Differential Effects of Insufflated, Subcutaneous, and Intravenous Growth Hormone on Bone Growth, Cognitive Function, and NMDA Receptor Subunit Expression

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The objective of this study was to characterize the effect of inhalable growth hormone (GH) delivered by an insufflator to the lungs of hypophysectomized Sprague Dawley rats. In the first cohort, the safety and efficacy of the insufflated GH were evaluated. Three experimental groups (n = 7 per group) were treated with GH for 15 d: One group received sc injection of GH daily at 200 μg/kg (SC200). Two other groups received GH by insufflation daily: 200 μg/kg (INS 200) and 600 μg/kg (INS 600). In the second set of experiments, GH was administered in three routes [SC200, INS200, intravenous (IV200)] (n = 10) for 5 d, and escape latency and N-methyl D-aspartate (NMDA) receptor expression were evaluated. In the first cohort, INS200 showed similar bioactivity as SC200 in growth promotion, tibial growth, as well as escape latency on the 12th day of treatment. Insufflated GH was well tolerated without significant inflammatory responses. In the second cohort, expression of the NMDA receptor 1 and 2B in hippocampus measured after 3 or 6 d of daily treatments were significantly higher in INS200 as compared to IV200, consistent with the improvement of the escape latency. In summary, the inhalable form of GH delivered by intratracheal insufflation was safe, and its bioactivity was comparable to sc injection both in promotion of growth and acquisition of learning ability. If applied properly to human, inhalable GH would be effective for growth promotion and possibly for several disorders caused by underexpression of NMDA receptors. (Endocrinology 151: 4418–4427, 2010)

Growth hormone (GH) deficiency, often caused by surgery or radiation of the hypothalamus pituitary axis, is treated by GH replacement therapy (1). GH is also used for several genetic disorders causing short stature such as Turner syndrome, Prader-Willi syndrome, and Noonan syndrome. The U.S. Food and Drug Administration recently approved GH treatment for idiopathic short stature in children (predicted adult height is less than 2.25 SD score) (2, 3). GH is also indicated for AIDS-associated wasting (4). Moreover, GH is known to influence the cognitive function in an animal model (5). Given the breadth of biological benefits of GH, its indications are likely to expand.

The effect of GH on the cognitive functions is implicated with increased transcription levels of N-methyl D-aspartate (NMDA) receptor (NR) 1 and NR2A in the hippocampus (6). The NMDA receptor is a heterotet-

Abbreviations: AUC, Area under the curve; DPPC, dipalmitoylphosphatidylcholine; FPF, fine particle fraction; GH, growth hormone; GHD, growth hormone deficiency; HYPOX, hypophysectomized; LSD, least significant difference; MMAD, mass median aerodynamic diameter; MWM, Morris Water Maze; NMDA, N-methyl D-aspartate; NR, NMDA receptor.
ramer consisting of two obligatory NR1 subunits and two of four possible NR2 subunits: NR2A, NR2B, NR2C, and NR2D. The NR2 subunits in the adult hippocampus and cortex are usually NR2A and NR2B, and the ratio of NR2B to NR2A decreases with age in diverse animal species, starting from or before the onset of sexual maturity. NR2 composition governs the properties of NMDA receptor channels and the extent of synaptic plasticity; a relative abundance of NR2B in the juvenile brain confers a greater plasticity than the adult brain (7).

In humans, GH are secreted up to eight to 10 pulses in a 24-h period (8), and the peak secretion occurs during deep sleep. The half-life of endogenously secreted GH in circulation is 18 ± 1.3 min (9). Therefore, for indications GH replacement is necessary, daily administration of GH is a common practice. Intravenous administration of GH several times a day would not be tolerated as a routine route of administration. Currently, GH is administered by sc injection. One of the challenges in the use of GH application is poor patient compliance. The requirement for daily sc injections often leads to early termination of the therapy, due to poor adherence particularly in pediatric patients (10).

