Pharmaceutical Research, Vol. 7, No. 7, 1990

Report

Nasal Absorption Enhancers for Biosynthetic Human Growth Hormone in Rats

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Received September 20, 1989; accepted December 18, 1989

The effects of several prospective absorption enhancers were assessed on the nasal absorption of biosynthetic human growth hormone (hGH) in the rat. These enhancers function by alternative mechanisms that include enzyme inhibition, reduction in mucus viscosity, and enhancement of membrane fluidity. The levels of plasma hGH achieved were determined by an enzyme-linked immunosorbent assay. The increase in peak height was calculated relative to nasal administration of hGH alone without any enhancers and the relative bioavailability was calculated with reference to subcutaneous injection data. A lysophospholipid, lysophosphatidylcholine, gave the highest peak concentration, with an increase in peak height of 450% and a relative bioavailability of 25.8%. However, the greatest increase in AUC (291%) was achieved with the aminopeptidase inhibitor, amastatin, which gave a relative bioavailability of 28.9%. A mucolytic agent, *N*-acetyl-L-cysteine, and a transmembrane fatty acid transporter, palmitoyl-DL-carnitine, were also found to promote the nasal absorption of hGH in this model, with relative bioavailabilities of 12.2 and 22.1%, respectively. Bestatin, an enzyme inhibitor, was not an effective absorption enhancer for hGH in this model.

KEY WORDS: human growth hormone (hGH); intranasal; absorption enhancers; rats; lysophosphatidylcholine.

INTRODUCTION

Until recently, growth hormone (GH), a protein drug (22K), was obtained by extraction from human pituitaries and consequently was available only in limited supply for the treatment of GH-deficient children. Disturbing reports on Creutzfeldt–Jakob disease in patients treated with pituitary-derived human growth hormone (hGH) seriously jeopardized the continuation of this therapy (1–3). However, developments in recombinant DNA technology have allowed the production of biosynthetic hGH (4), and treatment with biosynthetic hGH is now the stabilized treatment in GH-deficient children.

Although information on the use of hGH for conditions other than short stature is limited, the amount of hormone now available has allowed more detailed investigations of alternative therapeutic uses of hGH, such as for mineral metabolic disorders, metabolic bone disease, kidney failure, severe trauma, e.g., extensive burns, obesity, hyperlipidaemia, hemophilia, immunologic disorders, and hypothalamic and pituitary diseases (5).

Current therapeutic regimens for GH replacement in GH-deficient children require often painful injections of hGH three times a week. Consequently, alternative delivery systems employing nonparenteral routes of administration would be a considerable advantage and may encourage the use of hGH for alternative therapeutic purposes (6).

The nasal route is a potential method of administration for proteins and peptides. Since polypeptides are poorly absorbed from the nasal cavity, absorption enhancers were employed in attempts to increase the extent of peptide absorption. Effective absorption enhancers include nonionic surfactants, bile salts, fatty acids, and chelators (7). The precise mechanisms of action of the absorption enhancers are thought to be based on inhibition of proteolytic enzymes, reduction of mucus viscosity, "opening up" of tight junctions, and enhancement of "membrane fluidity" (7). However, many absorption enhancers, particularly the bile salts and nonionic surfactants, alter the membrane integrity and can permanently damage the membrane (8). Consequently, these materials are unacceptable for chronic use in humans. Nevertheless, since the potential therapeutic benefits are enormous, there is considerable interest in absorption enhancers that may be effective without evidence of topical or systemic toxicity following nasal administration (9).

The aim of the present study was to evaluate several prospective enhancers of nasal hGH absorption in the rat. The selected compounds were shown to inhibit enzymatic hydrolysis of peptides (amastatin and bestatin) and to reduce mucus visocity (*N*-acetyl-L-cysteine). The mechanisms of absorption promotion for lysophosphatidylcholine (LPC) and palmitoyl-DL-carnitine (PCC) are poorly understood, but both compounds possess "membrane activity" and have

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0724-8741/90/0700-0772$06.00/0

shown absorption enhancing properties previously. PCC has been reported to be a potent absorption enhancer after oral administration, without apparent damage to the mucosa at the concentration used (10). LPC has proven to be a potent absorption enhancer for insulin, when administered nasally to rats (11). Further, both PCC and LPC were potent absorption enhancers for gentamicin when administered vaginally to rats (12). hGH represents another high molecular weight model polypeptide drug for absorption studies.

