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Evidence for the Involvement of a Muscarinic Receptor in Ascorbic Acid Secretion in the Rat Stomach

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ABSTRACT. It has been demonstrated that ascorbic acid is present in human gastric juice at a very high level even in the fasting state. In this study, we confirmed such a physiological event in pylorus-ligated rats and investigated the mechanism for gastric ascorbic acid secretion using the rat *in vivo* perfusion method. Gastric juice from fasting rats after a 4-hr ligation contained a 6-fold higher level of ascorbic acid than that in sera, indicating the presence of its active transport system across the gastric wall. To identify an endogenous mediator, four kinds of gastric stimulants were compared for their secretory abilities. Although all four secretagogues used stimulated acid secretion to comparable levels, ascorbic acid secretion was stimulated only by carbamylcholine chloride (carbachol). Carbachol-stimulated ascorbic acid secretion was abolished by atropine pretreatment, showing that it is mediated via a muscarinic cholinergic receptor. This cholinergic stimulation was also demonstrated in a rat mutant that genetically lacks the ability to synthesize ascorbic acid in the liver, similar to humans. In addition, a potent inhibitor of the gastric proton pump, which is a final regulatory component in the mechanism of acid secretion, caused no inhibition of ascorbic acid secretion in Osteogenic Disorder Shionogi (ODS) rats. These results are the first evidence indicating that ascorbic acid, the reduced form of vitamin C, is actively and independently secreted into the gastric juice under regulation of a cholinergic receptor system. BIOCHEM PHARMACOL 53;4:553–559, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ascorbic acid; gastric ascorbic acid secretion; cholinergic stimulation; active transport; vitamin C; rat stomach

Ascorbic acid, one of the most important antioxidants *in vivo* [1], is known to be a multifunctional vitamin for humans [2], and has been further assessed by its promoting activity in the self-defense system *in vivo* [3]. We previously demonstrated its stimulatory effect on antibody production in mice and humans using an *in vitro* cell culture system in the presence of a stable ascorbate derivative, ascorbic acid 2-*O*-α-glucoside [4, 5]. In addition, we have revealed the enhanced production of the detoxifying protein metallothionein in the rat gastrointestinal mucosa following ingestion of ascorbic acid [6]. In addition to these effects, ascorbic acid may play a critical role in protecting against gastric cancer in humans [7]. However, a limited number of species, including humans, primates, and guinea pigs, are not able to synthesize ascorbic acid in their livers, so that they need to obtain it from foods. Moreover, its consumption in the body is greatly influenced by exposure to several kinds of environmental factors [8]. Considering the physiological importance of this vitamin, it is necessary to clarify its *in vivo* kinetics, e.g. absorption, distribution, accumulation, metabolism, and excretion.

It has been reported that the concentration of ascorbic acid in normal human gastric juice reaches levels six times higher than that in blood even in a fasting state [9]. This observation is suggestive of the presence of an active transport system of ascorbic acid from blood to gastric lumen. Moreover, the concentration of gastric ascorbic acid has been described to be reduced markedly in patients with acute or chronic gastritis, a gastric or duodenal ulcer [9–11], or infection of *Helicobacter pylori* [12, 13]. These facts, therefore, suggest that gastric ascorbic acid plays an important role in protecting the gastrointestinal tract against the occurrence of mucosal disorders and bacterial infection. To date, there has been no mechanistic evidence showing the active transport of ascorbic acid into gastric juice in any kind of animals. In the present study, we investigated the effects of potent gastric acid secretagogues to clarify the mechanism for ascorbic acid secretion in the rat stomach. We found only one cholinergic agent to have a potent stimulatory effect and proved the active transport mechanism for gastric ascorbic acid secretion.

