/-}$  mice, and three  $Car2^{+/+}/Car9^{+/-}$  mice were examined and compared with five normal ( $Car2^{+/+}/Car9^{+/+}$ ) C57BL/6J mice. The mice were fed with standard feed (R36 for mice and rats Lactamin, Stockholm) and housed in pathogen-free conditions. The health status of the animals was monitored on a regular basis, in accordance to the Federation of European Laboratory Animal Science Associations' recommendations, and none of the animals showed any signs of viral or bacterial infection, nor did any show helicobacteria in histopathological analysis of the gastric mucosa. The study protocols were approved by the Animal Care Committee of the Oulu University. The mice were transported to Uppsala University, where all experiments were approved by the Uppsala Ethics Committee for Experiments with Animals. An Additional six WT C57BL/6J mice were obtained from B & K Universal (Sollentuna, Sweden) and were used for control purposes. In the animal care unit of Uppsala University, the animals were maintained under standardized temperature and light conditions (12/12-h light/dark cycle at 21–22°C). The mice were kept in cages and had access to tap water and pelleted food (Ewos, Södertälje, Sweden) ad libitum.

**CA9 Genotyping.** DNA was isolated from mice ear markings by using NucleoSpin Tissue DNA extraction kit (Macherey-Nagel, Düren, Germany). The *Car9* gene was amplified with PCR by using Reddy Mix PCR Master Mix (ABGene, Surrey, U.K.). The template DNA for each reaction was 150 ng. The primers were 5'-CCAGTCAGCTGCATGGCC-3' and 3'-AGGAGCCTCGGGAGTCTCGA-5' for the WT allele, and 5'-AGGAGCAAAGCTGCTATTGG-3' and 3'-AGGAGCCTCGG-GAGTCTCGA-5' for the targeted *Car9* allele. The primers were chosen from the first exon that was disrupted in the knockout mice (23). The PCR program was 96°C for 5 min, 35 cycles of 96°C for 30 s, 56°C for 60 s, and 72°C for 60 s. The PCR products were characterized in 1.2% agarose gel (LE, analytical grade, Promega) containing 0.005% nucleic acid gel stain GelStar (BMA Biomedicals), and visualized by UV light.

**CA II Phenotyping.** CA activity was assayed from the blood sample (treated with EDTA, diluted 1:5,000) with imidazole-Tris technique (24). Because CA II constitutes the major fraction of CA activity in mouse blood, the test is considered reliable in monitoring CA II activity.

**Surgical Procedure.** The mice were anesthetized by spontaneous inhalation of isoflurane (Forene, Abbott). The inhalation gas was administered continuously through a breathing mask and contained a mixture of ≈30–40% oxygen, ≈60–70% nitrogen,

and 2.2 ± 0.2% isoflurane. Body temperature was maintained at ≈37.5°C by means of a heating pad controlled by a rectal thermistor probe. A catheter containing heparin (20 units/ml) dissolved in isotonic saline was placed in the left carotid artery to monitor blood pressure and for continuous infusion of an isotonic sodium carbonate solution (200 mM Na<sup>+</sup> and 100 mM CO<sub>3</sub><sup>2-</sup>) at 0.35 ml/h.

The bile and the pancreatic ducts were ligated very closely to their entrance into the duodenum to prevent pancreaticobiliary juice from entering the duodenum. It should be noted that the mouse pancreas has several excretory ducts, and, for this reason, small amounts of pancreatic juice may enter the duodenal lumen despite ligation of the pancreaticobiliary duct.

Silicone tubing was introduced through a hole made in the forestomach by electrical microcautery and was guided through the stomach and pylorus and secured by a ligature 2–3 mm distal to the pylorus. A PE-200 (Becton Dickinson) cannula was inserted into the duodenum ≈1.5 cm distal to the pylorus and secured by ligatures. The proximal duodenal tubing was connected to a peristaltic pump, and the segment was perfused with isotonic saline (150 mM NaCl) at 0.25 ml/min. Upon completion of surgery, the abdominal cavity was closed with sutures. After surgery, ≈30 min was allowed for stabilization of cardiovascular, respiratory, and gastrointestinal functions before experiments were commenced. Blood acid/base balance was checked (AVL Compact 3 blood gas analyzer, Graz, Austria) in 40-μl arterial blood samples taken at the end of the experiments.

