Enhancement of Nasal Salmon Calcitonin Absorption by Lauroylcarnitine Chloride in Rats

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Purpose. We investigated optimum formulation characteristics in the nasal absorption of salmon calcitonin (sCT) by incorporation of acylcarnitines.

Methods. Nasal sCT formulations were administered to anesthetized rats. Plasma calcium level was measured and pharmacological bioavailability (P.bioav) was calculated.

Results. Nasal sCT absorption was significantly enhanced by carnitines with acyl groups of 12 or more carbon atoms. Enhancement by lauroylcarnitine chloride (LCC) was observed at its critical micelle concentration and reached a plateau at the concentration of 0.1%. Optimal absorption was achieved at a molar ratio of LCC to sCT of 5:1. Enhancement was not influenced by osmolarity and maximum enhancement was obtained at pHs 3.1 and 4.0.

Conclusions. The 12-carbon LCC was the strongest enhancer among acylcarnitines. Micelle formation played a key role in this enhancement effect.

KEY WORDS: salmon calcitonin; nasal absorption enhancer; lauroylcarnitine; micelle formation.

INTRODUCTION

Calcitonin, a 32-amino acid sequence polypeptide hormone, is a therapeutic agent used in the treatment of hypercalcemia, Paget's disease and osteoporosis. Relative bioavailability of salmon calcitonin (sCT) in humans was recently reported to be poor, at only 1.6% that of intramuscular preparation (1).

To enhance the nasal absorption of sCT, various enhancers have been investigated including bile salts, sodium dihydrofusinate (2) and sodium tauro-24,-25-dihydrofusidate (1). However, most of these are associated with side effects such as irreversible changes in the nasal membrane. In a previous paper the authors suggested that some acylcarnitines may be potent nasal absorption enhancers (3).

The purpose of this study was to optimize the incorporation of acylcarnitines in the nasal absorption of sCT by investigating formulation characteristics such as the number of carbon atoms in acylcarnitines, acylcarnitine concentration, sCT dose, osmolarity and pH. We also wanted to speculate the mechanism by which acylcarnitines enhance the nasal absorption of sCT.

Acylcarnitines are endogenous amino acid-like compounds which play a role in the mitochondrial transport system in cells by carrying fatty acids across the mitochondrial membrane (4). Palmitoylcarnitine chloride (PCC, n = 16), for example, significantly enhances the in vivo mucosal absorption of poorly absorbed drugs after intestinal, rectal (5), vaginal (6), and nasal (3) administration in animals, although little is known about the effect of other acylcarnitines on nasal absorption or about the mechanism of this effect. The safety of PCC in intestinal mucosa has been demonstrated, with reversible enhancement of absorption and no induction of morphological changes (5, 8).

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MATERIALS AND METHODS

Chemicals

Salmon calcitonin (sCT) lot. Y2604 (4323 IU/mg) was obtained from Armour Pharmaceutical Co., Ltd. (now Rhône-Poulenc Rorer Ltd., USA). DL-Carnitine hydrochloride (CH), DL-hexanoylcarnitine chloride (HCC), DL-octanoylcarnitine chloride (OCC), DL-lauroylcarnitine chloride (LCC), myristoyl-DL-carnitine chloride (MCC), and palmitoyl-DL-carnitine chloride (PCC) were purchased from Sigma Chemical Co., Ltd. (USA). All other reagents used were of reagent grade.

Preparation of Drug Solutions

The basic formulation contained sCT (350 IU/ml) in isotonic citrate buffer (0.1 M, pH 4.0) and benzalkonium chloride (0.01%) and EDTA disodium salts (0.01%) as preservatives. After incorporation of sCT and carnitines, all solutions were adjusted for pH with NaOH or HCl and concentration according to each experiment. PCC was incorporated in the basic formulation before use. Osmotic pressure of all solutions was measured with an OM-6030 Osmotic Pressure Auto & Stat® (Kyoto Daichi Kagaku Co., Japan) and adjusted with NaCl.

