Vacuolating toxin from *Helicobacter pylori* activates cellular signaling and pepsinogen secretion in human gastric adenocarcinoma cells

Err-Cheng Chan*, Kuei-Tian Chen, Yuh-Ling Lin

Medical Biotechnology Laboratory, School of Medical Technology, Chang Gung College of Medicine and Technology, 259 Wen-Hua 1st Road, Taoyuan, Taiwan, ROC

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Abstract We investigated cellular signaling and pepsinogen secretion in the human gastric adenocarcinoma cell line AGS which was pretreated with the purified vacuolating cytotoxin from *Helicobacter pylori*. Results indicated that vacuolating toxin increased the levels of inositol phosphates, cytosolic free calcium concentration, adenosine 3′,5′-cyclic monophosphate, and phosphorylation of 31 kDa and 22 kDa proteins in the host cells. Moreover, pepsinogen secretion from AGS cells was stimulated with increasing concentrations of cytotoxin. We conclude that besides the *H. pylori* cytotoxin-induced cellular vacuoles, cytotoxin-stimulated signaling mediators and pepsinogen release are important factors involved in the etiology of chronic active gastritis and peptic ulcer disease.

Key words: Vacuolating toxin; *Helicobacter pylori*; Cellular signaling; Pepsinogen; Peptic ulcer; Phosphorylation

1. Introduction

The vacuolating toxin (cytotoxin; CT) of *Helicobacter pylori* has been proved to induce the formation of intracellular vacuoles in eukaryotic cells in vitro [1–3]. Since *H. pylori* strains isolated from patients with ulcer disease commonly produce CT, it has been proposed that CT may be a virulence factor in the pathogenesis of *H. pylori*-related gastric diseases [4–9]. However, there is still no direct evidence regarding the virulence role of *H. pylori* CT on in vivo pathogenesis. In animal model experiments, purified CT and *H. pylori* whole-cell extracts cause a similar tissue damage and ulceration when administered orally to mice, suggesting that CT may be responsible for *H. pylori*-related gastric diseases [10,11]. Contrary data reported by Eaton et al. showed that there were no differences in the colonization rate, inflammation, or epithelial vacuolization in gnotobiotic piglets given CT-positive or -negative strains, suggesting that CT is not an important pathogenic factor in this animal model [12,13]. Because the aforementioned conflicting data regarding the vacuolating activity of *H. pylori* CT on pathogenesis remain, further studies on CT are required to identify other phenotypic characteristics than vacuolating activity, which may be responsible for the specific *H. pylori*-related diseases.

Thus, the objective of this study is to characterize *H. pylori* CT at the effect on the transmembrane signaling pathway in cultured cells, and we report for the first time that purified *H. pylori* CT is able to activate major second messengers and increase pepsinogen secretion from human gastric adenocarcinoma cells (AGS).

It has been reported that pepsinogen levels are significantly increased in individuals with peptic ulcer diseases [14]. Therefore, the characteristics of CT to increase pepsinogen secretion from the host cells may be associated with the pathogenesis of *H. pylori*-related diseases.

2. Materials and methods

2.1. Materials

Brucella broth, chocolate agar and hemoglobin were purchased form Difco Lab. (Detroit, MI). Eagle medium, RPMI 1640 medium, fetal calf serum (FCS), and 50-cm^2 tissue culture flasks were obtained from Gibco Lab. (Grand Island, NY). Penicillin, streptomycin, trypsin, trypan blue, bovine serum albumin, pepsinogen, ammonium formate and isobutylmethylxanthine (IBMX) were purchased from Sigma (St. Louis, MO). HEPES, percholic acid, formic acid and ethylene glyco-bis(β-aminoethylether)-*N,N*-tetraacetic acid (EGTA) were purchased from Merck (Darmstadt, Germany). Fura-2/AM was obtained from Boehringer Mannheim (Mannheim, Germany). [^{32}P]Phosphoric acid and [^{3}H]inositol were obtained from Amersham Inc. (Buckinghamshire, UK).

