Mechanism of Nasal Absorption of Drugs. III: Nasal Absorption of Leucine Enkephalin

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Abstract □ The nasal absorption of a model peptide, leucine enkephalin (LE), was studied in rats using an in situ technique in which 4 mL of perfusion solution was circulated. Leucine enkephalin (LE) was found to undergo hydrolysis to its major metabolite *des*-tyrosine leucine enkephalin (DTLE). The addition of 1% sodium glycocholate (SGC) to the perfusion solution resulted in an increase in the overall rate of disappearance of LE and a decrease in the rate of formation of DTLE. When LE was added to nasal washings (i.e., Ringer's buffer that was precirculated through the nasal cavity to extract enzymes), LE was found to form DTLE. When SGC or puromycin was added to the nasal washings prior to the addition of LE, the rate of conversion of LE to DTLE was significantly reduced, suggesting that these two agents can inhibit peptidase enzyme activity in the nasal cavity. Since the volume of the solution has been shown to influence the kinetics of absorption of drugs administered nasally, a new experimental technique, the in vivo-in situ technique, which utilizes small volumes of solution and simulates realistic use of nose drops, was employed to further examine the mechanism of absorption and hydrolysis of LE in rats. Leucine enkephalin (LE) dissolved in 100 μL of Ringer's buffer was placed in the isolated nasal cavities of rats. The disappearance of LE and the appearance of DTLE were followed by rinsing the nasal cavity with fresh buffer. Disappearance of LE was always accompanied by appearance of DTLE, and the fraction of LE converted to DTLE decreased as the concentration of LE increased, suggesting a saturable enzymatic process. In the presence of the small peptides, L-tyrosyl-L-tyrosine and tri-L-tyrosine methyl ester, the hydrolysis of LE was reduced and less DTLE was formed, suggesting that the competitive inhibition of the nasal peptidases was caused by these small peptides. The results also suggested that as much as 30% of LE was absorbed in the first 10 min. In the presence of 1% SGC, the formation of DTLE was greatly reduced and the absorption of LE was greatly enhanced. After 10 min, no LE or DTLE remained in the nasal cavity, suggesting that SGC markedly inhibits enzymatic hydrolysis of LE and greatly accelerates absorption of both LE and DTLE into the systemic circulation.

Peptide drugs are among the most promising medicinal agents of modern times, but their hydrolytic instability in the gastrointestinal tract usually requires that they be administered parenterally. Although patients can be taught to inject themselves, it would be highly desirable to develop a noninvasive method for self administration of peptides. Nasal delivery has been considered as an alternative to the injection of peptides.

Recent data generated in our laboratories[1] and by other researchers[2-7] indicate that for peptides placed in the nasal cavity the rate of absorption is relatively rapid, the extent of absorption is small compared with other nasally administered drugs, and the bioavailability can be enhanced by coadministration of bile salts.

Recently,[8,9] it was shown that low concentration of dipeptides, such as L-tyrosyl-L-tyrosine and the pentapeptide leucine enkephalin (LE); Tyr-Gly-Gly-Phe-Leu), undergo rapid enzymatic hydrolysis in the nasal perfusate of rats. It was also shown[9] that the hydrolysis of LE in washings from the nasal cavities of rats was inhibited in the presence of peptidase labile peptides such as L-tyrosyl-L-tyrosine or L-tyrosyl-glycine.

In this report, the mechanism of nasal absorption and/or hydrolysis of LE is examined in detail. Specifically, the effects of the concentration of LE, of peptidase inhibitors, and of sodium glycocholate (SGC) on the stability and/or absorption of LE in the nasal cavity of the rat are reported.

Experimental Section

Chemicals and Equipment—Leucine enkephalin, *des*-tyrosine leucine enkephalin, sodium glycocholate, puromycin, L-tyrosyl-L-tyrosine, and try-L-tyrosine methyl ester were purchased from Sigma Chemical Company, St. Louis, MO. The high-performance liquid chromatography system consisted of a Waters model 6000A solvent delivery system, a Varian Vari-Chrom detector, a Rheodyne injector, and a Fisher Recordal Series 5000 recorder.

