In Vitro Binding Assay of ^{31}Methionine-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 CCK_B receptor, we evaluated the binding affinity of 4 oxidized forms of CCK8 for CCK_B 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 ^{28}Met and ^{31}Met caused to increase its binding affinity. The affinity of ^{31}Met 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 ^{31}Met with ^{31}Leu, ^{31}Lys, or ^{31}His.

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 (^{26}Asp-^{27}Tyr-^{28}Met-^{29}Gly-^{30}Trp-^{31}Met-^{32}Asp-^{33}Phe-NH_2) 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, ^{28}Met and ^{31}Met. We previously reported that the hydroxyl radical generated *in vitro* (Fenton's reaction) or hydrogen peroxide (H_2O_2) can oxidize ^{28}Met and ^{31}Met 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 ^{28}Met and ^{31}Met 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 CCK_B receptor was expressed in the cell surface of LoVo cells.[11] Further, we synthesized modified CCK8 molecules in which the ^{31}Met residue was replaced with other amino acids, namely, ^{31}His, ^{31}Lys, or ^{31}Leu, 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-

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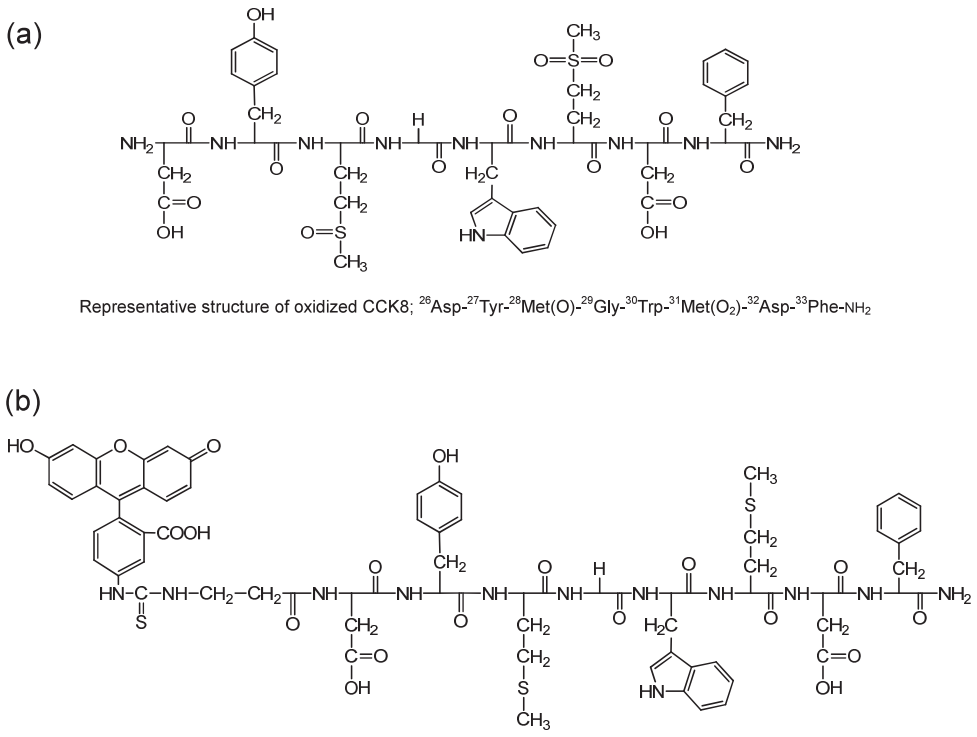

Fig. 1. Chemical Structures of Representative Oxidized CCK8 (a) and FL-CCK8 (b)

cal Industries Ltd. (Hiroshima, Japan). HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were obtained from Kanto Kagaku Co. Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan), respectively. Trifluoroacetic acid (TFA) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan); Dulbecco's modified Eagle's medium (DMEM), Gibco; fetal bovine serum (FBS), Invitrogen Corp. (Carlsbad, CA, U.S.A.); fluorescein isothiocyanate (FITC), Dojindo Laboratories (Kumamoto, Japan); penicillin, Banyu Pharmaceutical Co. Ltd. (Tokyo, Japan); and streptomycin, Meiji Seika Kaisha Ltd. (Tokyo, Japan). The water used in the experiments was purified using Milli-Q Labo equipment (Nihon Millipore Co. Ltd., Tokyo, Japan).