2.2. Purification of H. pylori CT

The cytotoxin-positive *H. pylori* strain K8 was a gift of Chang Gung Memorial Hospital at Taiwan. Bacterial cells were cultured on chocolate agar plates at 37°C in a microaerophilic atmosphere of 10% CO_2 in air and 95% humidity for 3 days. The grown colonies were used as inocula and were suspended in a 500-ml flask containing 150 ml of Brucella broth supplemented with 10% FCS and 0.2% β-cyclodextrin. Culture was incubated at 37°C with shaking (100 rpm) in a microaerobic environment by placing the flask in an anaerobic jar containing CampyPaks (Becton Dickinson) to generate the proper conditions. After 72-h incubation, the culture broth was harvested as the raw material for CT purification.

Culture broth was precipitated with saturated ammonium sulfate and resuspended in 20 mM Tris-HCl buffer (pH 7.7). The crude protein solution was applied to an isoelectric focusing cell (Mini-Rotofor Cell, Bio-Rad) and operated according to the instructions. Bio-Lyte (Bio-Rad) was prepared at a final concentration of 1% (v/v), and with a pH range of 5–8. The system was run at constant power 14 W at 4°C. Anion exchange chromatography was performed on an Econo-Pac Q cartridge (Bio-Rad), and proteins were eluted with 20 mM Tris-HCl containing a linear gradient of 0.3–0.5 M NaCl over 20 min. High pressure liquid chromatography (HPLC) with a preparative gel filtration column Bio-Sil SEC 250-5 (Bio-Rad) was performed with a mobile buffer containing 60 mM Tris-HCl and 0.1 M NaCl at a flow rate of 1 ml/min. The eluents were monitored for UV absorbance at 280 nm, and each collected fraction was determined for the vacuolating activity of CT.

2.3. Assay of CT activity

HeLa cells were cultured in the basal Eagle medium containing 5% FCS in a 96-well plate to produce a confluent monolayer after 24 h of incubation as described by Cover et al. [6]. The purified CT was serially diluted and 100 μl aliquots were incubated with adherent cells in 96-well plates for 18 h at 37°C. HeLa cell vacuolation was quantified spectrophotometrically at wavelength 540 nm using a neutral red uptake assay [15]. The unit of CT activity was defined as the amount of CT that increased the optical density by 0.01 in the neutral red assay, and the specific activity was defined as the total CT activity per mg protein.

*Corresponding author. Fax: (886) (3) 3288741.

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2.4. Determination of cytosolic free calcium concentration $[Ca^{2+}]_i$

Fluorescence assay with fura-2/AM as a probe was employed for measuring $[Ca^{2+}]_i$ in cultured cells [16]. The confluent AGS cells grown in RPMI 1640 medium were harvested from tissue culture flask by trypsinization (0.25% trypsin). Cells were resuspended in RPMI 1640 medium containing 4 μM fura-2/AM, and incubated at 37°C in 5% CO_2 incubator for 5 min. In the studies designed to determine the role of $[Ca^{2+}]_i$ in CT-induced AGS cells, a concentration of 1.25×10^6 cells/ml was incubated with a series of dilutions of CT in Krebs-Ringer buffer with 1.3 mM $CaCl_2$ or without extracellular Ca^{2+} (by adding 1 mM EGTA). Fluorescence was measured by a spectrofluorimeter (Jasco CAF-110). The average of $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. [17].

2.5. Determination of intracellular cAMP levels

AGS cells (2×10^6 cells/ml) were preincubated with 10 μM IBMX in RPMI 1640 with 0.2% bovine serum albumin at 37°C for 2 min. The indicated amount of CT was then added, and the reaction mixture was incubated for another 30 min. The reaction was stopped in boiling water for 5 min, and the concentration of cAMP in the solution was determined using an enzyme-linked immunoassay kit according to the manufacturer's instruction (Cayman Lab., Ann Arbor, MI).

2.6. Measurement of inositol phosphates

Total inositol phosphates were measured according to the method described by Jennings et al. [18]. The cultured AGS cells were incubated with [^{3}H]*myo*-inositol, washed with a Dulbecco-phosphate buffer, and resuspended in a Krebs-Ringer solution to a concentration of 2.3×10^6 cells/ml. Various amounts of CT were added to the cell solutions and incubated at 37°C for 5 min. The reaction was stopped by adding 3 volumes of $CHCl_3$:CH_3OH:HCl (100:200:2). Inositol monophosphate (IP_1), inositol biphosphate (IP_2), and inositol triphosphate (IP_3) were collected off a Dowex anion exchange column (AG 1-X8 resin, 100–200 mesh formate form) with 0.2, 0.5, and 1.0 M ammonium formate in 0.1 M formic acid, respectively. The amounts of IP_1, IP_2, and IP_3 were represented by the radioactivity (dpm), and their sum was the value of total inositol phosphate (IPs).

