Curcuma longa Extract Protects against Gastric Ulcers by Blocking H₂ Histamine Receptors

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Curcuma longa (C. longa), a common Indian dietary pigment and spice, has been used therapeutically in traditional folk medicine for a wide range of ailments, including wound healing, urinary tract infection, and liver ailments.1,2) Although evidence suggests that a C. longa extract also has considerable gastroprotective and antiulcerogenic effects,3,4) its mechanism of action remains unclear.

The genes encoding four histamine receptor subtypes (H₁, H₂, H₃ and H₄) have been cloned, and the receptors have been pharmacologically characterized, along with their signaling pathways.5) The H₂ histamine receptor (H₂R) is a seven transmembrane G protein-coupled receptor (GPCR) coupled to cAMP/protein kinase A/proton pump pathway.8) Hence, H₂R antagonists, such as histamine receptor agonists, induced intracellular cAMP production in U937 and HL-60 promyelocytes. Pretreatment with C. longa extract significantly blocked dimaprit-induced cAMP production in a concentration dependent manner, but had no effect on the elevation of cAMP levels triggered by isoproterenol-induced β₂-adrenoceptor activation in U937 cells. To identify the active component(s) of C. longa extract, we sequentially fractionated it by extraction with ethyl acetate, n-butanol and water. We found that the ethyl acetate extract showed the most potent H₂R antagonistic effect against dimaprit-induced cAMP production. However, curcumin, a major component of C. longa extract, showed no H₂R blocking effect. C. longa ethanol extract and ethylacetate extract also blocked the binding of [3H]-tiotidine to membrane receptors on HL-60 cells. These findings suggest that the extract from C. longa specifically inhibits gastric acid secretion by blocking H₂ histamine receptors in a competitive manner.

Key words Curcuma longa; H₂ histamine receptor; gastric ulcer; gastric juice secretion

Curcuma longa has been commonly used as a traditional remedy for a variety of symptoms such as inflammation, gastritis and gastric ulcer. When C. longa extract was administered per os to pylori-ligated rat stomachs, it reduced gastric acid secretion and protected against the formation of gastric mucosal lesions. We therefore tested whether C. longa extract inhibits gastric ulcers by blocking the H₂ histamine receptor. Dimaprit, a H₂ histamine receptor agonist, induced intracellular cAMP production in U937 and HL-60 promyelocytes. Pretreatment with C. longa extract significantly blocked dimaprit-induced cAMP production in a concentration dependent manner, but had no effect on the elevation of cAMP levels triggered by isoproterenol-induced β₂-adrenoceptor activation in U937 cells. To identify the active component(s) of C. longa extract, we sequentially fractionated it by extraction with ethyl acetate, n-butanol and water. We found that the ethyl acetate extract showed the most potent H₂R antagonistic effect against dimaprit-induced cAMP production. However, curcumin, a major component of C. longa extract, showed no H₂R blocking effect. C. longa ethanol extract and ethylacetate extract also blocked the binding of [3H]-tiotidine to membrane receptors on HL-60 cells. These findings suggest that the extract from C. longa specifically inhibits gastric acid secretion by blocking H₂ histamine receptors in a competitive manner.

Materials and Methods

Materials Dimaprit was purchased from Tocris (Bristol, U.K.). Histamine, ranitidine, Ro20-1724 were obtained from Sigma (St. Louis, MO, U.S.A.). [3H]-cAMP were obtained from NEN Life Science Products (Boston, MA, U.S.A.). Tiotidine was obtained from Tocris Cookson Inc. (Ballwin, MO, U.S.A.). [3H]-Tiotidine were obtained from Perkin Elmer Life Sci. (Wellesley, MA, U.S.A.). RPMI-1640, penicillin–streptomycin and fetal calf serum were purchased from JBI (Daegu, Korea). cAMP-binding protein (CBP) were obtained from NEURONEX (Pohang, Korea). Pure curcumin was kindly provided by Dr. M. J. Kim in POSTECH.

Preparation of the C. longa Extract The ground dried C. longa (100 g) was fluxed with 80% ethanol (EtOH) (400 ml) and shaken at room temperature for 24 h. The extraction was performed twice. After extraction, the mixture was concentrated with rotary vacuum evaporator (EYELA, Japan). The EtOH extract was dissolved in H₂O (100 ml) and fractionated with organic solvents, ethyl acetate (EA) and n-butanol (n-Buta). Each dried material resuspended in DMSO for cellular experiment and in saline for in vivo test.

Animals Male Sprague–Dawley (SD) rats were kept, under controlled temperature (23 °C) and lighting (12 h light: dark) conditions, with free access to water and food.

