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Histamine Release by Hydrochloric Acid is Mediated *via* Reactive Oxygen Species Generation and Phospholipase D in RBL-2H3 Mast Cells

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In order to investigate the underlying mechanism of HCl in oesophagitis, the inflammatory response to HCl was observed in RBL-2H3 mast cells. Rat basophilic leukemia (RBL-2H3) cells were used to measure histamine release, arachidonic acid (AA) release, reactive oxygen species (ROS) and peroxynitrite generation induced by HCl. Exogenous HCl increased the level of histamine release and ROS generation in a dose dependent manner, whereas it decreased the spontaneous release of [^{3}H] AA and the spontaneous production of peroxynitrite. Mepacrine (10 μM), oleyloxyethyl phosphorylcholine (10 μM) and bromoenol lactone (10 μM) did not affect both the level of histamine release and ROS generation induced by HCl. U73122 (1 μM), a specific phospholipase C (PLC) inhibitor did not have any influence on level of histamine release and ROS generation. Propranolol (200 μM), a phospholipase D (PLD) inhibitor, and neomycin (1 mM), a nonspecific PLC and PLD inhibitor, significantly inhibited both histamine release and ROS generation. Diphenyleneiodonium (10 μM), a NADPH oxidase inhibitor, and tiron (5 mM), an intracellular ROS scavenger significantly inhibited the HCl-induced histamine release and ROS generation. These findings suggest that the inflammatory responses to HCl is related to histamine release and ROS generation, and that the ROS generation by HCl may be involved in histamine release via the PLD pathway in RBL-2H3 cells.

Key words: Histamine, Reactive oxygen species, Peroxynitrite, Phospholipase

INTRODUCTION

Although hydrochloric acid (HCl) is not absolutely essential to the gastrointestinal function, it plays important roles including activating pepsinogen, killing of microorganisms and breaking down connective tissue and muscle fiber. Under pathological conditions, the reflux of gastric fluids particulary HCl causes oesophagitis (Biancani *et al.*,1997, Bell and Hunt, 1992). However, the underlying mechanism of HCl in oesophagitis remains undefined.

It is generally believed that the inflammatory responses are associated with phospholipase A_2 (PLA_2) activation, histamine release, reactive oxygen species (ROS) generation and nitric oxide production in the neutrophils, macrophages and mast cells (Attur *et al.*, 2000, Petrone *et al.*, 1980). There is a large body of evidences suggesting an interelationships between PLA_2 activation, ROS and histamine release. It has been reported that ROS stimulates arachidonic acid (AA) release in the vascular (Rao *et al.*, 1995) and pulmonary (Chakraborti *et al.*, 1995) smooth muscles. In macrophages, Ca^{2+}-dependent PLA_2 ($cPLA_2$) plays an important role in generating a respiration burst (Dana *et al.*, 1998). In addition, many investigators have reported that a PLA_2 inhibitor and DPI, a NADPH oxidase inhibitor, significantly inhibited both the histamine release from mast cells in response to antigen and A23187 (Varsani and Pearce, 1997, Matsui *et al.*, 2000).

The mast cells that are distributed widely in the connective tissues may be affected by gastric reflux. Therefore, in order to investigate the underlying mechanism of HCl in oesophagitis, the HCl-induced

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inflammatory responses, such as histamine release, AA release, and ROS and peroxynitirite generation were investigated in RBL-2H3 mast cells.

MATERIALS AND METHODS

Materials

Mepacrine, oleyloxyethyl phosphorylcholine (OPC), methyl arachidonyl fluorophosphonate (MAFP), bromoenol lactone (BEL), dithiothreitol (DTT), U73122, neomysin, propranolol, *o*-phthaldialdehyde, diphenyle-neiodonium chloride (DPI) and tiron were procured from Sigma (St. Louis, MO., USA). Dihydrorhodamine (DHR) and 2',7'-dichlorodihydrofluo-rescein diacetate (DCF-DA) were ob-tained from Molecular Probes (Eugene, OR, USA) and [^{3}H]arachidonic acid (AA) was purchased from Amersham Pharmacia (Bucks, UK).

