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Degradation of salmon calcitonin in rat kidney and liver homogenates

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Salmon calcitonin (sCT), a 32-amino-acid peptide, is the active component in many pharmaceuticals used for the management of bone diseases. In this study, the stability of sCT in rat kidney and liver homogenates were evaluated by LC-ESI-MS, and the structures of the major degradation products were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The results show that the half life of sCT was 13.18 min in rat kidney homogenate (2.5 mg/ml, protein concentration) and 43.07 min in rat liver homogenate (2.5 mg/ml, protein concentration). MALDI-TOF MS results indicated that sCT was initially cleaved at Leu^{9}-Gly^{10} and Gly^{10}-Lys^{11} bonds in rat kidney homogenate *in vitro*, at the same time, the major degradation fragment, Lys^{11}-Pro^{32}-NH_2 was metabolized at the C-terminal amide by deamidation, whereas in rat liver homogenate, the initial cleavage sites were at Val^{8}-Leu^{9} and His^{17}-Lys^{18}. The results indicated that the metabolism of sCT proceeds by initial endoproteolytic cleavage and subsequent exoproteolytic digestion.

1. Introduction

Salmon calcitonin (sCT) is a single polypeptide chain calcitropic hormone consisting of 32 amino acids (3,432 Da) with an N-terminal disulfide bridge between positions 1 and 7, and a C-terminal proline amide residue. It is currently formulated as either a sterile solution for intramuscular or subcutaneous injection, or as nasal spray in the management of several bone-related diseases including Pagets disease, hypercalcemia and osteoporosis as well as management of severe pain resulting from vertebral crush fractures (Azria 1989; Lee et al. 1991; Compston 1997). These cases generally require long-term therapy through a daily or alternate daily treatment with calcitonin.

Early PK studies in humans evaluated the disposition of sCT following intraveneous (IV), intramuscular (IM), and subcutaneous (SC) administration (Beveridge et al. 1976; Huwyler et al. 1979; Ardaillou et al. 1973; Nuesch et al. 1980). As a polypeptide, sCT is subject to chemical and enzymatic degradation and has a short biological half-life in the body, approximately 14 min (Segra and Dal Pra 1985; Lee et al. 1992, 1995; Hysing et al. 1992; Simmons et al. 1988; Lang et al. 1996; Windisch et al. 1997). Previous *in vivo* studies showed that the kidneys account for approximately two-thirds of the metabolism of calcitonin (Simmons et al. 1988). Earlier studies found that the kidney and liver, the main organs for metabolism of calcitonins (Scarpace et al. 1977), are believed to be the sites of internalization of the receptor-calcitonin complex from the cell surface into the intracellular matrix (Findlay et al. 1982, 1989) and subsequent hydrolytic decomposition in the lysosome (Hicke 1997). Kobayashi et al. (2000) evaluated the stability and metabolism character of sCT in rat liver and kidney subcellular *in vitro*, such as cytosolic and lysosome fractions. sCT in rat liver subcellular was stable. By using LC-ESI-MS/MS technology, they found two major degradants in kidney cytosolic fraction.

Since the presence of the disulphide bond at the amino terminus and the amide bond at the carboxyl terminus in the molecule makes calcitonins resistant to exoproteolytic hydrolysis, and endoproteolysis seems to be the initial process of the metabolism of calcitonin. However, the chemical structure and sequence of the primary metabolites of sCT in the liver and kidney has not been investigated yet. In order to investigate and compare the tissue specific metabolism in terms of extent of metabolism and metabolic pattern for sCT, we therefore evaluated stability of sCT in the major metabolism organs *in vitro* and investigated the major cleavage sites of it in kidney and liver homogenates by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

H-Cys^{1}-Ser-Asn-Leu-Ser^{5}-Thr-Cys-Val-Leu-Gly^{10}-Lys-Leu-Ser-Gln-Glu^{15}-Leu-His

-Lys-Leu-Gln^{20}-Thr-Tyr-Pro-Arg-Thr^{25}-Asn-Thr-Gly-Ser-Gly^{30}-Thr-Pro^{32}-NH_2

