Enhanced Intracellular Calcium Promotes Metabolic and Secretory Disturbances in Rat Gastric Mucosa during Ethanol-Induced Gastritis

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Changes in the Ca^{2+} homeostasis have been implicated in cell injury and death. However, Ca^{2+} participation in ethanol-induced chronic gastric mucosal injury has not been elucidated. We have developed a model of ethanol-induced chronic gastric injury in rats, characterized by marked alterations in plasma membranes from gastric mucosa and a compensatory cell proliferation, which follows ethanol withdrawal. Therefore, the present study explored the possible role of intracellular Ca^{2+} in the oxidative metabolism and in acid secretion in this experimental model. Glucose oxidation was greatly enhanced in the injured mucosa, as evaluated by CO_2 production by isolated mucosal preparations incubated with ^{14}C-radiolabeled glucose in different carbons. Oxygen consumption and acid secretion (aminopyrine accumulation) were also stimulated. A predominating secretory status was morphologically identified by electron microscopy in oxyntic cells of gastric mucosa from ethanol-treated rats. A coupling between secretory and metabolic effects induced by ethanol (demonstrated by an inhibitory effect of omeprazole in both parameters) was found. These ethanol-induced effects were also inhibited by addition of Ca^{2+} chelators to isolated gastric mucosa samples. Lanthanum, a Ca^{2+} channel blocker, inhibited ethanol-promoted increase of oxidative metabolism. In addition, a stimulated Ca^{2+} uptake by mucosal minces and increased *in vivo* Ca^{2+} levels in cytosolic and mitochondrial fractions, were also noticed. Enhanced glucose and oxygen consumptions were associated with higher ATP and NADP+ availability, whereas cytosolic NAD/NADH ratio (assessed by mucosal levels of lactate and pyruvate) was not significantly modified by the chronic ethanol administration. In conclusion, changes in Ca^{2+} homeostasis, probably mainly due to increased extracellular Ca^{2+} uptake, could mediate secretory and metabolic alterations found in the gastric mucosa from rats chronically treated with ethanol. Exp Biol Med 228:315–324, 2003

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We have developed a model in which prolonged ethanol administration elicits a histological profile of chronic gastric injury that is characterized by evident biochemical disturbances on plasma membrane functions. Such alterations include lower membrane fluidity, diminished activities of some membrane-associated enzymes, and decreased density of H_2-histaminergic receptors in isolated plasma membranes from gastric mucosa of rats chronically treated with ethanol (1, 2). In addition, lipid peroxidation has been implicated in both the already-mentioned alterations in plasma membranes, as well as in the in the compensatory mucosal proliferation triggered after ethanol withdrawal (2). Nonetheless, we have not fully clarified the underlying mechanisms involved in the ethanol-induced chronic damage in the rat gastric mucosa.

Ethanol has been considered to induce a number of changes in the gastric mucosa that seem to explain, at least in part, the mechanism of action of this agent. They include reduced gastric blood flow (3), disruption of the so-called gastric mucosal barrier (4, 5), accompanied by bicarbonate leakage (6), as well as metabolic alterations deeply linked to the acid secretory activity in the damaged gastric mucosa (7–10).

Gastric acid hypersecretion is an aggressive factor involved in the pathogenesis of some gastric and duodenal disorders (11), and acid secretion has been proven to be dependent on mucosal oxidative metabolism, which provides ATP for driving H^+-K^+-ATPase activity (12–14). In addition, Ca^{2+} is an intracellular messenger that participates in the coupling of stimulus-secretion in the normal mammalian gastric mucosa (15–17). The ion also mediates the regulation of some metabolic pathways in the gastric mucosa, such as increased glycogen breakdown induced by theophylline in amphibians (18, 19), as well as the cholin-

ergic activation of carbohydrate oxidation by rabbit gastric glands (20). Then, because intracellular levels of Ca^{2+} seem to be implicated in the preservation of gastric mucosal integrity (21), disturbances of Ca^{2+} homeostasis have been claimed to be a mechanism underlying gastric injury induced by several agents (22, 23).

Increased Ca^{2+} levels have been also involved in ethanol-induced acute gastric mucosal injury (24–26); however, the relationship between Ca^{2+} and ethanol-induced gastric damage has not been well established. Hence, the connection among ethanol-induced changes on gastric acid secretion, oxidative metabolism and intracellular Ca^{2+} mobilization is unknown.

Therefore, the present study was aimed to evaluate whether alterations on acid secretion and oxidative metabolism are involved in the pathogenesis of ethanol-induced chronic injury in rat gastric mucosa. Moreover, the possible role of intracellular changes on Ca^{2+} homeostasis was also assessed as a major factor inducing gastric mucosal damage.

Materials and Methods

Chemicals. D-[U-^{14}C] Glucose (sp. act. 260 mCi/mmol), D-[1-^{14}C] Glucose (sp. act. 260 mCi/mmol), glucose (sp. act. 55 mCi/mmol), and D-[6-^{14}C] glucose (sp. act. 56 mCi/mmol) were purchased from Amersham Co. (Arlington Heights, IL). Aminopyrine [dimethylamine-^{14}C] (sp. act. 100 mCi/mmol), [U-^{14}C] sucrose (sp. act. 600 mCi/mmol), and $^{45}Ca^{2+}$ were obtained from New England Nuclear Life Science Products, Inc. (Boston, MA). BAPTA-AM was obtained from Molecular Probes (Eugene, OR) and omeprazole (Losec®) from Astra Chemicals, S.A. (Mexico). Enzymes, coenzymes, EGTA and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal Model. The model of ethanol-induced subchronic gastric mucosal damage in male Wistar rats (230–270 g of body weight), has been reported in detail (1). Briefly, overnight fasted animals with free access to water received 1 ml of saline solution (control group), or 1 ml of 50% ethanol (gastritis group) by intragastric gavage, followed by free access to water (controls) or 5% ethanol in water (gastritis group). The treatment was continued for 5 days, and at the fifth day ethanol was withdrawn. After an overnight fast, animals were killed 2 to 3 hr after ethanol withdrawal under general anesthesia with sodium pentobarbital (40 mg/kg body weight). All procedures were conducted in accordance to our *Institutional Guide for Animal Experimentation* (National University of Mexico).

Isolation of Gastric Mucosa. Gastric mucosa from rats was isolated as previously reported (27). Briefly, rat stomachs were removed and cut open along the lesser curvature. The glandular area was dissected and cut to obtain minces or slices, which were rinsed three times and suspended in a medium containing (in mmol/l): 10 TES, 133 NaCl, 5 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 1 Na_2HPO_4, and 10 glucose, unless any change was indicated.

Glucose Oxidation. The rate of glucose oxidation through pentose phosphate and glycolytic acid citric cycle pathways, were estimated comparatively by measuring the production of $^{14}CO_2$ from [1-^{14}C], [6-^{14}C], or [U-^{14}C] glucoses as previously described, with minor changes (27): TES buffer-suspended minces of gastric mucosa were incubated in the presence of 0.4 to 0.8 μCi of ^{14}C-radiolabeled glucose in a final volume of 2 ml at 37°C, during 90 min. Thereafter, the $^{14}CO_2$ was trapped in 0.2 ml of 100 mmol/l hyamine, placed in a central wall of the flask. Then, hyamine was transferred to a vial containing scintillation liquid and counted. Appropriate controls were run simultaneously. All the experiments were performed in duplicate and the rate of glucose oxidation is expressed as nmol per hour per milligram of protein.