Cell culture

The rat basophilic leukemia (RBL-2H3) cells were grown in Dulbeccos modified Eagle minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) in 5% CO_2. The RBL-2H3 cells were harvested by incubating them in phosphate-balanced saline (PBS) containing 1 mM EDTA and 0.25% trypsin for 5 min at 37°C. Theses cells were used to measure the level of histamine release, AA release, and ROS and peroxynitirite generation.

Histamine assay

The harvested RBL-2H3 cells were washed with Krebs buffer (mM: NaCl 137, KCl 2.7, Na_2HPO_4 0.4, $MgCl_2$ 0.5, HEPES [pH 7.4] 10, $CaCl_2$ 1.8, glucose 5) and then suspended in Krebs buffer at a density of 10^6 cells/ml. The cells were incubated with various antagonists or inhibitors for 10 min, and histamine release was then induced by HCl (2-10 mM) for 30 min at 37°C. The released histamine was assayed using a fluorometric method (Shore *et al.,* 1959). After centrifugation, the histamine concentration in both the supernatant and the pellet were measured using 0.1 ml of 1% *o*-phthaldialdehyde in methanol. After 4 minutes, the reaction was teminated by adding 0.2 ml of 3 N HCl. The fluorescence intensity was measured using excitation and emission wavelengths of 355 and 455 nm, respectively with a fluorospectrophotometer (FL600, Microplate Fluorescence Reader, Bio-Tek).

Measurement of [^{3}H]AA release

The RBL-2H3 cells were suspended in 20 ml of Krebs buffer and incubated with [^{3}H]AA (0.2 μCi/ml) for 2 hr at 37°C. The cells were then washed twice with Krebs buffer containing 0.5 mg/ml bovine serum albumin to trap the liberated [^{3}H]AA. The cells were suspended in Krebs buffer at the density of 10^6 cells/ml and AA release was then induced by HCl for 60 min at 37°C. The radioactivity of the [^{3}H]AA released by HCl (2-10 mM) in the medium was measured with a scintillation counter (Jesus *et al.,* 1994).

Measurement of ROS generation

The intracellular peroxide level was quantified by fluorescence with DCF-DA. The RBL-2H3 cells were suspended in 20 ml of Krebs buffer and incubated with 20 mM DCF-DA for 1 hr at 37°C. The cells were washed twice with Krebs buffer and then suspended in Krebs buffer at the density of 10^6 cells/ml. The cells were incubated with various antagonists or inhibitors for 10 min, and HCl was then used to induced ROS generation for 30 min at 37°C. The fluorescence intensity was measured using the excitation and emission wavelengths of 485 and 535 nm, respectively with fluorospectrophotometer (Boland *et al.,* 2000). The values are expressed as a percentage of the fluorescence in control cultures.

Measurement of peroxynitirte generation

The level of intracellular peroxynitrite was quantified by fluorescence with DHR. The cells were suspended in 20 ml of Krebs buffer and incubated with 10 μM DHR for 30 min at 37°C. After washing with Krebs buffer twice, the cells were suspended in Krebs buffer at a density of 10^6 cells/ml, and adding HCl for 30 min was then done to induce peroxynitirite production. The fluorescence intensity was measured using the excitation and emission wavelengths of 488 and 515 nm, respectively with a fluorospectro-photometer (Palomba *et al.,* 2000). The values are expressed as a percentage of the fluorescence in the control cultures.

Data analysis

The results are represented as means ± S.D. and analyzed statistically by an analysis of variance (ANOVA). The differences between the groups were determined with a Newman-Keuls test. A p value < 0.05 was considered significant.

RESULTS

Effect of HCl on inflammatory responses in RBL-2H3 cells

Exogenous HCl increased the level of histamine release and ROS generation in RBL-2H3 cells in a dose dependent manner (Fig. 1), which suggests a close relationship between histamine release and ROS generation. However, the level of [^{3}H] AA release and peroxynitrite generation decreased with increasing HCl concentration in the basal state (Fig. 2).