**Measurement of Luminal Alkalinization.** The rate of luminal alkalinization was determined by back-titration of the perfusate to pH 4.90 with 10 mM HCl under continuous gassing (100% N<sub>2</sub>) by use of pH-stat equipment (Schott). The amount of titrated HCl was considered equivalent to duodenal HCO<sub>3</sub><sup>-</sup> secretion. The pH electrode was routinely calibrated with standard buffers before the start of the titration. The rates of luminal alkalinization are expressed as micromoles of base secreted per centimeter of intestine per hour (μmol·cm<sup>-1</sup>·h<sup>-1</sup>).

**Experimental Protocol.** The experiments were run as follows. Basal HCO<sub>3</sub><sup>-</sup> secretion in C57BL/6J mice was monitored continuously during 90 min and recorded at 10-min intervals, when perfused luminally with saline alone. In the test groups, 47 μM PGE<sub>2</sub> was present in the duodenal perfusate for 20 min (between the time points  $t = 30$  and  $t = 50$  min).

In all groups, the duodenal bicarbonate secretion, MAP, and body temperature were monitored continuously and recorded at 10-min intervals. Arterial blood samples for acid/base analysis were taken at the end of the experiment ( $t = 90$  min).

**Tissue Sections.** The mouse tissue specimens were fixed in 4% neutral-buffered formaldehyde for 8–27 days. The samples were then dehydrated and embedded in paraffin wax, and 4-μm sections were cut and placed on Superfrost microscope slides (Menzel, Braunschweig, Germany). The histological examination was performed after hematoxylin/eosin staining. The stained sections were examined by three investigators and photographed by using a Zeiss Axioskop 40 microscope.

**Statistical Analysis.** Descriptive statistics are expressed as means (±SEM), with the number of experiments given in parentheses. The statistical significance of data were tested by repeated-measures ANOVA. To test differences within a group, a one-factor repeated-measures ANOVA was used, followed by Fisher's probable least-squares difference PLSD post hoc test. Between groups, a two-factor repeated-measures ANOVA followed by a one-way ANOVA at each time point was used. If the ANOVA was significant at a given time point, a Fisher's PLSD post hoc analysis was used. All statistical analyses were per-





**Table 1. Blood gas analysis of WT and CA II- or/and CA IX-deficient mice**

Group	pH	pCO <sub>2</sub> , kPa	BE, mmol/liter	[HCO <sub>3</sub> <sup>-</sup> ], mmol/liter	pO <sub>2</sub> , kPa
WT, Sweden (n = 6)	7.36 ± 0.03	6.01 ± 0.53	0.92 ± 1.02	25.6 ± 1.14	12.6 ± 1.46
WT, Finland (n = 5)	7.35 ± 0.04	6.33 ± 0.74	-0.57 ± 0.92	26.3 ± 0.89	13.2 ± 1.27
WT, PGE <sub>2</sub> (n = 6)	7.38 ± 0.01	6.27 ± 0.21	2.02 ± 1.16	27.9 ± 1.27	12.3 ± 0.68
<i>Car2</i> <sup>-/-</sup> / <i>Car9</i> <sup>+/-</sup> (n = 5)	7.25 ± 0.03	7.85 ± 1.01	-3.47 ± 2.53	25.2 ± 3.10	11.7 ± 1.64
<i>Car2</i> <sup>-/-</sup> / <i>Car9</i> <sup>+/+</sup> (n = 3)	7.19 ± 0.01	8.88 ± 0.17	-2.20 ± 1.10	28.6 ± 1.45	10.5 ± 0.54
<i>Car2</i> <sup>-/-</sup> / <i>Car9</i> <sup>-/-</sup> (n = 1)	7.32 ± 0.00	7.75 ± 0.00	1.80 ± 0.00	29.5 ± 0.00	10.6 ± 0.00
<i>Car2</i> <sup>+/-</sup> / <i>Car9</i> <sup>-/-</sup> (n = 4)	7.38 ± 0.03	5.88 ± 0.67	0.43 ± 0.89	25.8 ± 1.43	12.8 ± 0.57
<i>Car2</i> <sup>+/-</sup> / <i>Car9</i> <sup>+/-</sup> (n = 5)	7.34 ± 0.03	7.43 ± 0.53	-1.34 ± 2.25	26.4 ± 2.23	10.2 ± 0.93
<i>Car2</i> <sup>+/-</sup> / <i>Car9</i> <sup>-/-</sup> (n = 3)	7.36 ± 0.02	6.06 ± 0.12	-0.10 ± 0.53	25.7 ± 0.39	12.3 ± 0.47