Animal Studies—*In Situ Nasal Experiment*—The surgical procedure and the experimental design for the in situ nasal absorption experiments were the same as those described by Huang et al.[10] A 4-mL portion of the drug solution was placed in a water-jacketed beaker maintained at 37 °C. The solution was circulated through the nasal cavity of the rat at a flow rate of 0.7 mL/min by means of a peristaltic pump. The perfusion solution was passed through the nasal cavity, out the nostrils, through a funnel, and returned to the water-jacketed beaker. The solution in the beaker was stirred constantly using a magnetic stirrer.

Samples of the drug solution were withdrawn with a micropipette. The extent of hydrolysis and/or absorption was determined over a period of 1 h by periodically analyzing the amount of drug remaining in the perfusion solution.

Perfusion Solutions—Ringer's buffer solution at pH 6.0 was prepared. Leucine enkephalin (LE) was dissolved in the above buffer to make a 60-μg/mL solution. *des*-Tyrosine leucine enkephalin (DTLE) was dissolved in the above buffer to make a 60-μg/mL solution. Sodium glycocholate (SGC; 1%) was added to the LE solution.

Recovery of Nasal Washings—Four milliliters of freshly prepared Ringer's buffer (pH 6.0; 37 °C) was circulated through the nasal cavity of a rat for 60 min. The washings were collected and stored in a refrigerator (5 °C) until used.

In Vitro Hydrolysis of Leucine Enkephalin in Nasal Washings—One milliliter of LE solution (60 μg/mL; 1.26×10^{-4} M) was added to 1 mL of nasal washings, and the mixture was incubated at 37 °C. Samples were withdrawn at 0, 10, 20, 30, 40, and 60 min, quickly frozen, and stored until analyzed for LE and DTLE using the HPLC method described below.

In Vitro Hydrolysis of LE in Nasal Washings in the Presence of Sodium Glycocholate—Solutions were prepared containing LE (60 μg/mL; 1.26×10^{-4} M) with SGC at concentrations of 1.25, 2.5, 5.0, and 10.0%. One-tenth-milliliter aliquots of these solutions were mixed with 0.2 mL of the nasal washings, incubated for 60 min at 37 °C, and analyzed for LE and DTLE using the HPLC method described below.

In Vitro Hydrolysis of Leucine Enkephalin in Nasal Washings in the Presence of Puromycin—Solutions were prepared containing LE (60 μg/mL; 1.26×10^{-4} M) with puromycin at concentrations of 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, and 1×10^{-6} M. One-tenth-milliliter aliquots of these solutions were mixed with a 0.2 mL of the nasal washings,

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incubated for 60 min at 37 °C, and analyzed for LE and DTLE using the HPLC method described below.

In Vivo-In Situ Nasal Experiment—Although it is most desirable to determine extent of absorption from plasma concentrations versus time profiles after nasal administration, lack of a reliable method of analysis for LE in rat plasma made it necessary to utilize the residual amounts of LE and its metabolite, DTLE, remaining in the nasal cavity for this determination. A new experimental technique, the in vivo-in situ technique, was employed to study the absorption and/or hydrolysis of LE under realistic conditions where small volumes are administered to the nasal cavity.

Figure 1 shows the experimental arrangement for the in vivo-in situ nasal experiments. The surgical procedure was similar to that described for the in situ recirculation studies, except that a glass tube (3 cm long and 0.3 mm in diameter with one end sealed) was inserted into the posterior nasal cavity through the esophagus to keep the solution in the nasal cavity. Prior to administering the drug, the nasal cavity was carefully washed with 10 mL of Ringer's buffer to remove all traces of blood.

One hundred-microliter aliquots of solutions containing LE with and without additives were placed in one nostril by means of a micropipette. At an appropriate time interval, the nasal cavity was rinsed with 3.9 mL of Ringer's buffer using a peristaltic pump, and the experiment was terminated.