2. Investigations and results

2.1. Rates of degradation of sCT in rat liver and kidney homogenates in vitro

To evaluate contrasts in the rates of metabolic cleavage between the investigated incubation systems, we assessed the metabolic degradation of sCT after incubation with the two main elimination organs homogenates: liver and kidney. Exclusively intact peptide was monitored. Time-courses of the residual amounts of sCT after incubation with rat liver and kidney homogenates *in vitro* are presented in Fig. 1. As a rough approximation, the obtained degradation profiles of sCT were evaluated according to

Table: Rates of proteolytic metabolism of sCT in different rat tissue homogenates

Tissue homogenate	Half life (min)
kidney	13.18
liver	43.07

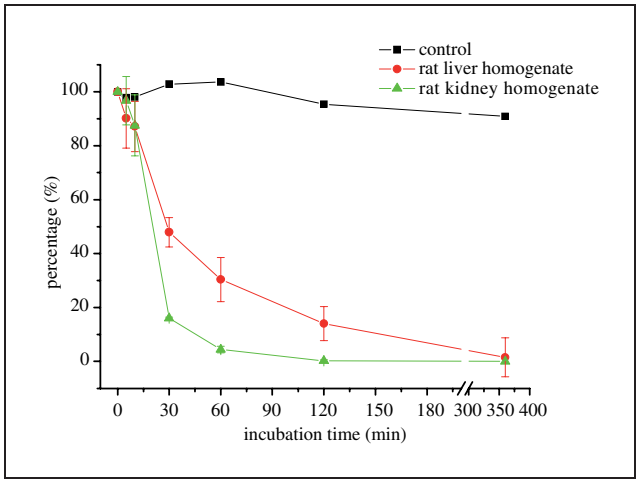

Fig. 1: Disappearance of sCT in rat liver and kidney homogenates. Data are expressed as mean ± SD (n = 3). Final protein concentration of these two homogenates: 2.5 mg protein/ml, final sCT concentration: 10 uM, pH 7.4. Residual rates of sCT at 0 min were considered 100%, and sCT solution without homogenates was used for the control