MATERIALS AND METHODS

Materials

Biosynthetic human growth hormone (Batch 180488), guinea pig anti-hGH IgG, and guinea pig anti-hGH F_{ab}-peroxidase were obtained from Novo-Nordisk, Denmark. L-α-Lysophosphatidylcholine (lysolecithin) (LPC), palmitoyl-DL-carnitine chloride (PCC), *N*-acetyl-L-cysteine (ACS), amastatin hydrochloride (AMA), bestatin hydrochloride (BES), *o*-phenylenediamine dihydrochloride (OPD), and human serum albumin (HSA) (Fraction V) were obtained from Sigma Chemical Company, Poole, England. All other chemicals were of reagent grade.

Experimental Methods

Animal Experiments

The rat *in vivo* experimental model as described by Hirai *et al.* (8) and modified by Fisher *et al.* (13) was used to study the effects of the potential absorption enhancers on the nasal absorption of hGH. Male Wistar rats (JABU, Sutton Bonnington) of about 200 g were fasted overnight and anesthetized by intraperitoneal injection of 75 mg/kg pentobarbitone (60 mg/ml). The rats were divided into groups of four, then tracheotomized, the esophagus was sealed, the carotid artery was cannulated, and 20 μl of hGH (1%, w/v; 1 mg/kg), with and without the enhancers, in 1/75 *M* phosphate buffer, pH 7.2, was instilled into the nasal cavity with a Hamilton syringe. The various enhancers and the concentrations employed are given in Table II. A dose of 0.5 mg/kg hGH was administered by subcutaneous injection into the flank of five individual animals.

Blood samples (500 μl) were collected into heparinized tubes containing 10 μl of heparinized saline (150 IU/ml), prior to administration and at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, and 300 min postadministration. The volume of blood collected was replaced with an equal volume of saline through a cannula in the jugular vein. The blood samples were kept on crushed ice before separation of plasma by centrifugation at 1300 rpm for 5 min on a Microcentaur centrifuge (MSE). The plasma samples were stored frozen at −20°C until assayed by enzyme-linked immunosorbent assay (ELISA).

Determination of Plasma Levels of hGH by ELISA

The plasma levels of hGH were determined by an ELISA which had previously been reported by Dinesen and Anderson (14), but standards were prepared in blank (preadministration) plasma. Briefly, 125 μl of guinea pig anti-hGH IgG, diluted 1/1000 in sodium carbonate buffer, pH 9.8, was incubated in a microtiter plate (NUNC Immunoplate I, NUNC Denmark) for 3 days at 4°C. The plate was triple washed in 0.9% NaCl, 0.1% Tween (SAL/TWE), 125 μl of the samples and the standards, in an equivalent dilution of plasma, diluted in sodium phosphate buffer, pH 7.4, containing 0.1 *M* NaCl, 0.5% human serum albumin (HSA), and 0.05% Tween 20 (SAL/TWE/HSA) was added to the wells, and the plate was incubated for 2 hr at room temperature (RT). Standard concentrations of hGH were prepared in diluted blank plasma (0.11–7.0 g/ml) and the samples were diluted to within this range for assay. The plate was triple washed in SAL/TWE and 125 μl guinea pig anti-hGH F_{ab}-peroxidase, diluted 1/1500 in SAL/TWE/HSA, was added to the wells and incubated for 2 hr at RT. The plate was triple washed in SAL/TWE, and 125 μl of enzyme substrate (40 mg OPD in 25 ml citrate/phosphate buffer, pH 5.0, and 20 μl 30% hydrogen peroxide) was added, and the plate was incubated in the dark for 1 hr. The reaction was stopped by the addition of 150 μl 2.5 *M* sulfuric acid to each well and the absorbance was read at 492 nm in a Titertek Multiscan (Flow Laboratories).

Table I. Mean Plasma Levels of hGH (ng/ml) with Time and Standard Errors of the Mean Achieved with Each Absorption Enhancer Used at the Concentrations Shown in Table II (n = 4), Without Any Enhancer (NE) (n = 4), and Following Subcutaneous Injection (SC) (n = 5)