Aminopyrine Accumulation. Accumulated aminopyrine in minces of gastric mucosa was measured as previously described by Chacín *et al.* (12), with minor modifications. Minces of gastric mucosa (6–8 mg dry weight) were gassed (95% O_2–5% CO_2) during 2 min and incubated at 37°C during 1 hr in TES solution containing 0.1 μCi/ml [^{14}C]-aminopyrine. Then, the pellets were obtained by centrifugation and immediately transferred to counting vials, weighed, and dried overnight at 80°C. The dried pellets were weighed, solubilized in 1 ml of 1N NaOH during 24 hr, neutralized and added to scintillation liquid, and counted for radioactivity. Intraglandular water content was determined in minces of gastric mucosa from both controls and ethanol-treated animals using [^{14}C]-sucrose as a marker for the extraglandular space, as previously described (12). Wet and dry weights were determined to calculate total water, and the extraglandular space was calculated from [^{14}C]-sucrose content of the tissue. The ratio of aminopyrine in intraglandular/extraglandular water was calculated and taken as an indicator of an intraglandular region relatively low pH (12). When BAPTA-AM was used, all the flasks were pre-incubated during 15 min in the absence of aminopyrine before allowing the incorporation of this reagent to the cell.

Ca^{2+} Uptake and Efflux by Isolated Gastric Mucosa. $^{45}Ca^{2+}$ uptake was performed as described previously (18), with some changes. Minces of gastric mucosa were incubated in the TES solution containing 0.2 μCi/ml $^{45}CaCl_2$ for different periods at 37°C. After incubation, the flasks content was immediately transferred to tubes containing 1 mmol/l $LaCl_3$ (in the absence of Ca^{2+}) and centrifuged again. The pellet was resuspended in TES solution containing 1 mmol/l EGTA and spun as indicated. The final pellets were dried overnight, weighed, solubilized in 1 ml of 1 N NaOH, neutralized, and counted. Uptake was calculated in terms of nmol of $^{45}Ca^{2+}$ per milligram protein on the basis of specific activity determinations. $^{45}Ca^{2+}$ efflux was also measured (18). Weighed slices of rat gastric mucosa were rinsed in TES solution (without Ca^{2+}) and incubated in the presence of 1.8 mM $CaCl_2$ containing 0.2μCi/ml $^{45}CaCl_2$. After incubating at 37°C for 2 hr, the tissue was gently

blotted on filter paper and transferred to the flasks containing 2 ml of Ca^{2+}-free TES solution. Mucosal slices were subsequently transferred to other flasks in series at different intervals to complete 90 min. Incubation was carried out at 37°C with shaking and oxygenation (95% O_2–5% CO_2). After washing, mucosa was digested, neutralized, and counted as described above. Each individual wash solution was dried at 80°C for 2 hr and the dried powder (dissolved in water) was counted for radioactivity. The total tissue radioactivity at the end of experiment was the sum of the total counts in each wash plus the digested tissue. Results are expressed as a percentage of total $^{45}Ca^{2+}$ incorporated.

Morphological Analysis by Electron Microscopy. Samples of gastric mucosa, isolated as described before, were fixed in a buffered solution containing 2.5% glutaraldehyde (pH 7.4). The tissues were then processed and analyzed according to Berglindh *et al.* (28). In a sample of 50 parietal cells, the nonsecreting and secreting cells were identified in accordance to their morphological characteristics as previously defined (28). The percentage of cellular area occupied by the intracellular canaliculus or lacunar structures was also determined and compared in both nonsecreting and stimulated parietal cells.

Quantification of Ca^{2+} in Subcellular Fractions. Gastric mucosa from controls and ethanol-treated animals were totally excised and homogenized in a buffer containing 0.25 mol/l sucrose and 10 mmol/l HEPES/KOH (pH 7.4). Subcellular fractions were obtained according to protocols described elsewhere (1, 2, 29). For total Ca^{2+} measurement, both mitochondrial and cytosolic fractions were deproteinized by adding $HClO_4$ (6–7% w/v final concentration) and centrifuged in the presence of 1% $LaCl_3$ and kept frozen until use. Ca^{2+} content from the deproteinized fractions was measured by atomic absorption flame photometry according to Díaz-Muñoz *et al.* (30).

Oxygen Consumption by Gastric Mucosa Minces. Minces of gastric mucosa (6–8 mg dry weight) contained in a 3 ml volume of TES solution were incubated at 25°C in the presence of 10 mmol/l glucose as substrate. The mixture was placed in a chamber equipped with a Clark-type oxygen electrode (Yellow Spring Instruments), and respiration was recorded polarographically for 10–15 min. Experiments with lanthanum were performed incubating gastric mucosa minces in the presence of 1 mmol/l $LaCl_3$, before the polarographic register. Oxygen uptake is expressed a $nAtO_2$/min/mg of tissue protein.

Analytical Procedures. Cytosolic activity of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was determined according to the method of Lörh and Waller (31). Succinate dehydrogenase (EC 1.2.1.16) was measured in the mitochondrial fraction, by the technique reported by King (32). In both, whole homogenate of gastric mucosa and isolated mitochondria, the activity of cytochrome oxidase (EC 1.9.3.1) was quantified according the method of Rafael (33). In neutralized acid extracts obtained from gastric mucosa, levels of ATP, ADP, lactate, and pyruvate were determined by the methods described elsewhere (34). The free pool of $NADP^+$ was also spectrophotometrically determined in these extracts, coupled to the reaction of the malic enzyme, as a modification of the technique described by Wise and Ball (35).

Calculations and Statistics. Mitochondrial recovery and the amount of protein per gram of gastric mucosa were calculated using the activity of cytochrome oxidase, as a marker enzyme, as previously described (36). All results are expressed as mean ± SE. The significance of the differences among groups was assessed by two-way ANOVA and, in the case of significance by *ad hoc* Newman-Keul's test.

Results

Changes in Mucosal Glucose Oxidation Induced by Chronic Treatment with Ethanol. Fulfillment of the metabolic requirements of the acid-secreting parietal cell under physiological circumstances requires a combination of substrates, but glucose seems to be the most effective in rats (13). Total glucose oxidation by isolated gastric mucosal minces, as assessed by $^{14}CO_2$ production from U-^{14}C-glucose, showed an apparent K_m of 1.3 ± 0.3 mM for glucose, as well as an apparent V_{max} of 11.1 ± 0.5 nmol of oxidized glucose·h^{-1}·mg^{-1} of protein, in control gastric mucosa. From these data, we used 10 mmol/l glucose as fixed substrate concentration for further incubations of gastric mucosal samples in the presence of radiolabeled glucose in different carbons (Table I). In animals subjected to chronic mucosal injury, glucose oxidation was doubled as

Table I. Oxidation of [1-^{14}C]-, [6-^{14}C]-, and [U-^{14}C]-Glucose by the Gastric Mucosa from Ethanol-Treated Rats

Treatment	Glucose isotope oxidation (nmols·h^{-1}·mg tissue $protein^{-1}$)					
	[1-^{14}C]	*n*	[6-^{14}C]	*n*	[U-^{14}C]	*n*
Control	9.9 ± 1.2	16	6.2 ± 0.7	8	8.9 ± 0.6	11
Ethanol (gastritis)	20.6 ± 2.0[a]	13	12.9 ± 1.7[a]	8	18.8 ± 2.1[a]	9
Control + omeprazole	—		—		7.3 ± 0.3[a]	4
Ethanol + omeprazole	—		—		12.0 ± 0.6[b]	4