Cell Culture The U937 and HL-60 cell line (American Type Culture Collection, Rockville, MD, U.S.A.) was cultured in suspension at 37 °C in RPMI 1640 medium supplemented with 10% (v/v) of heat-inactivated fetal calf serum and 1% (v/v) penicillin–streptomycin in a humidified atmosphere of 95% air and 5% CO₂.

Measurement of [3H]cAMP Intracellular cAMP generation was determined by using [3H]cAMP competition assay kit (Neuronex, Korea). U937 and HL-60 cells were stimulated with dimaprit or isoproterenol for 20 min in the presence of the phosphodiesterase inhibitor Ro20-1724 (5 μM),...
and the reaction was quickly terminated by three repeated cycles of freezing and thawing. The samples were then centrifuged at 2500×g for 5 min at 4 °C. The cAMP assay is based on the competition between [3H]-labeled cAMP and unlabeled cAMP present in the sample for binding to a crude cAMP binding protein. Free [3H]cAMP was adsorbed onto charcoal and removed by centrifugation. Bound [3H]cAMP in the supernatant was then determined by liquid scintillation counting. Each sample was incubated with 50 μl [3H]-labeled cAMP (5 μCi) and 100 μl binding protein for 2 h at 4 °C. Separation of protein-bound cAMP from unbound cAMP was achieved by adsorption of free cAMP onto charcoal (100 μl) followed by centrifugation at 12000×g at 4 °C. The 200 μl supernatant was then placed into an Eppendorf tube containing 1.2 ml scintillation cocktail to measure radioactivity. The cAMP concentration in the sample was determined based on a standard curve and expressed as picomoles per number of cells.

**[3H]-Tiotidine Binding** The binding of [3H]-tiotidine to intact HL-60 cells was quantified by the method described in previous report1 with some modification. Triplicate assays were performed in polyethylene tubes in 50 mM Tris–HCl, pH 7.4. [3H]-Tiotidine were incubated with 106 cells/tube in the absence or presence of ranitidine or C. longa extracts in a total volume of 200 μl. After 40 min at 4 °C, incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris–HCl, pH 7.4; rapid filtration onto Whatman GF/B glass-fibers filters was performed under reduced pressure, followed by three washes with 3 ml of ice-cold buffer. The amount of bound radioactivity was measured in a liquid scintillation cocktail. Specific binding was defined as the difference in the amount of radioactivity bound in the absence and presence of 1 mM unlabeled tiotidine.

**Measurement of Gastric Acid Secretion and Gastroprotective Activity in Vivo** The pylorus-ligated rat model first described by Shay et al. was used with some modification.10) SD rats weighing 200—250 g were used. Rats were deprived of food, but not water, for 18—24 h prior to each experiment. The test substances (vehicle, ranitidine, C. longa extract) dissolved in saline were administered orally (per os). After 1 h, rats were anesthetized by light ether, a small abdominal incision was made, the pylorus was ligated. The animals were sacrificed 8 h after ligation of the pylorus, the stomach was clamped at the oesophageal and duodenal junctions and then rapidly removed. The gastric juice was collected and the excised stomach was then filled with 15 ml of 4% formalin. After 24 h, the fixed stomach was opened along the greater curvature, gently rinsed in saline, and then pinned open to expose the gastric mucosa. The haemorrhagic and ulcerative lesions were counted and measured with a light microscope by an observer who was blinded to the treatment. The ulcer index was then calculated as the sum of the length of all lesions as previously described.11) The ulcer index was determined in six animals in each animal group. The gastric content was placed in tubes for later centrifugation at 1500×g for 30 min in a refrigerated centrifuge. Total acidity was determined by simple titration with 0.01 N NaOH using phenol-red as acid-base indicator.

**Histological Examination** The histological study of the stomach was performed following evaluation of the ulcer index. Samples of the corpus were excised and transferred to fresh formalin and later processed by routine techniques prior to embedding in paraffin. Sections (5 μm thick) were mounted on glass slides and stained with haematoxylin and eosin. Coded slides were examined by an experienced pathologist blinded to the treatment.

**Statistical Analysis** All quantitative data are expressed as mean±S.E.M. Comparisons between two groups were performed using Student’s unpaired t-test, and comparison among groups more than two was carried out using one-way analysis of variance (ANOVA). Differences were considered to be significant when the degree of confidence in the significance was 95% of better (p<0.05).

