In Vitro Binding Assay of 31Methionine-oxidized Cholecystokinin Octapeptide to the CCK B Receptor

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Methionine (Met) residues of cholecystokinin octapeptide (CCK8) are easily oxidized to produce Met sulfoxide and/or sulfone of CCK8. In order to investigate the effect of modification at Met of CCK8 for its CCKB receptor, we evaluated the binding affinity of 4 oxidized forms of CCK8 for CCKB receptor expressed in the plasma membrane of LoVo cells, by performing a flow cytometry-based competitive binding assay using fluorescein isothiocyanate (FITC)-labeled CCK8. Oxidative modification of CCK8 at 28Met and 31Met caused to increase its binding affinity. The affinity of 31Met sulfone CCK8 was strongest and was significantly higher (by 20–30%) than that of native CCK8 (p < 0.05). In contrast, the binding affinity of modified CCK8 was attenuated on replacing 31Met with 31Leu, 31Lys, or 31His.

Key words—— cholecystokinin octapeptide, flow cytometry, LoVo cells, methionine sulfone

INTRODUCTION

Cholecystokinin (CCK), expressed in the gastrointestinal tract and central nervous system, functions as a gastrointestinal hormone and an analgesic, and also plays a role in feeding behavior, memory, learning, etc.1–5) It was discovered 80 years ago and comprises 33 amino acids; CCK octapeptide (CCK8) and CCK tetrapeptide (CCK4), comprising 8 and 4 amino acids, respectively, have also been identified in living systems. CCK8 (26Asp–27Tyr–28Met–29Gly–30Trp–31Met–32Asp–33Phe–NH2) accounts for 60% of the CCK family expressed in the human body and exhibits stronger activity than CCK33; in rats, it induces gallbladder contraction and amylase secretion in the pancreatic acini.6–9)

Binding site of CCK with its specific receptors is known to exist around the C-terminal region, which involves two methionine (Met) residues, 28Met and 31Met. We previously reported that the hydroxyl radical generated in vitro (Fenton’s reaction) or hydrogen peroxide (H2O2) can oxidize 28Met and 31Met of CCK8 to produce a CCK8 sulfoxide or sulfone [Fig. 1 (a)].10) This suggests that reactive oxygen species can oxidize these Met residues to produce oxidized CCK8 in vivo. Thus far, few studies have described the physiological functions of oxidized CCK8 in living systems. To gain a deeper understanding in this regard, the physiological functions of oxidized CCK8 and native CCK8 should be compared. In this study, 4 kinds of oxidized CCK8 were synthesized by a peptide synthesizer; the molecules contained 28Met and 31Met sulfoxides and sulfones of CCK8 as described later. Using these standards of oxidized CCK8, binding affinities to CCK receptor were investigated in vitro by a flow cytometry, which employed CCK receptor-expressed cells. To evaluate the affinities, a fluorescence ligand, which can bind specifically to CCK receptor, should be necessary, and therefore, fluorescein-labelled CCK8 derivative (FL-CCK8) [Fig. 1 (b)] was newly synthesized as a fluorescence-emitting ligand of CCK. On the other hand, as for the CCK receptor-expressed cells, human cancer cell lines, LoVo cells were used in this study, because it has been reported that CCKB receptor was expressed in the cell surface of LoVo cells.11) Further, we synthesized modified CCK8 molecules in which the 31Met residue was replaced with other amino acids, namely, 31His, 31Lys, or 31Leu, and examined the affinity of these molecules for the CCK8 receptor.

MATERIALS AND METHODS

Chemicals—— CCK8 was purchased from Peptide Institute Inc. (Osaka, Japan). 9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids, including Fmoc-methionine sulfoxide and Fmoc-methionine sulfone, were purchased from Watanabe Chemi-
Synthesis of Standard Oxidized and Modified CCK8 — We used CCK8 molecules containing the following combinations: $^{28}$Met sulfoxide and $^{31}$Met sulfoxide [28Met (O) 31Met (O)], 28Met sulfone and 31Met sulfoxide [28Met (O2) 31Met (O)], 28Met sulfoxide and 31Met sulfone [28Met (O) 31Met (O2)], and 28Met sulfone and 31Met sulfone [28Met (O2) 31Met (O2)]. These molecules were synthesized using the PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan), according to the standard method. These CCK8 analogues were analyzed by reversed-phase (RP)-HPLC, electrospray ionization (ESI)-MS, and tandem mass spectrometry (MS/MS) in order to confirm their purity and their primary sequences. The purity of each peptide was 95–96%.

Synthesis of FL-CCK8 — We dissolved 2.0 mg CCK8-$\beta$Ala and 6.9 mg FITC in a tube containing 10 ml Na$_2$CO$_3$ buffer (pH 9.0) and incubated the tube at 4°C for 2 hr to allow the contents to react. Next, the reaction mixture was loaded onto a Sep-Pak$^\text{®}$ cartridge (C18 PLUS), initialized with 4 ml of MeOH followed by 4 ml of H$_2$O. The cartridge was washed with 2 ml H$_2$O, and FL-CCK8 was eluted with 2 ml of 30% MeOH. The purified FL-CCK8 in the eluate was detected by performing RP-HPLC. The FL-CCK8 concentration was adjusted to 15 $\mu$M with phosphate-buffered saline (PBS); this solution was immediately used in the binding assay. The purity of FL-CCK8 was approximately 95%.