Minces of gastric mucosa isolated from controls and ethanol-treated rats were incubated with 10 mmol/l glucose containing 0.4 to 0.8 μCi of ^{14}C-radiolabeled glucose in different carbons. The incubation was performed at 37°C for 90 min. Controls corresponded to rats treated with saline. Results are expressed as mean ± SE of *n* individual preparations. Statistics: [a] $P < 0.05$ vs controls; [b] $P < 0.05$ vs the ethanol group (gastritis).

compared with controls. The increased oxidation of the different glucose radioisotopes was of a similar magnitude in gastric samples from animals undergoing gastritis, suggesting that the enhanced capacity of chronically injured gastric mucosa for metabolizing glucose was not associated to changes in the relative contribution of each metabolic pathway (i.e., glycolysis, citric acid cycle or pentose phosphate shunt; Table I) Based on data taken from Table I, the production of $^{14}CO_2$ as a function of the ratio 1-^{14}C/6-^{14}C of glucose was of 1.6, indicating that rat gastric mucosa truly possesses an active pentose phosphate pathway activity for glucose oxidation, which agrees with metabolic considerations previously reported (27). The presence of an active pentose phosphate shunt in the rat gastric mucosa was additionally corroborated by measuring of glucose-6-phosphate dehydrogenase activity in cytosolic fractions of gastric mucosa: 333.0 ± 66.8 (ethanol group) vs 336.4 ± 47.6 nmol NADPH/min/mg of protein (control group). To evaluate the proportion of glucose oxidation directly linked to the acid secretory activity of the gastric mucosa, the H^+-K^+-ATPase inhibitor, omeprazole, was added *in vitro* to the incubation medium. As shown in Table I, omeprazole inhibited glucose consumption in both, mucosal samples from control and animals subjected to gastritis. However, the percentage of inhibition of glucose oxidation induced by omeprazole was of higher magnitude in mucosal samples of rats treated chronically with ethanol (36 ± 4 vs 18 ± 3% in controls; $P < 0.01$). These data suggest that a significant fraction of glucose oxidative metabolism is coupled to the activity of the H^+-K^+-ATPase.

Effect of Chronic Ethanol Treatment on the Secretory Acid Status in Gastric Mucosa. The accumulation of ^{14}C-aminopyrine was evaluated in mucosal samples obtained from our experimental groups, as a parameter indicative of the acid secretory function of the parietal cells. Basal aminopyrine accumulation (nonstimulated by secretagogues) was almost doubled in preparations obtained from ethanol-treated rats when compared to controls (Fig. 1). In control mucosal samples, either histamine or carbachol (gastric secretagogue which acts via Ca^{2+}) were able to stimulate acid secretion; however, both secretagogues failed to elicit an additional stimulation of acid secretion in rats subjected to gastritis (Fig. 1). The histaminergic H_2-receptor antagonist, cimetidine, had no effect in the basal aminopyrine accumulation in either, control or injured mucosal preparations. The BAPTA-AM, a chelator of intracellular Ca^{2+}, did lower the stimulated acid secretion recorded in mucosal samples from ethanol-treated rats (Fig. 1).

The results obtained from *in vitro* measurements of the acid secretory activity in gastric mucosa samples might be correlated with the morphologic changes of the parietal cells, induced *in vivo* by chronic ethanol administration. Figure 2A (control animals) shows a rat parietal cell, which has a characteristic conical shape with numerous mitochondria. Also there is a complex network of smooth-surface

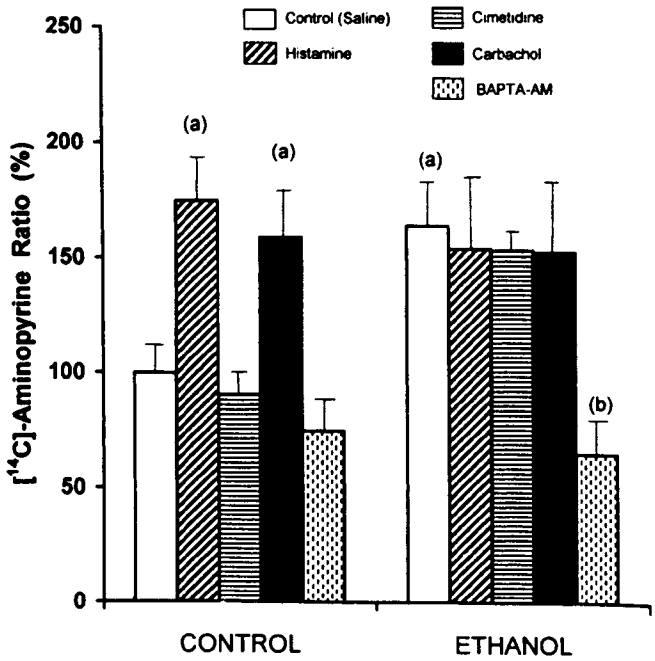

Figure 1. Effects of subchronic ethanol treatment on the aminopyrine accumulation (aminopyrine ratio). Minces of gastric mucosa from both controls and ethanol-treated rats were incubated in presence or in absence of histamine (10^{-4} M), carbachol (10^{-4} M), cimetidine (10^{-3} M), or BAPTA-AM (50 μM). The BAPTA-AM vehicle (DMSO) was added to control flasks (20 μl). For calculations, a mean value of 2.4 ± 0.4 (controls) and 2.6 ± 0.4 μl/mg dry wt (ethanol) was found for the intraglandular water. Values are means ± SE of 4–8 experiments. Control value: 0.92 ± 0.11 (100%). Statistics: (a) $P <$ 0.05 vs control group (saline); (b) $P < 0.05$ vs ethanol group.

intracytoplasmic membranes termed the vesicle tubular system (Fig. 2A). This morphological pattern represented the 69 ± 5% of the total population of oxyntic cells in control samples. When parietal cells were examined in mucosal samples from animals undergoing gastritis, it was observed that the vesicle tubules decreased in number and increased in volume, taking a vacuolar shape, whereas microvillus appeared on their secretory surface. Therefore, the apparent loss in surface area of the vesicletubular system seems to be compensated for by newly created microvillus (Fig. 2B). The last findings has been described before as a result of stimulating normal parietal cells with secretagogues (28); therefore, the increased number of vacuoles can be considered as expanded acid secretory canaliculus, which were present in the 50 ± 5% of parietal cells from ethanol-treated rats (Fig. 2B). Moreover, when comparing the percentage of cell surface occupied by the secretory canaliculus in oxyntic cells from both experimental groups, differences were quite evident. Few vacuoles were recorded in nonsecreting cells (i.e., controls animals) accounting for by 3.1 ± 0.2% of the total area. However, in stimulated oxyntic cells predominantly in the gastritis group, the vacuolar distribution represented up to 21 ± 2% of the total area calculated for the parietal cells.