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MATERIALS AND METHODS

Materials

Carbachol† and atropine sulfate were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Histamine dihydrochloride, tetragastrin, and dbcAMP were obtained from Nacalai Tesque (Kyoto, Japan), the San-a Pharmaceutical Co. (Tokyo, Japan), and Yamasa Shoyu (Choshi, Japan), respectively. All the chemicals mentioned above were freshly dissolved in saline before use. Urethane was a product of Tokyo Chemical Industry (Tokyo, Japan). Omeprazole was extracted with chloroform from Omepral tablets (Fujisawa Pharmaceutical Co., Osaka, Japan) in our laboratory. Ascorbic acid and other reagents employed were purchased from the Wako Pure Chemicals Co. (Osaka, Japan).

Animals

Male Wistar rats, weighing 160–200 g, were obtained from Japan SLC (Hamamatsu, Japan). ODS rats (6 weeks old) were purchased from Japan Clea (Osaka, Japan) and fed basal rat chow with water containing 0.1% ascorbic acid. Animals were used after 1 week of acclimation and fasted for 24 hr prior to the experiment.

Collection of Gastric Juice in Rats

Under ether anesthesia, an incision was made in the abdomen to ligate the pylorus. After 4 hr, the rats were anesthetized with ether again, killed by cardiac puncture, and then ligated at the orifice of the stomach. The stomachs were excised, and their contents were collected into test tubes. After centrifugation at 1000 *g* for 10 min, gastric juice was determined for volume, acidity, and ascorbic acid concentration. The acidity was measured by titration with 0.1 N NaOH up to pH 7.0 using a pH meter, and the ascorbic acid concentration was measured by an HPLC method described below.

Perfusion Study in Rat Stomach

Rats were anesthetized with a subcutaneous injection of urethane (1.2 g/kg), and the gastric fistula was prepared according to procedures described in a previous paper [14]. The stomach cavity was perfused with 0.9% NaCl adjusted to pH 5.0 at a flow rate of 0.25 mL/min, and the gastric effluent was collected for 10-min intervals. The acidity and ascorbic acid concentration of each effluent were measured. Both responses after administration of each agonist were calculated as the total amounts of acid or ascorbic acid secreted during 1 hr minus the preceding basal values. All chemicals were injected into the femoral vein at intervals of at least 90 min.

Determination of Ascorbic Acid

An aliquot of gastric juice, gastric effluent, or serum was mixed with the same volume of cold 10% metaphosphoric acid. Liver homogenates were prepared with 4 vol. of 2% metaphosphoric acid. After centrifugation at 12,000 *g* for 10 min, the supernatants were subjected to HPLC analysis according to a method described previously [15]. Briefly, a liquid chromatograph (Shimadzu LC-10AD) was equipped with a UV spectrophotometer (Shimadzu SPD-10) and a chromatopac (Shimadzu C-R6A). Separation was achieved by isocratic elution of a Shim-pack ODS column (6 × 150 mm) with 0.1 M potassium phosphate–0.1 M phosphoric acid (pH 3.0) at a flow rate of 0.7 mL/min. Each sample (20 μL) was analyzed at 243 nm and determined for its concentration based on its peak height. Total ascorbic acid concentration was measured following reduction of dehydroascorbic acid by dithiothreitol [16], and the concentration of dehydroascorbic acid was calculated by subtraction.

Statistical Analysis

Data were analyzed by Student's *t*-test with significant probability levels set at less than 0.05 or 0.01.

RESULTS

Determination of Ascorbic Acid Concentrations in Gastric Juice and Serum Collected from Fasting Rats

Following a 4-hr ligation of the pylorus, gastric contents from the individual fasting rats were collected together with their blood, and their resultant supernatants, gastric juice and serum, were then analyzed. The acidity and pH of gastric juice samples from 5 rats were 68.5 to 91.5 μEq/mL and 1.41 to 2.13, respectively. As shown in Fig. 1, there was a marked difference between the ascorbic acid concentrations in gastric juice and serum. Total ascorbic acid concentrations in both samples were 244.3 ± 64.1 μM (190.1 to 339.8 μM) and 40.7 ± 8.1 μM (32.0 to 52.3 μM), respectively, resulting in a ratio of 6:1. In all samples, ascorbic acid, the reduced form of vitamin C, comprised more than 95% of the total ascorbic acid. From this result, it was concluded that we could evaluate the kinetics of gastric ascorbic acid by analyzing its reduced form only.