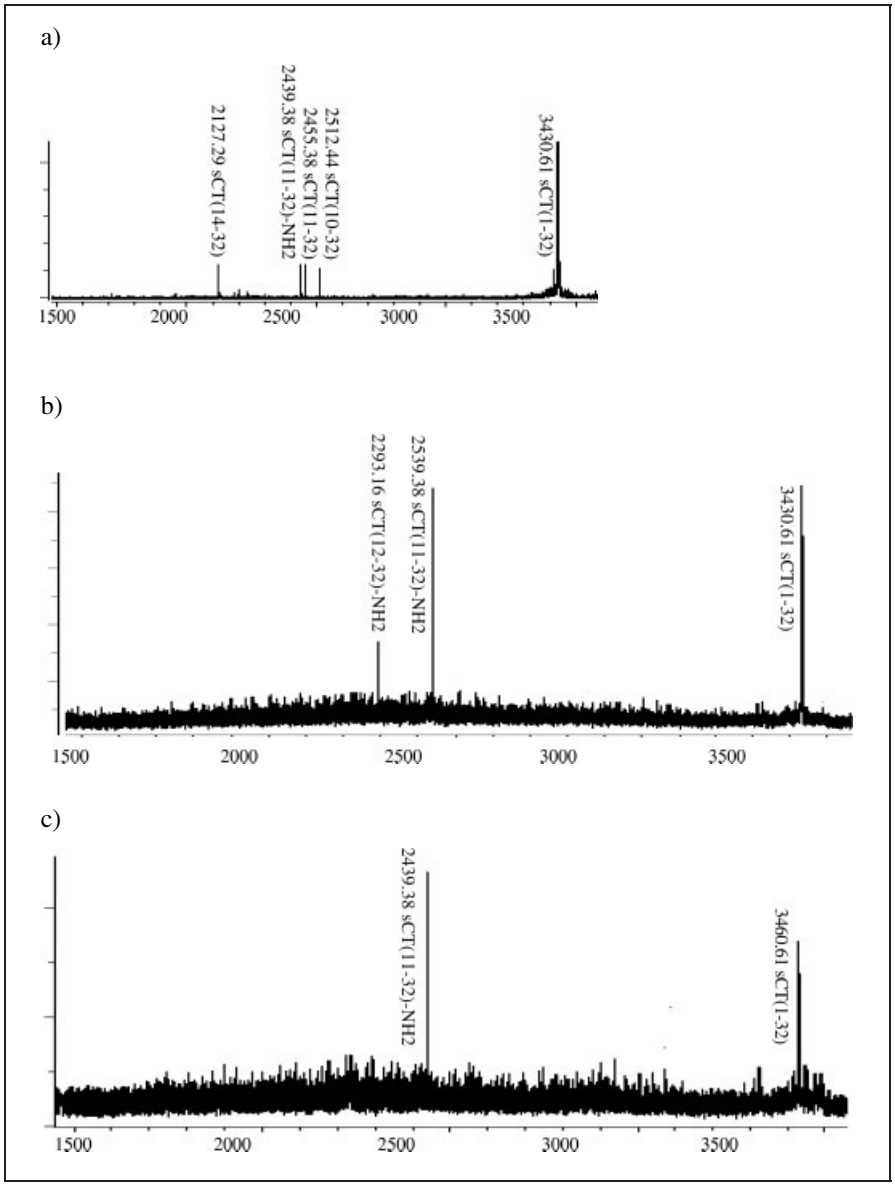

Fig. 2:
MALDI-TOF MS spectrum of degradation products of sCT incubated in the homogenates of rat kidney for (a) 5 min, (b) 60 min, (c) 360 min

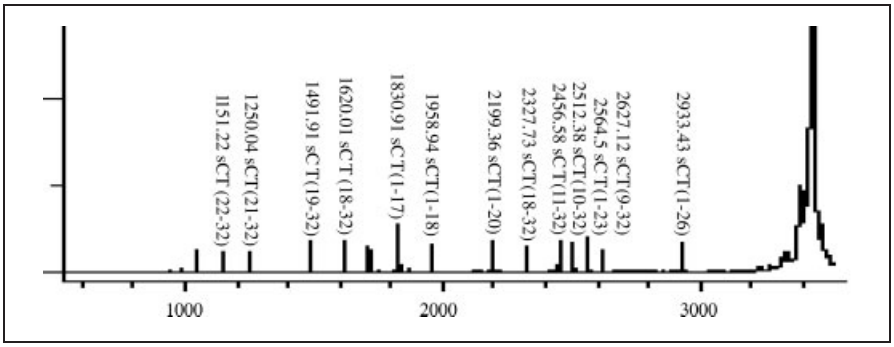

Fig. 3:
MALDI-TOF MS spectrum of degradation products of sCT incubated in the homogenates of rat liver for 10 min

pseudo-first-order kinetics. Normalized half-lives as defined in the experimental model were calculated. The results are given in the Table.

Among the tissues investigated, the kidney homogenate metabolized most rapidly (17% of intact compound left) and was classified as a high metabolic organ. Liver homogenates expressed intermediate activity against sCT (~48% remaining). The disposition rate of sCT was more than three times faster in the kidney compared with those of liver. In control experiments, no degradation was observed for the peptide during 360 min incubation.

2.2. Identification of metabolites of sCT produced in rat kidney and liver homogenate in vitro by MALDI-TOF MS

Spectra from controls showed that these metabolite peaks were neither present in rat kidney and liver homogenates that were not treated with sCT nor in sCT solutions treated under similar conditions but without contact with rat kidney and liver homogenates. These results indicated that the metabolite peaks were produced by enzymatic degradation in rat kidney and liver homogenates. The observed m/z values of the MALDI-TOF MS spectra correspond to $[M + H]^+$.

sCT was readily metabolized in rat kidney homogenate as indicated by various mass peaks of metabolites on the MALDI-TOF mass spectra of sCT incubated in rat kidney and liver homogenates (Figs. 2 and 3).

MALDI-TOF MS analysis was performed by analysis of samples containing metabolites emerging from the degradation of sCT incubated with rat kidney homogenates. From the results obtained, three typical MALDI-TOF MS spectra are shown in Fig. 2. After 5 min, the C-terminal metabolites sCT(10-32), sCT(11-32), sCT(14-32) and C-terminal amide deamidation fragment sCT(11-32) were detected (Fig. 2a). The fragment C-terminal amide deamidation fragment sCT(11-32) was monitored after 360 min (Fig. 2c). The fragments sCT(10-32), sCT(11-32), sCT(14-32) were undetectable after 1 h (Fig. 2b), suggesting that they could have been further degraded.

