Stability of PEGylated Salmon Calcitonin in Nasal Mucosa

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ABSTRACT: The purpose of this study was to evaluate the stabilization of salmon calcitonin (sCT) by PEGylation in nasal mucosa. Degradation of native sCT in the homogenates of rat nasal mucosa was investigated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The initial cleavage of sCT was due to tryptic-like endopeptidase activity, and the subsequent degradation followed the sequential pattern of aminopeptidase activity. To prepare PEGylated sCT resistant to the proteolytic degradation, the lysine residues susceptible to tryptic activity were selectively PEGylated by controlling reaction pH. The PEGylated sCT showed strong resistance against enzymatic degradation in rat nasal mucosa, with 56-fold prolonged half-life compared with that of native sCT. In the MALDI-TOF MS spectrum, the PEGylated sCT did not show any degradation peak for incubation of 120 min in the homogenates of rat nasal mucosa. The improved stability may be responsible for enhancing nasal absorption of PEGylated sCT. J Pharm Sci 93:256–261, 2004

Keywords: calcitonin; nasal absorption; PEGylation; peptide stability; conjugation; mucosal drug delivery

INTRODUCTION

Nasal delivery of therapeutic peptides has recently attracted considerable interest because it has several potential advantages including rapid absorption and avoidance of first-pass gastrointestinal and hepatic metabolism.[1,2] The easy accessibility of the nasal route facilitates self-medication, thus improving patient compliance compared with other parenteral routes.[3] Because of the large surface area of the nasal cavity and the highly vascularized nature of nasal mucosa, the plasma pharmacokinetic profiles of some drugs following nasal administration are similar to those obtained by bolus intravenous injection.[4] However, the bioavailabilities of nasally administered therapeutic peptides are often low, in most cases $<10\%$.[5] The low bioavailability can be attributed to degradation of therapeutic peptides by proteolysis in the nasal mucosa.[6] The nose is now well recognized as a site of metabolism.[7] Significant peptidase activity has been demonstrated, and both endopeptidases and exopeptidases are present in the nasal tissue.[8]

Nasal administration of salmon calcitonin (sCT) has been shown to be clinically effective in a variety of bone diseases characterized by increased bone resorption, such as Paget's disease of bone and postmenopausal osteoporosis.[9,10] However, the bioavailability of sCT from nasal spray formulation in human is very low, in the range 1–15%.[11,12] According to previous reports, sCT was readily metabolized by nasal enzymes[13] and the use of proteolytic enzyme inhibitors enhanced

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nasal absorption.[14] Therefore, enzymatic degradation is regarded as one of major barriers to the nasal absorption of sCT.

The covalent attachment of poly(ethylene glycol) (PEG), PEGylation, is a technique widely used to provide functional bioconjugates with improved biological stability by increasing resistance to proteolytic degradation.[15]

The effects of PEGylation also include better physical and thermal stability, increased solubility, reduced immunogenicity and antigenicity, and decreased toxicity.[16] In a previous report, PEGylation of sCT was shown to significantly increase the stability of sCT in tissue homogenates, probably due to protection from proteolytic enzymes by PEG molecules.[17] Also, regarding the hypocalcemic effects after intranasal administration to rats, the PEGylated sCT exhibited significantly prolonged serum calcium lowering effect compared with unmodified sCT.[18] The hypocalcemic effect reflects the amounts of sCT present in the systemic circulation and the sensitivity of the response to sCT, so it can be related to improved effects for the treatment of bone diseases such as Paget's disease and postmenopausal osteoporosis.

The purpose of this study was to evaluate the stability of PEGylated sCT in nasal membranes. The major degradation sites of native sCT in nasal membrane were investigated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and PEGylated sCT with better stability due to protection of the degradation sites was prepared. The stability of PEGylated sCT in rat nasal membrane was evaluated by reversed-phase high-performance liquid chromatography (HPLC) and MALDI-TOF MS.

EXPERIMENTAL

Materials

Salmon calcitonin (synthetic cyclic sCT) and succinimidyl propionate monomethoxy poly(ethylene glycol) (SPA-MPEG; MW, 2 kDa) were purchased from Bachem (Torrance, CA) and Shearwater Polymers (Huntsville, AL), respectively. A Micro BCA protein assay kit was obtained from Pierce (Rockford, IL). α-Cyano-4-hydroxy-*trans*-cinnamic acid (α-CHCA) and acetonitrile (HPLC grade) were obtained from Sigma (St. Louis, MO) and J.T. Baker (Philipsburg, NJ), respectively. Male Sprague-Dawley rats were purchased from the Experimental Animal Lab of Korean FDA (Seoul, Korea). All other chemicals were of analytical grade and were obtained commercially.

