expression was obtained by mapping β-gal activity in heterozygous FP +/− mice (Fig. 1*B*).

Segmental expression of the mFP receptor was most abundant in tubules that colabeled with antibodies to the TSC- and AQP2 antibody (collecting duct specific)-positive tubules. There was no evidence for hybridization of the FP antisense fragment to either proximal straight tubules or thick ascending limb (Fig. 2). No labeling of papillary or inner medullary structures was observed.

Extrarenal tissues. Abundant β-gal expression was detected in stromal surrounding the ureteral smooth muscle (Fig. 3). Epididymus possesses endogenous β-gal activity in control animals, complicating the interpretation of this tissues. However, this endogenous activity was not present in any other organs from wild-type animals examined including the distal vas deferens and luminal cells of the testis where low levels of β-gal activity were detected. In the female genital tract, β-gal expression was detected in ovary corpora luteal cells and the smooth muscle cells lining the fallopian tubule and uterus. Patches of intense β-gal labeling in tissues obtained from FP +/− mice were associated with dermal hair follicles. Liver failed to show any β-gal staining in hepatocytes, however, labeling of vascular tissue was detected.

DISCUSSION

The kidney is a site of robust prostaglandin synthesis and expresses abundant prostanoid receptors (8, 10,

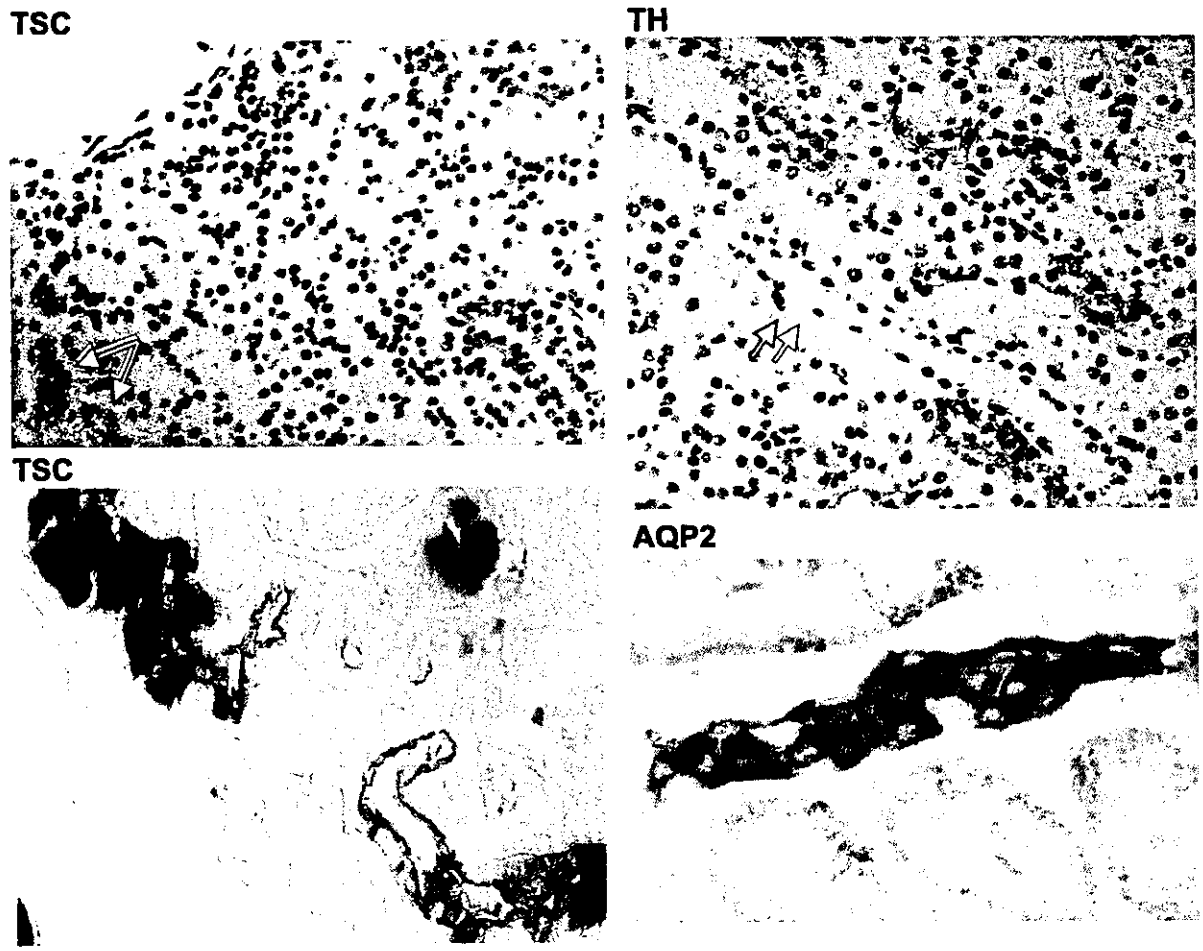

Fig. 2. Localization of FP receptor mRNA in kidney by β-galactosidase staining and in situ hybridization. Coimmunostaining using segment-specific antibodies was performed. For in situ hybridization, panels show tangential illumination combined with brightfield illumination of a mouse kidney section where the white grains depict sites of FP receptor riboprobe hybridization. Segments expressing β-galactosidase driven by the endogenous FP promoter are identified by the blue reaction product. Thiazide-sensitive cotransporter (TSC) immunoreactivity, characteristic for the distal convoluted tubule (DCT), colocalizes with either white grains or β-galactosidase, indicating expression of FP receptor mRNA in DCT. Tamm-Horsfall (TH) immunoreactivity, restricted to the thick ascending limb (TAL), does not colocalize with FP receptor mRNA and TH-positive TAL, whereas the FP receptor is expressed in TH-negative tubules. Aquaporin-2 (AQP2) specifically labels the collecting duct and colocalizes with FP receptor mRNA expressed in AQP-positive tubules.

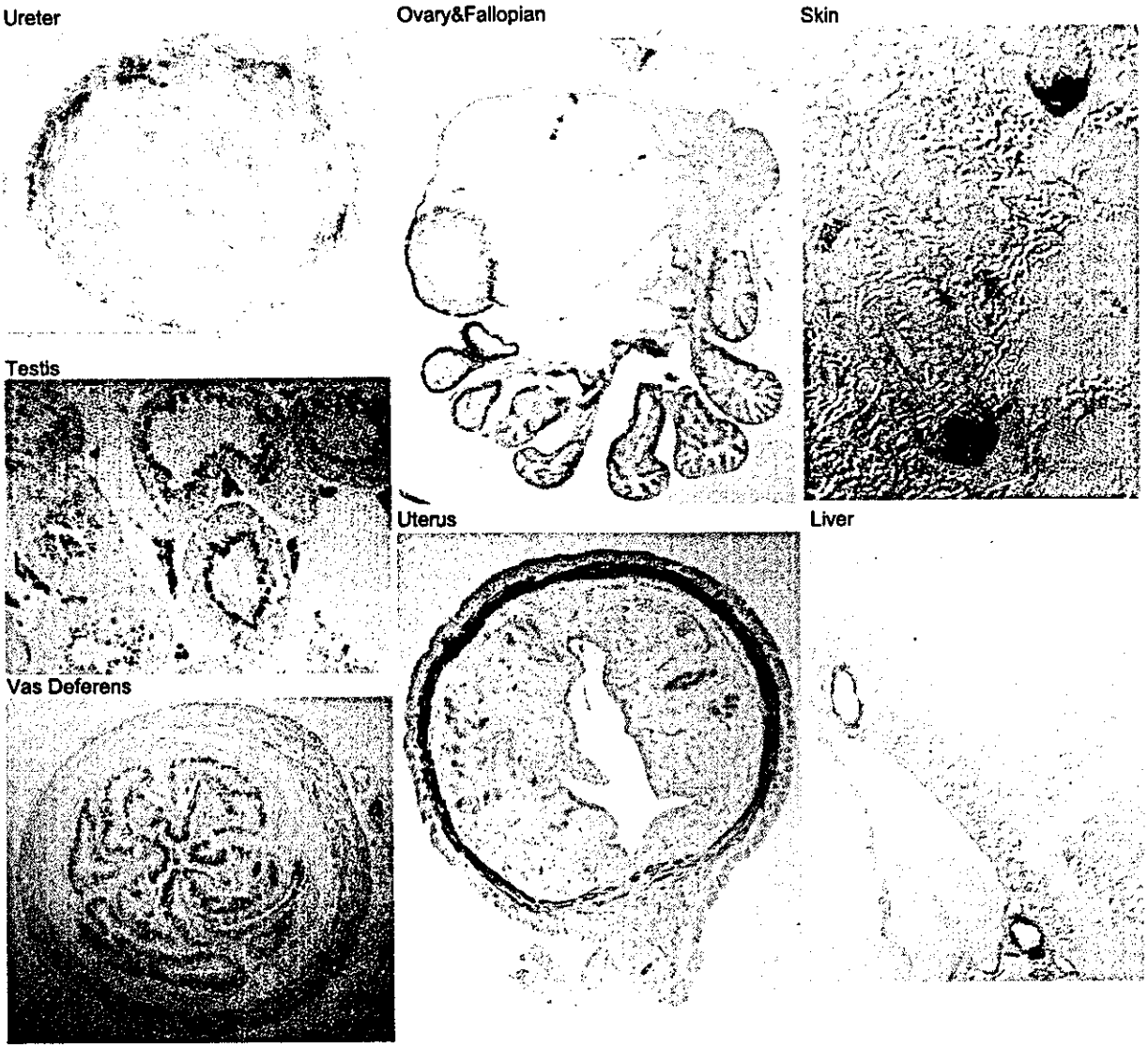

Fig. 3. β-Galactosidase expression in FP-lacZ knocked into tissues: ureter, testis, uterus, ovary, skin, and liver.

46). Renal expression of the FP, EP_1, EP_3, and TP receptor mRNAs is particularly high (13, 25, 46). Furthermore, many of the signaling pathways activated by this subset of receptors are similar (12, 33), allowing for the possibility that these receptors subserve functionally redundant roles. In this regard, it is of note that striking similarities between the renal effects of PGE_2 and $PGF_{2\alpha}$ exist. Similar to PGE_2, intrarenal infusion of $PGF_{2\alpha}$ is associated with natriuresis and diuresis, without altering glomerular filtration rate or renal hemodynamics (50). Furthermore, basolateral addition of either $PGF_{2\alpha}$ or PGE_2 can antagonize ADH-stimulated water absorption in microperfused collecting ducts (43). Nonetheless, because $PGF_{2\alpha}$ potently activates both prostaglandin FP and EP_3 receptors (1, 31), it is difficult to attribute these renal affects specifically to activation of the FP receptor. Furthermore, we are unaware of any published studies examining the renal effects of FP receptor-selective agonists. For these reasons, it is important that the present studies now demonstrate segmental expression of FP receptor mRNA along the mouse nephron.

The present studies used both in situ hybridization and a β-gal reporter knocked into the endogenous FP locus (47) to map the distribution of the FP receptor. FP receptor expression determined using these two different techniques was mutually supportive. In the kidney, the most intense labeling was detected over a subpopulation of cells in the cortex. The mouse FP receptor mRNA was most abundant in distal nephron segments colabeling with antibodies to the TSC1 and the vasopressin-stimulated water channel AQP2. In

mice, TSC1 is expressed only in the DCT, where it mediates NaCl absorption (15). FP receptor activation could inhibit salt absorption in this nephron segment, thereby contributing to the natriuretic effects of $PGF_{2\alpha}$. The DCT is also a major site of calcium absorption (32, 38), so it is also conceivable that $PGF_{2\alpha}$ plays a role in modulating Ca^{2+} absorption by the kidney. Similarly, the detection of the FP receptor in AQP2-immunoreactive cells demonstrates its expression in the collecting duct (20, 21), representing another site where its activation could contribute to $PGF_{2\alpha}$-induced natriuresis and diuresis.