To determine the validity of this technique and the efficiency of recovery of unabsorbed drug after nasal administration, 100-μL aliquots of solutions containing various concentrations of LE were placed in the nasal cavity. Immediately (within 1 min), the nasal cavity was washed with 3.9 mL of Ringer's buffer using a peristaltic pump. The volume was brought to 5 mL with normal saline, and the rinsings were analyzed. Up to 1 min following nasal administration, the rinsings contained a total of 100% of the administered dose in the form of LE and DTLE, as shown in Table I.

Preparation of the Administered Solutions—Leucine enkephalin (LE) was dissolved in Ringer's buffer (pH 6.0) to make 0.2-, 1-, and 25-mg/mL solutions. *des*-Tyrosine leucine enkephalin (DTLE) was dissolved in Ringer's buffer to make a 0.8-mg/mL solution. L-Tyrosyl-L-tyrosine (LTLT) was added to a 1-mg/mL LE solution to make a solution that was 9×10^{-3} M with respect to LTLT. Tri-L-tyrosine methyl ester (TLTME) was added to a 1-mg/mL LE solution to make a solution that was 6.1×10^{-3} M with respect to TLTME. Sodium glycocholate (SGC) was added to a 1-mg/mL LE solution to make a solution that was 1% with respect to SGC.

Analytical Procedures—*Determination of Leucine Enkephalin and des-Tyrosine Leucine Enkephalin during In Situ Nasal Perfusion Studies*—Aliquots (100 μL) of the perfusion solution initially containing 60 μg/mL of LE were withdrawn by means of a micropipette at 0, 5, 15, 20, 30, 40, and 60 min and diluted immediately with 50 μL of 0.2 M citrate buffer (pH 2.3) to quench hydrolysis. A 100-μL aliquot of the resulting solution was injected into a 4.6 × 250-mm column packed with ultrasphere-octyl reversed-phase support (5 μ, Altex Scientific RP-8) and eluted at a rate of 1 mL/min with a mobile phase consisting of 0.1 M phosphate buffer (pH 2.5):acetonitrile (34:10 by volume). The UV detector was set at 205 nm. A typical HPLC chromatogram for nasal perfusion solution containing the LE and DTLE is shown in Figure 2.

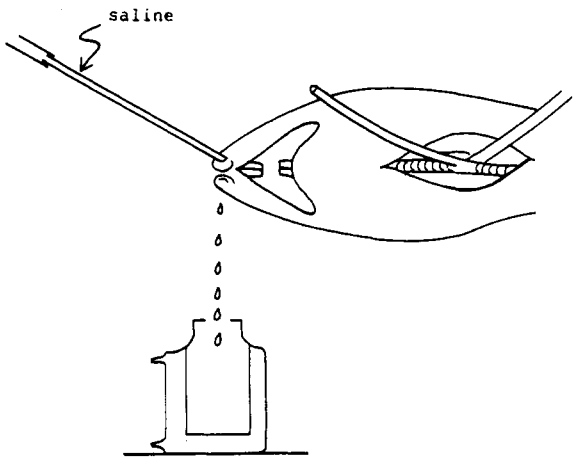

Figure 1—Experimental arrangement for the in vivo-in situ nasal absorption studies.

Table I—Recovery of Leucine Enkephalin (LE) and *des*-Tyrosine Leucine Enkephalin (DTLE) from the Rat Nasal Cavity following Exposure for One Minute or Less to Various Amounts of Leucine Enkephalin (In Vivo-In Situ Technique)[a]

Amount, μg	LE, μg	DTLE, μg	Percent Recovered
20	17.0	2.8	98.0
100	85.2	17.0	102.0
2500	2397.2	52.7	98.4

[a] n = 3.

The concentrations of the peptides in the perfusate were calculated by comparing their peak heights with a standard curve and correcting for dilution.