Preparation of PEGylated sCT

Mono-PEGylated sCT (mono-PEG-sCT) was prepared as described in our previous report with a slight modification.[19] Two milligrams of SPA-MPEG-2K was added to 0.2 mL of each sCT solution (5 mg/mL) in various buffer solutions with different pHs (pH 6, 7, 8, 9). The reaction mixtures were shaken gently at room temperature. After 1 h, the reactions were stopped by adding an excess of 1 M glycine solution. The mono-PEG-sCT fractions were then purified by size-exclusion chromatography using Superose HR 12 10/30 (Pharmarcia LKB, Sweden) eluted with 50 mM sodium citrate buffer (pH 5.0) at a flow rate of 0.4 mL/min. The ultraviolet (UV) absorbance was monitored at 215 nm. The mono-PEG-sCT fractions were collected and verified by MALDI-TOF MS.

Stability in Rat Nasal Mucosa

Male Sprague-Dawley rats weighing 230–260 g were maintained on a standard laboratory diet before surgery. The rats were fasted overnight with free access to water and then were euthanized by CO_2 inhalation. The heads were bisected along the nasal septum to expose the nasal cavity. The nasal mucosa was carefully excised from the nasal septum and turbinates and freed from the underlying cartilage and bone. The tissue was rinsed with an isotonic phosphate buffer and homogenized in 0.25 M sucrose in a glass homogenizer cooled by ice water. The homogenate was centrifuged at $9000 \times g$ for 5 min at 4°C, and the supernatant was used for analysis. Total protein concentration of the supernatant was determined by the micro BCA assay.[20] One hundred microliters of the homogenates at a protein concentration of 1.8 mg/mL was incubated with 100 μL of a 87 μM sCT or the equivalent mono-PEG_{2K}-sCT solution, prepared in an isotonic phosphate buffer, at 37°C for up to 120 min. Acetonitrile was added to each sample to obtain a 34% final concentration to terminate further degradation. Samples were centrifuged at $13{,}000 \times g$ at 4°C for 10 min to remove precipitated proteins. The supernatants were then analyzed for the remaining intact sCT or mono-PEG_{2K}-sCT by reversed-phase HPLC, and the degradation products were monitored by MALDI-TOF MS.

HPLC Analysis

The remaining content of intact sCT or mono-PEG_{2K}-sCT in the supernatants of nasal mucosa homogenates was determined by reversed-phase HPLC with a LiChrospher RP 18 column (4.0 × 25.0 mm, 5 μm, Merck, Darmstadt, Germany). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in distilled water (eluent A) and 80% acetonitrile containing 0.1% TFA (eluent B). The mobile phase was run with a linear gradient from 20 to 60% eluent B for 20 min at a flow rate of 1.0 mL/min, and the UV absorbance of the eluent was monitored at 215 nm. The injection volume was 40 μL. The column was calibrated with sCT solutions of known concentrations ranging from 10 to 250 μg/mL.

MALDI-TOF Mass Spectrometry

MALDI-TOF MS was performed with a Voyager-RP Biospectrometry Workstation (PerSeptive Biosystems, Cambridge, MA). The analysis was conducted in a linear mode, and data for 2-ns pulses of the 337 nm nitrogen laser were averaged for each spectrum. Linear, positive-ion TOF detection was performed using an acceleration voltage of 20 kV and a laser intensity of ~10% greater than threshold. The grid voltage and guide wire voltage were chosen for each spectrum to achieve the optimal signal-to-noise ratio. Spectra were obtained by summing >128 laser shots to obtain the comparable condition and smoothed with a 19-point Savitzky-Golay filter. The samples were mixed with α-CHCA in 50% acetonitrile in water containing 0.1% TFA as final concentration and dried on the sample holder. A mixture of peptide mass standards of angiotensin I, ACTH (clip 1-17), ACTH (clip 18-39), ACTH (clip 7-38), and bovine insulin was adapted for the external calibration. The sample-matrix solution was prepared by combining at a ratio of 1:1.