Fig. 4a illustrates a summary of the suggested cleavage pattern of sCT in contact with rat kidney homogenate. The major initial cleavage sites of sCT in rat kidney seems to occur at two different sites, namely between Leu^9-Gly^{10} and Gly^{10}-Lys^{11} (Fig. 2b), as documented by the C-terminal fragments. In addition to the enzymatic cleavage reaction happened in the sequence of sCT, the degradation fragment was further metabolized by deamidation at the C-terminal amide of sCT (11-32), which is a major and fairly stable degradation fragment in this system, and can be observed during 360 min of incubation with rat kidney homogenate as determined by MALDI-TOF MS (Fig. 2c). Other fragments seemed to originate from the major metabolites or intact sCT by sequential cleavage of aminopeptidase or carboxypeptidase activity; that is, Gln^{14}-Pro^{32}, Lys^{11}-Pro^{32} from Gly^{10}-Pro^{32} (Fig. 2b), Leu^{12}-Pro^{32} deamidation from Lys^{11}-Pro^{32} deamidation.

MALDI-TOF MS analysis was also performed by analysis of samples containing metabolites emerging from the degradation of sCT incubated with rat liver homogenates. From the results obtained after 10 min, representative MALDI-TOF MS spectra are shown in Fig. 3. Initial cleavage seems to occur at two different sites, namely between the His^{17}-Lys^{18} as demonstrated by the concomitant occurrence of N- and C-terminal counterparts, and between residues Val^8- Leu^9 (Fig. 3), documented by the C-terminal fragment. Subsequently, further truncations of

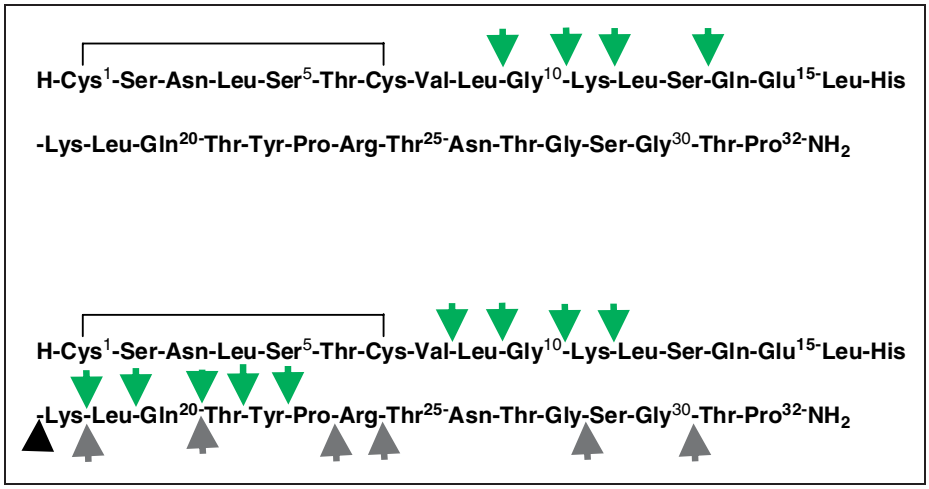

Fig. 4:
Scheme of suggested metabolic cleavage sites of sCT after incubation with rat kidney (a) and liver (b) homogenates.
Arrowheads indicate cleavage sites in sCT. Blank arrowheads, sites of concomitantly detected N- and C-terminal counterparts; grey arrowheads, only N-terminal fragment detected; open arrowheads, only C-terminal fragment detected

initial metabolites or intact sCT by sequential cleavage of aminopeptidase or carboxypeptidase activity, that is, sCT(1-30), sCT(1-28), sCT(1-26), sCT(1-24), sCT(1-23), sCT(1-20), sCT(1-18) and sCT(1-17) from intact sCT; sCT(10-32), sCT(11-32), sCT(12-32) from sCT(9-32); sCT(19-32), sCT(20-32), sCT(21-32), sCT(22-32) and sCT(23-32) from sCT(18–32). The resulting cleavage pattern is depicted in Fig. 4b.