Determination of Leucine Enkephalin and des-Tyrosine Leucine Enkephalin during In Situ Nasal Perfusion Studies in the Presence of Sodium Glycocholate—Aliquots (100 μL) of the perfusion solution initially containing 60 μg/mL of LE and 1% SGC were withdrawn by means of a micropipette at 0, 5, 15, 20, 30, 40, and 60 mins, diluted with 50 μL of Ringer's buffer solution, and immediately frozen until assayed using the above HPLC method.

Determination of Leucine Enkephalin and Its Metabolite (DTLE) During In Vivo-In Situ Studies—The peptides were determined using the following analytical technique. First, 100-μL aliquots of the nasal washings were quickly diluted to 5 mL with 0.2 M citrate buffer (pH 2.3) to quench the hydrolysis. A 25-μL aliquot of the resulting solution was injected onto a 4.6 × 250-mm stainless steel column packed with octadecylsilane reversed-phase support (Ultrasphere-ODS, 5 μ, Beckman Instruments, Irvine, CA) and eluted at a rate of 0.8 mL/min with a mobile phase consisting of 0.05 M phosphate buffer (pH 3.0):acetonitrile (7:3 by volume). The UV detector was set at 205 nm. The concentrations of the compounds were calculated by comparing their peak heights with standard curves after correcting for dilution.

Results and Discussion

In our previous communication,[9] it was shown that when 4 mL of a solution containing 60 μg/mL of LE (Tyr-Gly-

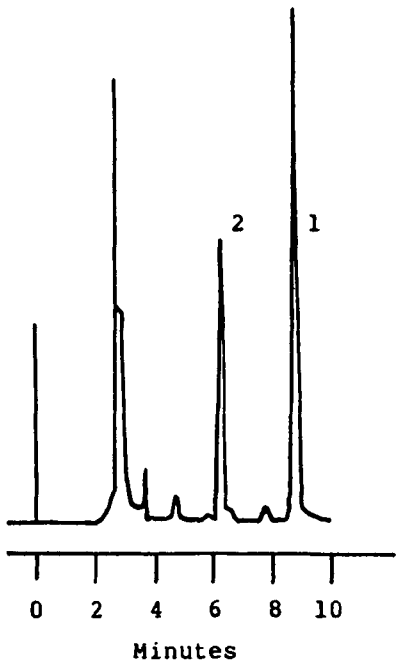

Figure 2—The HPLC chromatogram of nasal perfusion solution 60 min after beginning perfusion with Ringer's buffer containing 60 μg/mL of LE. Key: (1) LE; (2) DTLE.

Gly-Phe-Leu) was circulated through the nasal cavity of a rat, a rapid decline in the concentration of LE occurred; this was accompanied by the appearance of its metabolite DTLE (Gly-Gly-Phe-Leu), which itself subsequently disappeared. It was postulated that at very low peptide concentrations, the overall disappearance of LE and DTLE was the result of the following succession of hydrolytic decomposition reactions:

$$\text{LE} \xrightarrow{k_1} \text{DTLE} \xrightarrow{k_2}$$

Using this kinetic model, excellent agreement between the experimental and calculated data was observed.

In the same study, it was also shown that when Ringer's buffer was precirculated through the nasal cavity of the animal and the resulting mixture (nasal perfusate) was incubated with LE for 1 h at 37 °C, 50% of the LE was lost, primarily to form DTLE. It was also found that the extent of hydrolysis in the nasal perfusate was reduced by the addition of a 20-fold molar excess of small peptides, such as LTLT.[9]

The experiments described in this report were conducted to examine more thoroughly the effects of the concentration of LE, of SGC, and of other peptidase inhibiting agents on the rate and extent of hydrolysis and/or absorption of LE in the rat nasal cavity.