2.7. Pepsinogen measurement

AGS cells were suspended in cultured medium with a concentration of 2.3×10^6 cells/ml) and preincubated at 37°C for 30 min. Various amounts of CT were then added and incubated for another 30 min. After AGS cells were centrifuged, the supernatant was used for pepsinogen determination. Pepsinogen was measured by the method described previously [19,20] using an acid-denatured hemoglobin as the substrate. Porcine pepsinogen was used as a standard to quantify the samples' pepsinogen.

2.8. Phosphorylation of AGS proteins

The harvested AGS cells were preincubated with 1 mCi [^{32}P]phosphoric acid in 10 ml RPMI 1640 containing 10% FCS at 37°C for 45 min. Cells were then washed and resuspended in 2 ml Krebs-Ringer solution (pH 7.2). Various amounts of CT were added into the cell suspensions and incubated for 5 min. The reaction was stopped by boiling and aliquots were subjected to SDS-polyacrylamide (11%) gel electrophoresis in Laemmli buffer system [21]. Autoradiograms were obtained from the dried gel after exposure to Kodak BioMax MR film at −70°C. The phosphorylated proteins were quantified by scanning densitometry.

2.9. Statistical methods

Results are expressed as means ± S.E.; *n* indicates the number of experiments. The differences between treatment groups were determined using a random analysis of variance. The Student-Newman-Keuls test was used to compare the treatment means where applicable.

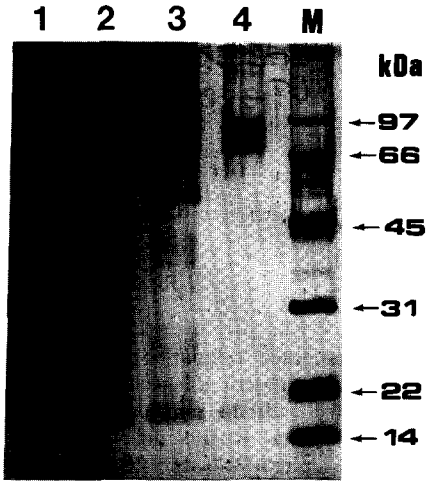

Fig. 1. SDS-PAGE (10% polyacrylamide) of *H. pylori* cytotoxin. Lane 1, proteins precipitated from a broth culture supernatant of CT-positive *H. pylori* with a 50% saturated solution of ammonium sulfate; lane 2, CT purified by IEF chromatography; lane 3, CT purified partially by cation exchanger; lane 4, a major band indicating purified $M_r = 87\,000$ toxin after gel filtration HPLC. Chromatography conditions were as described in the text. Lane M, a low molecular weight marker protein. Each lane contains 0.05 units of CT in the purified fraction.

3. Results

H. pylori CT in culture broth was precipitated with a saturated solution of ammonium sulfate. The precipitated proteins were resuspended and subjected to preparative isoelectric focusing chromatography, and CT activity was detected in the pH range 4.6–6.3 fractions that eluted from the column. These fractions were then chromatographed on an Econo-Pac Q anion exchange column, and CT activity was detected in a single peak. In the final purification step, the sample was subjected to HPLC preparative gel filtration, and CT activity was detected in the first peak. As summarized in Table 1, analysis of the specific activities at each stage in the purification process indicated that *H. pylori* CT was purified up to 5800-fold from the crude culture broth. The purified CT was then used as a stimulus in all the following experiments. The purity of CT was shown by 10% SDS-PAGE (Fig. 1). After gel filtration chromatography, all fractions with significant CT activity contained a band approximately at $M_r = 87\,000$. By heating the purified CT in a boiling water bath for 5 min, its

Table 1
Purification of vacuolating toxin from *Helicobacter pylori*

Purification step	Specific activity[a] (unit/μg)	Purification (-fold)
Broth culture supernatant	0.03	1
50% Ammonium sulfate precipitate	0.07	2
Isoelectric focusing	0.12	4
Econo-Pac Q chromatography	3.25	108
Bio-Sil SEC 250-5 high performance liquid chromatography	174.92	5830

[a]The unit of specific activity and chromatography conditions were as specified in the text.