RESULTS AND DISCUSSION

Degradation of sCT in Nasal Mucosa

Native sCT was readily metabolized in nasal membrane as indicated by various mass peaks of metabolites on the MALDI-TOF mass spectra of sCT incubated in rat nasal mucosa (Fig. 1). Spectra from controls showed that these metabolites peaks were neither present in nasal mucosa that was not treated with sCT nor in sCT solutions treated

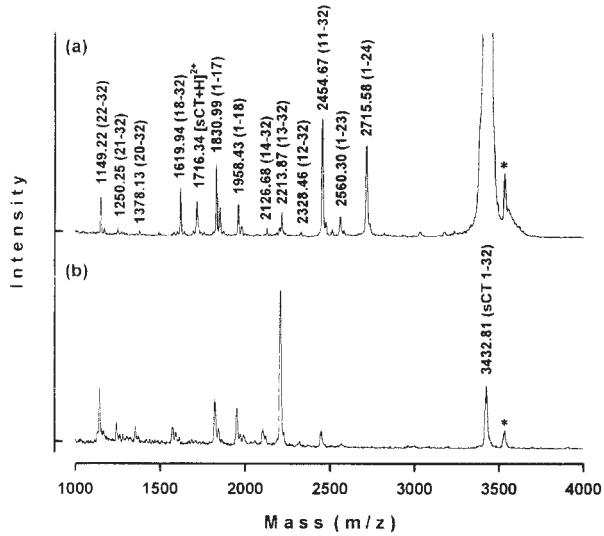

Figure 1. MALDI-TOF MS spectra of degradation products of sCT incubated in the homogenates of rat nasal mucosa for (a)10 and (b) 60 min (* is the mass peak originated from rat nasal mucosa).

under similar conditions but without contact with nasal mucosa. These results indicate that the metabolites peaks were produced by enzymatic degradation in rat nasal mucosa. The MALDI-TOF MS analysis showed that the major cleavage sites of sCT in rat nasal mucosa are Gly^{10}-Lys^{11}, His^{17}-Lys^{18}, Lys^{18}-Gln^{19}, and Arg^{24}-Thr^{25} (Fig. 2). The cleavages at Lys^{18}-Gln^{19} and Arg^{24}-Thr^{25} are indicative of tryptic activity, but those at Gly^{10}-$Lys^{11}$and His^{17}-Lys^{18} may occur by nonspecific endopeptidase activity. Other fragments seemed to originate from the major metabolites by sequential cleavage of aminopeptidase or carboxypeptidase activity; that is, Cys^{1}-Pro^{23} (*m/z* 2560.30) from Cys^{1}-Arg^{24} (*m/z* 2715.58), Ser^{13}-$ProNH_2^{32}$ (*m/z* 2213.87) from Lys^{11}-$ProNH_2^{32}$ (*m/z* 2454.67), and Tyr^{22}-$ProNH_2^{32}$ (*m/z* 1149.22) from Lys^{18}-$ProNH_2^{32}$ (*m/z* 1619.94). After 60 min of incubation, the Cys^{1}-Arg^{24} and Lys^{11}-$ProNH_2^{32}$ peaks disappeared and the Ser^{13}-$ProNH_2^{32}$ peak was distinctly observed. The importance of tryptic endopeptidase activity in the enzymatic degradation of nasal sCT has been reported in previous studies.[14] Lang et al.[13] reported that nasal sCT is degraded by tryptic-like endopeptidases, and the subsequent metabolic breakdown follows the sequential pattern of aminopeptidase activity. The Cys^{1}-Cys^{7} loop structure of sCT may lead to perfect stability of the N-terminus against aminopeptidase activity. Iwakawa et al.[21] reported that exoproteases such as aminopeptidase and carboxypeptidase did not cause significant degradation of eel calcitonin,

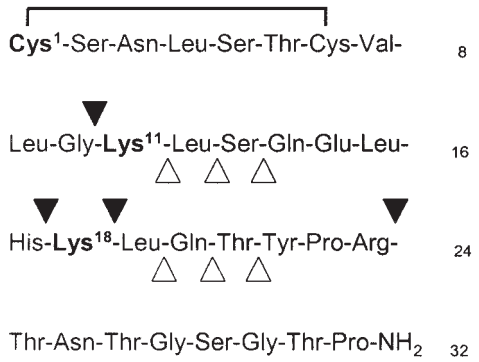

Figure 2. Major cleavage sites (▼) and the subsequent degradation sites (△) of native sCT during 60 min of incubation with rat nasal mucosa homogenates as determined by MALDI-TOF MS.

and endopeptidases such as trypsin or chymotrypsin caused a rapid loss of immunoreactive eel calcitonin. These data strongly indicate that nasal sCT is degraded by tryptic activity.