Effect of Acid Secretagogues on Gastric Ascorbic Acid Secretion in Perfused Rat Stomachs

To measure low levels of ascorbic acid accurately, we first examined its stability in the effluent from the perfused rat stomach. Even when 10 to 50 μM ascorbic acid solutions were kept at pH 3.0 to 5.0 and at room temperature for 1 hr, every recovery was more than 90% of the initial value. This means that oxidative degradation of ascorbic acid hardly

† *Abbreviations:* carbachol, carbamylcholine chloride; dbcAMP, dibutyryl cyclic AMP; urethane, ethyl carbamate; and ODS rat, Osteogenic Disorder Shionogi rat.

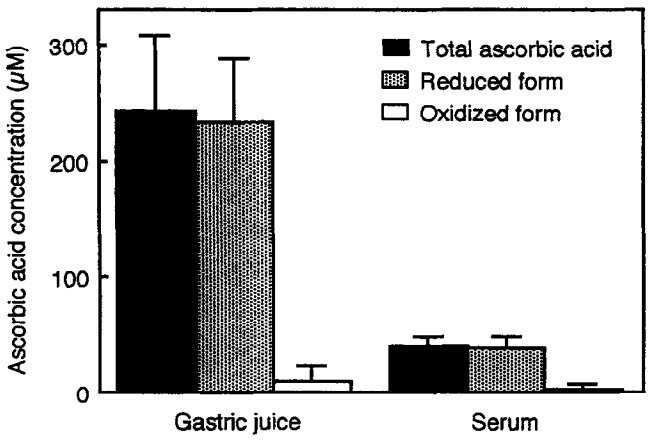

FIG. 1. Determination of ascorbic acid concentrations in gastric juice and serum of pylorus-ligated rats. Gastric juice and blood were collected 4 hr after pylorus ligation of fasting Wistar rats, and their resultant supernatants were assayed for ascorbic acid concentrations. Total ascorbic acid concentrations were determined following reduction of dehydroascorbic acid with dithiothreitol. Values are means ± SD from five rats.

occurred under the present experimental conditions. Using the perfused stomachs of Wistar rats, we compared the ascorbic acid-secretory response to several types of potent acid secretagogues. Figure 2 illustrates typical profiles showing the response to four such agents. A single injection of carbachol (10 μg/kg) effectively stimulated ascorbic acid secretion, whereas histamine (1 mg/kg), tetragastrin (10 μg/kg), and dbcAMP (20 mg/kg) showed little or no effect. On the other hand, gastric acid secretion was induced at comparable levels by these agents. Since all the responses were diminished within approximately 1 hr after their injection, the total output of gastric acid and ascorbic acid associated with each stimulation was calculated based on their contents in six 10-min effluents of each rat. Table 1 shows that the ascorbic acid-secretory response was apparently characteristic of carbachol alone. This stimulatory effect of carbachol was observed in a dose-dependent fashion at doses ranging from 1.25 to 20 μg/kg, as shown in Fig. 3.

To examine whether this secretion is mediated via a cholinergic receptor, we further studied the inhibition by atropine, a typical muscarinic receptor antagonist. As can be seen from the representative result in the perfusion method shown in Fig. 4, the carbachol-stimulated ascorbic acid secretion was abolished perfectly by an intravenous injection of atropine at a dose of 1 mg/kg. A similar result was obtained in the pylorus-ligated rats given an intraperitoneal injection of atropine (Table 2). Atropine significantly decreased the total output of acid and ascorbic acid in gastric juice. These findings revealed that carbachol stimulates gastric ascorbic acid secretion via a muscarinic cholinergic receptor. However, only the concentration of gastric ascorbic acid tended to be increased by atropine, though the reason was unclear.