Figure 3 shows that when a 4-mL solution containing 60 μg/mL of LE and 1% SGC is circulated through the nasal cavity, the bile salt not only altered the concentration–time profile of LE but also that of its metabolite DTLE. The differences in these profiles could be the result of one or more of the following effects attributable to SGC: (*1*) enhanced absorption of LE; (*2*) enhanced absorption of the DTLE; and/or (*3*) decreased formation of DTLE. It has been shown that SGC is an excellent enhancer of peptide absorption[3] and also an inhibitor of peptidases.[7]

To determine whether SGC affects the hydrolysis of LE in the nasal washings, we added various concentrations of SGC to nasal washings (precirculated buffer) and then studied the in vitro disappearance of LE and the appearance of DTLE in the washings. The results shown in Figure 4 indicate that SGC produces significant inhibition of the conversion of LE to DTLE at concentrations as low as 0.42%. The mechanism whereby the bile salt causes inhibition of hydrolysis is not clear at this time, but such inhibition was postulated by Hirai et al.[7] to be due to the effect of the bile salt on proteolytic enzymes.

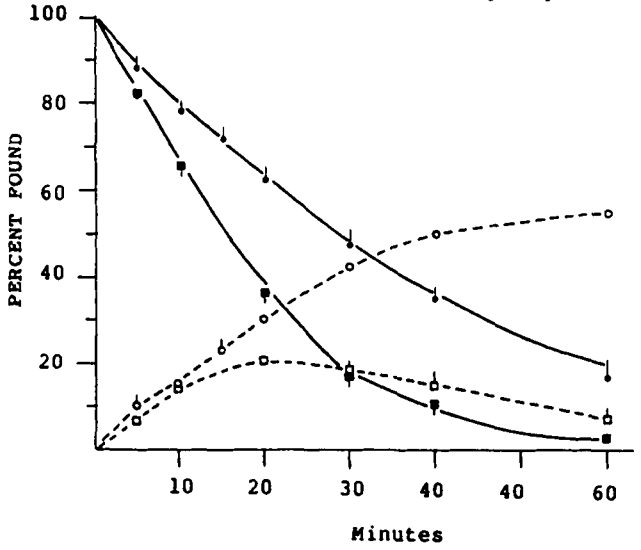

Figure 3—Plot showing the effect of 1% SGC on the disappearance of LE (60 μg/mL) and appearance of DTLE in the in situ perfusion solution. Key: (●) LE and (○) DTLE in the absence of SGC; (■) LE and (□) DTLE in the presence of SGC.

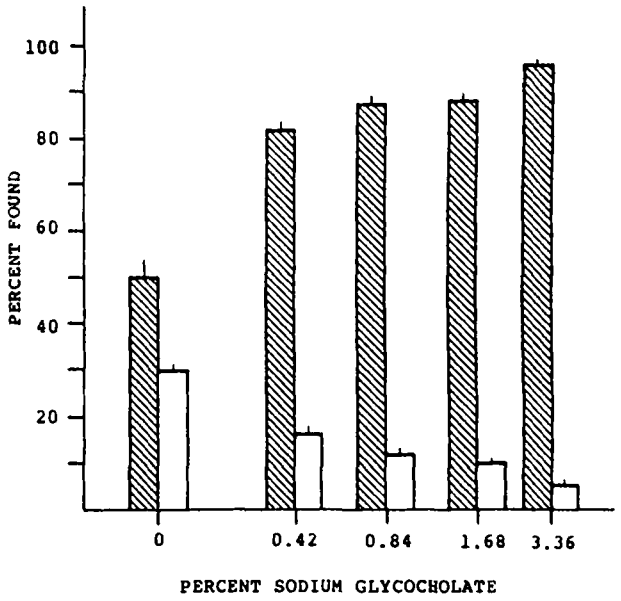

Figure 4—Effect of SGC on the in vitro hydrolysis of LE in nasal perfusate at 37 °C. Key: (▨) LE; (□) DTLE.