Animal Experiments

All experiments were conducted in adherence to the "Principles of the Animal Ethical Committee of Yamanouchi Pharmaceutical Co., Ltd." Male Sprague-Dawley rats (202–244 g, 7 weeks) were fasted for 20 h before administration. They were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal®; Abbott Laboratories, USA) at a dose of 50 mg/kg 10 min before administration and maintained with additional injections at 40 mg/kg of sodium pentobarbital. The nasopalatine was closed with a cyanoacrylate adhesive agent (Aron Alpha A®, Sankyo Co., Japan). No surgical operation was conducted. For intranasal administration, polyethylene tubing (PE10) connected to a 5-μl micro-syringe was inserted about 4 mm into the right nasal cavity. The sCT solution (14.3 μl/kg) was administered over a 30 sec. For subcutaneous administration, sCT solution (1.43 ml/kg) was injected under the back skin of the dorsum. Blood was taken periodically from the jugular vein with a heparinized syringe (0.15 ml/sample; total 0.75 ml). Plasma was separated by centrifugation at 3000 rpm for 10 min. Fifty microliters of plasma was used for plasma calcium assay with a Calcium E Test Wako® (Wako Pure Chemical Industries Ltd., Japan).

Calculation of Pharmacological Bioavailability

Plasma calcium level was calculated as a percentage of plasma calcium concentration as measured before administra-

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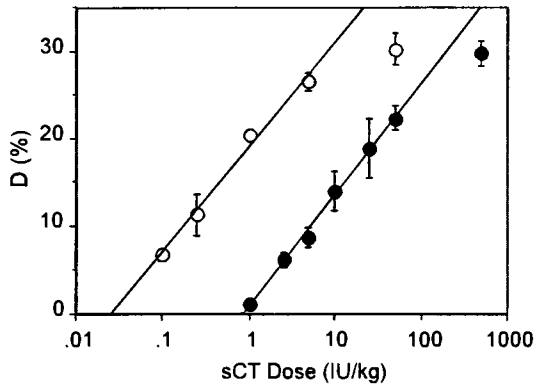

Fig. 1. Relationship between dose and pharmacological effect of salmon calcitonin after subcutaneous and intranasal administrations in rats. (○) subcutaneous (0.1–5 IU/kg):D = 11.91 × log(dose) + 18.88, $\gamma = 0.994$, $p < 0.01$, (●) intranasal (1–50 IU/kg): D = 12.53 × log(dose) + 1.27, $\gamma = 0.999$, $p < 0.01$. These lines were statistically parallel on analysis of covariance ($p < 0.05$). Each point represents the mean ± SE of 3 to 7 rats.

tion. The decrease in plasma calcium level for 0 to 4 h (D, total decrease) was calculated by a modification of Hirai *et al.* (9):

$$D(\%) = \frac{AUC_b - AUC_p}{AUC_b} \times 100 \quad (1)$$

where AUC_b is the area under the plasma calcium level versus time curve after administration of the basic formulation without sCT and AUC_p is that of the sCT formulation. In the dose range of 0.1–5 IU/kg and 1–50 IU/kg (subcutaneous (sc) and intranasal (in) administration, respectively), the plot of the logarithmic dose against D gave a straight line (Fig. 1) as follows:

$$D_{sc} = 11.91 \times \log(Dose_{sc}) + 18.88 \ (\gamma = 0.994, p < 0.01) \quad (2)$$

$$D_{in} = 12.53 \times \log(Dose_{in}) + 1.27 \ (\gamma = 0.999, p < 0.01) \quad (3)$$

where D_{sc} and D_{in} are the D values of sCT administered subcutaneously and intranasally, respectively, and $Dose_{sc}$ and $Dose_{in}$ are their respective doses. These lines were statistically parallel on analysis of covariance ($p < 0.05$). No experimental D_{in} value obtained exceeded the maximum value of 26.5% at 5 IU/kg of the sc dose, at which the line was straight. Therefore, pharmacological bioavailability (P.bioav) of sCT on in to sc administration can be defined by the following equation:

$$P.bioav(\%) = \frac{D_{in}/Dose_{in}}{D_{sc}/Dose_{sc}} \times 100 \quad (4)$$

$Dose_{sc\_calc}$ is defined as the calculated sc dose which induces the same D_{sc} as D_{in} obtained with in administration at $Dose_{in}$. Equation (2) gives

$$Dose_{sc\_calc} = 10^{((Din - 18.88)/11.91)} \quad (5)$$

Inserting $D_{sc} = D_{in}$ and $Dose_{sc} = Dose_{sc\_calc}$ into equation (4) gives

$$P.bioav(\%) = \frac{Dose_{sc\_calc}}{Dose_{in}} \times 100 \quad (6)$$

Inserting equation (5) into equation (6) gives

$$P.bioav(\%) = \frac{10^{((Din - 18.88)/11.91)}}{Dose_{in}} \times 100 \quad (7)$$

Equation (7) gives the P.bioav of sCT on in to sc administration in the dose range of 1–50 IU/kg of nasal sCT used in this study.