Values are means (±SEM). All blood samples for acid/base were taken at *t* = 90 min. BE, base excess.

alkalinization, a number of mice with various *Car2* and *Car9* genotypes were investigated. Stimulating the duodenal lumen for 20 min with 47 μM PGE<sub>2</sub> in double heterozygous *Car2*<sup>+/-</sup>/*Car9*<sup>+/-</sup> mice (*n* = 3) caused a significant increase in duodenal mucosal HCO<sub>3</sub><sup>-</sup> secretion, from 4.97 ± 0.67 μmol·cm<sup>-1</sup>·h<sup>-1</sup> to 11.6 ± 4.5 μmol·cm<sup>-1</sup>·h<sup>-1</sup>, *P* < 0.01 (Fig. 2). Neither the basal nor the stimulated secretions were significantly different from the control group. The genetic manipulation or PGE<sub>2</sub> did not affect the MAP, shown in Fig. 2.

**CA II-Deficient Mice.** The stimulatory effect of PGE<sub>2</sub> on DBS was completely absent in mice lacking the CA II enzyme. Two variants of CA II-deficient mice were tested, and PGE<sub>2</sub> did not change the DBS in either *Car2*<sup>-/-</sup>/*Car9*<sup>+/+</sup> mice (*n* = 3, *P* > 0.05) or *Car2*<sup>-/-</sup>/*Car9*<sup>+/-</sup> mice (*n* = 5, *P* > 0.05). Previous studies have reported that the CA inhibitors reduce basal bicarbonate secretion (12, 16, 17). However, the basal DBS in our *Car2*<sup>-/-</sup> mice was not significantly different from the WT control group or *Car2*<sup>+/-</sup> group. *Car2*<sup>-/-</sup> mice had a basal secretory rate of ≈4.5 ± 0.4 μmol·cm<sup>-1</sup>·h<sup>-1</sup> during the 90-min experimental period, as illustrated in Fig. 3. Neither the genetic manipulation nor PGE<sub>2</sub> affected MAP, illustrated in Fig. 3.

**CA IX-Deficient Mice.** In response to 47 μM luminal PGE<sub>2</sub>, *Car2*<sup>+/-</sup>/*Car9*<sup>-/-</sup> mice (*n* = 5) significantly increased the duodenal alkaline secretion. The increase was from 4.92 ± 0.55 μmol·cm<sup>-1</sup>·h<sup>-1</sup> to 13.8 ± 6.4 μmol·cm<sup>-1</sup>·h<sup>-1</sup> (*P* < 0.0001; Fig. 1). This increase was fairly similar to the effect that was monitored in the control or double-heterozygous mice. In mice with the CA genotype *Car2*<sup>+/-</sup>/*Car9*<sup>-/-</sup> (*n* = 4), both the net basal bicarbonate secretion and the net bicarbonate output during the PGE<sub>2</sub> stimulation were significantly increased compared with both the control group (Fig. 1) and the *Car2*<sup>+/-</sup>/*Car9*<sup>+/-</sup> mice. The basal secretion was 6.55 ± 0.95 μmol·cm<sup>-1</sup>·h<sup>-1</sup>, *P* < 0.001, and the peak secretory rate during PGE<sub>2</sub> exposure was 17.0 ±

3.9 μmol·cm<sup>-1</sup>·h<sup>-1</sup> (*P* < 0.0001; Fig. 1). Neither the genetic manipulation nor PGE<sub>2</sub> affected the MAP, as shown in Fig. 1.