3. Discussion

It has been reported that after intravenous administration, calcitonin is taken up rapidly by the liver and kidney, organs rich in calcitonin receptors (Blower et al. 1998). It is believed that after binding to the receptor, calcitonin is internalized and metabolized to the amino acid components or the peptide fragments by hydrolysis (Schneider et al. 1988). Therefore, in the present study, we investigated the metabolic stability of sCT in different rat tissue homogenates, such as liver and kidney. Furthermore, we identified typical metabolites and the related patterns of metabolic cleavage. Homogenates represent an easy and reliable biological system for metabolism studies in multiple organs and/or across species (Crooke et al. 2000; Ekins et al. 1999), in which enzymes from different metabolic compartments are mixed, and therefore the peptide substrate might be exposed to enzymes which are more abundance than the subcellular fractions isolated from organs.

In the present study, we found that the kidney had the higher metabolic activity toward sCT than liver. sCT involved two major metabolic pathways in rat kidney homogenates. Cleavage at the N-terminal of Gly^{10}, which were in accordance with its cleavage sites found in nasal homogenates (Dong et al. 2004) and cleavage sites found in rat renal cytosolic fraction (Kobayashi et al. 2000). The other pathway is the deamidation of the C-terminal amide of metabolite sCT(11-32), which is often observed for many peptides in kidney as well as in other tissues/*in vivo*. The cleavage position found in our studies, Leu^9-Gly^{10}, was consistent with studies of Kobayashi et al. (2000). Instead of using rat tissue homogenates, their investigation was determined in subcellular fractions isolated from rat liver and kidney, the important eliminating organs. In accordance to the data presented here sCT was shown to be cleaved at the Leu^9-Gly^{10} bond confirming the presence of a chymotryptic endopeptidase activity in rat kidney.

The importance of endopeptidase activity in the enzymatic degradation of sCT has been reported in previous studies (Kobayashi et al. 2000). Lang et al. (1996) reported that in rat nasal homogenate, sCT was degraded by tryptic-like endopeptidases, and the subsequent metabolic breakdown follows the sequential pattern of aminopeptidase activity. The Cys1-Cys7 loop structure of sCT may lead to perfect stability of the N-terminus against aminopeptidase activity. Furthermore, Kabayashi et al. (2000) observed that sCT incubated with the rat kidney cytosolic fractions was degraded as N-terminal metabolites, such as sCT(1-10) and sCT(1-9). Similiarly, we observed the subsequent digested fragment by aminopeptidase from the major degradation fragment in our studies.

Kabayashi et al. (2000) proposed the absence of the primary metabolites of sCT in rat liver. Whereas, in the present study, we found some metabolites in liver homogenate, indicating that the rate-determining step in the metabolism is endoproteolytic hydrolysis, and that the subsequent exoproteolytic digestion of the primary metabolites is very rapid. This difference may be explained by the experimental models applied. In the organ homogenate, enzymes from different metabolic compartments are mixed, and therefore the peptide substrate might be exposed to enzymes which are more abundant than the subcellular fractions isolated from organs. Since both the N- and C-termini of sCT are blocked, calcitonin is considered to be resistant to the exoproteolytic hydrolysis. Production of the metabolites primarily by the endoproteolytic hydrolysis supports this opinion.

In conclusion, in this study, the metabolite of the peptide hormones, sCT, produced by rat kidney and liver homogenates *in vitro* was successfully identified by MALDI-TOF MS. The results indicated that the metabolism of sCT proceeds by initial endoproteolytic cleavage and subsequent exoproteolytic digestion.

4. Experimental

4.1. Chemicals

sCT (Mr 3432, purity >99%, HPLC) was synthesized by our institute and was stored in a deep freezer (−70 °C). HPLC grade acetonitrile was obtained from Fisher (Fair Lawn, NJ, USA). Analytical grade trifluoroacetic acid (TFA) were purchased from Sigma (St, Louis, MO, USA). HPLC gade water was doubly purified with a Milli-Q (Millipore, Molsheim, France) system. α-Cyano-4-hydroxycinnamic acid was delivered by Sigma-Aldrich (Steinheim, Germany). All other reagents were of the highest grade commercially available.