RP-HPLC — An HPLC system comprising the LC10AD pump and SPD-10A UV detector was used for checking the purity of synthesized peptides. A YMC pack ODS-A302 column (150 mm
× 4.6 mm inner diameter, 5 μm; YMC Co. Ltd., Kyoto, Japan) was used for separation. We used aqueous solutions of 0.1% TFA (A) and 0.1% TFA along with 60% CH₃CN (B) as the mobile phases; these were pumped into the column at 1.0 ml/min. Elution was performed over a linear gradient as follows: 100% A pumped for 0–10 min; 100% B, for 10–40 min; and 100% B, for 40–45 min. The sample (20 μl) was injected into the HPLC column using a Rheodyne 7725i injector (Rheodyne).

**RESULTS AND DISCUSSION**

**FL-CCK8**

In the present study, a βAla derivative of CCK8 was fluorometrically labeled with FITC to obtain FL-CCK8 [Fig. 1(b)]. It has been reported that CCK binds to its receptor via its C-terminal end.¹⁴,¹⁵ Hence, ²⁶Asp that is present at the N-terminal of CCK8 was labeled with FITC. βAla was linked to this ²⁶Asp residue as a spacer molecule to prevent steric hindrance arising from the fluorescein moiety at the binding site. To determine whether FL-CCK8 can be used as a fluorescence ligand for the competitive binding assay, we first examined the specific binding of FL-CCK8 to CCKB receptor under a confocal laser scanning microscope. Figure 2 shows a representative photograph of the LoVo cells added with FL-CCK8. In this photograph, green fluorescence originated from FL-CCK8 was clearly observed around the cell surface. This result suggests that the synthesized FL-CCK8 could bind to the CCKB receptor expressed on the surface of LoVo cells. When the cells were incubated for more than 1 hr, gradual emission of fluorescence was ob-

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**Fig. 2. A Representative Photograph of LoVo cells Treated with 15 μM FL-CCK8, as Observed by Confocal Laser Scanning Microscopy (lower) and Its Differential Interference Contrast Mode (upper)**

Objective, × 40(W); filter, LP 505; wavelength, 488 nm; scale bar, 10 μm.
served from within the cells. This finding indicates that the cell membrane might be a collapse by incubation for more than 1 hr; hence, we set the incubation time to 1 hr. We plotted the fluorescence intensity observed by flow cytometry against the FL-CCK8 concentration added to the LoVo cells. Consequently, the degree of binding almost reached the saturation level when 15 µM FL-CCK8 was added (data not shown); hence, we selected this concentration for use in the competitive binding assay between oxidized CCK8 and FL-CCK8.

### Competitive Binding Assay

Figure 3 shows the RFI values of native and oxidized CCK8; these values show a significant difference \( p < 0.05 \). Compared to the RFI values of native CCK8, those of oxidized CCK8 were attenuated, indicating that oxidized CCK8 exhibited an enhanced binding affinity by 20–30% for its receptor. This enhanced binding affinity may be attributable to the oxidation of CCK8 at 28Met or 31Met. 31Met sulfone of CCK8 exhibited the strongest binding affinity; this suggested that compared to 28Met sulfone, 31Met sulfone bound more effectively to the receptor. These results indicate that hydrogen bonding or conformational distortion arising from the addition of an oxygen atom at 31Met in the sulfone moiety of CCK8 might enhance the binding affinity of CCK8 for its receptor. Accordingly, a suitable modification at 31st residue in CCK8 may lead to design a modified CCK8 with highly binding affinity to its receptor.

To clarify the role played by 31Met in the binding affinity of CCK8, we synthesized 3 artificially modified forms of CCK8, in which 31Met was replaced with other amino acids, namely, 31Leu, 31Lys, and 31His. We replaced 31Met with Leu to examine whether hydrophobicity or steric distortion at the 31st residue critically influences the binding of CCK8 to its receptor. In addition, since the isoelectric points of Lys and His rendered them positively charged in a phosphate buffer of pH 7.4, we selected basic amino acids for CCK8 modification in order to investigate the involvement of cationic charge to the binding of CCK8 to CCKB receptor.

The modified CCK8 molecules were evaluated by performing a competitive assay. Figure 4 shows the RFI values of modified CCK8. Compared to the RFI value of native CCK8, those of the modified forms
of CCK8 were elevated. In this case, the binding affinity of the modified CCK8 molecules for the receptor was completely weakened. This result indicates that steric hindrance or cationic charge at the 31st residue of CCK8 deteriorated the binding affinity to CCKB receptor. Therefore, negative polarity of Met sulfone may give an increased binding affinity of CCK8 to CCKB receptor. Modified CCK8, in which 31Met residue was replaced to negatively charged amino acids such as Asp or Glu, should be synthesized and the binding affinities should also be examined in future.

In conclusion, on the basis of the results of the study, we conclude that 28Met and 31Met were crucial for the binding of CCK8 to CCKB receptor. Especially, CCK8 interacting with the receptor via 31Met sulfone or sulfoxide clearly exhibits a stronger binding affinity than native CCK8. The physiological functions of these oxidized forms of CCK8 in vivo remain unclear, and should be investigated in the future, because Met sulfone or sulfoxide of CCK8 may be produced actually by ROS in vivo due to its high susceptibility to oxidation.

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REFERENCES