Time (min)	Absorption enhancer						
	NE	SC	LPC	PCC	AMA	BES	ACS
0	<1.1	8.0 (0.0)	<4.4	<2.2	<2.2	<1.1	<2.2
5	4.2 (1.0)	35.0 (11.3)	22.5 (2.8)	26.4 (1.2)	8.9 (2.4)	2.1 (0.6)	12.3 (2.3)
10	6.0 (1.7)	75.8 (11.5)	42.9 (0.5)	32.6 (2.7)	14.0 (2.4)	4.5 (1.6)	16.4 (3.5)
20	8.3 (1.3)	98.2 (17.0)	61.6 (3.9)	31.1 (2.7)	24.9 (7.1)	5.4 (2.0)	26.9 (2.4)
30	9.2 (2.0)	111.6 (16.9)	59.0 (6.0)	35.4 (3.2)	42.4 (15.2)	4.4 (1.7)	40.4 (11.1)
45	11.2 (1.9)	118.6 (15.9)	56.0 (9.0)	27.1 (2.3)	34.5 (9.8)	3.2 (0.9)	28.9 (4.4)
60	8.6 (1.8)	117.7 (19.3)	45.4 (4.1)	23.9 (1.5)	40.3 (16.3)	3.0 (0.9)	18.5 (3.6)
90	10.2 (2.5)	119.0 (23.2)	38.5 (7.0)	17.1 (1.9)	29.0 (9.6)	2.7 (0.8)	9.7 (2.1)
120	7.3 (2.8)	114.0 (22.9)	24.6 (3.6)	22.4 (2.7)	25.0 (7.8)	3.0 (1.0)	10.5 (3.8)
180	5.5 (2.7)	72.6 (14.1)	9.7 (1.8)	17.8 (3.3)	21.3 (7.9)	2.3 (0.9)	5.7 (1.9)
240	3.9 (1.5)	47.1 (10.4)	6.3 (0.5)	13.4 (4.4)	16.6 (8.6)	1.5 (0.3)	5.0 (1.9)
300	4.4 (1.5)	29.8 (6.4)	<4.4	<2.2	13.7 (6.0)	<1.1	3.8 (1.4)

Table II. Relative Potencies of the Different Absorption Enhancers for the Nasal Absorption of hGH (1 mg/kg) in Rats

Enhancer (%, w/v)	Peak concentration, ng/ml (SE)	Increase in peak height (%)	Increase in AUC[a] (%)	Bioavailability (%)[b]
NE	11.2 (1.9)	—	—	7.4
LPC (0.2%)	61.6 (3.9)	450	250	25.8
PCC (1.0%)	35.4 (3.2)	216	199	22.1
AMA (0.015%)	42.4 (15.2)	278	291	28.9
BES (0.015%)	5.4 (2.0)	—	—	1.9
ACS (20%)	40.4 (11.1)	260	64	12.2

[a] Area under the absorption curve.
[b] Bioavailability calculated relative to plasma levels achieved following subcutaneous injection of 0.5 mg/kg hGH.

Plasma levels of hGH were calculated using the Titersoft program (Flow) with reference to the standard curve constructed for the standard concentrations of hGH in plasma. The bioavailability achieved with each absorption enhancer was calculated relative to that achieved following subcutaneous injection.

RESULTS AND DISCUSSION

The mean plasma levels (ng/ml) and the standard errors of the means (n = 4) resulting fron nasal administration of hGH solution alone and hGH in combination with the absorption enhancers are shown in Table I. The respective areas under the absorption curves (AUCs), the percentage increases in peak plasma levels, the percentage increases in AUCs achieved with the enhancers, and the relative bioavailabilities are shown in Table II. The percentage increases were calculated with reference to the peak height and AUC which resulted from the nasal administration of hGH alone, without any enhancers.

LPC was an effective absorption enhancer for hGH, with an increase in peak height of 450%, an increase in AUC of 250%, and a relative bioavailability (BA) of 25.8% (Fig. 1). Lysophospholipids are surface active amphiphiles which are found naturally in most biological membranes at low concentrations. Their physical characteristics in relation to their biological properties were recently reviewed by Stafford and Dennis (15). LPC is a potent absorption enhancer, both nasally (11) and vaginally (12), in rats, and has also been shown to be capable of promoting the intestinal absorption of horseradish peroxidase in guinea pigs without apparent evidence of ultrastructural mucosal damage (16). However, in alternative studies, LPC has been reported to impair the mucosal barrier function and to enhance gut permeability to macromolecules by damaging intestinal mucosal cells (17–19). Although much of the reported damage may be a result of the synergistic effects of HCl and LPC in combination, as shown by Salo *et al.* (20), Richardson *et al.* (12) reported that LPC caused extensive desquamation of the vaginal epithelium in ovarectomized rats at a concentration of 0.5% (w/v). However, in the ovarectomized rat, the vaginal epithelium consists of only two layers of cells and, hence, is more susceptible to damage than epithelia in other animal models. Furthermore, LPC is an effective nasal absorption enhancer for hGH at lower concentrations than those used in this study (21).