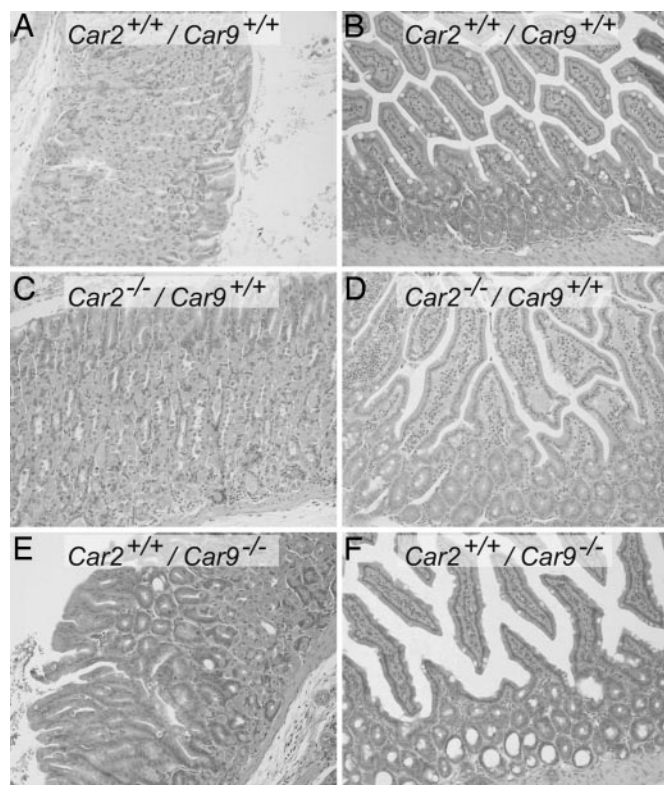
**CA II- and IX-Deficient Mouse.** The only mouse lacking both CA II and CA IX available for study did not show any change in the secretory response to PGE<sub>2</sub>. Although it is impossible to make any definitive conclusions from one experiment, in this experiment (Fig. 2) the basal secretory rate was lower than in controls, and the secretion stayed at the basal level when applying PGE<sub>2</sub> to the duodenal lumen. The MAP was the same as in controls (Fig. 2).

**Acid/Base Balance.** All samples for blood gas analysis were taken at the end of the experiments (*t* = 90 min). The individual parameters are shown in Table 1. Mice lacking CA II showed respiratory acidosis, as reported in ref. 25. This phenomenon is explained by the fact that CA II plays an important role in CO<sub>2</sub> exchange by erythrocytes. No other significant differences were observed between the animals in the following measured parameters: pH, pCO<sub>2</sub>, base excess, end-of-study plasma HCO<sub>3</sub><sup>-</sup>, or pO<sub>2</sub>.

**Morphological Changes.** In addition to the physiological findings described above, CA deficiencies were also associated with several morphological changes in the stomach and intestine (Table 2). The *Car2*<sup>-/-</sup>/*Car9*<sup>-/-</sup> mouse had mild pit-cell hyperplasia of the stomach and enlarged crypts of the small intestine. The pit-cell hyperplasia was noticed in all *Car9*<sup>-/-</sup> mice regardless of the *Car2* genotype (Fig. 4). Sixty percent had mild hyperplasia and 40% moderate hyperplasia. Forty percent of *Car9*<sup>-/-</sup> mice had mild glandular atrophy. In addition to the one double knockout mouse, two *Car9*<sup>-/-</sup> mice had enlarged small intestinal crypts. Interestingly, 50% of the mice with a genotype combination of *Car2*<sup>-/-</sup>/*Car9*<sup>+/+</sup> or *Car2*<sup>-/-</sup>/*Car9*<sup>+/-</sup> had mild pit-cell hyperplasia, and 13% had mild glandular atrophy. In addition, 50% had gastric cysts. Six mice