Although the presence of low levels of FP mRNA in the thick ascending limb cannot be excluded, it seems clear that the expression of FP transcripts in the thick ascending limb is markedly less than in either the DCT or cortical collecting duct (CCD). Interestingly, there appears to be a gradient for the intensity of FP gene expression along the distal tubule, with greater levels of expression in the DCT/connecting tubule, > CCD >>MCD. This is in contrast to EP_3 mRNA, which is more abundant in medullary CD than CCD and expressed in mTAL as well (9, 11, 45). Finally, the EP_1 receptor is most abundant in the papillary collecting duct (25, 45). This axial heterogeneity of the prostanoid receptors is consistent with a major role for $PGF_{2\alpha}$ action in the renal cortex as opposed to the medulla, where PGE_2 action may predominate.

The cellular effects of the FP receptor in distal renal epithelia remain uncharacterized. In fibroblasts, smooth muscle cells, or cells transfected with the FP receptor, $PGF_{2\alpha}$ activates a signaling pathway coupled to increased cell calcium and phosphatidylinositol hydrolysis (3, 23, 24). A similar signaling pathway is activated by the EP_1 receptor in the collecting duct, and this signaling pathway contributes the capacity of PGE_2 to inhibit vasopressin-stimulated water flow and sodium absorption (19, 25, 27). Activation of a Ca^{2+}-coupled signaling pathway by the FP receptor in the collecting duct could therefore contribute to natriuresis and diuresis caused by $PGF_{2\alpha}$ infusion. Other studies in transfected cells show that the FP receptor can activate a β-catenin-coupled signaling pathway (18), however, the significance of this pathway in differentiated renal epithelial is uncharacterized. Alternatively, of the known prostanoid receptors, the FP receptor protein sequence is most closely related to the EP3 receptor (37) that preferentially couples to Gi and inhibits vasopressin-stimulated cAMP generation and water flow via this pertussis toxin-sensitive mechanism (25, 27, 41). Additional studies will be required to determine which, if any of these pathways, is activated by the FP receptor in these nephron segments.

As previously reported, β-gal expression was abundant in ovarian corpus luteum where FP activation appears to play a critical role in parturition, initiating the perinatal decline in progesterone secretion (47). The expression of β-gal in corpora luteal cells provides additional validation for concordance of β-gal expression with FP mRNA expression since abundant expression FP mRNA has been demonstrated in corpora lutea of mice by both techniques (44, 47). FP receptor is also expressed in uterine smooth muscle (6), consistent with the present studies demonstrating β-gal in this tissue. Robust β-gal activity was also detected along the male genital tract, particularly in the epithelia lining the lumen of the epididymis and vas deferens. Because the epididymis possesses endogenous β-gal activity (17, 22) detected in the wild type (not shown), the significance of staining in this segment of the male genital tract remains uncertain. In contrast, we did not detect β-gal activity in wild-type testis, so FP recetor could be expressed in this tissue and its activation contributes to previous reports that in vivo administration of $PGF_{2\alpha}$ to mice causes atrophy of epididymal epithelium (39).

β-Gal expression was not apparent in hepatocytes, despite reported effects of $PGF_{2\alpha}$ on hepatic glucose output (34), consistent with the possibility of an unrelated receptor or pharmacological target for $PGF_{2\alpha}$ in mediating these effects. Interestingly, intense expression of β-gal activity was observed in hepatic vasculature, where it could mediate the capacity of $PGF_{2\alpha}$ to induce nitric oxide-dependent vasodilatation (4). Finally, the present studies also identified a restricted pattern of FP receptor in skin, particularly in the dermal papillae. This site of expression may be important in mediating the stimulatory effect of latanaprost, an FP-selective agonist, on hair growth (29).

In summary, the present studies demonstrate high levels of expression of mRNA for the FP receptor in kidney distal tubules, including the DCT and CCD. The intrarenal distribution of FP receptor mRNA corresponds with the known effects of $PGF_{2\alpha}$ on salt and water transport in the kidney. The FP receptor is expressed along both male and female genitourinary tracts.

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Crucial Role of Histamine for Regulation of Gastric Acid Secretion Ascertained by Histidine Decarboxylase-Knockout Mice

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ABSTRACT

Histidine decarboxylase (HDC) represents the sole enzyme that produces histamine in the body. The present work investigated the role of endogenous histamine in carbachol- and gastrin-induced gastric acid secretion with HDC-knockout ($HDC^{-/-}$) mice. Acid secretion was measured in either mice subjected to acute fistula production under urethane anesthesia or conscious mice that had previously undergone pylorus ligation. In wild-type mice, carbachol and gastrin significantly stimulated acid secretion, increasing gastric mucosal histamine. In contrast, in $HDC^{-/-}$ mice, carbachol and gastrin had little impact when either delivered alone or together. Nonetheless, the two agents achieved a synergistic effect when delivered together with exogenous histamine, stimulating acid secretion in $HDC^{-/-}$ mice. Such synergism was abolished by the histamine H_2-receptor antagonist famotidine. cAMP involvement in acid secretion was also examined with theophylline, a phosphodiesterase inhibitor, and forskolin, an adenylate cyclase activator. In wild-type mice, theophylline significantly increased acid secretion, enhancing carbachol- and gastrin-stimulated acid secretion. In contrast, in $HDC^{-/-}$ mice, theophylline failed to exert an effect on basal acid secretion, as well as carbachol- and gastrin-stimulated acid secretion. Although forskolin interacted with carbachol, allowing acid secretion in $HDC^{-/-}$ mice, similar results were not achieved with gastrin. Such results suggest that 1) histamine is essential for carbachol- and gastrin-stimulated gastric acid secretion in mice; and 2) histamine-induced cAMP production contributes to the in vivo response to carbachol or gastrin.

The regulation of gastric acid secretion involves a complex network of mediators that both stimulate secretion in response to meal ingestion and maintain gastric mucosal homeostasis (Hersey and Sachs, 1995; Hirschowitz et al., 1995; Aihara et al., 2003). Because histamine represents a potent stimulant for gastric acid secretion, H_2-receptor antagonists are widely used for the treatment of acid-related peptic disease. Histamine is synthesized from histidine by histidine decarboxylase (HDC) and stored in mast cells, enterochromaffin-like (ECL) cells, and enteric nerve fibers in the stomach (Hakanson et al., 1986; Prinz et al., 2003). Acetylcholine and gastrin also represent major mediators of gastric acid secretion (Wilkes et al., 1991). It has been suggested that ECL cells are stimulated by carbachol (an acetylcholine analog) and gastrin, resulting in histamine release from cytoplasmic granules, which in turn activates parietal cells to secrete acid (Lindstrom et al., 2001; Prinz et al., 2003). Because H_2-receptor antagonists inhibit vagus nerve- and gastrin-stimulated gastric secretion, histamine is thought to represent the final mediator of acid secretion (Grossman and Konturek, 1974). In contrast, in vitro studies have demonstrated that both carbachol and gastrin directly stimulate isolated parietal cells to result in acid secretion (Soll, 1980; Pfeiffer et al., 1990; Li et al., 1995). In addition, histamine amplifies parietal cell stimulation by carbachol and gastrin. Although the above-mentioned studies summarize the involvement of histamine release in carbachol- and gastrin-stimulated gastric acid secretion, until the present work, the role played by endogenous histamine for in vivo carbachol- and gastrin-induced gastric acid secretion remained poorly understood.

Previous reports have demonstrated that treatment with α-fluoromethylhistidine (α-FMH), an irreversible inhibitor of HDC, resulted in an 80% reduction in gastric mucosal histamine levels (Andersson et al., 1996). α-FMH is thought to act

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ABBREVIATIONS: HDC, histidine decarboxylase; $HDC^{-/-}$, histidine decarboxylase-knockout; ECL, enterochromaffin-like; α-FMH, α-fluoromethylhistidine; CCK_2 receptor, cholecystokinin subtype-2 receptor; $H_2R^{-/-}$, histamine H_2-receptor-knockout.

by depleting histamine stores in ECL cells, leaving mucosal mast cells apparently unaffected (Andersson et al., 1996). Although α-FMH treatment was found to inhibit basal acid secretion by >60%, ranitidine inhibited basal acid secretion by approximately 85%. In the same experiments, vagal stimulation was found to increase acid secretion in α-FMH-treated animals, although ranitidine prevented such a rise. These findings suggest that α-FMH treatment is not sufficient to eliminate histamine's influence on gastric secretion.

$HDC^{-/-}$ mice were recently generated by gene-targeting methods (Ohtsu et al., 2001, 2002; Tanaka et al., 2002). Such mice exhibited trace histamine levels and no de novo gastric mucosal histamine synthesis. Accordingly, $HDC^{-/-}$ mice represent an ideal model to assess the direct effects of carbachol and gastrin on parietal cells.

The present work examined the effects of carbachol, gastrin, and histamine, alone and in combination, on gastric acid secretion in $HDC^{-/-}$ mice. In addition, the effects of theophylline, a nonselective phosphodiesterase inhibitor, and forskolin, an adenylate cyclase activator, on carbachol- and gastrin-stimulated acid secretion were studied to elucidate the role played by cAMP in parietal cells.

Materials and Methods

Animals. Male and female $HDC^{-/-}$ mice, as well as control wild-type littermate mice, were generated on a mixed genetic 129/Sv × ICR background (Ohtsu et al., 2001; Tanaka et al., 2002) and raised until 10 to 12 weeks old. The animals were housed at 23°C with 13 h of light (7:00 AM–8:00 PM). Wild-type mice were fed ad libitum standard diet. $HDC^{-/-}$ mice were fed a low histamine diet (Nosan Co., Kanagawa, Japan). Before the experiments, both wild-type and knockout mice were deprived of food for 21 h and water for 2 h. Animal maintenance and experimental procedures were carried out in accordance with the guidelines of the Ethics Committee of Kyoto Pharmaceutical University.

Drugs. Drugs used were histamine 2HCl (0.1–10 mg/kg, histamine; Sigma-Aldrich, St. Louis, MO), carbamylcholine chloride (0.05 mg/kg, carbachol; Sigma-Aldrich), human gastrin-17 (1 mg/kg, gastrin; Sigma-Aldrich), theophylline (100 mg/kg; Nakarai Chemicals, Ltd., Kyoto, Japan), forskolin (5 mg/kg; Wako Pure Chemicals, Osaka, Japan), famotidine (10 mg/kg; Yamanouchi Pharmaceutical Co., Tokyo, Japan), and urethane (1.25 g/kg; Kyoto Kasei, Kyoto, Japan). Both theophylline and famotidine were suspended in 0.5% carboxymethylcellulose. Forskolin was first dissolved in dimethyl sulfoxide and then diluted with saline to the desired concentration. All other drugs were dissolved in saline. All agents and vehicle were prepared before administration and subcutaneously injected at a volume of 0.05 ml/10 g of body weight.

Measurement of Intragastric pH. Under ether anesthesia, each abdomen was opened and each stomach was removed and incised along the greater curvature. Intragastric pH was immediately measured with a pH meter (M-11; Horiba Co., Kyoto, Japan) that was directly placed on the fundic mucosa.

Measurement of Gastric Mucosal Histamine. Gastric mucosal histamine contents in wild-type and $HDC^{-/-}$ mice were measured according to a previously reported method (Kobayashi et al., 2000). In a limited number of mice, mucosal histamine levels were determined 1 h after carbachol or gastrin administration. In brief, each stomach was removed, rinsed with phosphate-buffered saline, weighed, and then homogenized in 0.01 M sodium phosphate buffer. The buffers contained 10^{-6} M semicarbazide HCl to prevent histamine degradation. Each homogenate was diluted 1:10 with sodium phosphate buffer and heated in boiling water for 10 min so as to release bound histamine. The homogenates were then centrifuged at 500*g* for 20 min, and the supernatants were used as samples. Quantitative determination of sample histamine levels was performed with the histamine enzyme immunoassay kit (SPI-bio, Massy Cedex, France).