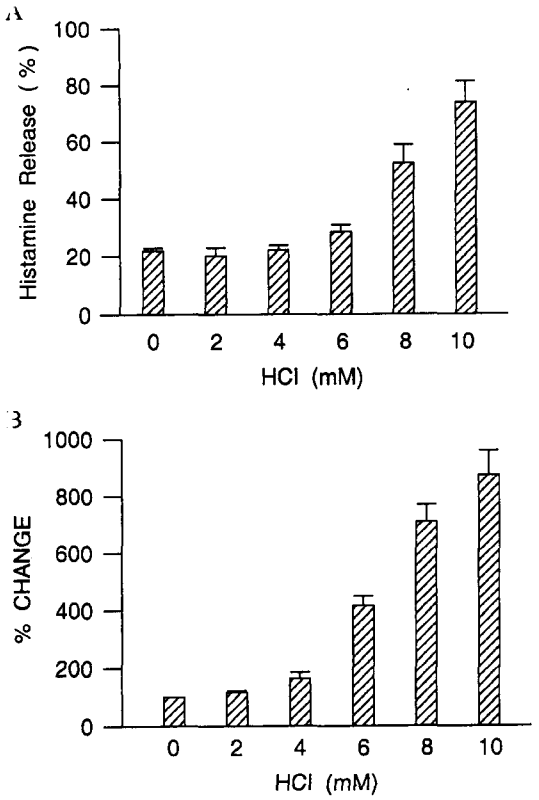

Fig. 1. Dose-responses of histamine release (A) and reactive oxygen species (ROS) generation (B) to HCl in RBL-2H3 cells. The RBL 2H3 cells were stimulated by 2-10 mM HCl for 30 min. The results are reported as a mean ± SD of 6 separate experiments and are expressed as the % release (A: histamine contents in supernatant / histamine contents in supernatant and pellet × 100) and % change of control (B).

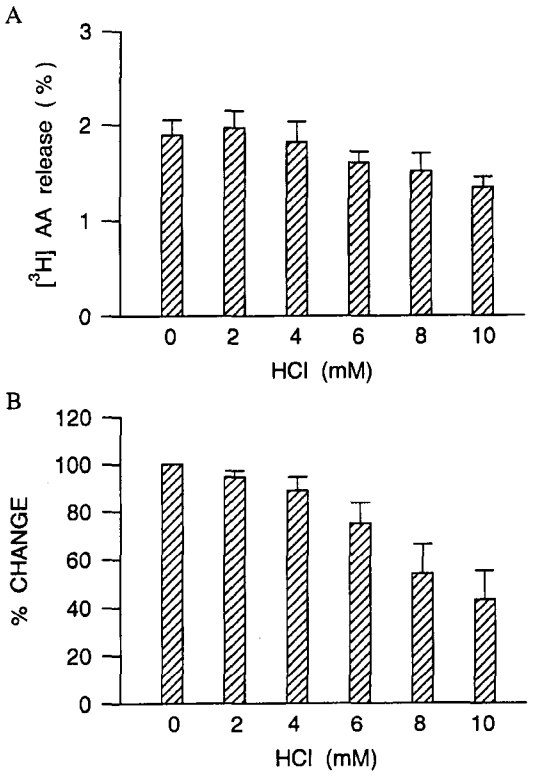

Fig. 2. Dose-response of [^{3}H]arachidonic acid (AA) release (A) and peroxynitrite generation (B) to HCl in RBL-2H3 cells. The RBL 2H3 cells were stimulated by 2-10 mM HCl for 30 min. The results are reported as a mean ± SD of 6 separate experiments and are expressed as the % release (A: radioactivity released in medium / total radioactivity in medium and pellet × 100) and % change of control (B).

Effects of PLA_2 inhibitors

In this study, exogenous HCl significantly decreased the spontaneous release of [^{3}H]AA. To determine if PLA_2 is involved in the inflammatory response stimulated by HCl the effects of the PLA_2 inhibitors on histamine release and ROS generation were observed. Mepacrine (10 μM) and OPC (10 μM), a secretory PLA_2 ($sPLA_2$) inhibitor, did not affect both the histamine release and ROS generation induced by HCl at a concentration of 8 mM and 6 mM, respectively (Fig. 3). Moreover, BEL (10 μM), a calcium-independent cytosolic PLA_2 ($iPLA_2$) inhibitor, did not inhibit the histamine release and ROS generation induced by HCl. However, MAFP (10 mM), a calcium-dependent cytosolic PLA_2 ($cPLA_2$) inhibitor, significantly increased the histamine release induced by HCl, but significantly decreased ROS generation. Antioxidant DTT (5 mM), which has recently been used as a $sPLA_2$ inhibitor, significantly inhibited both the histamine release and ROS generation induced by HCl (Fig. 3).