**Table 2. Morphological changes in stomach and intestine of CA II- or/and CA IX-deficient mice**

Genotype	Stomach			Intestine	
	Pit-cell hyperplasia	Glandular atrophy	Gastric cysts	Enlarged intestinal crypts	Intraepithelial lymphocytes in the duodenum
WT	0 of 5	0 of 5	0 of 5	0 of 5	0 of 5
<i>Car2</i> <sup>+/-</sup> / <i>Car9</i> <sup>+/-</sup>	1 of 3	0 of 3	1 of 3	0 of 3	1 of 3
<i>Car2</i> <sup>+/-</sup> / <i>Car9</i> <sup>-/-</sup>	4 of 4	0 of 4	0 of 4	1 of 4	0 of 4
<i>Car2</i> <sup>+/-</sup> / <i>Car9</i> <sup>+/-</sup>	5 of 5	2 of 5	0 of 5	1 of 5	0 of 5
<i>Car2</i> <sup>-/-</sup> / <i>Car9</i> <sup>+/+</sup>	1 of 3	1 of 3	2 of 3	0 of 3	1 of 3
<i>Car2</i> <sup>-/-</sup> / <i>Car9</i> <sup>+/-</sup>	3 of 5	0 of 5	2 of 5	0 of 5	4 of 5
<i>Car2</i> <sup>-/-</sup> / <i>Car9</i> <sup>-/-</sup>	1 of 1	0 of 1	0 of 1	1 of 1	0 of 1



**Fig. 4.** Morphological changes in the stomach and intestine. Hematoxylin/eosin staining of gastric (A, C, and E) and duodenal (B, D, and F) mucosa in WT (A and B), CA II-deficient (C and D), and CA IX-deficient (E and F) mice. The WT and CA II-deficient mice have normal histological structure of the mucosa. The CA IX-deficient mouse shows gastric pit-cell hyperplasia, glandular atrophy, and enlargement of intestinal crypts. Original magnifications,  $\times 200$ .

had intraepithelial lymphocytes in the duodenum; five had the genotype  $Car2^{-/-}/Car9^{+/+}$  or  $Car2^{-/-}/Car9^{+/-}$ , and one was a double heterozygote.

## Discussion

$HCO_3^-$  secretion by the duodenal mucosa is under central and local intestinal neurohumoral influence. Patients with duodenal ulcer disease have an impaired basal secretion and a markedly reduced secretory response to acid (2). Thus, deficiency of DBS has been suggested as a contributor in the pathogenesis of duodenal ulcer disease. The mechanisms by which this protective secretion is regulated have been studied extensively during the last two decades. A wide range of agonists has been shown to stimulate active  $HCO_3^-$  secretion, but acidification of the duodenal lumen by HCl seems to be the most important physiological stimulus. Acid-stimulation of the  $HCO_3^-$  secretion involves the release of a number of mediators, including  $PGE_2$ , vasoactive intestinal peptide, dopamine, acetylcholine (1), serotonin (26), and melatonin (27). A number of studies have shown that E-type prostaglandins and their derivatives protect the duodenal mucosa against ulcerations by stimulating  $HCO_3^-$  secretion. Furthermore, recent evidence suggests that the prostaglandin EP<sub>3</sub> receptor subtype plays a critical role in the physiological regulation of duodenal  $HCO_3^-$  response to both  $PGE_2$  and mucosal acidification (28).

In this study, we examined the importance of two CA isozymes, CA II and IX, in DBS. Both CA isozymes are highly expressed in both the gastric and duodenal epithelium (19) and may, thus, be the main CA isozymes involved in alkaline secretion. CA II is a soluble, cytoplasmic enzyme, and CA IX is

a cell-surface isozyme expressed mainly on the basolateral plasma membrane. In this investigation, we chose  $PGE_2$  as a stimulant of DBS. Low doses of  $PGE_2$  elicit secretory responses very similar to those induced by acid (17), and  $PGE_2$  is a well documented duodenal secretagogue in all species tested (1). Using  $PGE_2$ , we could more specifically study the involvement of CAs in the  $PGE_2$ -mediated increase of alkaline secretion. The  $HCO_3^-$  stimulatory action of  $PGE_2$  in the duodenum is suggested to involve both cAMP (5) and intracellular  $Ca^{2+}$  (6, 29), and both second messengers are thought to stimulate CA activity (12).