Determination of Gastric Acid Secretion in Mice with a Gastric Fistula. Each mouse was anesthetized with an intraperitoneal injection of urethane. After tracheotomy, a polyethylene 60 tube was inserted into the trachea to ensure a patent airway. Acid secretion was measured in the mice according to a previously reported method (Tanaka et al., 2002). In brief, the abdomen was incised and both the stomach and duodenum were exposed. An acute catheter (inside diameter of 2 mm), created with a polyethylene tube, was inserted into the stomach from a small incision made in the duodenum and secured with a ligature around the pylorus. At the beginning of each experiment, each stomach was rinsed several times with physiological saline and filled with 0.4 ml of saline through the catheter. Physiological saline (0.4 ml) was injected through the fistula and collected every 15 min. The acidity of the collected gastric fluid was determined by titration against 0.01 mol/l NaOH to a pH of 7.0 using an automatic titrator (Comtite 550; Hiranuma, Tokyo, Japan). Gastric acid output (volume × acidity) was expressed as microequivalents per 15 min. The cumulative acid output represents the sum of the acid output for 2 or 5 h after administration of each stimulant. In all experiments, basal gastric acid secretion was determined for at least 45 min before drug administration.

Determination of Gastric Acid Secretion in Mice Subjected to Pylorus Ligation. The effects on gastric secretion of subcutaneous administration of a single dose of histamine, carbachol, and gastrin were examined. In certain experiments, carbachol or gastrin was subcutaneously injected either immediately after or 45 min after histamine administration to $HDC^{-/-}$ mice. A histamine H_2-receptor antagonist, famotidine, was subcutaneously delivered 30 min before administration of histamine alone, histamine and carbachol, histamine and gastrin, or theophylline. To examine the role played by cAMP, theophylline or forskolin was subcutaneously injected 30 min before administration of the other agents. The theophylline dose was based on a previous report (Cheng et al., 1997). Control animals received vehicle alone. To compare results obtained with anesthetized mice to those obtained with conscious mice, the acid secretion-stimulating effects of histamine, carbachol, and gastrin were examined without urethane treatment. Each mouse was lightly anesthetized with ether for epigastric laparotomy and pylorus ligation. After recovery from anesthesia, histamine, carbachol, or gastrin was subcutaneously injected. Subsequently, 1.5 h after pylorus ligation, animals were lightly anesthetized with ether again and each stomach was removed to collect gastric fluid. Gastric fluid was analyzed with regards to volume and acidity. Control animals received vehicle alone.

Data Analysis. Data are presented as means ± S.E.M. Comparisons between control means and single treatment means were made with a two-tailed, paired, or unpaired Student's *t* test, as deemed appropriate. Comparisons of control means with multiple treatment means were made by one-way variance analysis followed by Dunnett's test. *P* values of <0.05 were regarded as significant.

Results

Intragastric pH in Wild-Type (WT) and $HDC^{-/-}$ Mice. To determine whether the lack of histamine affected basal gastric secretion, intragastric pH was determined. Intragastric pH in wild-type mice and $HDC^{-/-}$ mice was found to be 2.1 ± 0.2 and 4.7 ± 0.2, respectively; the difference was noted to be significant.

Effects of Carbachol and Gastrin on Wild-Type and $HDC^{-/-}$ Mice. In anesthetized wild-type mice subjected to acute fistula production, carbachol (0.05 mg/kg) and gastrin (1 mg/kg) significantly increased gastric acid secretion (Fig.

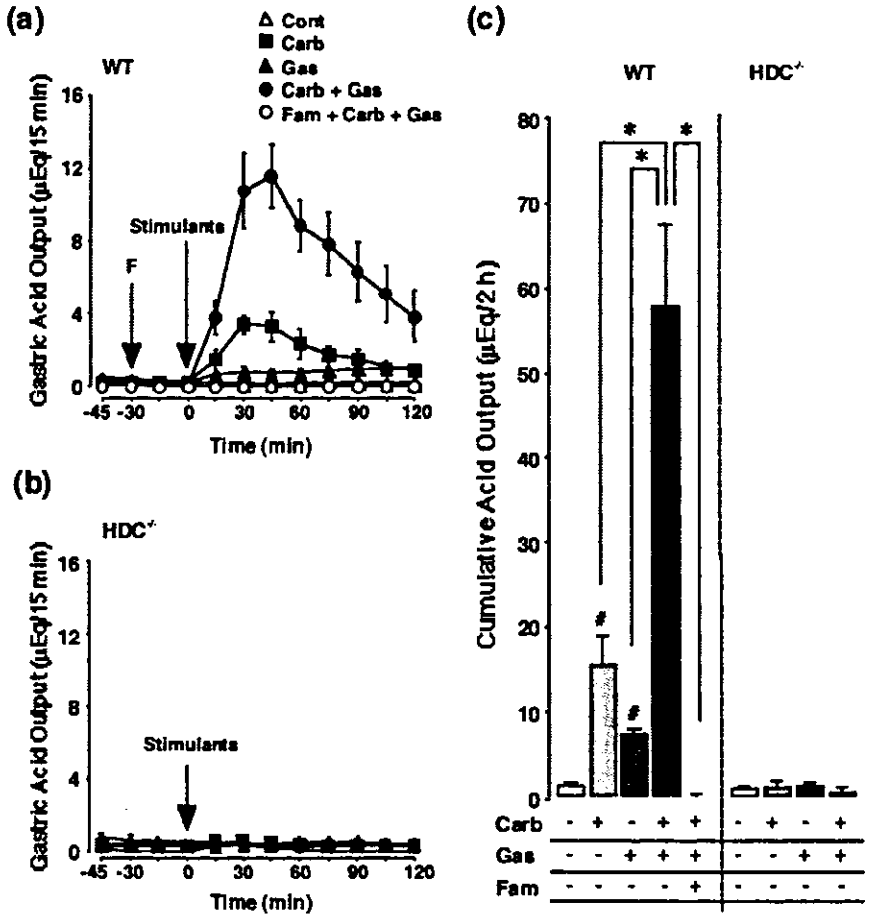

Fig. 2. Gastric acid secretion induced by carbachol combined with gastrin in WT and HDC$^{-/-}$ mice. Gastric acid secretion was measured with the gastric fistula method under urethane anesthesia. Carbachol (Carb), gastrin (Gas), or famotidine (Fam, F) was subcutaneously injected at doses of 0.5, 1, and 10 mg/kg, respectively. Control mice (Cont) were administered vehicle alone. Acid-output profiles for wild-type (a) and HDC$^{-/-}$ (b) mice are shown as means ± S.E.M. for four to six mice. The cumulative acid outputs after stimulation are as shown (c). #, $P < 0.05$, significant difference from vehicle-treated control mice (Dunnett's test). *, $P < 0.05$, significant difference between the indicated groups (Student's t test).

significantly stimulated acid secretion in wild-type mice, it failed to stimulate gastric acid secretion in HDC$^{-/-}$ mice (Fig. 10, a and b). Combined treatment with forskolin and theophylline tended to stimulate gastric acid secretion, yet the increase was not significant compared with forskolin alone. In HDC$^{-/-}$ mice, both forskolin and combined forskolin and theophylline did not significantly stimulate gastric acid secretion (Fig. 10b). Nonetheless, carbachol (0.05 mg/kg), but not gastrin (1 mg/kg), significantly enhanced acid secretion in HDC$^{-/-}$ mice treated with combined forskolin and theophylline (Fig. 10b).

Discussion

The above-mentioned results demonstrated that, although carbachol and gastrin individually exerted little influence on secretion in HDC$^{-/-}$ mice, the two secretagogues synergistically combined with exogenous histamine to stimulate acid secretion. The present study also revealed that although HDC$^{-/-}$ mice lack gastric mucosal histamine, such mice exhibit increased parietal cell sensitivity to exogenous histamine, which results in enhanced acid secretion. Although Tanaka et al. (2002) demonstrated slightly reduced basal acid secretion in HDC$^{-/-}$ mice (Tanaka et al., 2002), the present study found that intragastric pH was significantly higher in HDC$^{-/-}$ mice compared with wild-type mice. In addition, serum gastrin levels in HDC$^{-/-}$ mice were also significantly higher than those of wild-type mice, a result of the increased pH (data not shown).

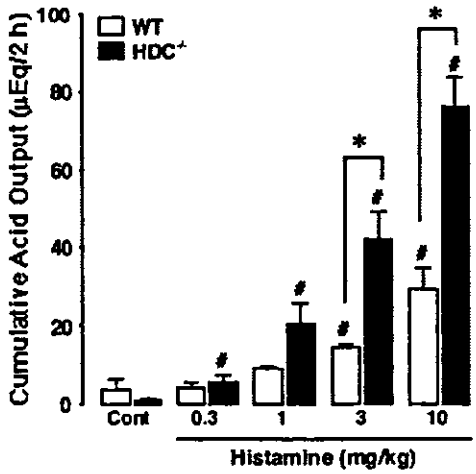

Fig. 3. Gastric acid secretion induced by histamine in WT and HDC$^{-/-}$ mice. Gastric acid secretion was measured with the gastric fistula method under urethane anesthesia. Histamine was subcutaneously injected at the indicated doses. Control mice (Cont) were administered vehicle alone. Cumulative acid output after stimulation for wild-type and HDC$^{-/-}$ mice are shown as means ± S.E.M. for five to six mice. # and *, $P < 0.05$, significant difference from vehicle-treated mice (Dunnett's test) and wild-type mice (Student's t test) treated with identical histamine doses, respectively.

Some disparities exist between the present study and a previous study. Tanaka et al. (2002) reported that carbachol stimulated weak and transient acid secretion in HDC$^{-/-}$

1, a and b). Combined treatment with carbachol and gastrin was found to synergistically stimulate acid secretion (Fig. 2, a and c). Cumulative analysis of acid output in such mice revealed that cotreatment with carbachol and gastrin significantly increased acid secretion compared with vehicle; wild-type mice treated with carbachol or gastrin alone exhibited similar effects (Fig. 2c). Pretreatment with famotidine (10 mg/kg) resulted in complete inhibition of acid secretion induced by both carbachol and gastrin (Fig. 2, a and c). In contrast, carbachol, gastrin, or carbachol simultaneously administered with gastrin did not stimulate gastric acid secretion in $HDC^{-/-}$ mice (Figs. 1, a and b, 2b). Based upon the above results, an effect of famotidine on gastric acid secretion was not observed in response to each secretagogue in $HDC^{-/-}$ mice (Fig. 2c).

Effects of Histamine on Carbachol- and Gastrin-Stimulated Gastric Acid Secretion in Wild-Type and $HDC^{-/-}$ Mice. In both wild-type and $HDC^{-/-}$ mice, gastric acid secretion was stimulated by exogenous histamine in a dose-related manner (Fig. 3). Nonetheless, $HDC^{-/-}$ mice exhibited hypersensitive gastric acid secretion in response to histamine; the increased secretion was significant at >3 mg/kg. Because histamine, at a dose of 0.3 mg/kg, led to a significant increase in acid secretion in $HDC^{-/-}$ mice, this dose was used in the following study.

To investigate the potentiative effect of histamine for carbachol and gastrin, the effects of low doses of histamine on the response to carbachol and gastrin were examined in $HDC^{-/-}$ mice. Combined treatment with carbachol (0.05 mg/kg) and histamine (0.3 mg/kg) clearly resulted in a synergistic increase in gastric acid secretion in $HDC^{-/-}$ mice (Fig. 4a). For 2-h cumulative acid secretion, the synergistic response was significantly greater than the response observed for controls and mice treated with histamine or carbachol alone (Fig. 4b). Famotidine (10 mg/kg) completely inhibited secretion after combined carbachol and histamine administration (Fig. 4b). Gastrin alone (1 mg/kg) had no effect on acid secretion in $HDC^{-/-}$ mice (Fig. 5a); however, gastrin (1 mg/kg) significantly augmented acid secretion 45 min after histamine treatment (0.3 mg/kg) (Fig. 5a). Famotidine (10 mg/kg) treatment completely inhibited enhanced acid secretion, resulting from combined gastrin and histamine (Fig. 5b).

The present study confirmed with conscious wild-type mice subjected to pylorus ligation that histamine significantly potentiated acid secretion in response to gastrin. In contrast, histamine only exhibited a tendency for potentiated secretion with carbachol stimulation (Fig. 6a). Nonetheless, histamine significantly potentiated acid secretion in response to both carbachol and gastrin in $HDC^{-/-}$ mice (Fig. 6b).

Effects of Carbachol and Gastrin on Gastric Mucosal Histamine Levels in Wild-Type Mice. Gastric mucosal histamine increased to 26.1 ± 1.5 and 22.0 ± 1.7 μg/g of wet tissue after treatment with carbachol and gastrin, respectively. Such levels were statistically greater than levels measured in control mice (15.6 ± 1.8 μg/g of wet tissue; Fig. 7).