DATA ANALYSIS

All data are expressed as the mean ± SE. Test and control values were statistically analyzed by Student's *t* test. To identify the source of any differences found, multiple comparisons of data were made by Scheffé's test using SAS program.

RESULTS AND DISCUSSION

Effect of Structural Differences in Acylcarnitines on Nasal Absorption of sCT

Figure 2 shows the pharmacological bioavailability (P.bioav) of 0.1% acylcarnitines with the number of carbon atoms in the acyl chain (n) as follows: n = 0 (CH), n = 6 (HCC), n = 8 (OCC), n = 12 (LCC), n = 14 (MCC), and n = 16 (PCC). CH induced little change in P.bioav. The P.bioav of CH and control (basic formulation) were 3.15 ± 0.70% and 4.30 ± 0.81%, respectively. Further, no statistically significant difference was seen among the P.bioavs of solutions containing CH, HCC and OCC. However, LCC, MCC and PCC strongly enhanced P.bioav, with respective values of 40.8 ± 2.0%, 24.5 ± 5.1%, and 28.5 ± 2.0% (LCC > PCC > MCC).

Similar effects for longer-chain acylcarnitines were reported in a rat rectal absorption study: cefoxitin was enhanced by longer chain acylcarnitines at 2% with a rank order of PCC > LCC > MCC (5). Further, PCC potentially enhanced absorption of gentamicin administered vaginally (6) and sCT administered nasally (3) to rats. In contrast, however, Fix *et al.* (5) observed a more erratic pattern on the rectal absorption of somatostatin analog; 2% of all acylcarnitines with 2–18 carbon atoms in the acyl chain except OCC (n = 8) enhanced absorption. These differences in enhancement by acylcarnitines might be due to differences in formula-

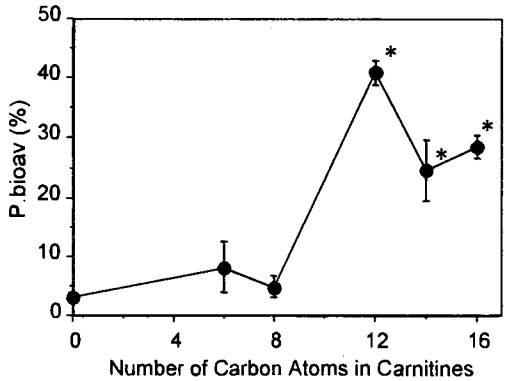

Fig. 2. Effect of the number of carbon atoms in the carbon chain of carnitines on nasal absorption of sCT (5 IU/kg) with 0.1% carnitines in rats. (*) Significantly different from C0, C6, and C8 by Scheffé's multiple comparison ($p < 0.05$). Each point represents the mean ± SE of 3 to 6 rats.

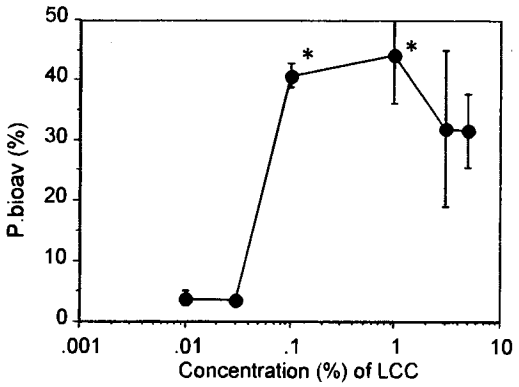

Fig. 3. Dependency of nasal sCT absorption on the concentration of lauroylcarnitine chloride (LCC) at a dose of 5 IU/kg in rats. (*) Significantly different from 0.01% and 0.03% by Scheffé's multiple comparison ($p < 0.05$). Each point represents the mean ± SE of 3 to 5 rats.

tion (drugs, buffer conditions) or in absorption site (nasal, intestinal, rectal, vaginal).

In order to optimize formulation characteristics in the nasal absorption of sCT, LCC was selected as the most preferable enhancer for the following reasons: (a) LCC had the best enhancing effect among the acylcarnitines tested (Fig. 4); (b) the formulation containing LCC was stable, while PCC (0.1%) exhibited precipitation not only in the basic formulation but also in simple isotonic buffer solution (pH 7.4) after storage for 1 day at 5°C or room temperature; and (c) LCC showed more rapid recovery of transepithelial electrical resistance in rat colonic mucosa than PCC, producing a histological evaluation value nearly equal to that of controls in contrast to PCC (7).