Evidence for activation of CAs by cAMP has not yet been reported for duodenal enterocytes, although it is well documented in several other tissues or cell types, including the brain (30) and erythrocytes (31). Furthermore, cAMP has been found to increase transcriptional activity of the *Car2* gene promoter (32). The results we obtained in this study are consistent with these prior reports. We speculate that  $PGE_2$  probably increases cAMP and/or  $Ca^{2+}$  in the duodenal enterocytes in a CA-mediated response but that, in CA II deficiency, this response was not seen, and the DBS stayed at the basal level.

Both the basal and  $PGE_2$ -stimulated secretory rates observed in this study were higher than reported in refs. 17, 33, and 34. The basal secretory rate observed in our control experiments was  $5.6 \pm 0.65 \mu\text{mol}\cdot\text{cm}^{-1}\cdot\text{h}^{-1}$ , which is more than two times higher than that observed in a recent study by Hirokawa *et al.* (17). Furthermore, we also observed a much larger increase in DBS in response to luminal  $PGE_2$ . The difference might be explained by either biological or experimental variations. Anesthesia is known to influence basal parameters and might also alter normal physiological functions in the animal, such as blood pressure and acid/base status. In this study, we document that both the MAP and acid/base balance in the mice studied remain in the physiological range, despite the quite extensive experimentation.

Our results showed that basal  $HCO_3^-$  secretion was not significantly affected in CA II-deficient mice compared with the control animals. These results contrast with results in previous studies showing a decrease in basal secretion of bicarbonate after CA inhibition (12). It is important to note the difference in the experimental conditions between the previous study and this study. The prior studies applied CA inhibitors, which more or less inhibit all major CA isozymes expressed in the gastrointestinal epithelia. In this study protocol, we were able to eliminate a single isozyme by a genetic approach, without affecting any other isoform. To our knowledge, however, compensatory up-regulation of another CA, such as CA I or XIII, might occur. Even though we obtained only one double-knockout mouse, the results on this mouse showed that the basal bicarbonate secretion was low compared with all other groups. One possible explanation for this phenomenon is that CA IX may partially compensate for missing CA II activity. Thus, double deficiency may more closely approximate the condition that was created by CA inhibitors, i.e., uncatalyzed bicarbonate production by duodenal enterocytes. In the previous studies showing acetazolamide-induced decrease in the secretion, the basal CA-expression might have been activated by experimentally induced conditions, such as inflammatory processes starting during abdominal surgery (resulting in release of endogenous prostaglandins and histamine). As a consequence, the bicarbonate secretion rate may have been artificially stimulated to a higher level, and this higher level of secretion was effectively inhibited by the CA inhibitor.

CA IX deficiency produced only a mild physiological change. Interestingly, the mice with a  $Car2^{+/+}/Car9^{-/-}$  genotype showed higher basal and  $PGE_2$ -induced DBS rates compared with the control measurements (illustrated in Fig. 1). Even though we cannot explain the physiological mechanism for this phenomenon, the results suggest that CA IX, indeed, influences bicarbonate homeostasis in the normal gastrointestinal tract. CA IX

deficiency also produced morphological changes in the stomach and intestine. In the stomach, pit-cell hyperplasia and glandular atrophy were noted in a fashion similar to the previous study (23). In addition, some CA IX-deficient mice showed enlarged crypts in the small intestine that may be attributed to the role of CA IX in cell adhesion (35). Another interesting observation was mild pit-cell hyperplasia, mild glandular atrophy, and gastric cysts in the *Car2*<sup>-/-</sup> mice. These results suggest that CA II may also play some role in normal gastric morphogenesis.

In conclusion, we have investigated the functional interaction between the secretagogue PGE<sub>2</sub> and CA in the process of duodenal secretion. Our study provides convincing evidence that CA II deficiency in the mouse produces a clear gastrointestinal

phenotype. We conclude that CA II is essential for exertion of PGE<sub>2</sub>-mediated stimulation of duodenal HCO<sub>3</sub><sup>-</sup> secretion in the mouse *in vivo*. Even though CA II is the most abundant isozyme in the alimentary tract, no gastrointestinal symptoms have yet been associated with CA II-deficient patients. Based on the results reported here, further studies are warranted to investigate the possible gastrointestinal manifestations in CA II-deficient patients.

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