Effects of Theophylline and Forskolin on Gastric Acid Secretion in Wild-Type and $HDC^{-/-}$ Mice. It has been suggested that theophylline augments the secretory response to histamine via inhibition of phosphodiesterase, because H_2-receptors are Gs protein-coupled receptors that are known to increase cAMP. To further investigate the role of cAMP in histamine-mediated acid secretion, the effects of theophylline and gastric secretagogues on basal and stimulated-acid secretion were examined in anesthetized wild-type and $HDC^{-/-}$ mice. Theophylline (100 mg/kg) significantly stimulated 5-h cumulative gastric acid secretion in wild-type mice but failed to achieve a similar effect in $HDC^{-/-}$ mice (Fig. 8, a–c). Famotidine completely prevented theophylline-stimulated acid secretion in wild-type mice (Fig. 8, a and c). In addition, theophylline tended to increase mucosal histamine levels in wild-type mice stomachs (Fig. 8d). Moreover, theophylline enhanced gastric acid secretion induced by carbachol, gastrin, and histamine in wild-type mice (Fig. 9a). In contrast, theophylline administered together with either carbachol or gastrin failed to affect acid secretion in $HDC^{-/-}$ mice. Nonetheless, upon concurrent administration of theophylline and histamine (0.3 mg/kg), significantly enhanced secretion was observed (Fig. 9b). Theophylline delivered with 10 mg/kg histamine, however, did not result in further enhanced acid secretion; thus, it seems that maximal stimulation was achieved with the lower dose (Fig. 9b).

The effect of forskolin, an adenylate cyclase activator, was examined to further study the role played by cAMP for histamine-mediated acid secretion. Although forskolin (5 mg/kg)

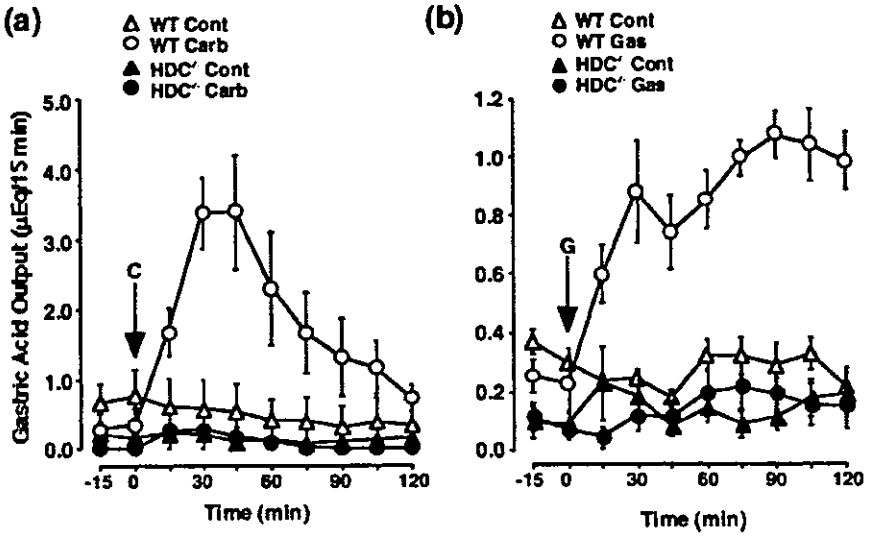

Fig. 1. Gastric acid secretion induced by carbachol (a) or gastrin (b) in WT and $HDC^{-/-}$ mice. Gastric acid secretion was measured with the gastric fistula method under urethane anesthesia. Carbachol (Carb, C) and gastrin (Gas, G) were subcutaneously injected at doses of 0.05 and 1 mg/kg, respectively. Control mice (Cont) were administered vehicle alone. The acid output profiles are shown as means ± S.E.M. for five to eight mice.

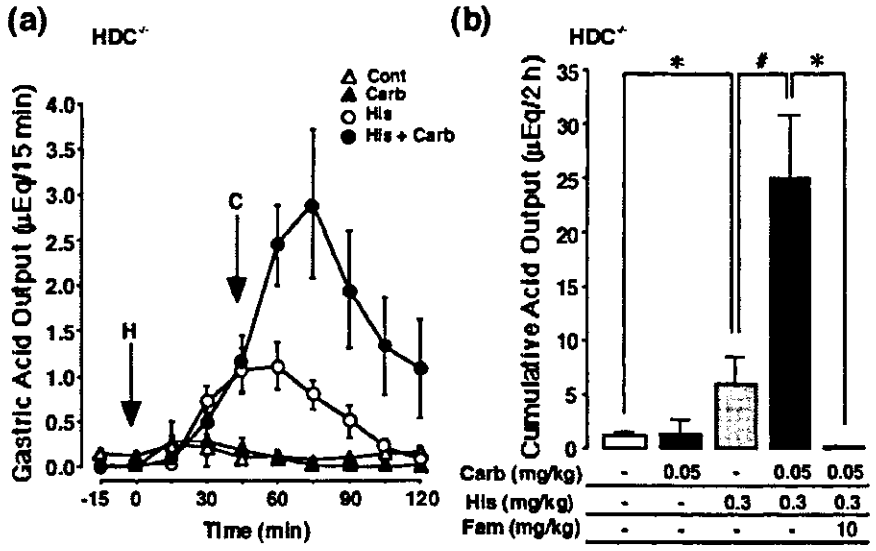

Fig. 4. Gastric acid secretion stimulated by histamine combined with carbachol in HDC$^{-/-}$ mice. Gastric acid secretion was measured with the gastric fistula method under urethane anesthesia. In dual agonist studies in HDC$^{-/-}$ mice, carbachol (Carb, C) was subcutaneously injected at a dose of 0.05 mg/kg 45 min after histamine (His, H) treatment with a dose of 0.3 mg/kg (a). Famotidine (Fam; 10 mg/kg) was subcutaneously injected 30 min before histamine treatment with a dose of 0.3 mg/kg. Control mice (Cont) were administered vehicle alone. The acid output profiles are shown as means ± S.E.M. for five to seven mice. The cumulative acid outputs after stimulation of HDC$^{-/-}$ mice are as shown (b). *, $P < 0.05$, significant difference from mice treated with either vehicle or carbachol (0.05 mg/kg) combined with histamine (0.3 mg/kg) (Student's t test). #, $P < 0.05$, significant difference from mice treated with histamine (0.3 mg/kg) (Dunnett's test).

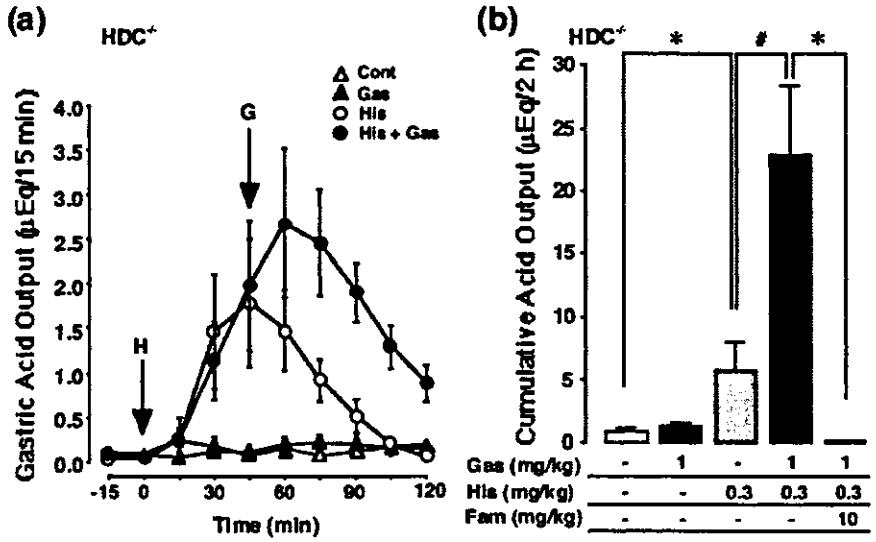

Fig. 5. Gastric acid secretion stimulated by histamine combined with gastrin in wild-type and HDC$^{-/-}$ mice. Gastric acid secretion was measured with the gastric fistula method under urethane anesthesia. In dual agonist studies in HDC$^{-/-}$ mice, gastrin (Gas, G) was subcutaneously injected at a dose of 1 mg/kg 45 min after histamine (His, H) treatment with a dose of 0.3 mg/kg (a). Famotidine (Fam; 10 mg/kg) was subcutaneously injected 30 min before histamine treatment with a dose of 0.3 mg/kg. Control mice (Cont) were administered vehicle alone. The acid-output profiles are shown as means ± S.E.M. for five to seven mice. The cumulative acid outputs after stimulation of HDC$^{-/-}$ mice are as shown (b). *, $P < 0.05$, significant difference from mice treated with either vehicle or gastrin (1 mg/kg) combined with histamine (0.3 mg/kg) (Student's t test). #, $P < 0.05$, significant difference from mice treated with histamine (0.3 mg/kg) (Dunnett's test).

mice. Nonetheless, in the present study, carbachol did not stimulate acid secretion in HDC$^{-/-}$ mice. Recently, existence of a histamine-uptake mechanism for mast cells was reported (Ohtsu et al., 2002). Given the possibility that HDC$^{-/-}$ mice might uptake and store exogenous histamine obtained from food into mast cells, potentially influencing acid secretion, the difference observed in the results might stem from the fact that a diet low in histamine was used for the present work.

It has been suggested that carbachol and gastrin stimulate histamine release from ECL cells, inducing acid secretion. In the present study, the finding that carbachol and gastrin synergistically stimulated acid secretion in the presence of exogenous histamine, but not in the absence of exogenous histamine in HDC$^{-/-}$ mice, should be stressed. In addition, such stimulated acid secretion was completely blocked by pretreatment with famotidine. Considering that histamine release from ECL cells is absent in HDC$^{-/-}$ mice, such results indicate that carbachol and gastrin can directly act on parietal cells, but require H_2-receptor activation to initiate acid secretion. We postulate that both sufficient intracellular cAMP, an effect achieved with histamine, and Ca^{2+}, an effect achieved with carbachol and gastrin, in parietal cells is required to initiate acid secretion. Accordingly, in HDC$^{-/-}$ mice fistula experiments, if histamine, i.e., cAMP, is present, carbachol and gastrin are able to potentiate acid secretion more than with histamine stimulation alone.

It is important to note that, in wild-type mice, gastrin significantly stimulated acid secretion, maintaining an elevated level 2 h after administration. In contrast, gastrin-stimulated acid secretion in histamine-pretreated HDC$^{-/-}$ mice gradually declined to baseline after peaking at a maximal level. Accordingly, gastrin seems to stimulate acid secretion in wild-type mice by causing a continuous release of histamine from ECL cells. Such results agree with previous reports (Gerber and Payne, 1992; Prinz et al., 1993). It has been reported that, although gastrin was found to induce histamine release from ECL cells via interaction with CCK_2 receptors, carbachol did not stimulate such histamine release (Norlen et al., 2001). Interestingly, the neurotransmitter pituitary adenylate cyclase-activating polypeptide was noted to exert a strong stimulatory effect on ECL cell histamine mo-

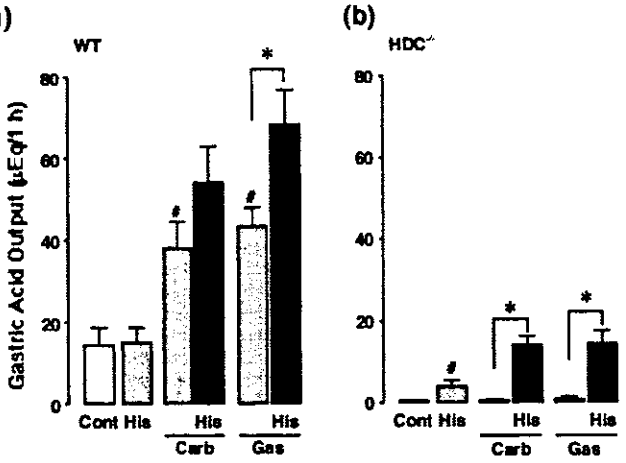

Fig. 6. The effects of histamine on responses to carbachol and gastrin in wild-type and $HDC^{-/-}$ mice. Gastric acid secretion was measured using the pylorus-ligation method (1.5 h). Histamine (His), carbachol (Carb), or gastrin (Gas) was subcutaneously injected at doses of 0.3, 0.05, and 1 mg/kg, respectively. Control mice (Cont) were administered vehicle alone. Data are expressed as means ± S.E.M. for six to nine mice. #, $P < 0.05$, significant difference from vehicle-treated control mice (Dunnett's test). *, $P < 0.05$, significant difference between the indicated groups (Student's t test).