Optimal Concentration of LCC for Nasal Absorption of sCT

P.bioavs of 0.1% and 1% LCC were significantly greater than those with 0.01% and 0.03% ($p < 0.05$) (Fig. 3). However, this enhancement declined at the concentrations of 3% and 5%. As maximum enhancement was obtained at 0.1%, this concentration was used to investigate the dose-dependency of nasal absorption of sCT (1–50 IU/kg) as shown in Fig. 4. D reached plateau at 5 IU/kg of sCT and did not have clear dose-dependency. Optimal sCT absorption was achieved at a molar ratio of LCC to sCT of 5:1. Interestingly, this result is consistent with the optimal nasal absorption for insulin in sheep at a molar ratio of sodium taurodihydrofusidate to insulin of 5:1 (10).

Effects of Osmolarity and pH on Nasal Absorption of sCT with or Without LCC

According to pathological condition, osmotic pressure in the nasal mucosa varies from hypotonic to hypertonic. Figure 5 shows the effect of osmolarity on the nasal absorption of sCT with or without 0.1% LCC. Osmotic pressure ratio was defined as the ratio of sample osmotic pressure to isotonic osmotic pressure (289 mOsm/kg). With an increase in osmolarity from 0.7 to 5.0, P.bioav in the absence of LCC increased from 3.48 ± 0.86% to 13.0 ± 2.9%. In contrast, P.bioav in the presence of 0.1% LCC ranged from 38.2% to 48.1%. P.bioav was significantly increased in all formulations with varying osmolarity compared to those without LCC ($p < 0.05$), but no significant differences existed among the formulations which incorporated LCC.

sCT is known to be stable only in acidic conditions. In the absence of LCC, nasal sCT absorption decreased with increasing pH; P.bioav was 15.5 ± 1.3% at pH 2.5, but only 0.48 ± 0.05% at pH 8.2. With the addition of 0.1% LCC, P.bioav was significantly improved at every pH compared to those without LCC ($p < 0.05$). Optimal P.bioav was obtained at pHs 3.1 and pH 4.0, with values of 43.5 ± 6.0% and 40.8 ± 2.0%, respectively.

It was concluded that incorporation of 0.1% LCC at a molar ratio of LCC to sCT of 5:1 in isotonic citrate buffer at pH 4.0 was the optimum nasal sCT formulation. This optimized formulation was physically stable and no significant change in P.bioav was observed on storage for more than 1 year (13 months) at 5.0°C.

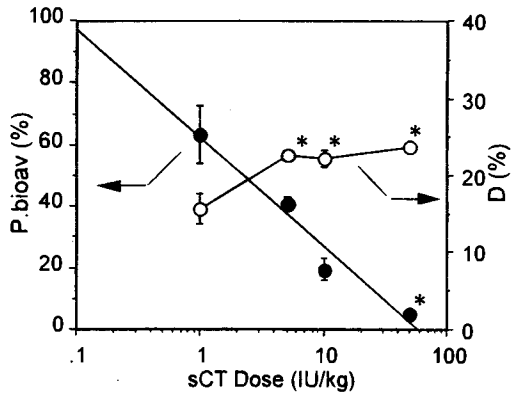

Fig. 4. Effect of sCT dose at a concentration of 0.1% of lauroylcarnitine chloride(LCC) on nasal sCT absorption in rats. (●) pharmacological bioavailability: P.bioav (%) = −35.2 × log(sCT dose) + 62.0 ($r = 0.980$, $p < 0.05$). (○) decrease in plasma calcium level (D). * Significantly different from 1 IU/kg by Scheffé's multiple comparison ($p < 0.05$). Each point represents the mean ± SE of 3 to 5 rats.

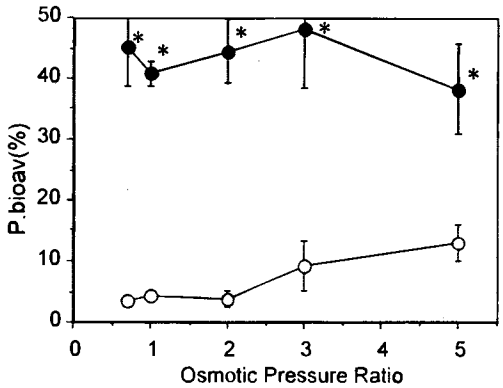

Fig. 5. Effect of osmolarity on the enhancement of nasal absorption of sCT (5 IU/kg) by lauroylcarnitine chloride (LCC) in rats. (●) with 0.1% LCC and (○) without LCC. Osmotic pressure ratio (x-axis) was calculated as 289 mOsm/kg was 1. (*) Significantly different ($p < 0.05$) from the same osmotic pressure ratio without LCC by Scheffé's multiple comparison. Each point represents the mean ± SE of 3 to 5 rats.