Effects of the PLC and PLD inhibitors

The effects of PLC and PLD inhibitors on both histamine release and ROS generation were observed in order to determine if PLC and PLD were involved in the inflammatory response stimulated by HCl. U73122 (specific PLC inhibitor, 1 μM) did not have any influence on histamine release and ROS generation. Both propranolol (PLD inhibitor, 200 μM) and neomycin (nonspecific PLC and PLD inhibitor, 1 mM) significantly inhibited both histamine release and ROS generation (Fig. 4).

Effects of DPI and tiron

As shown in Fig. 1, exogenous HCl dose-dependently increased the level of histamine release and ROS generation. To determine whether the HCl-induced ROS generation was related to histamine release, the effects of DPI and tiron on HCl-induced histamine release were measured. DPI (10 μM), an NADPH oxidase inhibitor, significantly inhibited the HCl-induced histamine release and ROS generation (Fig. 5). Tiron (5 mM), an intracellular ROS scavenger, blocked both the histamine release and ROS generation induced by HCl (Fig. 5).

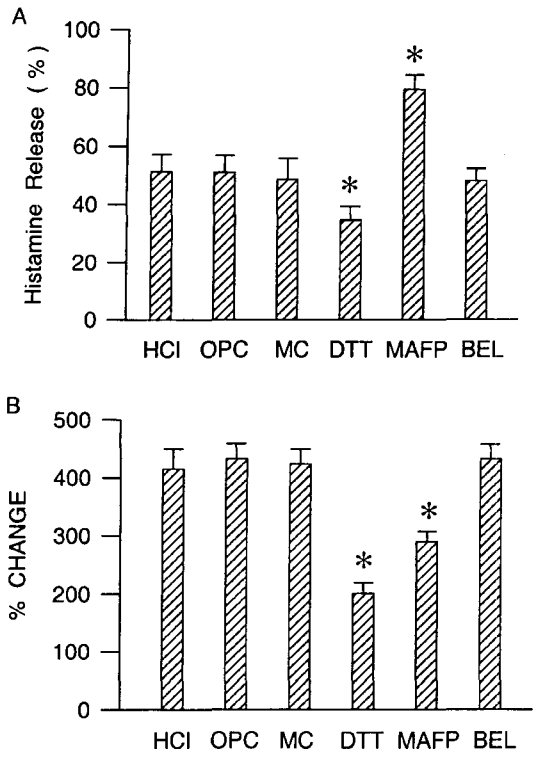

Fig. 3. Effects of phospholipase A_2 inhibitors on HCl-induced histamine release (A) and ROS generation (B). The cells were preincubated with oleyloxyethyl phosphorylcholine (OPC, 10 μM), mepacrine (MC, 10 μM), dithiothreitol (DTT, 5 mM), methyl arachidonyl fluorophosphonate (MAFP, 10 μM) and bromoenol lactone (BEL, 10 μM) for 10 min. Histamine release was induced by 8 mM HCl for 30 min and ROS generation was induced by 6 mM HCl for 60 min. The results are reported as a mean ± SD of 6 separate experiments. *P < 0.05 vs. HCl alone.