Preparation of Mono-PEGylated sCT

There are three possible sites for PEGylation in the chemical structure of sCT; they are, the primary amino group of the N-terminus (Cys^1) and two lysine residues (Lys^{11} and Lys^{18}). PEGylation of sCT produces seven possible PEGylated sCTs; they are, three mono-PEG-sCTs (N-terminus-; Lys^{11}-; and Lys^{18}-), three di-PEG-sCTs (N-terminus- and Lys^{11}-; N-terminus- and Lys^{18}-; and Lys^{11}- and Lys^{18}-), and one tri-PEG-sCT (N-terminus-, Lys^{11}-, and Lys^{18}-). In our previous study, the nasal absorption of PEGylated sCT was inversely related to the molecular weights of the PEG attached.[18] When one PEG molecule of molecular weight 2 kDa was conjugated to sCT (mono-PEG_{2K}-sCT), a significant high and prolonged hypocalcemic effect was observed. The mono-PEG_{2K}-sCT was purified from the reaction mixture (molar ratio of PEG to sCT = 3:1) by size-exclusion chromatography and analyzed by the reversed-phase HPLC method reported previously. A representative chromatogram of mono-PEG_{2K}-sCTs composed of three positional isomers is shown in Figure 3.

As shown in the stability study of native sCT in nasal mucosa, the degradation pattern of sCT suggests that Lys^{11} and Lys^{18} of three PEGylation sites are important for enhancing the stability of sCT in the nasal mucosa. The production of mono-PEG-sCTs modified at Lys^{11} and Lys^{18}, except the N-terminus, could be carried out by taking

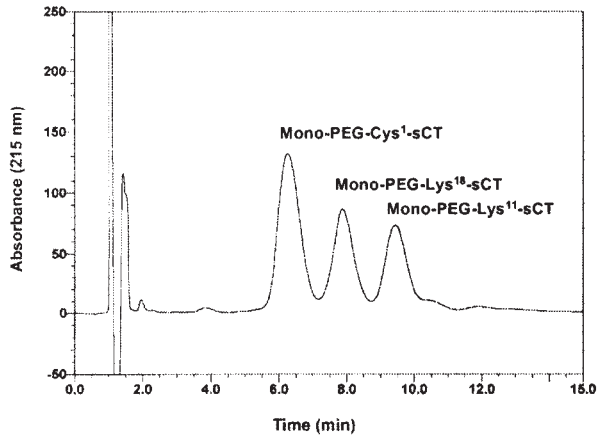

Figure 3. Reversed-phase HPLC chromatogram of mono-PEG_{2K}-sCTs prepared at reaction pH 7.0 (0.1 M phosphate buffer).

advantage of reactivity difference between the α-amino group of the N-terminus and the ε-amino group of the Lys residue depending on the reaction pH. At a reaction pH of 6, predominantly N-terminus-modified mono-PEG-sCT was produced compared with Lys-modified mono-PEG-sCTs; however, the amount produced was <10% of the three mono-PEG-sCTs produced at the reaction pH of >8 (Fig. 4). At pH > 8, the primary amine was completely deprotonated and the more nucleophilic ε-amino group of the Lys residue was generally more reactive toward electrophilies than the α-amino group of the N-terminus. On the contrary, at a lower pH (6.0), the more acidic α-amino group was more deprotonated and reactive toward the electrophilic group of PEG. In this

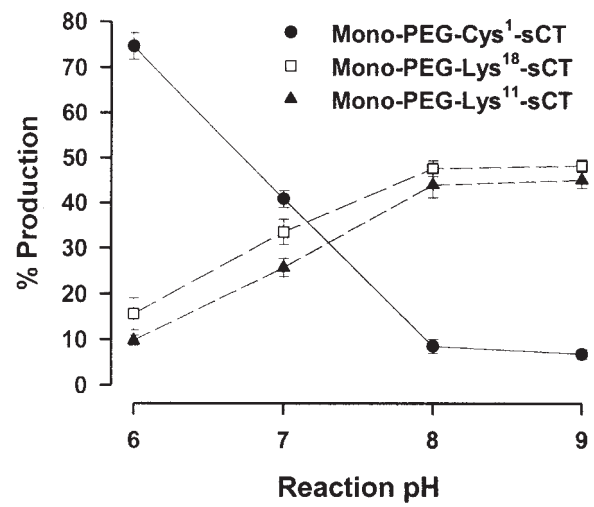

Figure 4. Production of positional isomers of mono-PEG_{2K}-sCTs at different reaction pHs.

study, mono-PEG-sCT prepared at reaction pH of 8 was used for the stability study in nasal mucosa.