Table 2
Effect of CT on the production of [^{3}H]inositol phosphates in AGS cells[a]

CT (μg/ml)[b] challenge	(% of control)		
	[^{3}H]IP_1	[^{3}H]IP_2	[^{3}H]IP_3
Basal control	100.0 ± 2.6	100.0 ± 3.6	100.0 ± 3.8
0.18	101.2 ± 1.3	166.2 ± 9.7	97.5 ± 5.4
0.36	100.6 ± 2.3	178.5 ± 14.2	110.6 ± 6.9
0.72	104.1 ± 7.3	205.3 ± 22.1	130.1 ± 9.8
1.0	90.3 ± 2.1	200.4 ± 18.5	258.3 ± 26.1
1.8	97.3 ± 6.9	186.5 ± 20.4	270.9 ± 30.1

[a]Values given are means ± S.E.M. from at least 3 separate experiments.
[b]There are 175 units per μg of CT.

cellular vacuolating effect and cellular signaling induction were completely inactivated.

To examine the effect of CT on phosphoinositide metabolism, we measured the levels in cellular IP_1, IP_2, and IP_3, the metabolites of inositol phospholipid hydrolysis. In the presence of calcium, CT dose-response studies were performed, and the results indicated that CT induced increases in IP_2 and IP_3 but not IP_1 (Table 2).

In cultured AGS cells, the mean basal $[Ca^{2+}]_i$ was 45 ± 3 nM ($n = 5$), and addition of the purified CT induced a transient and rapid increase in $[Ca^{2+}]_i$. As shown in Table 3, $[Ca^{2+}]_i$ was stimulated in a CT dose-dependent manner. In order to distinguish the source of the increased $[Ca^{2+}]_i$, the effect of CT was examined in EGTA-supplemented cell suspension. Removal of extracellular Ca^{2+} using EGTA reduced basal $[Ca^{2+}]_i$ from 45 to 22 nM but did not affect the response to CT. This indicates that CT-induced Ca^{2+} mobilization in AGS cells is not only dependent on extracellular Ca^{2+} sources but also on intracellular stores (Table 3).

In cultured AGS cells, 60 μg/ml CT induced a 4-fold increase in cAMP formation (Table 3), and the stimulatory effect of CT on cAMP formation was dose-dependent.

To investigate whether phosphorylation of host cell proteins was stimulated by CT, extracts were made of [^{32}P]phosphoric acid-preincubated AGS cells alone and after CT treatment. These extracts were separated by SDS-PAGE and followed by autoradiography. Phosphorylation of two host proteins was identified, a major one of 31 kDa and a minor of 22 kDa, as being induced in CT-pretreated AGS cells (Fig. 2).

Table 3
Effect of CT on $[Ca^{2+}]_i$ and [cAMP] in AGS cells

CT (μg/ml) challenge	$CaCl_2$	$[Ca^{2+}]_i$ (nM)	[cAMP] (pmol/10^6 cells)
Control	+	45 ± 3	10.2 ± 0.3
5	+	60 ± 9	10.7 ± 0.1
10	+	79 ± 2	12.0 ± 0.6
20	+	110 ± 10	13.5 ± 1.1
40	+	201 ± 23	25.5 ± 1.3
60	+	289 ± 29	38.3 ± 1.8
Control	−	22 ± 4	
5	−	38 ± 5	
10	−	65 ± 9	
20	−	89 ± 18	
40	−	156 ± 29	
60	−	182 ± 21	

For $[Ca^{2+}]_i$ measurements the AGS cells were stimulated with CT in the presence or in the absence of extracellular Ca^{2+}. The rise in $[Ca^{2+}]_i$ was evaluated as described in the text. Data represent the mean ± S.E.M. of 5 different experiments.

The ability of CT to stimulate pepsinogen secretion from AGS cells was examined with or without the presence of calcium. As shown in Table 4, in the presence of EGTA (without calcium) and in the presence of 1.3 mM calcium, CT caused increases in pepsinogen secretion to the same extent. It is evident from these data that a small amount of calcium (from CT-induced intracellular calcium pools) is enough to enhance CT-induced pepsinogen secretion.