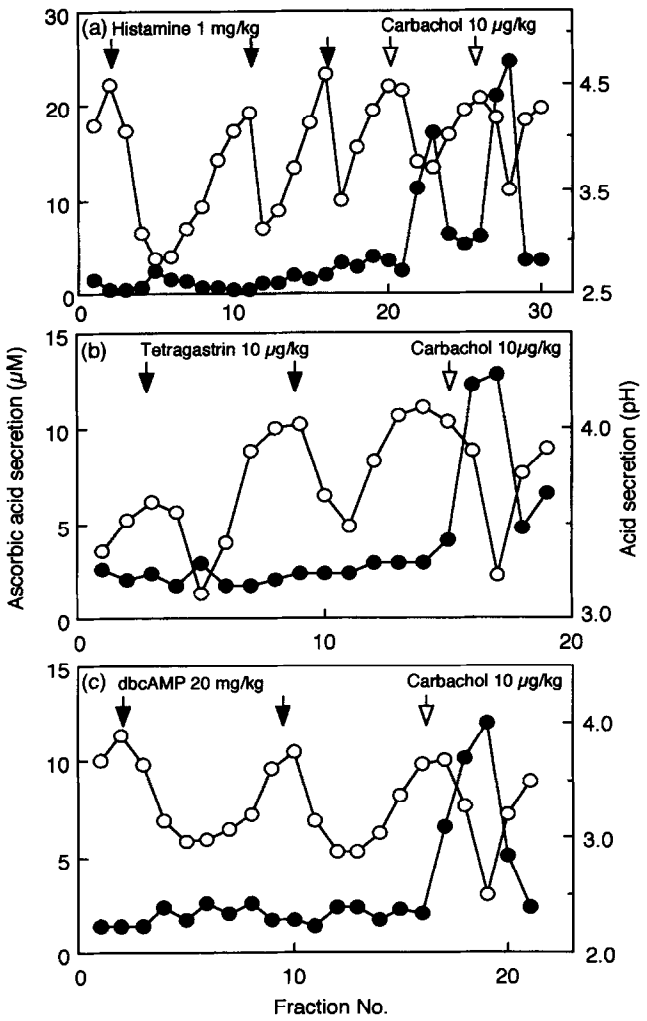

FIG. 2. Typical profiles of carbachol-, histamine-, tetragastrin-, and dbcAMP-induced acid and ascorbic acid secretion in rat stomachs. Using a gastric perfusion model of Wistar rats, a single intravenous injection of each acid secretagogue was given to rats, and 10-min fractions were analyzed for pH () and ascorbic acid concentration (●). At the points indicated with black arrows, histamine (a), tetragastrin (b), or dbcAMP (c) was injected repeatedly, and carbachol was also injected at the points indicated with white arrows.

Gastric Ascorbic Acid Secretion in ODS Rats

To evaluate the physiological involvement of gastric ascorbic acid secretion in humans, we next investigated this responsiveness in ODS rats, which genetically lack the ability to synthesize ascorbic acid, similar to humans. Under the same experimental conditions, carbachol was found to stimulate gastric ascorbic acid secretion in ODS rats. As shown in Fig. 5, this responsiveness was just a little weaker than that in Wistar rats, whereas the carbachol-induced acid secretion was enhanced in ODS rats compared with Wistar rats (32.8 ± 9.2 vs 8.7 ± 3.4 μEq/hr). This may be due to considerably lower levels of ascorbic acid in livers and sera of ODS rats compared with those of Wistar rats. These lines of evidence emphasize the universality of the gastric ascorbic acid-secretory system throughout mammalian species.