4.2. MALDI-TOF MS

Mass spectrometric detection of peptides from prepared homogenates samples were carried out in the reflection positive ion mode on a Bruker REFLEX III MALDI-TOF-MS (Bruker-Franzen, Bremen, Germany). Samples were mixed with an equal volume of matrix solution (a-cyano-4-hydroxycinnamic acid) following the common protocol of the dried-droplet technique. External mass calibration of the mass spectrometer was performed with the peptide standards bradykinin, human secretin and ubiquitin (Sigma, St Louis, MO, USA).

4.3. HPLC-ESI-MS

LC experiments were conducted using a Finnigan Surveyor™ HPLC system (Thermo Electron, San Jose, CA, USA). Separation of sCT was achieved using a 100 × 2.1 mm SB300C18 column (Angilent, USA) packed with on-line filters. Mobile phase compositions were [A] 0.1% (v/v) TFA in water and [B] acetonitrile. A linear gradient method was employed where the percent B mobile phase was varied from 10% at t = 0.0 min to 70% at t = 15.0 min. After a one minute hold at 70% B, the column was recycled to 10% B and equilibrated for 10 min. The flow rate during the gradient separation was 0.2 mL/min and column equilibration. The injection volume for all LC experiments was 10 μL.

A Finnigan TSQ Quantum Discovery (Thermo Electron, San Jose, CA, USA) was used for detection of sCT. Both Finnigan Surveyor™ HPLC system and Finnigan TSQ Quantum Discovery were controlled using Xcalibur® version 1.2 software. The optimal mass spectrometer parameters for the Finnigan TSQ Quantum Discovery (Thermo Electron, San Jose, CA, USA) were as follows:

Source: ESI; ion polarity: positive; ESI needle voltage: 4800 V; sheath gas pressure: 35 arb units; auxillary gas pressure: 5 arb units; ion transfer capillary temperature: 250 °C; tube lens offset: −90 V (at m/z 859); source CID offset: 20 V; scan mode: selected ion monitoring.

4.4. Preparation of homogenates of rat liver and kidney

Male Sprague-Dawley rats (240–260 g) were purchased from the Beijing Vital River Laboratories (Beijing, China). The animals were fasted overnight prior to the study but had free access to water. Three Sprague-Dawley male rats were exsanguinated and decapitated. The livers and kidneys were quickly removed and immediately collected.

Excised tissue was weighed, minced and suspended in a 4-fold volume of ice-cold incubation buffer (50 mM Tris-HCl pH 7.4). Tissue was homogenized using a Teflon digital homogenizer, then centrifuged (2000 g) for 10 min at 4 °C. The supernatant was stored at −80 °C until further analysis. *In vitro* assays were performed within 8 days of tissue collection.

Protein concentration in tissue homogenates was quantified using the bicinchoninic acid (BCA) colorimetric assay (BCA Protein Assay Kit, Pierce Biotechnology, Inc, Rockford, Ill, USA) with bovine serum albumin as a reference standard. Homogenates were diluted to 2.5 mg protein/ml as necessary.

4.5. Experimental procedures

Rate of metabolism of sCT was measured as the rate of decrease of the parent compound in the presence of kidney and liver homogenates. The homogenates were diluted with Tris-HCl (50 mM, pH 7.4) buffer (final protein concentration 2.5 mg protein/ml). A 2 ml aliquot of each fraction was pre-incubated for 15 min at 37 °C, and the reaction was initiated by addition of sCT at a final concentration of 10 μM. The reaction was stopped at 0, 5, 10, 30, 60, 120, and 360 min by the addition of a 4-fold volume of ice-cold acetonitrile. The reaction mixture was centrifuged at 10,000 × g in an Eppendorf centrifuge, and the supernant fraction was evaporated to dryness under nitrogen and then stored at −20 °C until analysis. The residue was re-dissolved in a 100 μl volume of the HPLC mobile phase and analyzed by LC-ESI-MS for the metabolism rate and MALDI-TOF MS for the metabolite identification.

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