4. Discussion

In this study, we used the purified *H. pylori* CT to investigate its signaling effect and pepsinogen secretion on a gastric cell line. The high purity of CT was illustrated on SDS-PAGE (Fig. 1). The heat inactivation of the CT preparation abolishes the vacuolation and signaling effects indicating that the preparation contains a highly purified CT but not LPS or some cell wall materials which might interfere in the investigation. The results in the present study indicate that in AGS cells,

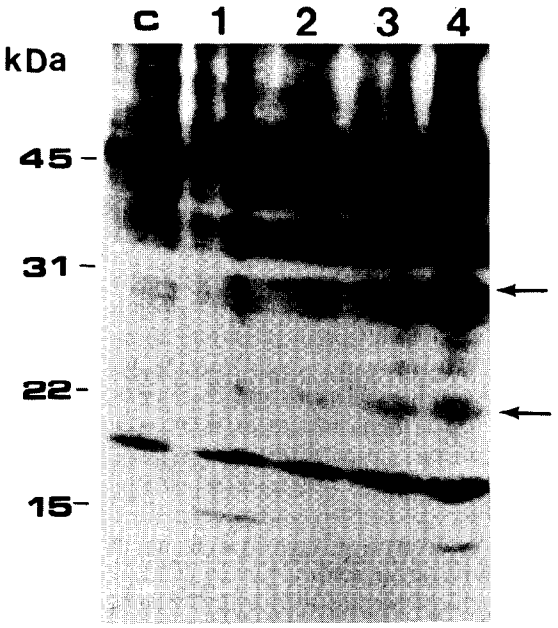

Fig. 2. The phosphorylation of proteins in AGS cells stimulated by CT. The AGS cells with [^{32}P]phosphoric acid were preincubated for 1 min at 37°C, then the various amounts of CT (lanes 1–4: 2, 5, 10, and 20 μg/ml; lane C: control) were added for another 5 min. Proteins were separated by a 11% SDS-PAGE.

Table 4
Effect of CT preincubation on pepsinogen secretion from AGS cells

CT challenge (μg/ml)	Pepsinogen secretion (μg/10^6 cells)	
	−EGTA	+EGTA
Control	1.50 ± 0.04	1.44 ± 0.05
10	3.15 ± 0.06	3.18 ± 0.06
20	8.25 ± 0.34	8.11 ± 0.12
50	11.25 ± 0.68	10.89 ± 0.85
70	15.39 ± 0.36	14.58 ± 0.65

Values given are means ± S.E.M. from at least 3 separate experiments. AGS cells were preincubated with various amounts of CT with or without 1.5 mM extracellular Ca^{2+} (adding 1.0 mM EGTA to deprive extracellular Ca^{2+}).

H. pylori CT acts as a classical first messenger to activate the two signaling pathways that are currently thought to mediate agonist-induced pepsinogen secretion [22]. One pathway is mediated by CT-activated phospholipase and a consequent increase in IP_3. It has been reported that increased IP_3 can stimulate $[Ca^{2+}]_i$ released from intracellular stores [23]. Therefore, we suggest that *H. pylori* CT can be classified as agents like cholecystokinin, carbachol, and gastrin which are reported to interact with cell membrane receptors to activate IP_3 production and a consequent increase in $[Ca^{2+}]_i$ [23–25]. The other pathway is mediated by CT-activated adenylcyclase and a consequent increase in cellular level of cAMP. Therefore, *H. pylori* CT has similar characteristics to agents like secretin, vasoactive intestinal peptide, prostaglandins, and cholera toxin which can interact with the cell membrane to increase the cellular level of cAMP [22,26,27].

Increases in both cellular levels of cAMP and calcium result in activation of protein kinases and/or phosphatases, and consequently mediate the final steps that result in pepsinogen secretion through a currently unknown mechanism [22]. Pepsinogen is known to be increased in serum of duodenal ulcer patients [14]. In the present study, we observed that CT induces phosphorylation of host proteins and pepsinogen secretion from AGS. Further work is required to identify the phosphated proteins and to determine their mechanism in mediating pepsinogen secretion in AGS cells.

In conclusion, we have demonstrate that *H. pylori* CT is able to activate transmembrane signals in host cells, such as increases in the levels of IPs, $[Ca^{2+}]_i$, cAMP, and protein phosphorylation, and consequently stimulates pepsinogen secretion. In the presence of gastric acid, pepsinogen is converted to an active proteolytic enzyme, pepsin. We propose that increased luminal pepsin activity resulting from elevated *H. pylori* CT concentration could degrade the gastric mucus layer, which might enable gastric acid to injure the underlying epithelium and consequently to form chronic active gastritis and peptic ulcers.

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