**RESULTS**

**Effect of C. longa on Gastric Acid Secretion and the Formation of Gastric Mucosal Lesions** Using the Shay ulcer model, which causes gastric hypersecretion,12) we investigated the effects of C. longa extract on gastric acid secretion and mucosal injury, and compared them with the effects of ranitidine. Ranitidine has been shown to have a marked anti-ulcer effect and has been used in the treatment and prevention of a variety of gastrointestinal disorders associated with gastric acid secretion.13) Histopathological examination of gastric lesions showed discontinuation of the surface epithelium in the vehicle treated group. In contrast, C. longa extract protected the gastric mucosal layer as effectively as ranitidine (Fig. 1A). Using the pylori-ligated rat model, we found that per os administration of C. longa extract significantly inhibited gastric acid (Fig. 1D), gastric juice secretion (Fig. 1B), and ulcer formation (Fig. 1C), comparable to the effects of ranitidine. The ethanol extract of C. longa reduced the volume of gastric acid secreted after pylorus ligation, indicating that this may be the mechanism by which C. longa extract protects the gastric mucosa.

**C. longa Extract Inhibits H2 Histamine Receptor (H2R)-Mediated Intracellular cAMP Production in U937 and HL-60 Promyelocytes** H2R is the primary target of anti-ulcer drugs,14) and H2R antagonists have been shown to inhibit gastric acid secretion in many animal model systems.15,16) H2R is also expressed not only on gastric parietal cell but also on immune cells,17) including the U937 and HL-60 promyelocytic cell lines, which have been widely used as model systems for H2 histamine receptor activity.13,15) We therefore tested the effect of dimaprit, a selective H2R agonist, on the generation of cAMP in U937 and HL-60 cells. Application of various concentrations of dimaprit triggered cAMP production in a concentration-dependent manner (Fig. 2A), and with an EC50 of 5.2±0.9 μM. The effect of 10 μM dimaprit was inhibited by 10 μM of the specific H2 receptor antagonist, ranitidine (10 μM), and by C. longa extract in a concentration-dependent manner (Fig. 2B, closed circle). In addition, C. longa extract also suppressed histamine-induced cAMP production in a concentration-dependent manner (Fig. 2B). Since C. longa extract may block Gsα-protein activity rather than H2R, we tested the involvement of Gsα-protein in the activity of C. longa extract by treating the U937 cells with isoproterenol (500 nM), an agonist of the Gsα-protein coupled β2-adrenoceptor, following treatment with C. longa extract.18,20) C. longa did not affect isoproterenol-induced cAMP production (Fig. 2B, closed box), indicating that the
Fig. 1. Effect of \textit{C. longa} Extract on Gastric Ulcer

(A) Microscopic appearance of pylori-ligation induced gastric lesions. The lesion formed in the gastric mucosa of vehicle treated (a), 5 mg/kg ranitidine treated (b), 100 mg/kg \textit{C. longa} extract treated (c) rats. No mark infiltration of inflammatory cells was observed. Hematoxylin and eosin staining (original magnification: objective×100).

(B) Effects of 80% ethanol extract of \textit{C. longa} (stripe bar) and of ranitidine (gray bar) on the total gastric juice secretion after pylorus ligation in SD rats. (C) Protection effect of \textit{C. longa} extract on the formation of gastric mucosal lesion. Each bar represents lesion score of Shay-model induced gastric damage. Test substances (vehicle (closed bar), ranitidine (gray bar), \textit{C. longa} extract (stripe bar)) were injected through \textit{per os} and then rats were killed 8 h after the surgery. (D) Effects of 80% ethanol extract of \textit{C. longa} (stripe bar) and of ranitidine (gray bar) on the gastric acid secretion after pylorus ligation in SD rats. Each column represents the mean±S.E.M. (n=4—9 per group) and the difference between groups was determined by ANOVA. The marked column showed a significant difference in a comparison with the control: *p<0.05, **p<0.01 and ***p<0.001.

Inhibitory effect of \textit{C. longa} extract on the dimaprit-induced cAMP generation was through direct inhibition of H$_2$R. We could not detect significant cellular damage (by MTT assay) in cells treated with vehicle or pretreated with ranitidine or \textit{C. longa} extract (data not shown), suggesting that the \textit{C. longa} inhibition of dimaprit-induced cAMP responses was not due to cell death.