Stability of Mono-PEGylated sCT in Nasal Mucosa

PEGylation of sCT showed resistance against tryptic-like or nonspecific enzymatic degradation in rat nasal mucosa. The degradation kinetics of native sCT and mono-PEG_{2K}-sCT are shown in Figure 5. Recoveries of added sCT and mono-PEG_{2K}-sCT from nasal homogenates, determined by HPLC, were 91.5 and 93.2%, respectively. The native sCT was rapidly degraded with only ~15% of sCT remaining after 60 min of incubation in rat nasal mucosa homogenates. The degradation of mono-PEG_{2K}-sCT was, however, dramatically reduced, with >90% remaining after 120 min of incubation. The degradation half-life of mono-PEG_{2K}-sCT (1386.0 min) was prolonged 56-fold compared with that of native sCT (24.7 min). In the MALDI-TOF MS spectrum of mono-PEG_{2K}-sCT incubated in the homogenates of rat nasal mucosa for 120 min, the apparent degradation peak was not observed (Fig. 6). These results indicate that the mono-PEG_{2K}-sCT is very stable against various proteolytic enzymes present in the nasal mucosa. In addition, this study strongly supports the idea that the enhanced and sustained hypocalcemic effect of nasally administered mono-PEG_{2K}-sCT demonstrated in our previous study[18] might be mainly due to improved stability of mono-PEG_{2K}-sCT in nasal membrane compared with native sCT.

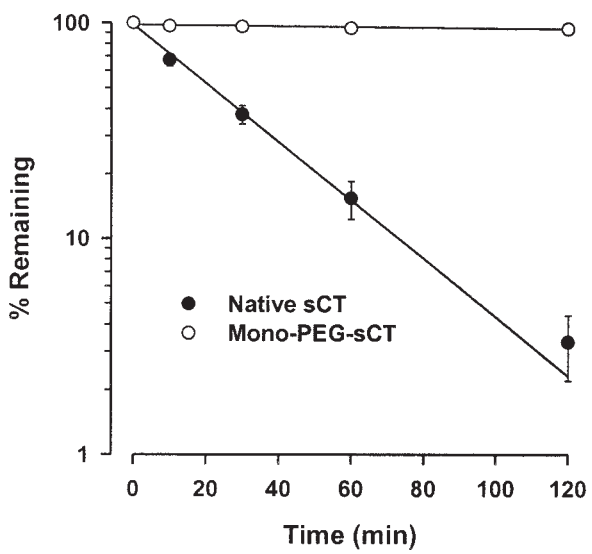

Figure 5. Stability of native sCT and mono-PEG_{2K}-sCT in homogenates of rat nasal mucosa.

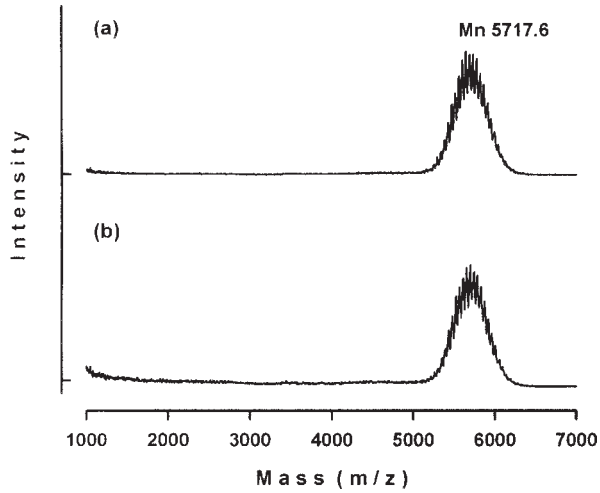

Figure 6. MALDI-TOF MS of mono-PEG_{2K}-sCT incubated in rat nasal homogenates for 120 min (a: at initial time; b: after 120 min of incubation).

CONCLUSIONS

MALDI-TOF MS analysis of native sCT incubated in the homogenates of rat nasal mucosa revealed that the metabolic cleavage is mainly due to tryptic-like endopeptidases activity. The Lys residues of sCT susceptible to tryptic activity were selectively PEGylated by controlling reaction pH, and the PEGylated sCT showed strong resistance against enzymatic degradation in rat nasal mucosa. The improved stability may be responsible for enhancing nasal absorption of the drug and improving therapeutic efficacy.

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