TABLE 1. Effect of acid secretagogues on gastric acid and ascorbic acid secretion in the perfused stomach of Wistar rats

Agent	Dose	No. of rats	Acid secretion (μEq/hr)	Ascorbic acid secretion (nmol/hr)
Carbachol	10μg/kg	6	8.71 ± 3.37 (3.50–13.50)	66.63 ± 39.31 (29.93–131.63)
Histamine	1mg/kg	6	9.10 ± 4.56 (4.50–16.75)	9.37 ± 7.54 (0–18.07)
Tetragastrin	10μg/kg	4	3.81 ± 1.93 (2.00–6.50)	4.24 ± 3.86(0–8.13)
dbcAMP	20mg/kg	4	7.50 ± 2.65 (3.88–9.88)	8.70 ± 7.25 (0–16.83)

Using the perfused rat stomach, both acid and ascorbic acid contents in each 10-min effluent were analyzed during 1 hr following the administration of agents. Responses were evaluated as the total amounts of acid or ascorbic acid secreted minus the preceding basal values. All data represent means ± SD with the variation from the lowest to highest values noted in parentheses.

Dissociation of Ascorbic Acid-Secretory Response from Acid-Secretory Response

Although ascorbic acid present in the gastric juice of humans in the fasting state has been assumed to be washed out together with acid and water secretion, there has been no distinct observation until now. We next examined whether these two physiological events occur simultaneously by the same mechanism. Omeprazole is a potent inhibitor of gastric H^+, K^+ -ATPase which is responsible for acid secretion from the parietal cells, resulting in its strong inhibition at the terminal process of secretory response. As shown in Fig. 6, a single intraperitoneal injection of omeprazole (60 mg/kg) to ODS rats induced a significant reduction of carbachol-stimulated acid secretion. However, no inhibition was observed of carbachol-induced ascorbic acid secretion and, furthermore, there was a tendency for it to be enhanced following this treatment.

Participation of Passive Transport of Ascorbic Acid Across the Gastric Mucosa

In this experiment, a mechanistic pathway of passive transport of ascorbic acid from blood to the gastric lumen was assessed using the perfused rat stomachs. By a single intravenous injection of sodium ascorbate at a dose of 10 mg/rat, the serum ascorbic acid level was elevated immediately to about 1.1 mM, which was more than twenty times higher than the normal level, and it decreased very rapidly to about 200 μM just after 30 min. Under this experimental condition, ascorbic acid concentrations in six 10-min effluents after administration were nearly equal to the basal value (data not shown). Thus, such an elevation of serum ascorbic acid concentration had no effect on spontaneous secretion of ascorbic acid into the gastric juice. In addition, there was no significant difference in carbachol-induced responses of ascorbic acid secretion between these rats showing relatively high levels (100–200 μM) of serum ascorbic acid and normal rats (data not shown). Consequently, there existed no passive transport system of ascorbic acid in the stomach.

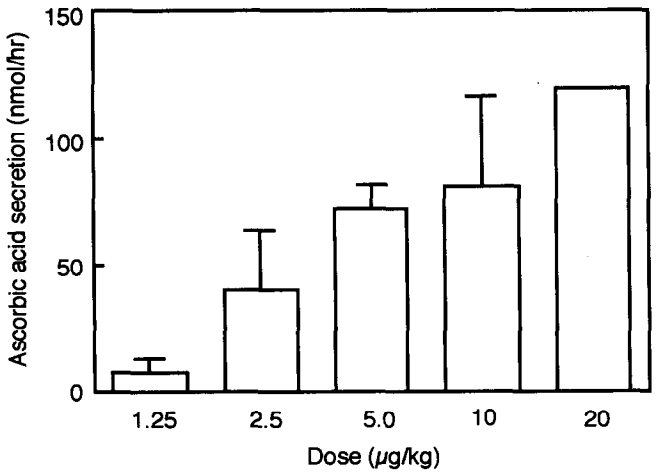

FIG. 3. Dose response of carbachol on ascorbic acid secretion in the perfused rat stomachs. Carbachol was administered intravenously at different doses to rats at 1.5-hr intervals. The total output of ascorbic acid secreted by each injection was calculated and expressed as means ± SD of three separate experiments for 1.25 μg/kg, three experiments for 2.5 μg/kg five experiments for 5 μg/kg, six experiments for 10 μg/kg and two experiments for 20 μg/kg.