Role of Ca^{2+} in Ethanol-Induced Metabolic and Secretory Changes. Both the inhibitory effect of BAPTA-AM and the absence of carbachol stimulation on

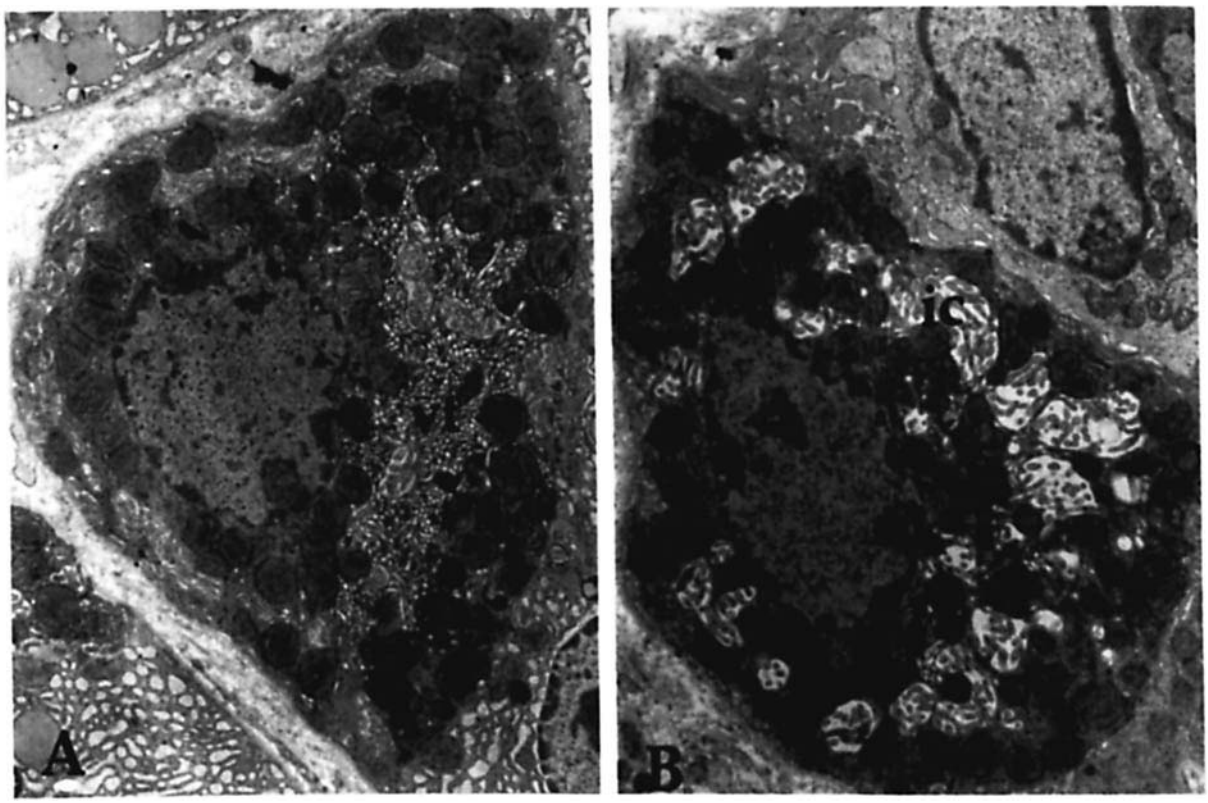

Figure 2. Electron micrographs of parietal cells. The pictures are representative of at least three stomachs per group. (A) control animal. (B) Ethanol-treated animal. Pieces of gastric mucosa freshly isolated were immediately placed in a cooled solution of 2.5% glutaraldehyde in 100 mM of phosphate buffer, pH 7.4, and processed for transmission electron microscopy. vt, vesicletubular system; ic, Intracellular canaliculus and microvillus; m, mitochondria. Magnification ×4000.

the aminopyrine accumulation were detected in the gastric mucosa from ethanol-treated rats (Fig. 1). Such effects led us to examine the possible role of Ca^{2+} in the regulation of carbohydrate metabolism in normal and injured gastric mucosa. For this, agents capable of modifying cell Ca^{2+} homeostasis were used *in vitro*. Using EGTA to sequester extracellular Ca^{2+}, glucose oxidation was reduced as compared with the basal glucose utilization by preparations from control animals. Addition of BAPTA-AM to the incubation medium with EGTA, elicited an even stronger inhibitory effect on glucose oxidation (Fig. 3). In rats subjected to gastritis, EGTA significantly reduced the ethanol-induced stimulation of mucosal glucose oxidation, while the incubation in presence of both Ca^{2+} chelators, completely abolished the chronic effect of ethanol (Fig. 3). The muscarinic agonist carbachol, stimulated the glucose oxidation in control animals, but this agent did not modify the glucose catabolism in the ethanol-treated rats (Fig. 3). Mucosal glucose oxidation in the presence of 1 mmol/l $LaCl_3$ was inhibited in a similar extent, either in samples obtained from control or from animals subjected to gastritis (Table II). These data strengthen the dependence of gastric mucosa oxidation of glucose and the extracellular calcium, indeed supporting to that found in the experiments using EGTA (Fig. 3).

To get a better understanding of the putative effect of chronic ethanol on Ca^{2+} dynamics, uptake and efflux of $^{45}Ca^{2+}$ were determined in mucosal samples of our experimental groups (Fig. 4). Mucosal Ca^{2+} uptake was progressively increased and saturated at 30 min of incubation. Despite a quite similar profile of Ca^{2+} uptake was found in gastric mucosa isolated from both control and rats undergoing gastritis, it was clear that injured gastric mucosa incorporated more radioactive Ca^{2+} than the normal one (Fig. 4A). On the contrary, Ca^{2+} release was similar in mucosal samples obtained from both experimental conditions (Fig. 4B). These results are in agreement with those obtained by measuring total Ca^{2+} in subcellular fractions (Table III). In both subcellular fractions, cytosol and mitochondria, there was an enhanced Ca^{2+} accumulation in mucosal samples from ethanol-treated rats (Table III).

Oxygen Consumption and Production of ATP and Lactate in Gastric Mucosa from Control and Ethanol-Treated Animals. Despite that calculated amount of mitochondrial protein per gram of gastric mucosa (36) was lower in animals undergoing gastritis (32.4 vs 44.5 mg of mitochondrial protein/g of mucosa, in controls; $P < 0.01$), samples of damaged mucosa had high oxygen consumption (Table II). This could be explained by increased activities of mitochondrial enzymes, such as that of succinate dehydrogenase, which was enhanced in animals subjected to gastritis (11.1 ± 0.8, against 7.4 ± 1.0 nmol·min^{-1}·mg^{-1} of mitochondrial protein, in controls; $P < 0.02$). Interestingly, oxygen uptake by gastric mucosal slices was