Relatively noninvasive alternative routes of GH delivery would, therefore, significantly improve the GH replacement therapy. Oral administration of GH is not a viable option because of inactivation of the protein by proteases in the gastrointestinal tract. There are not many alternative parenteral formulations amenable to systemic delivery of protein drugs. Transdermal delivery is limited because of the large molecular weight of protein drugs, unless accompanied by physical or chemical means to enhance their skin penetration. In this regard, an inhalable formulation is considered a promising alternative. Several features make the lung a unique portal for systemic drug delivery. These include a relatively large surface area for absorption (~100 m² in adults) (11), high epithelial permeability, and vascular perfusion (12, 13). Lower pulmonary activity of drug-metabolizing enzymes translates to a smaller first-pass effect, compared with oral administration (11, 15). This latter property is particularly beneficial for delivery of protein drugs, such as GH and insulin. Accordingly, the lung has gained increasing attention as a site for systemic delivery of protein drugs, as evident from recent development of inhalable insulin (16–18). Although eventually withdrawn from the market due to concerns for lung cancer in patients with prior history of smoking (http://mediaroom.pfizer.com/news/pfizer/20080409005647/en/Pfizer-Statement-Exubera-Labeling-Update-United-States), patient satisfaction with inhaled insulin was quite high, compared with injections, particularly among patients with needle anxiety (19). Inhalable GH is also promising from a safety standpoint. A recent study comparing inhaled and sc GH in children with the GH deficiency found that inhalation was well tolerated and did not result in significant changes in pulmonary functions (20).

In establishing the inhalable GH as a potential alternative to injectable GH, it is critical to compare the bioactivity of GH administered in different routes. The bioactivity of inhaled GH was previously compared with GH delivered via alternative routes in pediatric patients (20). However, bioactivity could not be compared at similar doses, because of the significant loss of dose during inhalation. Comparison between studies is also difficult, because the results vary with the device performance and inhalation techniques.

Therefore, the aim of this study was to evaluate various biological effects of an inhalable form of GH delivered by intratracheal insufflation, which minimized dose loss during delivery, and to compare the potency of the inhaled GH with those obtained with injections in surgically hypophysectomized (HYPOX) rats, a common animal model for evaluation of GH efficacy. In addition, we demonstrate the effect of different routes of GH administration on the memory acquisition and expression levels of NMDA receptors, principal cellular machinery responsible for initiating many forms of synaptic plasticity in different areas of the brain (7).

**Materials and Methods**

Recombinant human GH (Growtropin) was a gift of Dong-A Pharmaceutical Co., Ltd. (Yongin-si, Korea). BSA was obtained from Sigma (St. Louis, MO), Lactose monohydrate from Mallincrodt (Paris, KY), and dipalmitoylphosphatidylcholine (DPPC) from Lipoid GmbH (Ludwigshafen, Germany).

**Preparation and characterization of spray-dried GH powder**

**Powder preparation**

The GH solution received from the supplier was first purified by ultrafiltration (molecular weight cut-off: 10,000) to remove the inactive ingredients. GH powder was produced by the LabPlant SD-05 spray dryer (Lab Plant Ltd, Huddersfield, UK). Control powder containing BSA instead of GH was prepared as a control. For both proteins, lactose and DPPC were dissolved in the ratio of approximately 1:1:3 in 70% ethanol to make a 2-mg/ml feed solution. Briefly, DPPC was first dissolved in 95% ethanol and then combined with the aqueous phase containing lactose with GH or BSA. Each solution was maintained at 40 C and constantly stirred during the spray-drying process. The solutions were introduced to the spray-dryer at 17 ml/min and atomized instantly through a 1-mm orifice nozzle using compressed air. The inlet temperature was 120–150 C.

**Anderson cascade impactor**

Aerodynamic particle size distribution was determined using an eight-stage Mark II Anderson Cascade Impactor (ACI). Pow-
under samples (10 mg) were manually loaded into hard gelatin capsules (size 3), put in a Rotahaler, and split-open to release the particles. Glass fiber filters were placed on the ACI stages to prevent particle bounce or reentrainment (21). Each set of powders was drawn through the induction port into the ACI operated at a flow rate of 28.3 liter/min for 10 sec. The amount of particles deposited at individual impaction stage was determined by measuring the difference in weight of glass fiber filters (for the filter stage, pore size < 1 μm, ThermoFisher; for all other stages, pore size 1 μm, Pall Corp.) placed on the stages. The effective cut-off aerodynamic diameters for each stage were: Stage 0, 9 μm; Stage 1, 5.8 μm; Stage 2, 4.7 μm; Stage 3, 3.3 μm; Stage 4, 2.1 μm; Stage 5, 1.1 μm; Stage 6, 0.65 μm; Stage 7, 0.43 μm. The fine particle fraction (FPF) was defined as the quotient of the amount of powder with an aerodynamic size < 4.7 μm (particles deposited at stage 3 and lower) divided by the initial total powder loaded in the Rotahaler (10 mg, nominal dose). A calibration curve was prepared by plotting the effective cut-off diameter vs. the cumulative mass of powder smaller than effective cut-off diameter divided by the total recovered mass in the ACI. The mass median aerodynamic diameter (MMAD) was defined on this graph as the particle size at which the line crossed the 50th percentile.