Mechanism of Nasal Absorption Enhancement of sCT by Acylcarnitines

Yalkowski *et al.* (11) reported that the critical micellar concentration (CMC) of acylcarnitines in 0.2 M KCl (25°C) was as follows: OCC, 3.3%; LCC, 0.029%; MCC, 0.0037%; and PCC, 0.00037%. Notably, we found that acylcarnitines only promoted the nasal absorption of sCT at the concentration of 0.1% which was greater than their CMCs (Fig. 2). Furthermore, LCC showed clear dose-dependency in its enhancing effect on sCT absorption corresponding to its CMC (0.03%) and reached maximum at 0.1%, about 3 times the CMC (Fig. 3). Interestingly, these results are consistent with those obtained with different enhancers for insulin: enhancement by sodium deoxycholate in humans (12) and sodium taurodihydrofusidate in sheep (10) was triggered at their CMCs and plateaued at 3–4 times these values. Optimal P.bioav was achieved between pH 3.1 and 4.0 (Fig. 6). Because LCC has a pK value for the zwitterionic micelle of about 4.85, the cationic micelle of LCC at low pH may be better able than neutral micelles to interact with the negative charge of the nasal epithelium. However, this enhanced absorption by LCC was not affected by variation in osmolarity (Fig. 5). This result may reflect the finding that the surface potential of LCC micelle decreases only slightly with a high degree of protonation under an osmotic pressure ratio range of 0 to 2.1 (11). Thus, micelle formation is considered to be an important factor in the mechanism of enhancement of acylcarnitines on the nasal absorption of sCT.

The contribution of inhibition of proteolytic enzyme by acylcarnitine to the transport seems low; maximum inhibition by aprotinin (trypsin inhibitor) was very recently reported only 1.4 times enhancement in the nasal sCT absorption at pH 4.0 (13) which is difficult to explain 9.5 times enhancement by LCC. Regarding the transcellular route, Gordon *et al.* (12) proposed that sodium deoxycholate enhances nasal insulin absorption via the formation of polar channels within nasal membranes with sodium deoxycholate reverse micelles. LeCluyse *et al.* (14) reported that long-chain acylcarnitines perturb the lipid structure of the brush border membrane of rat small intestine. In our study, the finding of saturation of sCT transport with LCC suggests that local membrane saturation or interaction is involved. Paracellular transport involvement was recently reported (8). In a human adenocarcinoma cell line in vitro, PCC decreased transepithelial electrical resistance reversibly and enhanced the absorption of hydrophilic markers by a paracellular route. PCC also caused transient structural alterations to the tight junction. These effects of PCC were Ca ion-dependent, and it had no disruption of actin filaments distribution in Caco-2 cells. On the contrary, it may decrease the permeability of tight junction which relates to the contraction of the actomyocin complex because PCC inhibits protein kinase C (15). These experiments were conducted at the concentration beyond the CMC but strongly support our postulation that micellar formation of LCC is a key mechanism of its enhancing effect of nasal sCT.

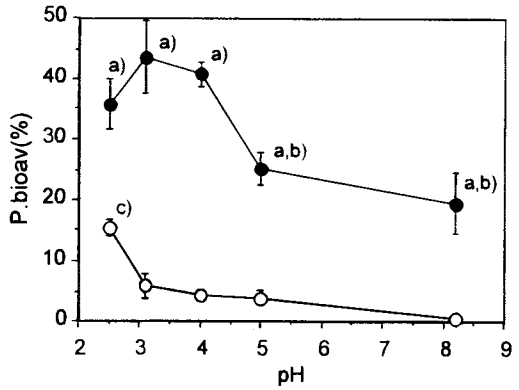

Fig. 6. Effect of pH on the enhancement of nasal absorption of sCT (5 IU/kg) by lauroylcarnitine chloride (LCC) in rats. (●) with 0.1% LCC and (○) without LCC. Significantly different ($p < 0.05$) from a) the same pH without LCC, b) pH 3.1 with LCC, and c) pH 8.2 without LCC by Scheffé's multiple comparison. Each point represents the mean ± SE of 3 to 7 rats.

In conclusion, the 12-carbon LCC, the optimal enhancer for sCT absorption, had the strongest enhancing effect only at the concentration of 0.1%. This enhancement was not influenced by osmolarity and pH. The LCC formulation was stable, with no precipitation for more than 1 year on storage at 5°C. It is also suggested that acylcarnitines micelle formation plays a key role in their enhancement of the nasal sCT absorption.

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