Effects of the Ethyl Acetate Fraction of \textit{C. longa} and Curcumin on H$_2$R Signaling We subjected the 80% ethanol extract of \textit{C. longa} to successive solvent extractions\textsuperscript{21} and obtained the ethyl acetate (EA), \textit{n}-butanol (\textit{n}-Buta), and water extracts. To determine the H$_2$R blocking activity of these extracts, U937 cells were pretreated with each extract, and the cells were subsequently treated with dimaprit. We found that both the EA and \textit{n}-Buta extracts significantly inhibited dimaprit-induced cAMP production, but the effect of EA extract was more pronounced (Fig. 3A). Since the active constituent(s) may have accumulated in the EA extract, we compared the H$_2$R inhibitory activity of the EA and ethanol extracts of \textit{C. longa}. We found that both extracts significantly inhibited H$_2$R in a concentration-dependent manner (Fig. 3B), with IC$_{50}$ of 6.0±0.5 μg/ml and 30.00±0.78 μg/ml, respectively. These findings indicate that substance in \textit{C. longa} that acts H$_2$R is probably contained in the EA extract. Since curcumin is the major component of \textit{C. longa},\textsuperscript{23} we investigated the effect of curcumin on dimaprit-induced H$_2$R signaling. Curcumin, however, showed no significant effect on H$_2$R activity (Fig. 3C). Curcumin alone has no effect on cAMP formation (Fig. 3C).

$[^3]$H-Tiotidine Binding Assay with \textit{C. longa} Extract To determine whether the effects of \textit{C. longa} extract were due to its ability to block histamine binding to H$_2$R, we tested the effects of \textit{C. longa} extract on $[^3]$H-tiotidine binding to H$_2$R in undifferentiated HL-60 cells.\textsuperscript{9} We found that both ranitidine and \textit{C. longa} ethanol and EA extract significantly blocked $[^3]$H-tiotidine binding to undifferentiated HL-60 cells (Fig. 4), and 10-fold low concentration of EA extract showed more potency rather than ethanol extract.

DISCUSSION

\textit{C. longa} has been widely used as anti-ulcer remedy in tra-
its EA fraction (open circle) on H2R-mediated cAMP production in U937 cells, as reported, but its action mechanism is still unclear. In the present study, we have provided a possibility that the C. longa ethanol extract prevent the development of gastric ulcers by blocking H2R.

The C. longa extract induced inhibition of cAMP signaling is due to blocking of histamine binding to H2R, rather than acceleration of degradation, since the inhibition was observed in the presence of the phosphodiesterase inhibitor Ro20-1724. In addition, the inability of C. longa extract to inhibit β2-adrenoceptor mediated cAMP production (Fig. 2B) indicates that the active component(s) of C. longa extract are highly selective, inhibiting only the binding of histamine (or dimaprit) to H2R (Fig. 4). In addition, EA fraction of C. longa extract showed prominent H2R antagonistic effect rather than other fractions, suggesting that specific activity was increased (about 4-fold) by successive extraction with EA (Fig. 3B), indicating that further fractionations and purifications of EA fraction will give a possibility to find active single compound more potent that ranitidine.

In previous report showed that the C. longa extract has curcumin, demethoxycurcumin, bisdemethoxycurcumin, and ar-turmerone. Among these, the curcumin is the major component of C. longa and purified curcumin was more active than either demethoxy- or bisdemethoxycurcumin. In our study, however, pure curcumin showed no significant effect on dimaprit-induced H2R signaling in our system (Fig. 3C). These results demonstrate that unidentified minor constituent in C. longa extract might be associated with H2R blocking. There are two previous reports about the constituents in the C. longa EA extract. In these studies, curcumin I (1), curcumin II (2), and curcumin III (3) were isolated as active principles in the EA extract. Therefore, further experiment is required whether these compounds are related with H2R regulation and gastric acid secretion. This will help us to determine the critical compound in C. longa extract related with H2R blocking and anti-ulcer effect.

Antagonism of H2R has been the cornerstone of an immense market for pharmacological treatments of acid-peptic disorders of the gastrointestinal tract. The selective inhibition by C. longa of H2R and gastric acid secretion indicate that C. longa extract or its active component(s) may be promising therapeutic candidates for the treatment of gastric ulcers and other H2R-related diseases.

H2R antagonists have widely been used for the treatment of gastric-ulcer. Despite H2R antagonists are relatively safe drugs, cimetidine, however, is known to interfere with other drugs through hepatic metabolism, the concentration of co-committant drugs such as warfarin, benzodiazepine tend to be elevated. Nevertheless further experiments are required, we expect that C. longa extract or its active component(s) may replace and reduce the cimetidine related side effects.

In conclusion, our data suggest that C. longa extract shows selective and competitive H2R antagonistic effects, indicating a mechanism for the reduction in gastric ulceration observed for C. longa extract. By blocking H2R, this extract would provide another remedy for the treatment of gastric ulcers.

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