**Scanning electron microscopy**

Dry powders were attached to specimen stubs using double-sided tape and coated with gold-palladium in the presence of argon gas using a Hummer I sputter coater (Anatech Ltd.). Dry powders were imaged with a JEOL JSM-840 scanning electron microscope (JEOL USA, Inc.) using a 5-kV accelerating voltage, a 10-mm working distance, a 70-μm objective aperture, and a probe current of 6 x 10^-11 amps.

**Analysis of GH recovered from dry powder**

Five milligrams of GH powder was accurately weighed, suspended in 1 ml PBS (pH 7.4), and incubated at 37°C with constant stirring. At 1, 3, and 24 h, the particle suspension was centrifuged at 8000 rpm for 5 min, and 0.8 ml of supernatant was sampled for analysis. After each sampling, fresh PBS was replaced. This release method was used to maximize the drug recovery from the powder without bringing the drug concentration below the detection limit. This method does not necessarily predict the drug release kinetics in the respiratory tract, where the inhaled powder would be placed at the interface of air and moist lung tissues covered with mucus rather than in liquid. The concentration of GH in the supernatant was determined using High Pressure Liquid Chromatography (1100 series, Agilent Technologies, Palo Alto, CA) and a gel filtration column [TSK G3000SWXL (300 x 7.8 mm; particle size 5 μm)]. The mobile phase was a mixture of 0.06 M phosphate buffer and isopropyl alcohol in the ratio of 97:3. The flow rate was 0.6 ml/min. Five microliters of each sample was injected into the preequilibrated column followed by 30 min of wash with the mobile phase. The UV detector was set at 214 nm. Retention time of GH was 17.8–17.9 min.

**Animals**

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee, Laboratory Animal Research Center, Samsung Biomedical Research Institute (Seoul, Korea).

**Efficacy and safety testing**

**GH administration**

Male HYPOX Sprague Dawley rats (hypophysectomized at 4 weeks of age), weighing approximately 80–100 g, were purchased from Japan SLC, Inc. Total body weight was monitored during a 7-d acclimatization period, and only animals with body weight change of <10% were selected for study. Animals were housed under standardized conditions (12-h light, 12-h dark cycle, constant temperature and humidity). Animals had free access to standard rat chow and water. No substitution with thyroxine or cortisol was given.

**Experimental design**

For the first part of the study, rats were randomized by weight to five experimental groups (n = 7). One group of animals received sc injection of GH daily at 200 μg/kg (SC200) for 15 d. Two other groups received two doses of insufflated GH daily for 15 d: 200 μg/kg (INS600) and 600 μg/kg (INS600). The last two groups served as controls and received either sc saline in the volume equivalent to the SC200 group or powder equivalent to the INS600 group. The Morris Water Maze (MWM) performance test was conducted beginning on the experimental d 11 and continued for five consecutive days. On the 16th day, animals were euthanized by CO2 asphyxiation and subjected to evaluation of proximal tibial growth and lung toxicity.

Before each treatment, animals were anesthetized with sc ketamine (50 mg/kg) and xylazine (5 mg/kg). Because ketamine is known to have effects on the memory acquisition and learning process (22, 23), animals receiving sc GH were also anesthetized with ketamine and xylazine, although it was not necessary for the procedure, to minimize the variation due to the ketamine anesthesia. The GH powder and control powder were delivered directly into the trachea via a powder insufflator (Model DP-4; Penn-Century, Philadelphia, PA). Dry powder containing GH or BSA was loaded in the insufflator. The powder mass was quantified by subtracting the weight of empty device from the device loaded with the sample. The anesthetized animals were intubated using a 16-gauge 2-inch-long IV indwelling cannula, which would approach the carina, as an endotracheal tube. The delivery tube of the insufflator was placed at the entry of the cannula, and the powders were then introduced to the lungs by supplying 1 ml of air through a syringe connected to the insufflator. It was assumed that 100% of the discharged dose was delivered to the airways below carina. The animals were treated in this manner for 15 d, monitoring the weight gain during the first 10 d. The body weight was measured daily at 0800 h.