Synthesis of Standard Oxidized and Modified CCK8 —— We used CCK8 molecules containing the following combinations: ^{28}Met sulfoxide and ^{31}Met sulfoxide [^{28}Met (O) ^{31}Met (O)], ^{28}Met sulfone and ^{31}Met sulfoxide [^{28}Met (O_2) ^{31}Met (O)], ^{28}Met sulfoxide and ^{31}Met sulfone [^{28}Met (O) ^{31}Met (O_2)], and ^{28}Met sulfone and ^{31}Met sulfone [^{28}Met (O_2) ^{31}Met (O_2)]. These molecules were synthesized using the PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan), according to the standard method.[12,13] 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-βAla and 6.9 mg FITC in a tube containing 10 ml Na_2CO_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® cartridge (C18 PLUS), initialized with 4 ml of MeOH followed by 4 ml of H_2O. The cartridge was washed with 2 ml H_2O, 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 μM 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% CH_3CN (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).

ESI-MS/MS —— The samples were analyzed on an LCQ ion-trap mass spectrometer (Thermo Fisher Scientific, Kanagawa, Japan) equipped with an ESI source, operated in the positive-ion mode. The samples were injected with a syringe pump at a constant flow rate of 5 μl/min. The experimental conditions were as follows: spray needle voltage, 5.5 kV; heated capillary temperature, 200°C; and sheath gas flow rate, 35 arbitrary units. Mass spectra were obtained in the full ion scan mode over an m/z range of 200–2000. To identify the amino acid oxidation site, we operated the mass spectrometer in the MS/MS mode (further MS^n operation, if necessary). Collision-induced dissociation (CID) spectra were obtained with the isolation width set at 1.5 m/z. The fragmentation efficiency of the precursor ions was maximum when the relative collision energy was 20–40% (instrument parameter).

Binding Assay —— LoVo cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The LoVo cells (4×10^5 cells/ml) were transferred to sterilized plastic tubes (capacity, 1.5 ml) and washed with PBS and 0.1% bovine serum albumin (BSA) in PBS. We added 50 μl of 15 μM FL-CCK8 and 50 μl of 40 μM CCK8 or oxidized CCK8 (total volume, 100 μl) to the cells and incubated this reaction mixture at 37°C for 1 hr. Next, the cells were washed with 0.1% BSA in PBS and PBS and suspended in 200 μl PBS. The final cell suspension was quantified using the flow cytometer (Cyto-ACE 300; Jasco Co. Ltd., Tokyo, Japan). The following equation was used to calculate the binding affinity of CCK8.

$$\text{Relative fluorescence intensity (RFI; \%)} = (Flu_{\text{test}}/Flu_{\text{CCK8}}) \times 100 \quad (1)$$

Where Flu_{test} and Flu_{CCK8} are the fluorescence intensities of a tested peptide such as oxidized CCK8 and native CCK8, respectively. Each RFI (%) was represented as mean ± S.D. ($n = 5$).

Statistical Analysis —— Welch's t test was used to analyze the statistical significance of the data. A p value of < 0.05 was considered significant.

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, ^{26}Asp that is present at the N-terminal of CCK8 was labeled with FITC. βAla was linked to this ^{26}Asp 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 CCK_B 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 CCK_B receptor expressed on the surface of LoVo cells. When the cells were incubated for more than 1 hr, gradual emission of fluorescence was ob-