DISCUSSION

In this study, we demonstrated for the first time that fasting gastric juice collected from pylorus-ligated rats contained very high levels of ascorbic acid, and that the resultant concentration went up to about six times that of the serum

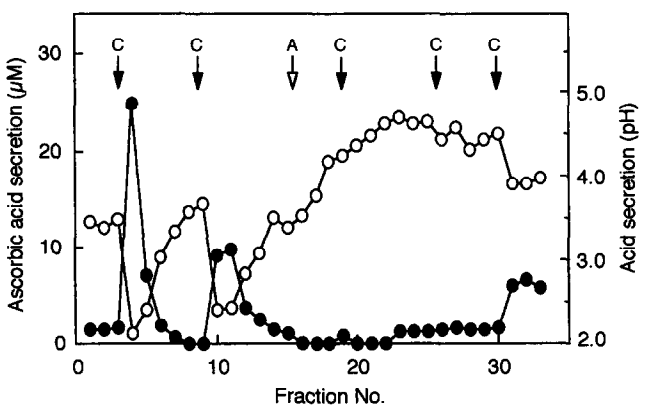

FIG. 4. Inhibitory effect of atropine on carbachol-induced ascorbic acid secretion in the rat stomach. The gastric perfusion model of the Wistar rat received a repeated intravenous injection of carbachol (10 μg/kg, black arrows) with an intravenous injection of atropine (1 mg/kg, white arrow). Each perfusate was measured for pH () and ascorbic acid concentration (●).

TABLE 2. Effects of atropine on gastric acid and ascorbic acid secretion in pylorus-ligated rats

Treatment	Dose	Acid secretion		Ascorbic acid secretion	
		Concentration (μEq/mL)	Total output (μEq)	Concentration (μmol/mL)	Total output (μmol)
Saline		63.2 ± 6.2	189.2 ± 23.1	0.38 ± 0.05	1.14 ± 0.29
Atropine	10 mg/kg	43.2 ± 19.7	40.2 ± 53.2*	1.26 ± 0.97	0.51 ± 0.15*

Atropine was injected i.p. just after pylorus ligation of fasting Wistar rats. Gastric juice during the 4-hr ligation was collected and assayed for volume, acidity, and ascorbic acid concentration. All data represent means ± SD of three rats per group. *Significantly different from saline-treated group, $P < 0.05$.

level. Such a concentration gradient from gastric juice down to blood is almost consistent with the results obtained in normal human subjects [9]. Since both assessments (in rats and humans) were performed following food deprivation we can rule out the possibility that high amounts of ascorbic acid in gastric juice are derived from the diet. Moreover, this concordant result obtained from both species, which have a drastic difference in their ascorbic acid-synthesizing abilities, suggests the universal presence of an ascorbic acid-specific transport system in the stomachs of most mammals. Therefore, we concluded that rats could be used for investigating the mechanism of ascorbic acid secretion in the stomach. A marked elevation of serum ascorbic acid concentration in rats caused neither spontaneous secretion of ascorbic acid into the stomach nor amplification of inducible secretion by carbachol. These findings indicate that gastric ascorbic acid is not secreted through a passive transport pathway, but through an active transport system. However, Sobala *et al.* [17] have demonstrated an elevation of gastric ascorbic acid concentrations after an intravenous injection of ascorbic acid in a man. This difference may be due to the experimental conditions such as species tested, anesthesia, and stress, but it remains to be clarified. An active secretory mechanism of gastric ascorbic acid has been speculated in humans [9, 10, 18]. More interestingly, the reduced form of vitamin C comprised more than 95% of total ascorbic acid contents in both gastric juice and serum. This means that ascorbic acid, which exists mostly as a reduced form in the blood, could be secreted