In the second part of the study, escape latency and NMDA receptor expression were evaluated while administering GH in 3 different routes. Rats were randomized by weight to 3 groups (each n = 10). One group of animals received sc injection of GH daily at 200 μg/kg (SC200). The other groups received same dose of inhalable GH daily until they were killed: 200 μg/kg (INS200) and another group received intravenous GH daily (IV200). Of the 30 rats used in this experiment, 15 rats (5 rats per group) were killed at d 3 of GH treatment for evaluation of NMDA receptor expression. The remaining 15 rats were treated with GH for 5 d and were killed at d 6. MWM performance test were performed from d 3 to d 5 of GH treatment. Therefore, d 1 in MWM test pertains to d 3 of GH treatment.
Morris water maze performance test
The MWM test used a circular water maze tank (157 cm diameter × 60 cm height) filled with the nontransparent, dilute milk solution (24 ± 1°C). A transparent Plexiglas platform (10 cm diameter × 47 cm height) was placed 3 cm below the water surface in a fixed location in one of the quadrants. The maze was located in the center of a well-lit room and surrounded by black curtains (placed at 50 cm from the pool periphery) with four distinct external visual cues. The swimming path of each rat was monitored by an overhead video camera connected to a computer and analyzed by an automated tracking system (Smart v.20; Panlab SL, Barcelona, Spain).

During a test, the animals were required to locate the hidden Plexiglas platform in relation to the external visual cues. Each daily session consisted of three trials. In each trial, the rat was placed in the water facing the maze wall in one of the three quadrants except for the target quadrant containing the hidden platform. The order of entry into each quadrant was randomized daily. Each trial ended once the animals found the platform. If the rats were unable to locate the platform within 90 sec, they were guided toward it by the experimenter. Once they have found or been placed on the platform, the rats were allowed to stay for 30 sec. The rat was then wiped dry with a towel and positioned at a different starting point for the next trial. In each trial, the time taken to reach the platform (escape latency in seconds), the length of the swim path (distance in cm), and the swimming speed (cm/sec) were recorded. At the end of a session, the rat was wiped dry and returned to the home cage. The daily GH administration was continued during the test period.

Evaluation of proximal tibial growth
After euthanasia, tibias were harvested and fixed in 10% neutral buffered formalin. The fixed tibias were split along the frontal plane at the proximal end and processed for paraffin embedding, sectioned, and stained with hematoxylin & eosin. The width of the proximal tibial growth plate was measured from the left tibia. Three measurements per section (medial, central, and lateral) were made at the magnification of ×100 under a light microscope. Measurements were averaged to determine the mean width of the proximal tibial growth plate.

Evaluation of lung toxicity of the dry powder
Immediately after euthanasia, the chest was opened and the trachea exposed and ligated with a 3-0 suture. The lungs were harvested and carefully trimmed of nonpulmonary tissue. The left lungs were weighed, dried in an oven at 95°C for 48 h, and weighed again. The lung water content was estimated as the ratio of the difference between the wet lung weight and the dry lung weight relative to total body weight (24). The rat lungs were harvested, fixed in 10% neutral buffered formalin, processed for paraffin embedding, sectioned, and stained with hematoxylin & eosin. The level of lung injury was scored according to a previously reported system (25, 26) with modification. The extents of edema and infiltration of leukocytes were scored in the 0 (not observed) to 3 (severe) scales, respectively. The two scores were summed to a final score ranging from 0 to 6. The examiner was blinded as to the identity of the specimen.

Hippocampal tissue was harvested from the euthanized HYPOX animals and homogenized in TRizol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated according to the manufacturer’s protocols. The cDNAs were synthesized by SuperScript III reverse transcriptase (Invitrogen). Real-time PCR (RT-PCR) was performed to quantify the mRNA levels of NMDA receptor subunits using an ABI PRISM 7900HT system and TaqMan gene expression assays (Applied Biosystems, Foster City, CA). NR1 (Rn01436030_m1), NR2A (Rn00561342_m1), NR2B (Rn00561352_m1), AMPA (Rn00691897_g1), and GAPDH (Rn01462662_g1) were labeled with FAM. The mRNA levels were expressed relative to the expression of GAPDH. The 2-DDCT method was used to analyze the data using SDS2.3 software (Applied Biosystems).

Pharmacokinetic studies
GH administration and sampling
Normal male Sprague Dawley rats (300–320 g) were anesthetized with sc ketamine (50 mg/kg) and xylazine (5 mg/kg), and catheters were implanted in the jugular veins of rats for blood sampling and intravenous injection. The rats then received GH once by the intratracheal insufflation of a dry powder (INS) or sc injection. The amounts of powder and solution were adjusted according to the rat weight to provide 200 µg/kg of GH. Blood samples (0.2 ml) were collected from the jugular vein and placed in heparin-treated tubes. Samples were obtained at 5, 10, 20, 30, 45, 60, 90, 120, 180, 270, 340, 380, and 450 min. The GH concentration in the plasma was measured using the Immuno-Radiometric assay (IRMA, DiaSorin, Saluggia, Italy), which was specific to human GH and not cross-reactive with rat GH. The detection range of the immunoassay was 0.5–50 ng/ml, and the interassay relative sd was 5.2%. Plasma samples were assayed in duplicate after dilution. At the end of blood sampling, rats were euthanized by CO2 asphyxiation.
Pharmacokinetic analysis