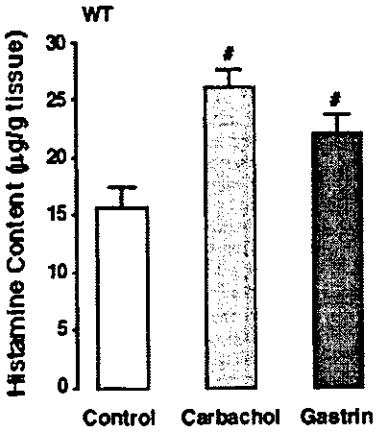

Fig. 7. Effects of carbachol and gastrin on gastric mucosal histamine levels in WT mice. Either carbachol or gastrin was subcutaneously injected at doses of 0.05 or 1 mg/kg, respectively. Control mice (Cont) were administered vehicle alone. Data are expressed as means ± S.E.M. for five mice. #, $P < 0.05$, significant difference from vehicle-treated control mice (Dunnett's test).

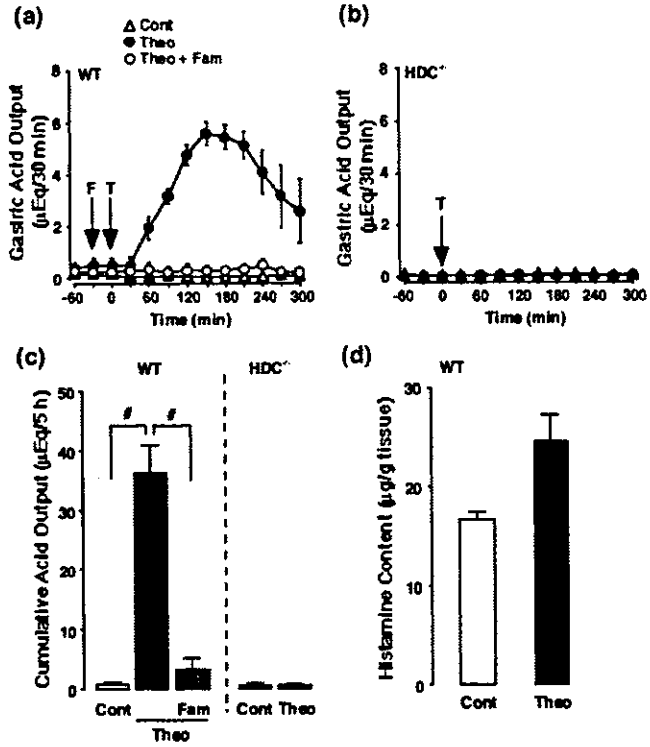

Fig. 8. Effects of theophylline on gastric acid secretion and mucosal histamine levels in WT and $HDC^{-/-}$ mice. Gastric acid secretion was measured with the gastric fistula method under urethane anesthesia. The acid output profiles for wild-type (a) and $HDC^{-/-}$ (b) mice are shown as means ± S.E.M. for three to four mice. Theophylline (Theo, T) or famotidine (Fam, F) was subcutaneously injected at doses of 100 or 10 mg/kg, respectively. Control mice (Cont) were administered vehicle alone. The cumulative acid outputs after stimulation are as shown (c). Data are expressed as means ± S.E.M. for three to four mice. Gastric mucosal histamine levels 1 h after treatment with theophylline (100 mg/kg) were measured (d). #, $P < 0.05$, significant difference from theophylline-treated mice (Dunnett's test).

bilization (Norlen et al., 2001; Sandvik et al., 2001). Consequently, the mechanism underlying carbachol-induced acid secretion might be partially mediated by pituitary adenylate cyclase-activating polypeptide.

It is of interest that theophylline treatment resulted in markedly different acid secretory responses in wild-type and $HDC^{-/-}$ mice. Theophylline significantly stimulated acid secretion in wild-type mice, but not $HDC^{-/-}$ mice. Moreover, famotidine significantly inhibited acid secretion induced by theophylline in wild-type mice, despite the fact that theophylline only tended to increase histamine levels. Such results suggest that endogenous histamine remains essential for theophylline-stimulated acid secretion. Because increases in intracellular cAMP have been suggested to result in histamine release (Miyazaki et al., 1992; Prinz et al., 1993), theophylline might induce histamine release from either ECL or mast cells.

Theophylline treatment in wild-type mice extensively increased gastric acid secretion in response to histamine, carbachol, and gastrin. Nonetheless, theophylline treatment in $HDC^{-/-}$ mice only significantly increased acid secretion in response to histamine; no increase in secretion was observed with concurrent administration with carbachol or gastrin. Such results imply that, in wild-type mice, carbachol, gastrin, and histamine stimulate acid secretion by increasing cAMP levels in parietal cells. In theophylline-treated $HDC^{-/-}$ mice, however, carbachol and gastrin could not stimulate acid secretion, because endogenous histamine was not present. The results strongly suggest that histamine is required for cAMP-mediated acid secretion by carbachol and gastrin.

It is of interest that pretreatment with forskolin and theophylline resulted in different acid secretory responses in wild-type and $HDC^{-/-}$ mice. Namely, forskolin, delivered either with or without theophylline, significantly stimulated acid secretion in wild-type mice, but not $HDC^{-/-}$ mice. It remains likely that such distinct responses represent the product of whether or not endogenous histamine is present in the gastric mucosa. Previous studies have demonstrated that histamine induced an increase in intracellular Ca^{2+}, as well as intracellular cAMP, via interaction with H_2-receptors (Delvalle et al., 1992a,b). Accordingly, it seems that histamine stimulates acid secretion by facilitating an increase in both intracellular cAMP and Ca^{2+}. In contrast, forskolin,

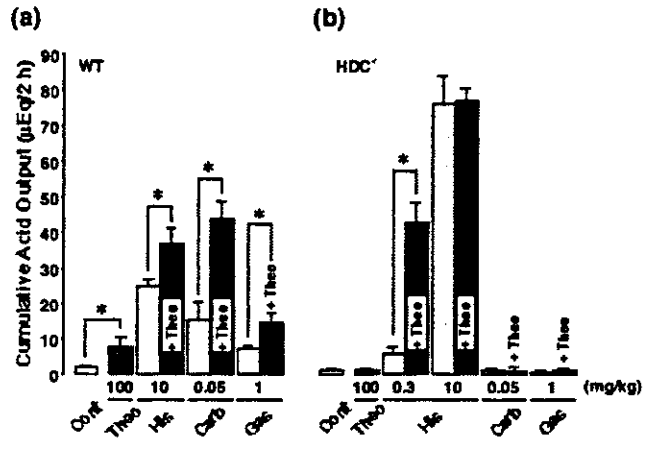

Fig. 9. Effects of theophylline on histamine-, carbachol-, and gastrin-stimulated gastric acid secretion in WT (a) and $HDC^{-/-}$ (b) mice. Gastric acid secretion was measured with the gastric fistula method under urethane anesthesia. The cumulative acid outputs after stimulation are as shown. Theophylline (Theo), histamine (His), carbachol (Carb), or gastrin (Gas) was subcutaneously injected at doses of 100, 0.3, 0.05, and 1 mg/kg, respectively. Control mice (Cont) were administered vehicle alone. Data are expressed as means ± S.E.M. for four to five mice. *, $P < 0.05$, significant difference between the indicated groups (Student's t test).

combined with theophylline, failed to increase acid secretion in $HDC^{-/-}$ mice. Such findings suggest that an increase in cAMP alone is insufficient to stimulate acid secretion. The mechanism by which carbachol potentiates acid secretion to a small degree in $HDC^{-/-}$ mice treated with combined forskolin and theophylline might stem from increased second messenger levels, i.e., cAMP and Ca^{2+}, in parietal cells. The reason underlying the inability of gastrin to increase acid secretion in $HDC^{-/-}$ mice most likely results from the inability of gastrin to significantly induce elevation of second messenger levels compared with carbachol.

Current genetic technology has allowed generation of knockout mice lacking genes for such receptors as CCK_2 and H_2, as well as such mediators as gastrin. The functional and morphological changes in the gastric mucosa of such knockout mice has been reported previously (Nagata et al., 1996; Langhans et al., 1997; Friis-Hansen et al., 1998; Kobayashi et al., 2000; Ogawa et al., 2003). A study of gastrin-knockout mice demonstrated that histamine or carbachol administration failed to stimulate acid secretion (Friis-Hansen et al., 1998). Nonetheless, such a study demonstrated that gastrin administration for 6 days reversed the effects of gastrin deficiency, leading to an increase in the number of mature H^+, K^+-ATPase-positive parietal cells and a partial restoration of acid secretion. Such results strongly suggest that deficiency of mature parietal cells contributes to impaired acid secretion in gastrin-knockout mice. Decreased acid secretion in CCK_2 receptor knockout mice is also thought to result from a deficiency of mature parietal cells (Nagata et al., 1996; Langhans et al., 1997). In contrast, because the histological makeup and appearance of the mucosal surface of $HDC^{-/-}$ mice closely resembles that of wild-type mice, $HDC^{-/-}$ mice can be used to investigate the mechanisms underlying acid secretion.

H_2-receptor knockout ($H_2R^{-/-}$) mice were generated and analyzed in terms of gastric functional and morphological changes (Kobayashi et al., 2000; Ogawa et al., 2003). The basal intragastric pH of $H_2R^{-/-}$ mice was normal (<3.0). It should also be noted that carbachol, but not gastrin, clearly stimulated acid secretion in such mice. Accordingly, Kobayashi hypothesized that in $H_2R^{-/-}$ mice, a cholinergic pathway might allow maintenance of basal acid secretion in the absence of a histamine-mediated pathway. As stated above, the present study has advanced the understanding of the mechanisms underlying acid secretion by demonstrating that carbachol stimulation of acid secretion is dependent on increased levels of intracellular cAMP induced by histamine.

In conclusion, studies with $HDC^{-/-}$ mice demonstrated that carbachol and gastrin directly stimulate parietal cells to secrete gastric acid only in the presence of gastric mucosal histamine. In addition, it was also found that high intracellular cAMP levels are requisite for carbachol, gastrin, and histamine to stimulate gastric acid secretion.

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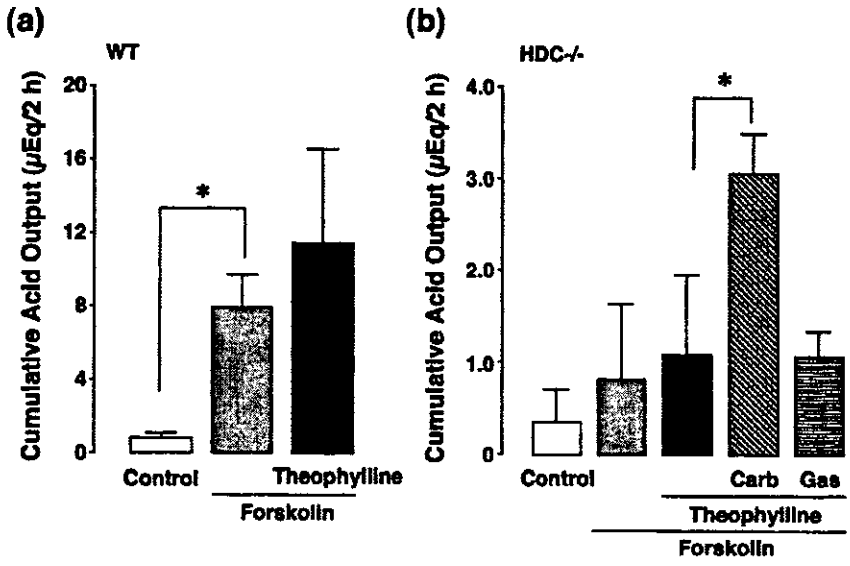

Fig. 10. Effects of forskolin on carbachol- and gastrin-stimulated gastric acid secretion in WT (a) and $HDC^{-/-}$ (b) mice. Gastric acid secretion was measured with the gastric fistula method under urethane anesthesia. The cumulative acid outputs after stimulation are as shown. Forskolin, theophylline, carbachol (Carb), or gastrin (Gas) was subcutaneously injected at doses of 5, 100, 0.05, and 1 mg/kg, respectively. Control mice were administered vehicle alone. Data are expressed as means ± S.E.M. for four to six mice. *, $P < 0.05$, significant difference between the indicated groups (Student's t test).