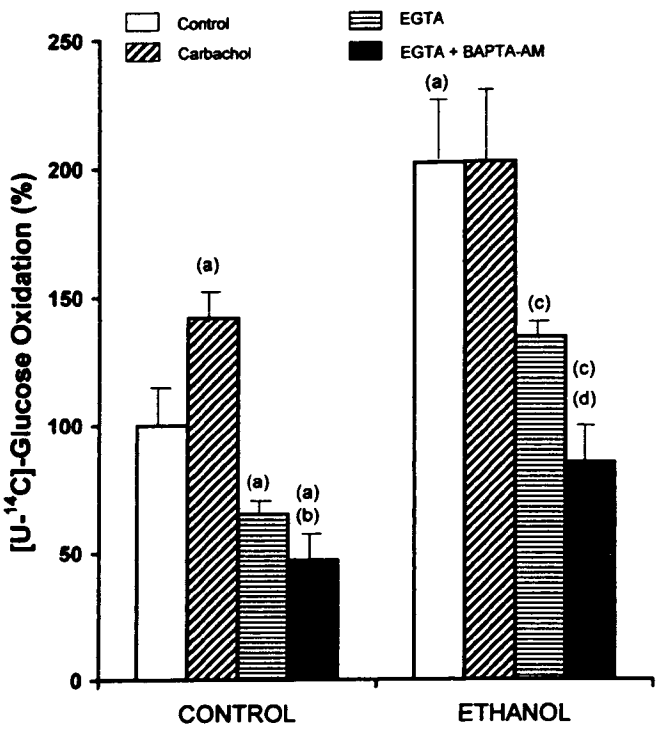

Figure 3. Effects of carbachol and Ca^{2+} quelators on the rate of glucose oxidation. Minces of gastric mucosa from controls and ethanol-treated animals, were incubated with 10 mM glucose plus 0.4 μCi [U-^{14}C]-glucose, in absence or presence of carbachol 10^{-4} M, EGTA 1 mM, or EGTA + BAPTA-AM 50 μM. $CaCl_2$ was omitted from TES solution in experiments with EGTA or EGTA + BAPTA-AM. Control value: 10.4 ± 1.8 nmol of glucose oxidized·h^{-1}·mg^{-1} of protein. Results are mean ± SE of 4–10 experiments. Statistics: (a) $P < 0.05$ *vs* controls (saline); (b) $P < 0.05$ vs EGTA; (c) $P < 0.05$ vs ethanol-treated animals; (d) $P < 0.05$ vs EGTA in ethanol-treated animals.

largely modified by addition of lanthanum or omeprazole. Pre-incubation with 1 mmol/l $LaCl_3$ inhibited oxygen consumption by control mucosa samples (up to 55%); the inhibiting effect of lanthanum in the stimulated oxygen uptake by slices of damaged gastric mucosa was even bigger (72%; Table II). Coinciding with its effect on glucose oxidation by mucosal preparations (Table I), addition of omeprazole led to an inhibition of 39.5% and of 51.3% in the rate of gastric mucosal oxygen consumption by control and ethanol-treated rats, respectively.

Although animals treated chronically with ethanol showed lower mitochondrial content in the gastric mucosa, these preparations produced more ATP than controls after incubation with glucose (Table IV). Because gastric mucosal ADP content was not significantly modified in either, control and ethanol-treated animals, total adenine nucleotides were augmented in gastric mucosa at the onset of gastritis. Despite increased oxidation of glucose was noted in mucosal samples of animals with gastritis, no significant changes were found in the level of lactate and on the lactate/pyruvate ratio (Table IV). Because glucose flux through the pentose phosphate pathway was increased in gastric mucosa of ethanol-treated rats, in the absence of changes in glucose-6-phosphate dehydrogenase, the content of $NADP^+$ was measured. As expected, mucosal samples from rats undergoing gastritis showed a significant higher $NADP^+$ level than in controls (Table IV); therefore, enhanced activity of pentose phosphate shunt in the damaged mucosa could be caused by, at least in part, to the increased mucosal $NADP^+$ availability.

Discussion

The present study was aimed to discern whether changes in oxidative metabolism and acid secretory activity, are participating in the mechanisms of gastric mucosal injury induced by *in vivo* chronic treatment with ethanol. Additionally, the role of Ca^{2+} homeostasis as a mediator of those changes was also investigated. Our data strongly suggest that chronic gastric mucosal injury *in vivo* occurs with changes in Ca^{2+} mobilization, disturbing its homeostasis. These events exert a profound influence in the oxidative catabolism of carbohydrates and the energy supply for the stimulated acid secretion by parietal cells after mucosal injury induced by chronic ethanol treatment.

Glucose has been considered as the main oxidative substrate for rat gastric mucosa, in function of its efficiency in supporting the acid secretory activity (13, 27). In the present study, kinetics of glucose by gastric mucosa from control animals were very similar to those reported in other species (14, 18). Total glucose oxidation corresponding to the different glucose oxidative pathways (glycolysis, pentose phosphate shunt, citric acid cycle), was significantly stimulated in gastric mucosa from animals subjected to gastritis (Table I). It should be noted that present experiments were conducted in the absence of ethanol because ethanol and its

Table II. Effects of 1 mM $LaCl_3$ on the Rate of Oxygen Uptake and Glucose Oxidation in the Ethanol-Treated Rat Gastric Mucosa

Treatment	Addition	O_2 uptake nATO_2·min^{-1}·mg tissue^{-1}	Rate of 6-^{14}C-glucose oxidation nmols·hr^{-1}·mg tissue protein^{-1}
Control	None	47.3 ± 4.0	6.11 ± 0.6
	Lanthanum	21.5 ± 1.8*	4.17 ± 0.4*
Ethanol	None	75.2 ± 8.4	11.6 ± 1.1
	Lanthanum	21.1 ± 3.0*	7.5 ± 0.9*

Aliquots of gastric mucosa samples were incubated for 90 min at 37°C in the presence of 10 mmol/l glucose containing 0.5 μCi of 6-^{14}C-glucose to assess the rate of glucose oxidation. Other aliquots from the same samples were assayed for oxygen consumption, as described in the Materials and Methods section. *$P < 0.05$ vs control.

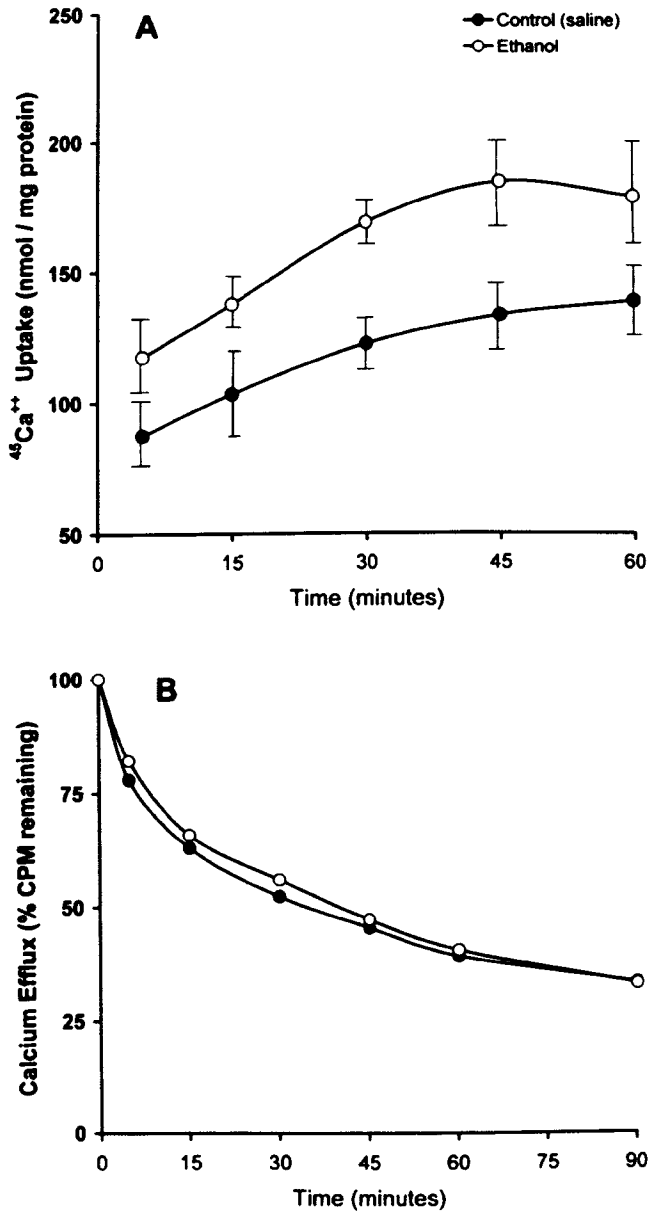

Figure 4. Effects of ethanol on $^{45}Ca^{2+}$-influx (A) and -efflux (B) by the gastric mucosa of rats. Experiments were performed as described in Materials and Methods section. Results are means ± SE of four experiments. Values in ethanol-treated group are statistically significant vs the corresponding point in the control group.