PCC was a significantly less effective enhancer for hGH, with an increase in peak height of 216%, an increase in AUC of 199%, and a BA of 22.1%. PCC has been reported to be an effective absorption enhancer in the intestine for drugs that are normally poorly absorbed, without causing any apparent change in mucosal structural integrity (10). The mechanism of absorption enhancement for PCC is thought to be related to the ability of carnitine to act as a "carrier" molecule to transport fatty acids across mitochondrial membranes (10). The work of Richardson *et al.* (12) showed that PCC at a concentration of 1% was a more potent absorption enhancer for gentamicin than LPC (0.5%), the nonionic surfactant laureth-9 (1%), or citric acid (10%), following intra-

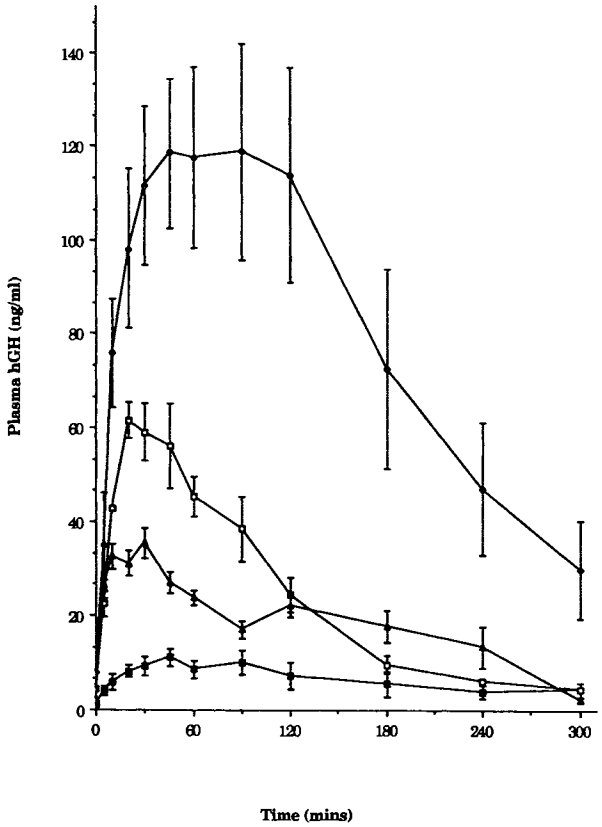

Fig. 1. Mean plasma levels of hGH (ng/ml) (n = 4) following intranasal administration of 1 mg/kg hGH solution alone (■), in combination with two absorption enhancers, 0.2% lysophosphatidylcholine (□) and 1% palmitoyl-DL-carnitine (▲), and following subcutaneous injection of 0.5 mg/kg hGH (♦).

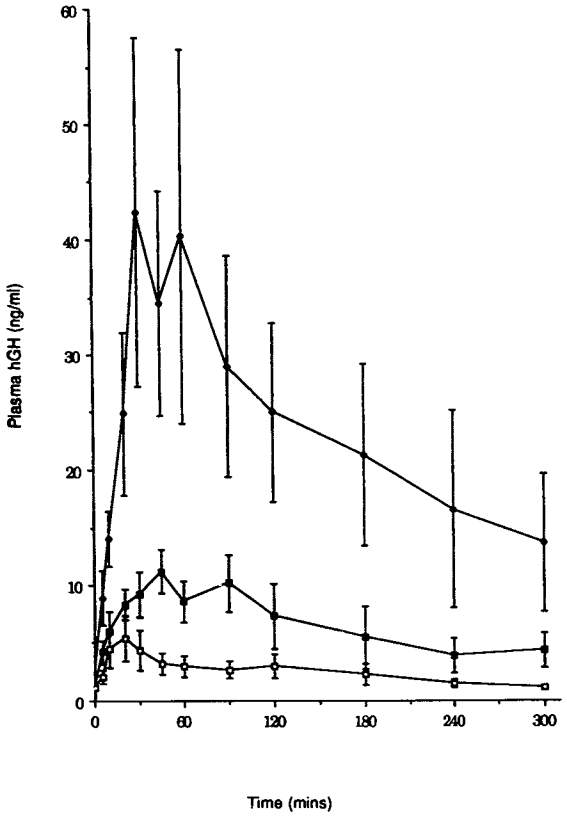

Fig. 2. Mean plasma levels of hGH (ng/ml) (n = 4) following intranasal administration of 1 mg/kg hGH solution alone (■) and in combination with two aminopeptidase inhibitors, 0.015% amastatin (♦) and 0.015% bestatin (□).