Puromycin is a known inhibitor of the soluble proteolytic enzymes in brain tissue which hydrolyze LE.[11] To determine if puromycin can also inhibit rat nasal peptidases, we studied its influence on the hydrolysis of LE in nasal washings. Figure 5 shows that puromycin inhibits the hydrolysis of LE in nasal washings, and the inhibition is virtually complete at a puromycin concentration of 1×10^{-3} M. It is interesting to note that the inhibition by puromycin is approximately equivalent to that of LTLT when they are compared at

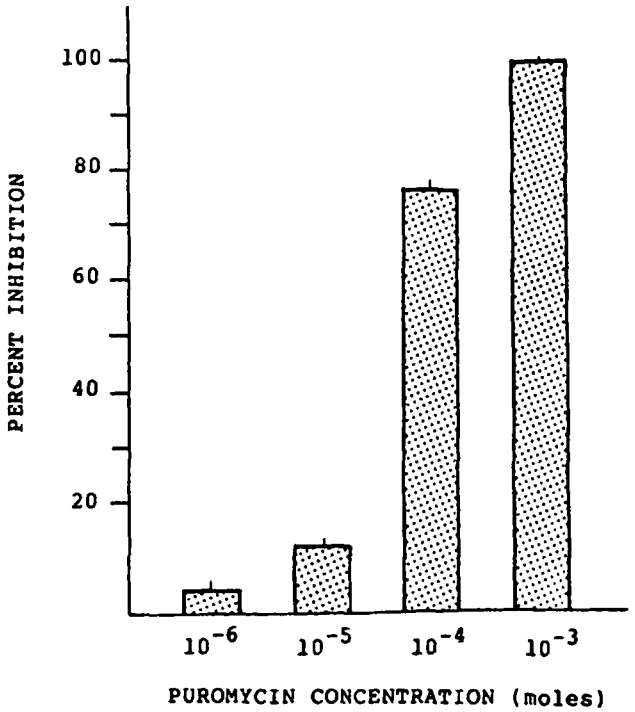

Figure 5—Effect of puromycin on the in vitro hydrolysis of LE in nasal perfusate at 37 °C.

equimolar concentrations (5×10^{-3} M).[9]

The results of the in situ studies suggest that when a relatively large volume (4 mL) of a low concentration of LE is circulated through the nasal cavity of rats, the disappearance of the pentapeptide occurs mostly by hydrolysis (probably catalyzed by peptidases) rather than by absorption of the pentapeptide into the systemic circulation. This is probably the result of the susceptibility of LE to enzymatic hydrolysis, coupled with the relatively slow absorption rate due to the large volume employed in the in situ technique.[10]

In order to demonstrate the influence of concentration and volume on the extents of absorption and hydrolysis of LE in the rat nasal cavity, and to examine the mechanism of nasal absorption under more realistic conditions, the "In Vivo-In Situ Technique" was devised. When 100 μL of solutions containing 0.2, 1.0, and 25 mg/mL of LE were administered and left in the nasal cavity for different time intervals prior to rinsing, the concentration–time profiles for LE and DTLE shown in Table II were obtained. The results show that the disappearance of LE was always accompanied by the appearance of DTLE, and a greater fraction of LE was converted to DTLE at the lower concentrations of LE.

In a similar experiment, the disappearance of DTLE itself from the nasal cavity was studied. The results are shown in Table III. Examining the data in Table II, when the LE dose was 20 μg, only 14.6% of the recovered drug was LE, while 61.4% was DTLE after 10 min. On the other hand, when the dose was 2500 μg, 60.7% of the dose was recovered as LE and 8.2% as DTLE after 10 min. These results suggest that conversion of LE to DTLE is a saturable process. Anik et al.[2] reported that the nasal bioavailability of an LH-RH analogue in monkeys increased about fourfold when the dose was doubled, and they attributed this behavior to saturation of metabolic enzymes in the nasal cavity.

The extent of hydrolysis of LE was found to be reduced considerably when LTLT or its more lipophilic methyl ester was added to the LE solution (Table IV). The fraction of DTLE formed was also very small. It appears that these dipeptides inhibited the hydrolysis of LE by competing for peptidase enzymes in the nasal cavity.

It is interesting to note than when hydrolysis was inhibited at high concentrations of LE or in the presence of peptidase inhibitors (Tables II and IV), there was still significant disappearance (~30% during the first 10 min) of LE from the nasal cavity. This might have been due to absorption of either LE or its metabolite DTLE. Recent studies[12] have shown that desmopressin is relatively well absorbed from the human nasal cavity when it is administered in relatively high concentrations.