Table III. Content of Total Ca^{2+} Determined by Atomic Absorption Spectrometry in the Cytosolic and Mitochondrial Fractions from the Rat Gastric Mucosa

Treatment	Total calcium content (nmol Ca^{2+}·mg tissue protein^{-1})	
	Cytosol	Mitochondria
Control	0.49 ± 0.03	1.86 ± 0.15
Ethanol (gastritis)	0.65 ± 0.05*	2.85 ± 0.31*

Both cytosolic and mitochondrial fractions were isolated from homogenates of gastric mucosa, deproteinized by 6% $HCLO_4$, and centrifuged in presence of 1% $LaCl_3$. Ca^{2+} total content was measured by atomic absorption flame photometry. Results are mean ± SE of three determinations from pooled gastric mucosa. *$P < 0.05$ vs controls.

metabolites were practically undetectable at the moment of animal's death (1). Then, changes on glucose oxidative metabolism, found in this model, were a consequence of the chronic mucosal injury, instead due to ethanol oxidation during *in vitro* incubations. In agreement with this statement is the finding of a very low capacity of rat gastric mucosa to oxidize ethanol, at concentrations below 150 mmol/l (37).

Our data, using radiolabeled glucose in different carbons, clearly showed that an active pentose phosphate shunt is present in the rat gastric mucosa (Table I). This agrees qualitatively with the effect reported by Sernka and Harris (27), despite the magnitude of glucose oxidation was lower than the effect described by these authors. Because we detected glucose-6-phosphate dehydrogenase activity, this further support that a fraction of glucose oxidation is derived to the pentose phosphate shunt in the rat gastric mucosa. Indeed, chronic ethanol treatment substantially increased glucose flux through this metabolic pathway in the injured mucosa, effect seemed to be due an increased availability of $NADP^+$ (Tables I and IV). A similar stimulation of the pentose phosphate shunt has been observed during early stages of liver fibrosis induced by carbon tetrachloride (38). Here, we have suggested that increased pentose phosphate shunt activity could participate in the replenishment of reduced glutathione and probably in the synthesis of deoxyribonucleotides, stimulating cell replication (38). In this context, we have observed a normalization of the mucosal glutathione level and increased DNA synthesis, during the early recovery period after ethanol withdrawal in the experimental present model (2). Hence, both processes requiring NADPH (DNA synthesis and glutathione replenishment) would favor the oxidative flux of glucose through pentose phosphate shunt.

Increased glucose oxidation can be associated to an enhanced demand of metabolic energy for the by H^+-K^+-ATPase activity, leading to an acid hypersecretory state in the injured gastric mucosa. The inhibitory effects of omeprazole on the glucose oxidation in the gastric mucosa of ethanol-treated rats (Table I), and on oxygen consumption, strongly suggest a coupling between metabolic and secretory activities in this model of mucosal injury (Figs. 1 and 2). A similar dependence between secretory and metabolic effects has been reported before in the normal gastric mucosa (12–14, 39). Aminopyrine accumulation seemed to be a good indirect index of the acid secretion in the rat gastric mucosa. Chronic ethanol treatment *in vivo* induced a significant increase on the acid secretory activity, as assessed by aminopyrine accumulation and electron microscopy data (Figs. 1 and 2). Therefore, these data show that chronic ethanol treatment also occurs with a mucosal hypersecretory state, which resembles the stimulatory action of ethanol on this parameter, found after its acute exposure (5, 8–10, 40). In addition, ethanol seemed to directly stimu-

Table IV. Effect of Ethanol *in Vivo* Administration on the Tissue Level of Lactate, Pyruvate, ATP, ADP, and $NADP^+$ in Incubated Slices of Rat Gastric Mucosa

Treatment	Total tissue content (μmols·g wet weight^{-1})				
	Lactate	Pyruvate	ATP	ADP	$NADP^+$
None (control)	2.1 ± 0.2	0.16 ± 0.02	2.4 ± 0.2	1.6 ± 0.2	0.015 ± 0.001
Ethanol (gastritis)	2.4 ± 0.2	0.17 ± 0.01	3.4 ± 0.3*	1.9 ± 0.2	0.027 ± 0.002*

Slices of gastric mucosa isolated from both control and ethanol treated rats were incubated for 90 min (at 37°C), in the presence of 10 mmol/l glucose (pH 7.4). Immediately after incubation, slices were immediately deproteinized with 6% $HClO_4$. Extracts were further neutralized, and the content of lactate, pyruvate, ATP, ADP, and $NADP^+$ were determined as described in Materials and Methods. Results are mean ± SE of five individual experiments. Statistics as indicated in the Table II.

late the functional expression of gastric H^+-K^+-ATPase (41).

The close relationship among glucose oxidation, ATP generation, and acid secretory activity found in the normal and ethanol-injured mucosa seems to be regulated by changes in intracellular levels of Ca^{2+} as a possible physiological mediator. Although Ca^{2+} has been claimed to be a mediator triggering cell death (42), changes in Ca^{2+} homeostasis are also involved in the compensatory cell proliferation that follows partial hepatectomy in rats (30). In the normal gastric mucosa, the physiological role of Ca^{2+} in the regulation of metabolic and secretory processes has been established (15–17, 19, 20). However, the role of Ca^{2+} during generation of gastric mucosal injury has not been fully clarified, since information is lacking on *in vivo* effects of this cation on gastric mucosa.

The metabolic inhibition induced by absence of Ca^{2+} (+EGTA), or by the presence of lanthanum in the incubation medium, clearly indicates that extracellular Ca^{2+} participates in the regulation of oxidative metabolism in gastric mucosa. Indeed, the stimulation of glucose oxidation in ethanol-injured rat gastric mucosa was particularly sensitive to the blockade of Ca^{2+} entry to mucosal cells. However, the addition of the intracellular Ca^{2+} chelator, BAPTA-AM, induced an extra inhibition of the glucose oxidation, suggesting that Ca^{2+} mobilization from internal stores also mediates the metabolic ethanol effect (Fig. 3). Despite carbachol did not induce additive stimulation of glucose oxidation and aminopyrine accumulation in mucosa from ethanol-treated rats, it did stimulate glucose oxidation in control animals (Figs. 1 and 3). $LaCl_3$ was a stronger inhibitor of calcium-induced effects on energy metabolism in the chronically ethanol-injured mucosa, as compared with control rats (Table II). These data indicate that a significant fraction of glucose oxidation depends on Ca^{2+} mobilization, from both extracellular medium and internal stores, being this dependence stronger in gastric mucosa from rats subjected to gastritis. Similar results have been reported in guinea pig parietal cells treated with ethanol *in vitro*, where carbachol also failed in stimulating the acid secretion. This has been attributed, at least in part, to high levels of intracellular Ca^{2+} and activation of protein kinase C, presumably induced by ethanol oxidation (43). The above results strongly suggest that ethanol-induced chronic mucosal damage could be associated to disturbances on Ca^{2+} homeostasis, characterized by increased levels of intracellular Ca^{2+} in the rat gastric mucosa.