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Apoptosis and Related Proteins in Placenta of Intrauterine Fetal Death in Prostaglandin F Receptor-Deficient Mice[1]

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ABSTRACT

The present study investigated whether the increase of apoptosis in the placenta is associated with intrauterine fetal death in prostaglandin F receptor-deficient mice. Apoptosis was demonstrated within placental and decidual tissue by the TUNEL method. The majority of apoptosis was found in syncytiotrophoblast tissues. Enhanced TUNEL-positive staining in the syncytiotrophoblast layer was scattered in the placental tissues in clusters of apoptotic cells in the death group. Marked TUNEL-positive cells were identified in decidua of both groups. The rate of apoptosis in the placenta and decidua in the death group was higher than that in the survival group ($P < 0.05$). Immunohistochemical analysis showed that the level of active caspase-3 protein expression in the placenta in the death group was much higher than that in the survival group. The level of Bcl-2 protein expression in the placenta in the death group was much lower than that in the survival group. Western blot analysis demonstrated that increased expression of the active form of caspase-3 was detected in the placenta and decidua in the death group compared with that in the survival group. In contrast, a decrease in the expression of Bcl-2 was detected in the placenta and decidua in the death group compared with that in the survival group. Enhanced expression of Bax:Bcl-2 ratio was detected in placenta and decidua in the death group compared with that in the survival group. Thus, significantly increased apoptosis in the mouse placenta and decidua might be involved in the pathophysiologic mechanism of intrauterine fetal death.

apoptosis, placenta, pregnancy, trophoblast

INTRODUCTION

Intrauterine fetal death is an alarming event in the practice of obstetrics. Postterm pregnancy is associated with an increased risk of perinatal mortality and morbidity [1, 2]. However, the mechanisms leading to fetal and neonatal death in postterm pregnancies or term pregnancies are unclear.

Clearly, apoptosis is important in many aspects of reproduction. Apoptosis occurs in all placental cell types, increases from first to third trimester, and is believed to be physiologically important for normal placental growth and development [3, 4]. Aberrant placental apoptosis may affect placental function, resulting in complicated pregnancies. Increased trophoblast apoptosis has been documented in the placentas of growth-restricted fetuses, and maternal smoking has been shown to be associated with decreased placental apoptosis at term [5, 6]. Placental apoptosis is also increased during spontaneous abortion in the first trimester [7], preeclamptic pregnancy [8, 9], and postterm pregnancy [10].

Apoptotic signaling is processed by highly regulated and specific proteolysis via caspase. Caspases are synthesized as inactive precursors that must be cleaved autocatalytically or by other caspases for activation. Triggering of apoptosis results in a cascade of caspase activation, in which the last caspases to be activated are those that digest cellular substrates, resulting in morphological changes and death of the cell. Bcl-2 of antiapoptotic regulators was first discovered in human follicular lymphoma and was regarded as a protooncogene [11]. It is reported that Bcl-2 has the function of inhibiting apoptosis [12, 13]. Bcl-2 plays an important role in preventing apoptosis in the syncytiotrophoblast [14]. Bax of proapoptotic regulators appears to accelerate the cell-death signal. Overexpression of Bax promotes apoptotic cell death, and conversely, Bcl-2 promotes this type of cell survival [15, 16]. The ratio of Bax to Bcl-2 is an important determinant of cell death or survival [17].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), one of the most abundant prostaglandins, is particularly involved in reproductive functions, such as ovulation, luteolysis, and parturition [18]. Sugimoto et al. [19] developed mice lacking the prostaglandin F receptor. These mice do not deliver fetuses at term and, instead, continue their pregnancy, although these mice are normal with respect to other aspects of reproductive physiology. No abnormalities in the weight or histology of placentas at term were observed in the $PGF_{2\alpha}$ receptor-deficient mice. These fetuses gradually died in uterus with prolonged pregnancy and were reabsorbed. Therefore, this $PGF_{2\alpha}$ receptor-deficient mouse provides a good animal model for studying intrauterine fetal death in postterm pregnancy [10, 19]. The present study investigated whether the increase of apoptosis in the placenta and decidua is associated with intrauterine fetal death in $PGF_{2\alpha}$ receptor-deficient mice.

MATERIALS AND METHODS

All animals were maintained in accordance with institutional guidelines for the care and use of laboratory animals. Mice were housed under standard photoperiod (12L:12D) and temperature (23 ± 1°C). Mice were also given free access to a nutritionally balanced diet and tap water. The $PGF_{2\alpha}$ receptor-deficient mice were obtained as described previously [19]. Nor-

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mal adult female mice with the +/− genotype bred in our animal facility were mated with either $PGF_{2\alpha}$ receptor-deficient male mice or male mice with the +/− genotype, and the resulting female $PGF_{2\alpha}$ receptor-deficient mice were used in the present experiment. Mouse genotypes were identified by polymerase chain reaction.

The day that a copulation plug was found was considered to be Day 0 of pregnancy. Placentas were removed from fetuses on Days 18–23 of pregnancy. At the same time, the survival and weight of fetuses were recorded. At least three pregnant mice were used for each day of pregnancy. All fetuses were divided into the survival and death groups. Fetal death was determined by lack of movement, respiratory activity, or reaction to painful stimuli. The placentas from fetuses in the death group were excluded when partial or complete body maceration, skin wrinkling, and discoloration of skin were noted. The placentas on Days 21 and 22 of pregnancy were used for analysis of apoptosis, caspase-3, Bcl-2, and Bax. Half the placentas from the same mice were fixed in a 10% formaldehyde neutral buffer solution and embedded in paraffin. Five sections were cut for each sample. The other placentas were isolated by gently separating placental and decidual tissues. The tissues were flash-frozen and stored at −80°C until processed for Western blot analysis.

In Situ Detection of DNA Nicking

Detection of DNA fragmentation was performed using the TUNEL technique. The TUNEL procedure was performed using an Apop Tag kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions. The tissue sections were deparaffinized, and protein was digested with 20 μg/ml of proteinase K for 15 min at room temperature. Endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS. After washing with PBS, an equilibration buffer was applied directly to the specimen. Terminal deoxynucleotidyl transferase (TdT) enzyme and dUTP-digoxigenin were added and incubated at 37°C for 1 h in a humidified chamber. The reaction was then stopped with a stop/wash buffer supplied with the kit, and the slides were incubated with an anti-digoxigenin-peroxidase solution for 30 min at room temperature, colorized with diaminobenzidine (DAB)/H_2O_2, and counterstained with methyl green. Negative controls were processed with labeled dUTP in the absence of the TdT enzyme. Sections of normal rodent mammary gland were used as positive controls. For each section, six fields of view were examined, and the number of TUNEL-positive stained nuclei was expressed as a percentage of the total number of nuclei counted.

Immunohistochemistry

Tissue samples were embedded in paraffin, and sections (thickness, 5 μm) were cut with a microtome and placed on coated slides. Paraffin was removed from the tissue sections with xylene, and the sections were rehydrated in graded ethanol solutions. Antigen retrieval was performed by heating in 10 mM citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. After blocking with 5% normal rabbit serum, sections were incubated with the primary antibody for Bcl-2 (polyclonal antibody N-19 at 1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and for caspase-3 (polyclonal antibody D-175 at 1:100 dilution; Cell Signaling Technology, Beverly, MA) at 4°C overnight. They were rinsed in PBS and incubated with biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA), followed by avidin-biotin-peroxidase solution (Vectastain ABC Elite kit; Vector Laboratories). Next, DAB with 0.003% H_2O_2 in PBS was added to each slide. The tissues were lightly counterstained with hematoxylin and examined by light microscopy. Negative controls were performed with normal rabbit serum instead of primary antibody with all other steps unchanged.

Western Blot Analysis

Each sample was homogenized in a lysis buffer (0.01 M Tris [pH 7.8], 0.1 M NaCl, 0.1 mM EDTA, 1 mM PMSF, 2 μg/ml of pepstatin, 1 μg/ml leupeptin, and 2 μg/ml of chymostatin) and centrifuged at 12 000 × *g* for 10 min at 4°C, and the supernatants were stored at −80°C. Then, the protein concentration of the lysates was determined with a BCA protein assay kit (Pierce, Rockford, IL), and samples (25 μg) were denatured in a gel loading buffer at 100°C for 3 min and loaded on a 4%–20% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes. Blocking was performed with PBST (PBS containing 0.1% Tween 20) containing 5% nonfat dried milk solution for 1 h. The transferred membrane was incubated with the primary antibody for caspase-3 (polyclonal antibody D-175 at 1:500 dilution; Cell Signaling Technology) and for Bcl-2 and Bax (polyclonal antibody N-19 and P-19, respectively, at 1:1000 dilution; Santa Cruz Biotechnology, Inc.) overnight at 4°C. After washing, horseradish peroxidase-conjugated anti-rabbit immunoglobulin G was applied at a 1:1000 dilution for 1 h at room temperature. The blot was washed in PBST three times and visualized with an enhanced chemiluminescence Western blotting detection system (Amersham, Arlington Heights, IL) and exposed to x-ray film. The same membranes were reprobed with anti-glyceraldehyde phosphate dehydrogenase antibody and used as a control to ascertain that equivalent amounts of proteins had been transferred. Quantitative densitometry of Western blots was performed using the Scion Image Program (Eastman Kodak, Rochester, NY).

Statistical Analysis

Values are expressed as the mean ± SEM. Statistical analysis between the groups was performed using the Student *t*-test and Mann-Whitney *U*-test. Values were considered to be significant at $P < 0.05$.

RESULTS

To assess reproductive function in $PGF_{2\alpha}$ receptor-deficient mice, we evaluated the number of pups, survival, weight, and amniotic fluid in the previous studies [10, 19]. In the present study, wild-type mice delivered normal fetuses at term, but $PGF_{2\alpha}$ receptor-deficient mice were unable to deliver the fetuses. The fetuses gradually died in the uterus in $PGF_{2\alpha}$ receptor-deficient mice after Day 18 of pregnancy. The rate of intrauterine fetal death was lower on Days 19 and 20 of pregnancy but increased to 31.5% on Day 21 and to 60.7% on Day 22 of pregnancy. All fetuses died in the uterus by Day 23 of pregnancy. No significant differences were observed in the weight of the fetus, placenta, and decidua on Days 21 and 22 of pregnancy between the survival and death groups.

Apoptosis

Apoptosis was demonstrated within placental and decidual tissue by the TUNEL method. The TUNEL-positive cells were found in the trophoblast and stromal cells. The majority of apoptosis was found in syncytiotrophoblast tissues in the placenta. Increased TUNEL-positive staining in the syncytiotrophoblast layer was shown in the placenta in the death group (Fig. 1b) compared with that in the survival group (Fig. 1a). Elevated TUNEL-positive staining in the syncytiotrophoblast layer was scattered throughout the placenta, showing clusters of apoptotic cells in the death group. Marked TUNEL-positive cells were identified in the decidua in both groups (Fig. 1, c and d). The rate of apoptosis in the placenta and decidua in the death group was higher than that in the survival group ($P < 0.05$) (Fig. 2).