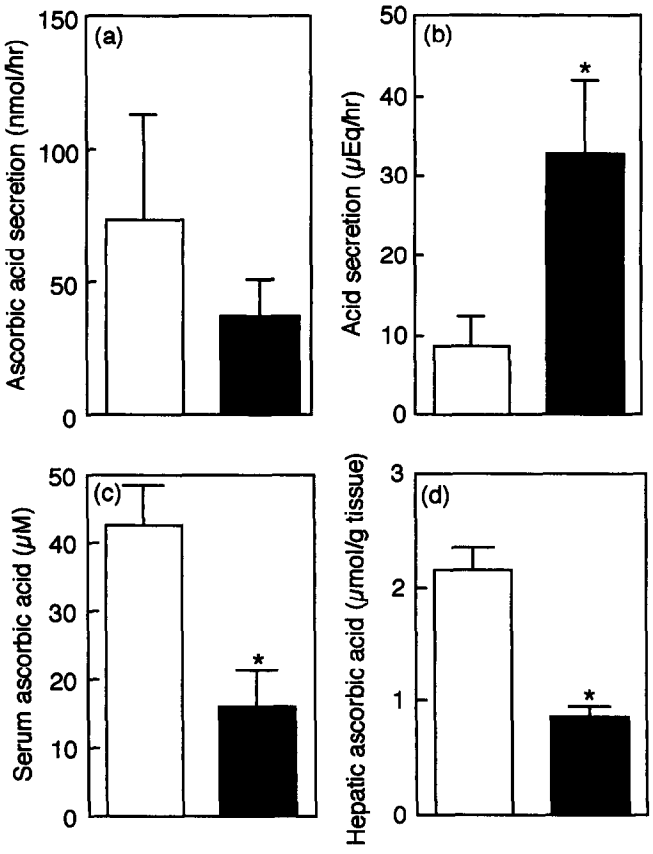

FIG. 5. Stimulatory effect of carbachol on ascorbic acid secretion in the stomachs of Wistar and ODS rats. Carbachol-induced ascorbic acid secretion (a) and acid secretion (b) were evaluated in the perfused stomachs of Wistar (open columns, N = 3) and ODS (closed columns, N = 5) rats. Ascorbic acid concentrations in serum (c) and liver (d) were assayed in each rat just after the perfusion experiments. All data are means ± SD. Key: *Significantly different from the corresponding values for Wistar rats, $P < 0.01$.

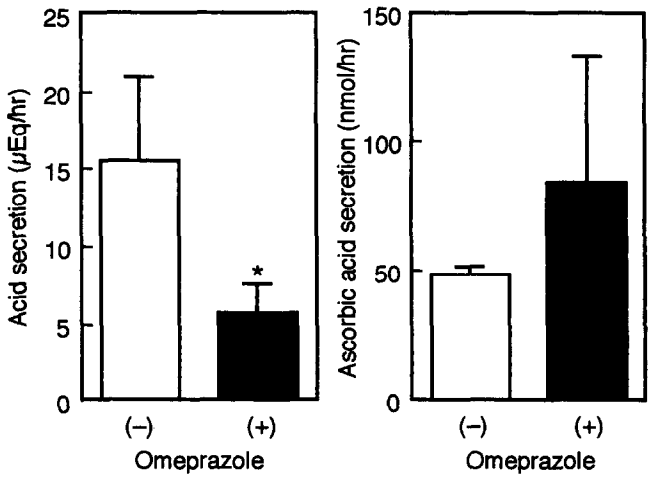

FIG. 6. Effect of a proton pump inhibitor (omeprazole) on carbachol-induced acid and ascorbic acid secretion in the stomachs of ODS rats. Carbachol (10 μg/kg)-induced secretory responses were assessed in the perfused stomachs of ODS rats before (open columns) or after (closed columns) subcutaneous injection of omeprazole at a dose of 60 mg/kg. All data are means ± SD from four rats. Key: *significantly different from saline-treated group, $P < 0.05$.

into the gastric juice in its intact form. Therefore, we can conclude that this ascorbic acid-secretory system in the stomach should be specific only to its reduced form.