Confirming the aforementioned, we have the following findings in ethanol-injured gastric mucosa: 1) in both cytosolic and mitochondrial fractions, total calcium content is increased (Table II), 2) mucosal minces incorporated more actively the added external Ca^{2+} (Fig. 4). This ion acts as a regulator in of some mitochondrial enzyme activities in the rabbit gastric mucosa, influencing the metabolism of carbohydrates and its derivatives (20). Indeed, levels of total mitochondrial Ca^{2+} reported herein correspond to those proposed as capable of affecting mitochondrial dehydrogenase activities in other tissues (44). Linked to the above mentioned, Ca^{2+} also seems to mediate the mechanism of secretory stimulation by of H_2-receptor agonists in the parietal cell (17, 45). Hence, high levels of intracellular Ca^{2+} induced by chronic treatment with ethanol could promote enhanced activity of mitochondrial enzymes, such as succinate dehydrogenase. The latter might explain the enhanced oxidative metabolism of the injured mucosa, even thought that mitochondrial protein was found reduced in gastric mucosa from animals with gastritis.

Controversy exists regarding the participation of Ca^{2+} in the pathogenesis of gastric mucosal lesions induced *in vitro* and *in vivo* by acute ethanol exposure. Ethanol treatment increases intracellular Ca^{2+} concentration, leading to a gastric mucosal lesion (26). *In vitro*, external Ca^{2+} exacerbates ethanol-induced damage in gastric mucosal samples (46), and Ca^{2+} channel blockers exert protection for gastric mucosa against the deleterious effect of ethanol (26, 47, 48). In contrast, it has also been reported that Ca^{2+} could diminish the extent of ethanol-induced gastric injury (49), and that verapamil, a Ca^{2+} channel antagonist, indeed worsened the ethanol-induced gastric mucosal damage (24). Despite the fact that nature of this discrepancy is still unknown, this could be related, at least in part, to the different experimental models used (*in vivo* vs *in vitro*), ethanol and Ca^{2+} concentrations, time of exposure, and rate of ethanol metabolism, among others. The present work shows that Ca^{2+} intracellular levels could mediate metabolic and secretory effects, which were more evident in gastric mucosa isolated from animals subjected to gastritis.

Ethanol effects have been reported in gastric mucosa

metabolism. Acute treatment with ethanol produces a significant decrease in ATP content (50), accompanied of elevated lactate/pyruvate ratio in the gastric mucosa (10). In addition, ethanol can also decrease glucose oxidation by the gastric mucosa, mainly through inhibition of the pentose phosphate pathway (51). Indeed, it has been proposed that topical ethanol exposure alters oxidative phosphorylation, coincident with its damaging effect on gastric mucosa, most likely via perturbations in tissue blood flow (52). Because we did not find the above-described effects of ethanol in this present model of chronic mucosal injury, we could rule out the participation of acute ethanol effects. Therefore, the changes in oxidative metabolism, acid secretion and in intracellular Ca^{2+} content, seemed to be characteristic in rat gastric mucosa during progression of a chronic mucosal damage induced by ethanol treatment.

In conclusion, for the first time we have demonstrated that changes in Ca^{2+} mobilization leading to a disturbed Ca^{2+} homeostasis occur in a model of chronic gastric mucosal injury *in vivo* after ethanol withdrawal. These events can be related to metabolic adjustment to maintain the oxidative catabolism of carbohydrates and the energy supply for the acid secretion stimulated in the parietal cells. However, it is also possible that Ca^{2+} could mediate preparative changes that are driving the cell proliferative response that follows to discontinuation of ethanol in this model (2). In a similar context, changes of intracellular Ca^{2+} participate in the metabolic adjustment (energy and redox potentials) that occurs at the onset of liver regeneration induced by partial hepatectomy in rats (30, 34).

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1. Hernández-Muñoz R, Montiel-Ruíz F. Reversion by histamine H2-receptor antagonists of plasma membranes alterations in ethanol-induced gastritis. Dig Dis Sci **41**:2156–2165, 1996.
2. Hernández-Muñoz R, Montiel-Ruíz C, Vázquez-Martínez O. Gastric mucosal cell proliferation in ethanol-induced chronic mucosal injury is related to oxidative stress and lipid peroxidation. Lab Invest **80**:1161–1169, 2000.
3. Guth PH. Gastric blood flow in ethanol injury and prostaglandin cytoprotection. Scand J Gastroenterol **21**:86–91, 1986.
4. Davenport HW, Warner HA, Code CF. Functional significance of gastric mucosal barrier to sodium. Gastroenterology **47**:142–152, 1964.
5. Davenport HW. Ethanol damage to canine oxyntic glandular mucosa. Proc Soc Exp Biol Med **126**:657–662, 1967.
6. Dayton MT, Kauffman GL, Schlegel JF, Code CF, Steinbach JH. Gastric bicarbonate appearance with ethanol ingestion. Mechanism and significance. Dig Dis Sci **28**:449–455, 1983.
7. Sernka TJ, Gilleland CW, Shanbour LL. Effects of ethanol on active transport in the dog stomach. Am J Physiol **226**:397–400, 1974.
8. Lenz HJ, Ferrari-Taylor J, Isenberg JL. Wine and five percent ethanol are potent stimulants of gastric acid secretion in humans. Gastroenterology **85**:1082–1087, 1983.
9. Singer MV, Leffmann C, Eysselein VE, Calden H, Goebell H. Action of ethanol and some alcoholic beverages on gastric acid secretion and release of gastrin in humans. Gastroenterology **93**:1247–1254, 1987.
10. Chacín J, Cárdenas P, Lobo P, Hernández I. Secretory and metabolic effects of ethanol in the isolated amphibian gastric mucosa. Gastroenterology **100**:1288–1295, 1991.
11. Wolfe MM, Soll AH. The physiology of gastric acid secretion. N Engl J Med **319**:1707–1715, 1988.
12. Chacín J, Prieto A, Cárdenas P. Substrate-level energy dependence of acid secretion in the isolated human gastric mucosa. Gastroenterology **89**:525–531, 1985.
13. Shaw GP, Anderson NG, Hanson PJ. Metabolism and gastric acid secretion. Substrate-dependence of aminopyrine accumulation in isolated rat parietal cells. Biochem J **277**:223–229, 1985.
14. Fryklund J, Gedda K, Scott D, Sachs G, Wallmark B. Coupling of H^+-K^+-ATPase activity and glucose oxidation in gastric glands. Am J Physiol **258**:G719–G727, 1990.
15. Soll AH. Extracellular calcium and cholinergic stimulation of isolated canine parietal cells. J Clin Invest **68**:270–278, 1981.
16. Muallem S, Sach G. Changes in cytosolic free Ca^{2+} in isolated parietal cells. Differential effects of secretagogues. Biochim Biophys Acta **805**:181–185, 1984.
17. Chew CS, Brown MR. Release of intracellular Ca^{2+} and elevation of inositol triphosphate by secretagogues in parietal and chief cells isolated from rabbit gastric mucosa. Biochim Biophys Acta **888**:116–125, 1986.
18. Chacín J, Cárdenas P, Lobo P, Subero O. Role of calcium in secretory and metabolic effects of substrates in the gastric mucosa. Am J Physiol **251**:G161–G168, 1986.
19. Subero O, Lobo P, Chacín J. Ca^{2+} requirement for metabolic effects of secretagogues in the amphibian gastric mucosa. Am J Physiol **257**:G969–G976, 1989.
20. Hernández I, Chacín J. Mechanism of cholinergic stimulation of glucose oxidation in isolated gastric glands. Am J Physiol **267**: G227–G234, 1994.
21. Slomiany BL, Liu J, Kawai T, Czajkowski A, Slomiany A. Effect of ebrotidine on gastric mucosal calcium channel activity. Am J Gastroenterol **88**:881–886, 1993.
22. Glavin GB. Calcium channel modulators: Effects on gastric function. Eur J Pharmacol **160**:323–328, 1989.
23. Tepperman BL, Soper BD. Effect of extracellular Ca^{2+} on indomethacin-induced injury to rabbit dispersed gastric mucosal cells. Can J Physiol Pharmacol **72**:63–69, 1994.
24. Koo MW, Cho CH, Ogle CW. Verapamil worsens the ethanol-induced gastric ulcers in rats. Eur J Pharmacol **120**:350–355, 1986.
25. Glavin GB, Szabo S. Effects of the Ca^{2+} chelators EGTA and EDTA on ethanol- or stress-induced gastric mucosal lesions and gastric secretion. Eur J Pharmacol **233**:269–273, 1993.
26. Kokoska ER, Smith GS, Deshpande Y, Wolf AB, Rieckenberg C, Miller TA. Calcium accentuates injury induced by ethanol in human gastric cells. J Gastrointest Surg **3**:308–318, 1999.
27. Sernka TJ, Harris JB. Pentose phosphate shunt and gastric acid secretion in the rat. Am J Physiol **222**:25–32, 1972.
28. Berglindh T, Dibona DF, Ito S, Sachs G. Probes of parietal cell function. Am J Physiol **238**:G165–G176, 1980.
29. Aguilar-Delfín I, López-Barrera F, Hernández-Muñoz R. Selective enhancement of lipid peroxidation in plasma membrane in two experimental models of liver regeneration: Partial hepatectomy and acute CCl_4 administration. Hepatology **24**:657–662, 1996.
30. Díaz-Muñoz M, Cañedo-Merino R, Gutiérrez-Salinas J, Hernández-Muñoz R. Modifications of intracellular calcium release channels and calcium mobilization following 70% hepatectomy. Arch Biochem Biophys **349**:105–112, 1998.
31. Löhr GW, Waller HD. Glucose-6-phosphate dehydrogenase. In: Bergmeyer HU, Ed. Methods of Enzymatic Analysis, 2nd edition. New York: Weinheim and Academic Press, pp744–751, 1974.
32. King TE. Preparation of succinate dehydrogenase and reconstitution of succinate oxidase. Methods Enzymol **10**:322–331, 1967.