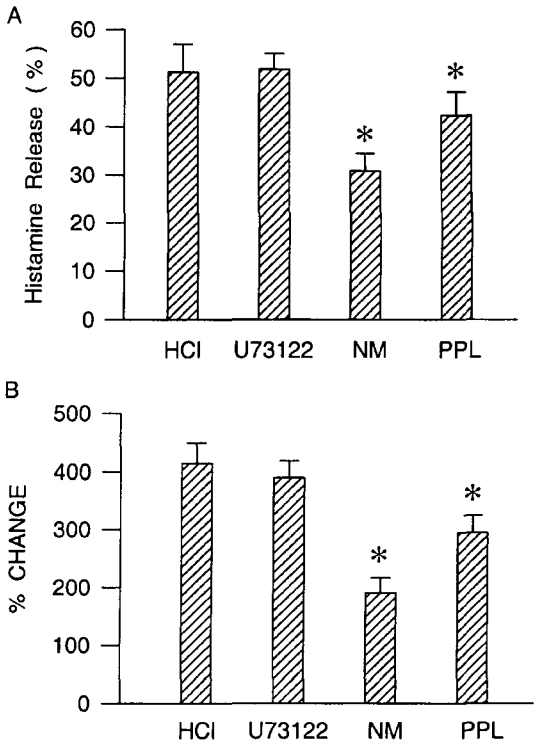

Fig. 4. Effects of phospholipase C and D inhibitors on HCl-induced histamine release (A) and ROS generation (B). The cells were preincubated with U73122 (a specific phospholipase C inhibitor, 1 mM), neomycin (nonspecific phospholipase C and D inhibitor, 1 mM) and propranolol (a phospholipase D inhibitor, 200 μM) for 10 min. Histamine release was induced by 8 mM HCl for 30 min and ROS generation was induced by 6 mM HCl for 60 min. The results are reported as a mean ± SD of 6 separate experiments. *P < 0.05 vs. HCl alone.

DISCUSSION

Under pathological conditions, the underlying mechanism of HCl in oesophagitis caused by gastric reflux remains unclear. Mast cells are widely distributed in the connective tissues. It is possible that regurgitated HCl may have an influence on mast cells in the mucosa or smooth muscles of the oesophagus.

The proliferation of RBL 2H3 cells treated with 2-10 mM HCl for 48 hrs was investigated in a preliminary study to confirm the cytotoxicity of HCl. The proliferation of RBL 2H3 cells was not affected by < 8 mM HCl, whereas the level of proliferation decreased by 40% at a concentration of 10 mM. To exclude the possibility of the cytotoxic effect of HCl, exogenous HCl at concentrations < 8 mM with 30 min treatment were used in this study.

To investigate the underlying mechanism of HCl in oesophagitis, the inflammatory responses to HCl were observed in the RBL-2H3 mast cells. Exogenous HCl dose-dependently increased the level of histamine release and ROS generation in the RBL-2H3 cells, whereas it decreased the spontaneous release of [^{3}H] AA and the spontaneous production of peroxynitrite generated by the interaction between superoxide and nitric oxide. HCl did not cause nitric oxide production in the RBL 2H3 cells, and both the level of histamine release and ROS generation induced by HCl were unaffected by nitric oxide synthase inhibitors (data not shown). This suggests that the inflammatory responses to HCl in the RBL-2H3 cells may be related to the level of histamine release and ROS generation rather than the level of PLA_2 activation and peroxynitirte generation.

There is a large body of evidence suggesting an interrelationship between ROS and PLA_2 activation. It has been reported that ROS stimulate AA release in the vascular (Rao *et al.*, 1995) and pulmonary (Chakraborti *et al.*, 1995) smooth muscle. In macrophages, $cPLA_2$ may play an important role in generating a respiration burst (Dana *et al.*, 1998). In this study, the effects of PLA_2 inhibitors on the level of histamine release and ROS generation were observed on order to determine the role PLA_2 plays in the inflammatory responses stimulated by