Caspase-3, Bcl-2, and Bax Expression

Immunohistochemical analysis of active caspase-3 protein expression was demonstrated in the syncytiotrophoblasts in the mouse placenta in both the survival and death groups. Active caspase-3 protein in the placenta in the death group (Fig. 3b) was much more abundant than in the survival group (Fig. 3a). Immunohistochemical analysis demonstrated Bcl-2 protein expression in the cytoplasm of syncytiotrophoblasts. The nuclei were never immunostained for Bcl-2. No immunostaining for Bcl-2 protein was detected in fetal capillaries. Bcl-2 protein in the placenta in the death group (Fig. 3d) was much less abundant than in the survival group (Fig. 3c). Bax protein in the placenta in the death group was more abundant than in the survival group (data not shown).

Western blot analysis demonstrated specific bands for

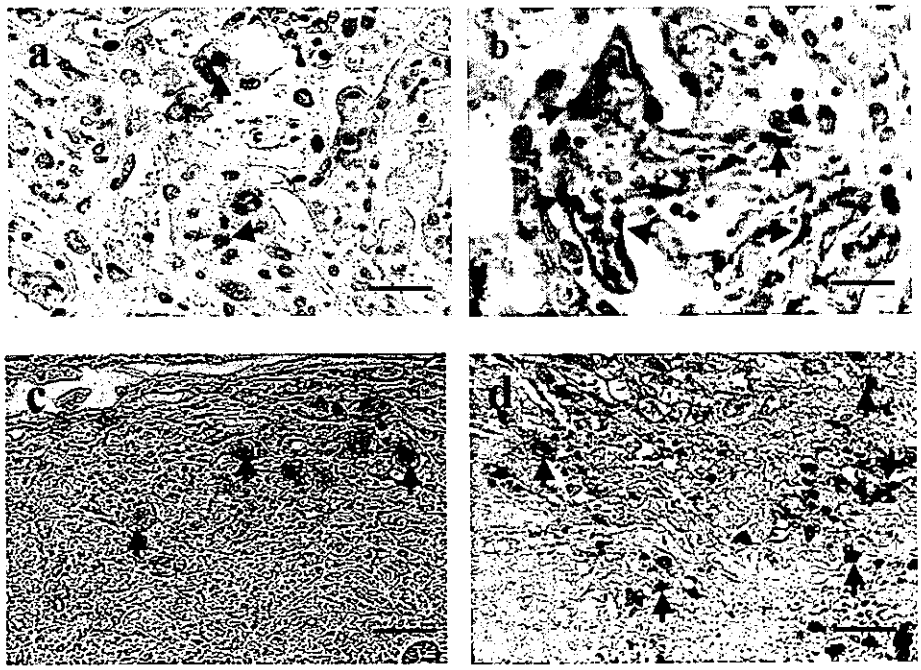

FIG. 1. TUNEL staining in placenta and decidua of the survival and death groups. Dark-brown staining indicates a positive reaction. Tissues were counterstained with 1% methyl green. The TUNEL-positive cells (arrows) in the placenta (a) and decidua (c) at Day 22 of pregnancy in the survival group are shown. Increased TUNEL-positive staining (arrows) was seen in the syncytiotrophoblast layer (b) and decidual tissues (d) at Day 21 of pregnancy in the death group. Bars = 50 μm.

caspase-3, Bcl-2, and Bax in placental and decidual tissues. Increased expression of the active form of caspase-3 was detected in the placenta and decidua in the death group compared with the survival group (Fig. 4). In contrast, a decrease in the expression of Bcl-2 was detected in the placenta and decidua in the death group compared with the survival group (Fig. 5). The high levels of Bax expression were clearly observed in both placenta and decidua in the survival and death groups. The enhanced expression of Bax was detected in placenta in the death group compared with that in the survival group (Fig. 6). The expression of the Bax:Bcl-2 ratio increased approximately 3.4-fold on placenta and 2.3-fold on decidua in the death group compared with the survival group (Fig. 7). These results are in agreement with the findings of direct immunostaining of the placenta in the survival and the death groups.

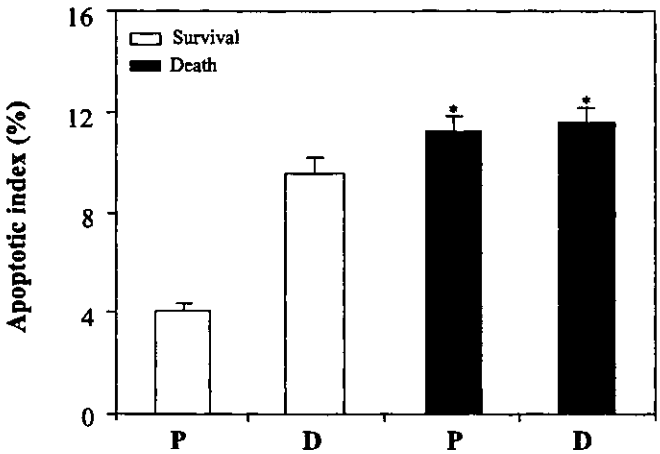

FIG. 2. Comparison of incidences of apoptosis in the placenta (P) and decidua (D) from the survival group and death group at Days 21–22 of pregnancy. The data represent the mean ± SEM of 8–12 samples from different fetuses. *$P < 0.05$, compared with survival group.

DISCUSSION

The many possible causes of fetal death generally can be categorized as fetal, placental, and maternal causes. The clinical history is often unremarkable, and often no detectable, preexisting antepartum medical or obstetric complication can be directly associated with the human fetal death. Obvious abnormalities are uncommon on gross inspection of the fetus or placenta [20]. Little is known regarding the environmental and genetic controls that regulate programmed cell death in the placental trophoblast. The trophoblast regulates maternal-fetal gas, nutrient, and waste product exchange. Homeostasis of syncytiotrophoblast is crucial to the maintenance of normal transport and secretary functions of the placental villous. Increases in trophoblast apoptosis were found in placentas from women with pregnancies complicated by preeclampsia and intrauterine growth restriction compared to controls [5, 21]. Therefore, the identification of apoptosis in this key cellular interface highlights the importance of understanding what controls apoptosis in the placenta [22]. However, to our knowledge, no reports concerning apoptosis and related proteins in intrauterine fetal death have been published.

We previously reported that increases in apoptosis and the apoptotic staining of the syncytiotrophoblast layer were found in a few postterm placentas in cases in which the fetus died within 24 h [10]. In the present study, we investigated this phenomenon and demonstrated that a significant increase of apoptosis in the syncytiotrophoblast layer of the mouse placenta might be involved in intrauterine fetal death. Increased TUNEL-positive staining in the syncytiotrophoblast layer was shown in the placenta of the death group compared with that of the survival group. Syncytiotrophoblast apoptosis has also been suggested to precede the breaks in the trophoblast covering villi and the loss of integrity in the syncytium [23]. A recent study demonstrated increased apoptosis in syncytiotrophoblast cells in either severe preeclamptic term placentas or in term placentas of intrauterine growth-restricted fetuses compared with normal

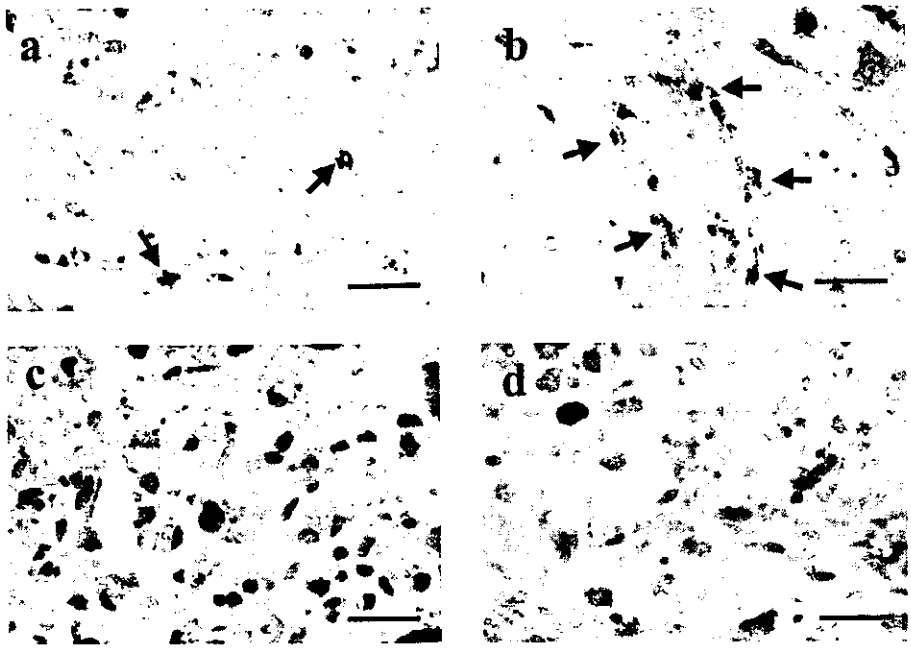

FIG. 3. Immunohistochemical staining for active caspase-3 and Bcl-2 protein in sections of the placentas from the survival and death groups at Day 22 of pregnancy. Active caspase-3 positive immune reactivity (arrows) was seen in the survival group (a) and the death group (b). Bcl-2-positive immune reactivity was seen in the survival group (c) and the death group (d). Brown staining indicates a positive reaction. Tissues were counterstained with hematoxylin. Bars = 50 μm.

term placentas [24]. Dysregulation of the apoptotic process probably causes a variety of diseases [25, 26]. An obvious question raised by the present study is what happens to placental function when placental apoptosis is increased in term pregnancy of preeclamptic placentas or placentas from intrauterine growth-restricted fetuses [5, 21]. The elevation of apoptosis in syncytiotrophoblast cells of the postterm pregnant placenta may be the cause of the damage of the syncytiotrophoblast layer. The syncytiotrophoblast plays a key role throughout pregnancy, being the site of many placental functions that are required for growth and development. Placental dysfunction leads to the loss of nutrition of the fetuses and contributes to the increased morbidity associated with postterm pregnancies.

The present study also showed the expression of apo-

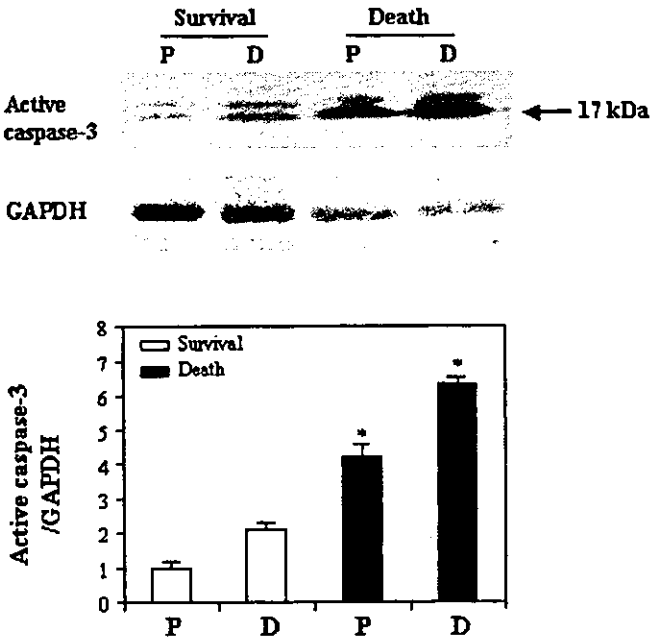

FIG. 4. Expression of active caspase-3 in the placental and decidual tissues on Days 21–22 of pregnancy. **Top**) A representative Western blot of active caspase-3 expression. This antibody detects only the large fragment of activated caspase-3 (17–19 kDa); it does not recognize endogenous levels of full-length caspase-3 or other caspases. Data were representative of five independently performed experiments. **Bottom**) A summary of densitometric values normalized to glyceraldehyde phosphate dehydrogenase (GAPDH). The density of the placenta from survival group bands was set arbitrarily at 1.0. Values shown represented the mean ± SEM from five separate experiments. D, Decidua; P, placenta. $^*P < 0.05$ compared with the survival group.