33. Rafael J. Cytochrome c oxidase. In: Bergmeyer HU, Bergmeyer J, Eds. Methods of Enzymatic Analysis, vol. 3. Weinheim, Germany: Verlag Chemie, pp266–273, 1983.
34. Gutiérrez-Salinas J, Miranda-Garduño L, Trejo-Izquierdo E, Díaz-Muñoz M, Vidrio S, Morales-González JA, Hernández-Muñoz R. Redox state and energy metabolism during liver regeneration. Alterations produced by acute ethanol administration. Biochem Pharmacol **58:**1831–1839, 1999.
35. Wise EM, Ball EG. Malic enzyme and lipogenesis. Biochemistry **52:**1255–1263, 1964.
36. Hernández-Muñoz R, Díaz-Muñoz M, Chagoya de Sánchez V. Effects of adenosine administration on the function and membrane composition of liver mitochondria in carbon tetrachloride-induced cirrhosis. Arch Biochem Biophys **294:**160–167, 1992.
37. Caballería J, Baraona E, Lieber CS. The contribution of the stomach to ethanol oxidation in the rat. Life Sci **41:**1021–1027, 1987.
38. Hernández-Muñoz R, Díaz-Muñoz M, López V, López-Barrera F, Yánez L, Vidrio S, Aranda-Fraustro A, Chagoya de Sánchez V. Balance between oxidative damage and proliferative potential in an experimental rat model of CCl4-induced cirrhosis: Protective role of adenosine administration. Hepatology **26:**1100–1110, 1997.
39. Hersey SJ, Miller M, Owirodu A. Role of glucose metabolism in acid formation by isolated gastric glands. Biochim Biophys Acta **714:**143–151, 1982.
40. Eysselein VE, Singer MV, Went H, Goebell H. Action of ethanol on gastrin release in the dog. Dis Dis Sci **29:**12–18, 1984.
41. Klaassen CH, Swarts HP, De Pont JJ. Ethanol stimulates expression of functional H^+,K^+-ATPase in SF9 cells. Biochem Biophys Res Commun **210:**907–913, 1995.
42. Schanne FA, Kane AB, Young EE, Farber JL. Calcium dependence of toxic cell death: A final common pathway. Science **206:**700–702, 1979.
43. Niki S, Rokutan K, Nakamura K, Ogihara S, Kutsumi H, Saitoh Y, Aoike A, Kawai K. Calcium-dependent signaling of acid secretion in isolated parietal cells from guinea pigs and its modification by ethanol. Nipp Shok Gak Zas **89:**1484–1490, 1992.
44. McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol Rev **70:**391–425, 1990.
45. Chew CS. Cholescystokinin, carbachol, gastrin, histamine and foskolin increase $[Ca^{2+}]_i$ in gastric glands. Am J Physiol **250:**G814–G823, 1986.
46. Arakawa T, Fukuda T, Kobayashi K, Tarnawski A. Prostaglandin-induced protection of cultured rat gastric cells against ethanol is inhibited by a microtubule inhibitor. Digestion **57:**41–46, 1986.
47. Ghanayem BI, Matthews HB, Maronpot RR. Calcium channel blockers protect against ethanol- and indomethacin-induced gastric lesions in rats. Gastroenterology **92:**106–111, 1987.
48. Ostrowski J, Pesta J, Linnik D, Butruk E. The influence of calcium antagonists (verapamil, nifedipine, and $MgCl_2$) on rat gastric damage induced by ethanol *in vivo* and *in vitro*. J Physiol Pharmacol **44:**273–281, 1993.
49. Takeuchi K, Nobuhara Y. OkabeS. Role of luminal Ca^{2+} on normal and damaged gastric mucosa in the rat. Dig Dis Sci **30:**1072–1078, 1985.
50. Tague LL, Shanbour LL. Effects of ethanol on bicarbonate-stimulated ATPase, ATP and cyclic AMP in canine gastric mucosa. Proc Soc Exp Biol Med **154:**37–40, 1977.
51. Cho C, Pfeiffer CJ, Misra HP. Ulcerogenic mechanism of ethanol and the action of sulphanilyl fluoride on the rat stomach in vivo. J Pharm Pharmacol **43:**495–498, 1991.
52. Victor BE, Taegtmeyer H, Miller TA. Gastric mucosal high-energy phosphate metabolism. Influence of ethanol and PEG_2. Dig Dis Sci **40:**120–127, 1995.