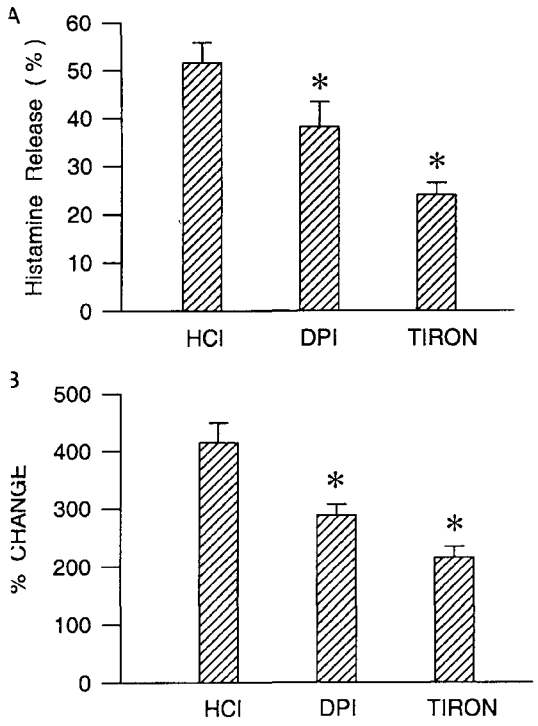

Fig. 5. Effects of diphenyleneiodonium chloride (DPI) and tiron on HCl-induced histamine release (A) and ROS generation (B). The cells were preincubated with DPI (an NADPH oxidase inhibitor, 10 μM) and tiron (an intracellular ROS scavenger, 5 mM) for 10 min. Histamine release was induced by 8 mM HCl for 30 min and ROS generation was induced by 6 mM HCl for 60 min. The results are reported as a mean ± SD of 6 separate experiments. *P < 0.05 vs. HCl alone.

HCl. Mepacrine (10 μM), OPC (10 μM) and BEL (10 μM) did not affect both the level of histamine release and ROS generation induced by HCl. However, MAFP (10 μM) significantly increased the level of histamine release induced by HCl but significantly decreased the level of HCl-induced ROS generation, which contrasts with a previous reports on the interrelationship between ROS and PLA_2 activation. It has been reported that the inhibition of MAFP is due to the blockage of central serine for catalysis (Huang *et al.*, 1996). In whole cell systems, MAFP will likely interact with many different proteins. The effect of MAFP on histamine release and ROS generation in RBL-2H3 cells may be due to its action as an inhibitor of other enzymes having a serine catalytic site rather than a PLA_2 inhibitor.

The antioxidant, DTT (5 mM), has been used as a $sPLA_2$ inhibitor since $sPLA_2$ contains several disulfide bonds (Raguenes-Nicol *et al.*, 1999). In this study, DTT significantly inhibited both the histamine release and ROS generation induced by HCl. However, the other $sPLA_2$ inhibitors, mepacrine and OPC, had no effect on both the level of histamine release and ROS generation induced by HCl. The effect of DTT on the level of histamine release and ROS generation in RBL-2H3 cells might be due to its action as an antioxidant rather than a PLA_2 inhibitor. Moreover, HCl dose-dependently decreased the level of spontaneous release of [^{3}H]AA in RBL-2H3 cells. Overall, it is unlikely that PLA_2 is involved in the HCl-induced histamine release and ROS generation in RBL-2H3 cells.

The effects of the PLC and PLD inhibitors on histamine release and ROS generation were examined to determine whether PLC and PLD are involved in inflammatory responses stimulated by HCl. U73122 (1 μM), a specific PLC inhibitor did not have any influence on the level of histamine release and ROS generation. Propranolol (200 μM), a PLD inhibitor (Sohn *et al.*, 1997), and neomycin (1 mM), a nonspecific PLC and PLD inhibitor (Cha *et al.*, 1998), significantly inhibited both histamine release and ROS generation. These results suggest that PLD, but not PLC, is involved in the HCl-induced histamine release and ROS generation in RBL-2H3 cells.

In order to determine whether HCl-induced ROS generation is related to histamine release, the effects of DPI and tiron on the level of HCl-induced histamine release were measured. DPI (10 μM), an NADPH oxidase inhibitor, significantly inhibited the HCl-induced histamine release and ROS generation. This result is concurs with a previous report suggesting that DPI suppressed the histamine release and ROS generation in response to an antigen and A23187 (Matsui *et al.*, 2000). Furthermore, tiron (5 mM), an intracellular ROS scavenger (Bass and Berk, 1995), significantly blocked both the histamine release and ROS generation induced by HCl, suggesting that ROS generation by HCl may be involved in histamine release. In contrast, HCl-induced ROS generation was unaffected by histamine antagonists such as pyrilamine and cimetidine (data not shown), suggesting that histamine may have little influence on the HCl-induced ROS generation.

In conclusion, the inflammatory responses to HCl are related to the level of histamine release and ROS generation, and that ROS generation by HCl might be involved in histamine release via the PLD pathway in RBL-2H3 cells.

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