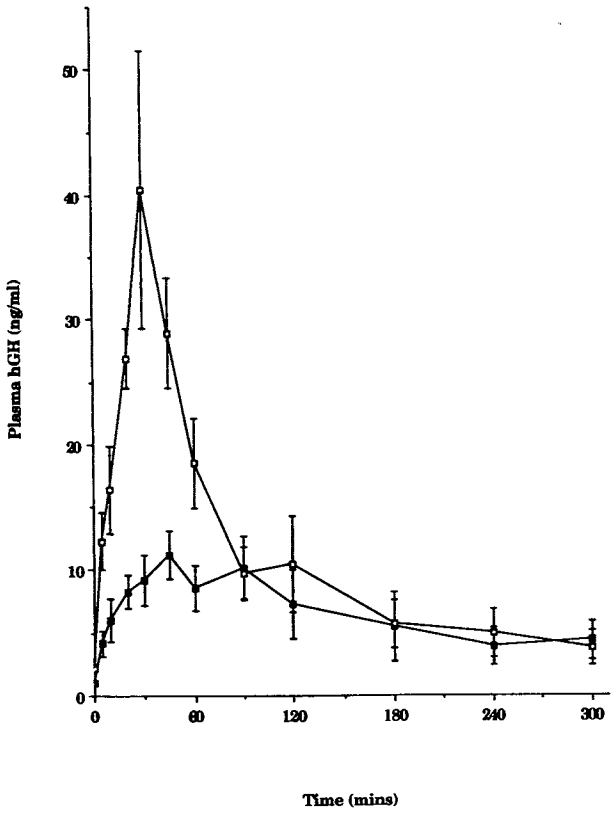

Fig. 3. Mean plasma levels of hGH (ng/ml) (n = 4) following intranasal administration of 1 mg/kg hGH solution alone (■) and in combination with a mucolytic agent, 20% *N*-acetyl-L-cysteine (□).

vaginal administration to rats. Therefore, it was expected that this compound would also prove to be a potent enhancer for the nasal route.

Of the two enzyme inhibitors used in this study, amastatin appeared to be an effective absorption enhancer, with a peak height increase of 278%, an increase in AUC of 291%, and a BA of 28.9%. However, bestatin did not enhance the absorption of hGH and, in fact, seemed to reduce the extent of absorption (Table II, Fig. 2). The reason for this phenomenon is not clear, but this result could be partially explained by the lower sensitivity of the ELISA at low plasma concentrations of hGH. However, it is possible that bestatin could compete with hGH for transport sites or interact specifically with the molecule to reduce absorption. Amastatin is a potent inhibitor of leucine aminopeptidase and a less potent inhibitor of aminopeptidase A, while bestatin is a very potent inhibitor of aminopeptidase M (22). We are unaware of any previous studies on the use of enzyme inhibitors as absorption enhancers for hGH. However, Hanson *et al.* (23) showed a general correlation between the ability of various compounds to inhibit the proteolytic activity of a rat nasal extract *in vitro* and the extent of absorption enhancement of salmon calcitonin achieved by these same compounds *in vivo*. Bestatin was not an effective absorption enhancer for calcitonin, and amastatin was not tested. Stratford and Lee (24) have demonstrated the presence of potent aminopeptidase activity in homogenized nasal mucosa. Our data suggest that inhibition of this enzymatic activity can lead to absorption enhancement, and further, inhibition of aminopeptidase M may be less important for nasal absorption enhancement of hGH than alternative aminopeptidases. In the normal state, there appears to be a dynamic equilibrium among naturally occurring proteinases, peroxidases, and proteinase inhibitors, which may be disturbed by an excess of proteinases (25). The possible long-term consequences of disturbing this balance between enzymes and inhibitors in nasal drug delivery systems are not known.

N-Acetyl-L-cysteine (ACS) is a potent mucolytic agent which is used clinically at a concentration of 20% in Airbron to reduce both the viscosity and the tenacity of mucus and to facilitate its removal in bronchopulmonary disease. At this concentration, ACS was an absorption enhancer for hGH, with a peak height increase of 260%, an increase in AUC of 64%, and an increase in BA of 12% (Fig. 3).

Several of the compounds tested proved to be potent nasal absorption enhancers for hGH in the rat, most notably amastatin and LPC. However, the potency of these enhancers for hGH in alternative animal models with intact cilia function needs to be determined. The extent of local and systemic toxicity following intranasal administration also needs to be assessed. It will be interesting to determine if synergism of absorption enhancement can be achieved, allowing enhancers with different mechanisms to be used at lower concentrations.

ACKNOWLEDGMENTS

This work was funded by Novo-Nordisk. We would like

to thank Elisa Brelsford and Ann Biggerstaff for performing the ELISA analysis on the plasma samples.

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