Since SGC was found to inhibit the hydrolysis of LE in nasal washings, both in vitro and in situ, a study was undertaken to determine the effect of SGC on the disappearance of LE using the "In Vivo-In Situ Technique". The results in Figure 6 show that the addition of 1% SGC to the LE solution caused a significant reduction in the amount of DTLE produced during the first 5 min. After 10 min of treatment, neither LE nor DTLE existed in any significant concentrations in the nasal cavity. These results strongly suggest that SGC not only inhibited the hydrolysis of LE, but it also enhanced the absorption of both LE and its metabolite(s). Similar enhance-

Table II—Percent of Leucine Enkephalin (LE) Remaining and *des*-Tyrosine Leucine Enkephalin (DTLE) Formed in the Nasal Cavities of Rats (In Vivo-In Situ Technique)

Time, min	0.2 mg/mL[a,b]			1.0 mg/mL[a,b]			25.0 mg/mL[a,c]		
	LE	DTLE	Total	LE	DTLE	Total	LE	DTLE	Total
0	100	0	100	100	0	100	100	0	100
2.5	27.0	65.3	92.2	50.1	53.5	103.6	83.7	4.7	88.4
10	14.6	61.4	76.0	10.6	58.4	69.0	60.7	8.2	68.9
20	n.d.[d]	28.8	28.8	2.1	34.2	36.3	53.5	11.8	65.3
60	n.d.[d]	n.d.[d]	n.d.[d]	1.4	11.8	13.2	19.4	17.8	37.2

[a] Initial concentration of LE. [b] n = 2–3. [c] n = 2–4. [d] Not detected.

Table III—Concentration of *des*-Tyrosine Leucine Enkephalin (DTLE) Remaining in the Nasal Cavities of Rats (In Vivo-In Situ Technique)

Time, min	Concentration Found, μg/mL[a]
0	16.0[b]
1	14.3
2.5	12.3
5	9.0
10	4.4
15	3.0

[a] Values expressed as mean, n = 3. [b] Theoretical initial concentration obtained after administration of 0.1 mL of a 0.8-mg/mL LE solution nasally, followed by rinsing with 3.9 mL of Ringer's buffer.

Table IV—Concentrations of Leucine Enkephalin (LE) Remaining and *des*-Tyrosine Leucine Enkephalin (DTLE) Formed in the Nasal Cavities of Rats in the Presence of Dipeptides (In Vivo-In Situ Technique)

Time, min	L-Tyrosyl-L-Tyrosine (9×10^{-3} M)		Tri-L-Tyrosine Methyl Ester (6.1×10^{-3} M)	
	% LE Remaining[a]	% DTLE Found	% LE Remaining[a]	% DTLE Found
1	96.0	3.5	98.7	0.0
2.5	78.4	13.6	89.1	4.3
5	74.7	13.2	83.2	8.5
10	58.7	24.0	75.2	13.9
12.5	—	—	53.9	16.5
15	44.8	27.2	49.1	16.5

[a] Based on the initial concentration obtained after administration of 0.1 mL of 10×10^2-μg/mL LE solution nasally, and rinsing with 3.9 mL of Ringer's buffer.