It has been clarified that gastric acid secretion is mediated by endogenous chemical mediators, such as acetylcholine, histamine, and gastrin, via three different types of receptors on parietal cells [19]. These agonists stimulate their corresponding receptors to elevate intracellular mediators such as cAMP and Ca^{2+} in the cells. Such intracellular signal transduction can activate the final step of acid secretion, a proton pump on a canalicular membrane of the parietal cell [20]. The present study demonstrated that among four acid secretagogues that exerted comparable levels of acid secretory response, only carbachol had a potent stimulatory effect on ascorbic acid secretion, indicating that gastric ascorbic acid secretion is regulated by a mechanism distinct from that of acid secretion. This is also supported by the following two findings: dbcAMP showed almost no activity in gastric ascorbic acid secretion and a proton pump inhibitor had no effect on this secretion. The involvement of an acetylcholine receptor in the regulation of ascorbic acid secretion was further confirmed by the inhibitory effect of atropine. However, the result that atropine increased the ascorbic acid concentration in gastric juice of pyrolus-ligated rats seems to have little fundamental significance, because atropine efficiently inhibited ascorbic acid secretion in the perfusion method. It is likely that pylorus ligation in conscious rats causes a vagal excitation that may participate in ascorbic acid secretion. From these results, gastric ascorbic acid secretion was determined to be physiologically controlled via a muscarinic cholinergic receptor. Consequently, we are able to presume that this receptor may associate with an unknown transporter or channel specific to ascorbic acid. This physiological phenomenon may be a universal event that functions all day for living mammals, although we have determined such a response during the fasting period.

Since gastric cells do not have the capacity to synthesize ascorbic acid even in rats, this compound must be transported through the blood stream to unknown gastric cells. Therefore, ODS rats that genetically lack L-gulono-γ-lactone oxidase [21], a terminal enzyme for the synthesis of ascorbic acid, are a good animal model for evaluating the physiological significance of gastric ascorbic acid secretion in place of humans. In ODS rats, we observed a significant enhancement of gastric acid secretion induced by carbachol, compared with that in Wistar rats. A genetic difference in such a responsiveness of acid secretion has not been described. However, carbachol-induced gastric ascorbic acid secretion in ODS rats was still observed, with just a little decrease compared with that in Wistar rats. This reduction is considered to be due to low levels of serum and hepatic ascorbic acid in ODS rats. These results indicate that the gastric ascorbic acid-secretory potential is independent of its synthesizing ability.

In humans, ascorbic acid is an essential nutrient that has multifarious functions even in the gastrointestinal tract. In particular, it plays a critical role in the stomach of inhibiting the formation of nitrosoamines, which are considered to increase the risk of gastric cancer [22–24]. A high level of ascorbic acid ingested together with food can effectively prevent the formation of these reactive substances in the stomach. However, it is suggested that a considerable amount of reactants can be generated continually even in the empty stomach, since saliva contains a large amount of nitrate that is easily reduced to nitrite by microorganisms [25]. Since gastric ascorbic acid secretion is regulated through a cholinergic nerve system, it may be evaluated as one of the important intragastric defense systems during the fasting period for mammals. Furthermore, recent epidemiologic evidence indicates that *H. pylori* infection increases the risk of gastric cancer [26]. This infection causes a reversible lowering of gastric ascorbic acid concentrations, and its eradication can improve the secretion into gastric juice [13, 18, 27]. Infection with *H. pylori* is characterized by infiltration of lymphocytes, polymorphonuclear leukocytes, and macrophages in the gastric mucosa, and these cells produce reactive oxygen radicals to induce various disorders in the gastric epitheliums. Since intragastric ascorbic acid may be a promising scavenger of these oxygen radicals, mechanistic dysfunction of ascorbic acid secretion originating with *H. pylori* infection must be clarified in the future. To further assess the physiological significance of this secretory mechanism in humans, both the molecular demonstration of this transport system and its detailed regulatory mechanism should be investigated.

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