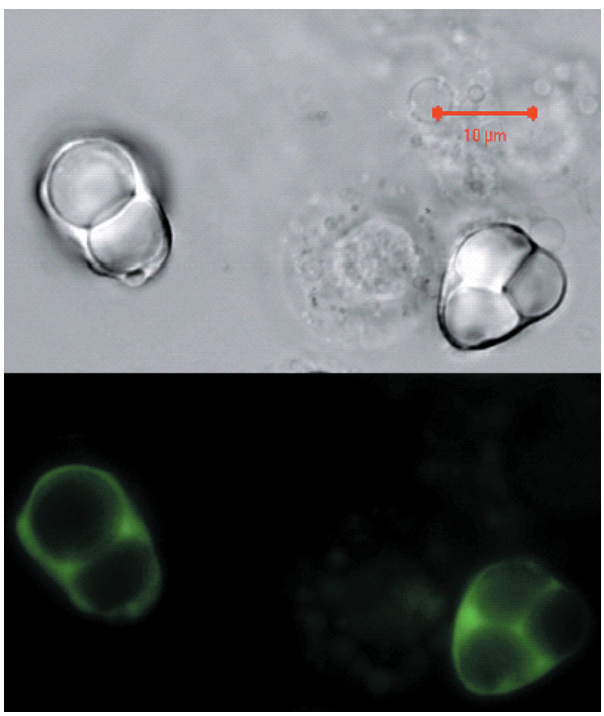

Fig. 2. A Representative Photograph of LoVo cells Treated with 15 μM FL-CCK8, as Observed by Confocal Laser Scanning Microscopy (lower) and Its Differential Interference Contrast Mode (upper)

Objective, × 40 (W); filter, LP 505; wavelength, 488 nm; scale bar, 10 μm.

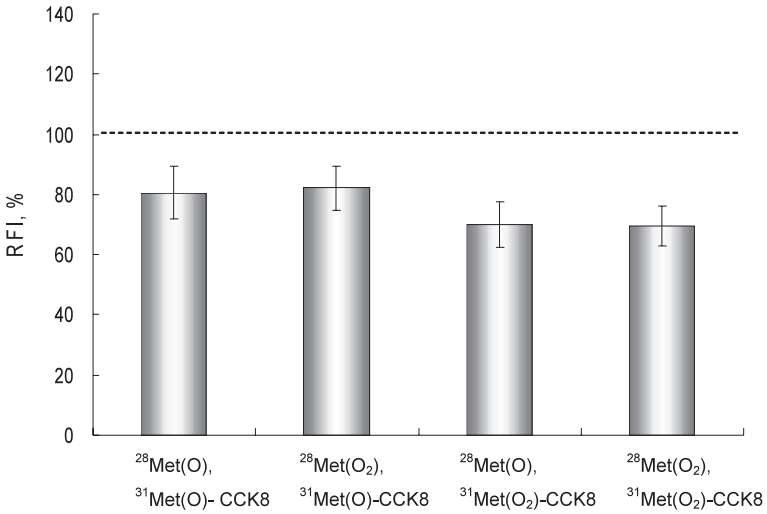

Fig. 3. Relative Fluorescence Intensity of Oxidized CCK8
RFI of 100 (dotted bar) means the same binding affinity of native CCK8.

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 RFIs of 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 ^{28}Met or ^{31}Met. ^{31}Met sulfone of CCK8 exhibited the strongest binding affinity; this suggested that compared to ^{28}Met sulfone, ^{31}Met 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 ^{31}Met 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 ^{31}Met in the binding affinity of CCK8, we synthesized 3 artificially modified forms of CCK8, in which ^{31}Met was replaced with other amino acids, namely, ^{31}Leu, ^{31}Lys, and ^{31}His. We replaced ^{31}Met 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 CCK_B receptor.

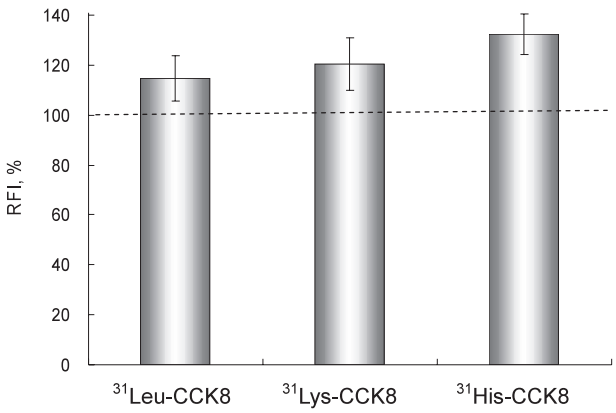

Fig. 4. Relative Fluorescence Intensity Arising from ^{31}Leu, ^{31}Lys, and ^{31}His of CCK8
RFI of 100 (dotted bar) means the same binding affinity of native CCK8.

The modified CCK8 molecules were evaluated by performing a competitive assay. Figure 4 shows the RFIs 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 CCK_B receptor. Therefore, negative polarity of Met sulfone may give an increased binding affinity of CCK8 to CCK_B receptor. Modified CCK8, in which ^{31}Met 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 ^{28}Met and ^{31}Met were crucial for the binding of CCK8 to CCK_B receptor. Especially, CCK8 interacting with the receptor via ^{31}Met 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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