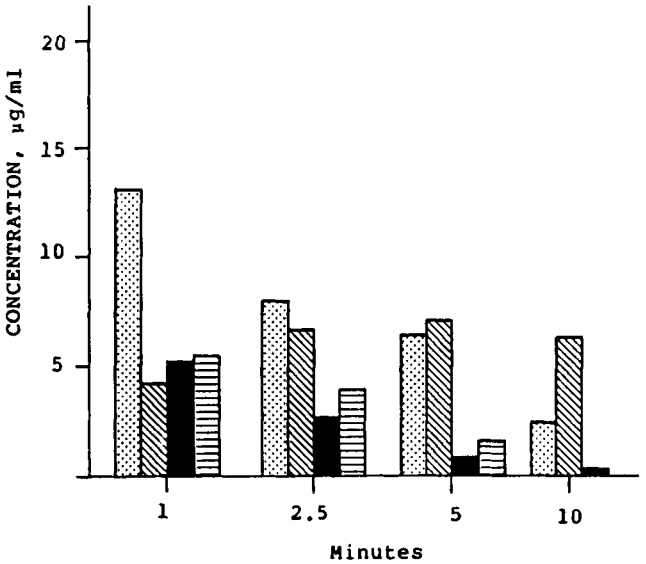

Figure 6—Effect of 1% SGC on the hydrolysis and absorption of LE in the nasal cavities of rats (in vivo-in situ technique). Key: LE with (■) and without (▨) SGC; DTLE with (▤) and without (▩) SGC (initial concentration of LE = 18.75 μg/mL.)

ment of the nasal absorption of peptides by SGC has been reported by Hirai et al.[7] in their studies with insulin and by Hussain[13] in his studies with a thyrotropin releasing hormone analogue. In both cases, the bioavailabilities of peptides, as determined from blood level data, were increased significantly in the presence of SGC.

Since SGC enhances the absorption of both small and large peptides and also large, enzymatically stable, hydrophilic molecules, such as dextran[14] and gentamicin,[15] it is possible that surfactants such as SGC exert their effects by affecting the integrity of the membrane and possibly creating larger pores in the nasal mucosa.

Published reports and our own observations on the nasal absorption of peptides in humans,[12] monkeys,[2] and rats indicate that although the fraction of the dose absorbed varies from one peptide to another, in each case the process of absorption is rapid. For example, in humans, 30% of a nasal dose of desmopressin is absorbed in 15 min. In monkeys, 2% of a nasal dose of LH-RH is absorbed in 15 min. Thus, the initial rate of nasal absorption of peptides in a variety of animal species is as rapid as the absorption of lipophilic molecules, such as propranolol, whereas the total amount of peptide absorbed is usually small and appears to be self limited. If it is assumed that peptides pass through aqueous pores in the nasal membrane, as do amino acids, the limitation to peptide absorption may be the result of blocking of the pores, either by the peptides themselves or by their hydrolysis products. This may also explain why compounds such as SGC, which have a great effect on the integrity of membranes, are so effective at enhancing both the rate and extent of absorption of peptides.

Conclusions

Using a standard in situ technique in which 4 mL of a 60-μg/mL solution of LE was circulated through the rat nasal cavity, LE was rapidly and almost quantitatively converted to DTLE. Using the same technique, when SGC was added to the LE solution, the disappearance of LE and the subsequent appearance and disappearance of DTLE were both altered. This may be due to inhibition of peptidase enzymes by SGC which slows the hydrolysis of LE and its metabolite DTLE. Addition of SGC or puromycin to nasal washings prior to the addition of LE inhibited hydrolysis of LE to DTLE in vitro. The inhibition was virtually complete at the higher concentrations of these two agents.

In the in vivo-in situ technique employing small volumes of LE solutions, the rate and extent of formation of DTLE were dependent on the concentration of LE administered, suggesting that the peptidase enzymes that hydrolyse LE became saturated at higher concentrations. When either LTLT or TLTME were added to the solutions of LE in the in vivo-in situ experiments, the formation of DTLE was reduced, suggesting that these two agents compete for the same peptidase enzymes. In the presence of these two agents, mass balance calculations left 30% of the administered dose of LE unaccounted for after 10 min. This was presumed to be absorbed. When SGC was added to LE solutions in the in vivo-in situ experiments, the formation of DTLE was reduced considerably and the disappearance of both LE and DTLE from the nasal cavity were virtually complete in <10 min, suggesting that SGC inhibits the hydrolysis and promotes the absorption of LE and possibly also DTLE.

It can be concluded that the in vivo-in situ technique yields important information about the nasal absorption of peptides under conditions that simulate use of nose drops in humans. The resulting data are more realistic than data obtained with the in situ technique.

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