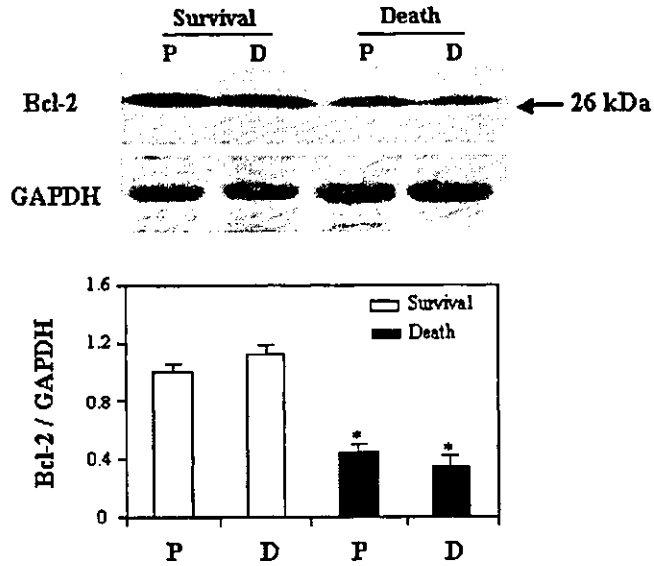

FIG. 5. Expression of active Bcl-2 in the placental and decidual tissues on Days 21–22 of pregnancy. **Top**) A representative Western blot of Bcl-2 expression. Data are representative of five independently performed experiments. **Bottom**) A summary of the densitometric values normalized to glyceraldehyde phosphate dehydrogenase (GAPDH). The density of the placenta from survival group bands was set arbitrarily at 1.0. Values shown represent the mean ± SEM from five separate experiments. D, Decidua; P, placenta. $^*P < 0.05$ compared with survival group.

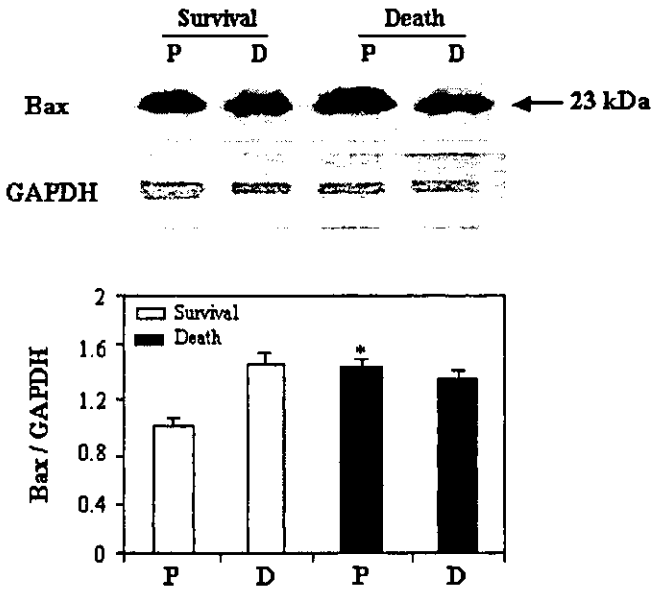

FIG. 6. Expression of Bax in the placental and decidual tissues on Days 21–22 of pregnancy. **Top**) A representative Western blot of Bax expression. Data are representative of three independently performed experiments. **Bottom**) A summary of the densitometric values normalized to glyceraldehyde phosphate dehydrogenase (GAPDH). The density of the placenta from survival group bands was set arbitrarily at 1.0. Values shown represent the mean ± SEM from three separate experiments. D, Decidua; P, placenta. $^*P < 0.05$ compared with survival group.

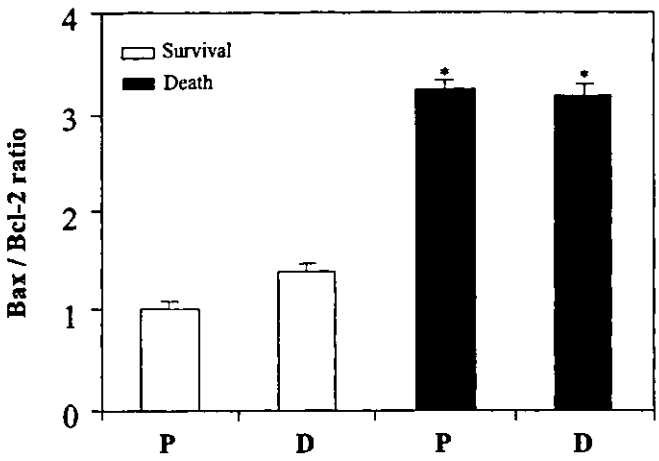

FIG. 7. Expression of Bax:Bcl-2 ratio in the placental (P) and decidual (d) tissues on Days 21–22 of pregnancy. Values were determined from Figures 5 and 6 and represent the mean ± SEM from three to five separate experiments. $^*P < 0.05$ compared with survival group.

ptosis and related proteins in decidual tissues. A higher level of apoptosis was detected in the death group compared with the survival group. Increased active caspase-3, increased Bax:Bcl-2 ratio, and decreased Bcl-2 were found in the decidua of the death group compared to that of the survival group. The decidua is thought to provide nutrition to the developing embryo, to protect the embryo from the immunologic responses of the mother, and to regulate trophoblast invasion into the uterine stroma. Apoptosis was detected in decidua of early pregnancy in several studies [7, 27]. Apoptosis in the decidua increased with gestational day in mice [10]. The decidual weight begins to decrease during later pregnancy in mice [10], and the histological regression of decidual basics begins at approximately Day 14 of pregnancy in rats [28]. Increased apoptosis with advancing gestational age suggested that apoptosis is a normal placental aging process [3]. Increased apoptosis in decidua of postterm pregnancy represents an acceleration of the aging process. The increased apoptosis in the decidua in cases of spontaneous abortion might cause apoptosis in the syncytiotrophoblast [7]. The increase in trophoblast apoptosis in postterm pregnancy possibly is a consequence of decidual apoptosis; thus, both trophoblast cell death and fetal mortality may relate to increased dysfunction of the decidua.

We found that the expression of Bcl-2 was decreased in the placenta in the death group compared with that in the survival group. High expression of Bcl-2 in the syncytiotrophoblast would protect this key layer of placental villi from apoptosis [29]. The lower Bcl-2 expression in the placenta of the death group might be related to the induction of apoptosis by increasing the expression of active caspase-3. Active caspase-3 has been demonstrated in human and mouse placentas [30, 31]. Caspase activity is regulated by the Bcl-2 family of proteins [32–35]. Lea et al. [36] examined the immunostaining of Bcl-2, a proto-oncogene thought to inhibit apoptosis, in biopsy specimens from first-trimester failing pregnancies. Immunostaining of Bcl-2 was less intense in failing pregnancies. The decreased Bcl-2 expression in complete hydatidiform mole, severe preeclamptic, and intrauterine growth-retarded placentas was considered to be a biological change that precedes apoptosis [24, 37]. Smith et al. [3] showed ultrastructural evidence of apoptosis in the syncytiotrophoblast, suggesting that Bcl-2 does not completely protect the syncytium from self-destruction. Therefore, the lack of protection by Bcl-2 in the syncytiotrophoblast layer leads to a great increase in apoptosis in the placenta at fetal death.

In the present study, a high Bax:Bcl-2 ratio in placental and decidual tissues was found in the death group compared with the survival group. These results provide evidence that the Bax:Bcl-2 "rheostat" may be a critical factor in regulating apoptosis in the placenta of postterm pregnancy. These findings are in agreement with those of Qiao et al. [37], who reported that an increased Bax:Bcl-2 ratio in complete hydatidiform mole might contribute to the high level of apoptosis. Bax is a likely candidate for regulating apoptosis to the effector stage in syncytiotrophoblast [38]. No significant difference was found in Bax of decidua between groups. Therefore, the Bax:Bcl-2 ratio and/or Bcl-2 may be more useful in assessing cell death via apoptosis.

Hormones have been implicated in the stimulation or inhibition of apoptosis. To our knowledge, little PGF_α-receptor signaling was detected in trophoblasts, although it was found in myometrium (unpublished data). As reported previously, no significant differences were found in apoptosis of the placenta and decidua between $PGF_{2\alpha}$ receptor-deficient and wild-type mice [10, 31]. Therefore, trophoblast apoptosis in this mouse model was unlikely to have been caused by the lack of prostaglandin signaling. We did not measure the levels of progesterone and estrogen to directly assess their effect on apoptotic expression in the present study. However, some studies have demonstrated that the level of progesterone and estrogen was associated with apoptosis in decidua of rat [39, 40]. Gu et al. [41] suggested that progesterone did not play a role in preventing decidual apoptosis in the rat. In the present study, the maternal plasma concentration of progesterone and estrogen was similar for the fetuses in the two groups, because they were from same maternal mouse. The difference of the apoptotic ex-

pression in the survival and death groups was not affected by the change of endocrine capacity in maternal plasma levels. It is unknown whether the local endocrine capacity on the trophoblast or decidual component influences apoptosis in fetal death.

The question is whether the increased apoptosis in the placenta and decidua is a cause or a result of intrauterine fetal death. We found increased apoptosis in placenta and decidua of the death group compared with the survival group in postterm pregnancy. A higher level of apoptosis was found in placentas from pregnancies complicated by preeclampsia and intrauterine growth restriction compared to placentas from pregnancies with normal fetal growth [5, 21]. The dysregulation of the apoptotic process probably causes a further increase in apoptosis in the placenta and decidua of fetal death, resulting in the extreme placental and decidual senescence. In this situation, the placenta may be hard-pressed to meet the metabolic requirements of fetal growth and development. The compensatory process is known to occur in an area of relatively poor placental perfusion, resulting in hypoxic ischemia of the placental circulation. Hypoxia is a known trigger of apoptosis in different tissues, and hypoxia may trigger apoptosis in the placenta as a possible mechanism of pregnancy-related complication [42, 43]. Again, placenta may respond to hypoxic stress by increasing apoptosis; thus, the increased apoptosis aggravates the senescence. This process may be a vicious circle in placenta of fetal death. The dysregulated apoptosis may lead to dysfunction of placenta, resulting in intrauterine fetal death. If so, then apoptosis in such conditions might be occurring as a cause of the pathologic processes leading to fetal death. Alternatively, if the increase in apoptosis is an intrinsic component of trophoblast and decidual differentiation that is programmed to begin at the "end" of the trophoblast differentiation program, then the increased trophoblast apoptosis in postterm pregnancy could be a result of the finalization of the differentiation program and could not be a direct contributor to fetal death.

The design of the present study had a few limitations. First, we could not precisely identify the time of fetal death in the mouse uterus. The placentas of fetal death which included body maceration, skin wrinkling, and skin discoloration were excluded from the death group. Therefore, the remaining placentas included in the death group were considered to have been collected shortly after fetal death. In a future study, we will attempt to evaluate the condition of the mouse fetuses early, using high-frequency flow imaging [44]. The second limitation was the use of the TUNEL method for identifying apoptosis. Although it has been reported that necrosis might cause false-positive staining in this method [45, 46], it appeared to be a reliable and highly sensitive method for showing apoptosis [40, 47]. Our previous results showed that findings using the TUNEL method were similar to those using DNA fragmentation assay [10, 31]. It has also been demonstrated that this method involves less false-positive staining from necrosis after fetal death [48]. Edwards et al. [48] reported that a large number of brain cells undergo apoptosis in infants who suddenly die after intrauterine insults but that a significantly lower number undergo necrosis. The third limitation was the possibility that the increase in placental apoptosis in the death group occurred after fetal death. In general, placental cells do not die soon after fetal death. Furthermore, no further increase in apoptosis was shown in placentas after fetal death at later time points compared with the level in the death group in the present study (data not shown). Because placental apoptosis increases with gestation [3, 10, 49], the increases in apoptosis that we observed were unlikely to have been the result of placental changes after fetal death. Our group will attempt to further identify the relationship between fetal death and placental apoptosis in future studies.

In conclusion, apoptosis, active caspase-3, Bcl-2, and Bax were found in the mouse placentas and decidua of both the survival and death groups. Apoptosis has a positive relationship with active caspase-3 and the Bax:Bcl-2 ratio and a negative relationship with Bcl-2 in the placenta and decidua. Increased apoptosis, active caspase-3, and Bax:Bcl-2 ratio in the placenta and decidua were found in the death group compared with the survival group. In contrast, a decrease in the expression of Bcl-2 was detected in the death group compared with the survival group. A significant increase in apoptosis in the mouse placenta and decidua might be involved in the pathophysiologic mechanism of intrauterine fetal death.

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