The area under the plasma concentration vs. time curve (AUC) from zero to the final detectable growth hormone concentration was computed by the linear trapezoidal rule. The AUC was extrapolated to infinity as the quotient of the last measured concentration and the terminal elimination rate constant. The terminal elimination rate constant was estimated from the terminal slope of the linear regression line fitted to the log plasma concentration-time data by the method of linear least squares.

Statistical analysis

All results are expressed as mean ± SEM, unless specified otherwise. One-way ANOVA test and least significant difference (LSD) test were performed as a post hoc test to demonstrate statistical differences (P < 0.05), using the Sigma-stat for Windows (SPSS Inc., Chicago, IL).

Results

Preparation and characterization of spray-dried GH powder

Inhalable dry powder of GH, containing lactose and DPPC as inactive ingredients, was produced by spray drying as described in the Materials and Methods. The
Excipients used here are known to be safe and provide the aerodynamic properties suitable for inhalation (27, 28). The inactive ingredients are either approved by the U.S. Food and Drug Administration for inhalation (lactose) or endogenous to the lung (DPPC). Dry powders containing protein, lactose, and DPPC showed low density, providing a MMAD in the range of 1–5 μm, found to be desirable for human inhalation (27, 28). It is uncertain whether different particle size requirement exists for small animals like rats due to the anatomical difference. However, the particles designed for human inhalation have frequently been used in preclinical studies using rats achieving good lung deposition (29). It is at least unlikely that passage of particles through the lower airways would be limited due to the particle size, given that the average diameter of respiratory bronchioles of rats is as large as 0.5 mm (30).

Consistent with the existing studies (25, 26), the GH powder was highly dispersible in air, resulting in the MMAD of 5.4 ± 0.2 μm and the FPF of nominal dose of 25.7 ± 0.7%. This value indicates that when administered via a dry powder inhaler, approximately 26% of the dose would reach the airways below trachea in human. As shown in Fig. 1A, the GH powder was spherical and hollow, which explains the smaller MMAD than the geometric diameter of approximately 10 μm observed by scanning electron microscopy. The control powder (containing BSA instead of GH) had similar aerodynamic properties (MMAD: 4.5 ± 0.4 μm; FPF: 27.5 ± 5.7%; both P > 0.05 vs. GH powder by t test).

To test the integrity of spray-dried GH, the protein was recovered from the powder and analyzed by the size-exclusion HPLC according to the assay method described in the U.S. Pharmacopeia (31). GH recovered from the powder was similar to that of original GH (Fig. 1B) in molecular weight, indicating that the spray drying process did not cause aggregation or degradation of GH. The HPLC analysis revealed that 1 mg of powder contained 0.128 ± 0.01 mg of GH. The amount of powder required to provide the desired dose of GH was determined based on this measurement.

Efficacy and safety of insufflated GH

Figs. 2 and 3 show the results of the first cohort. When the net weight gain by the 10th day of GH administration was compared, no statistical difference was noted between
SC200 and INS200 ($P = 0.26$, Fig. 2A). The difference between INS200 and INS600 was not statistically significant ($P = 0.068$), but the difference between SC200 and INS600 was significant ($P = 0.005$). The increase in the tibial growth plate (Fig. 2B) showed a similar trend. There was no difference between INS200 and SC200 group ($P = 0.247$). INS600 group showed significantly more weight gain and tibial growth plate than INH 200 ($P < 0.001$) as well as sc ($P < 0.001$).

The water content in the lungs increased in all groups receiving inhalable powders, including control powder, compared with sc administrations ($P < 0.01$) (Fig. 2C). When the inflammatory cell infiltration in the lung tissues was graded semiquantitatively, the inflammatory cell infiltration score ranged from 1 to 2, much lower than the score obtained with administered phorbol myristate acetate (score $5.62 \pm 0.24$) (26) and there was no difference among the groups (Fig. 2D; $P = 0.569$, Kruskal-Wallis test).

**Insufflated GH had a positive influence on the memory/learning in the HYPOX rats**

As shown in Fig. 3A, the animals receiving GH demonstrated significant improvement in the escape latency especially on the second day of the MWM test (control powder vs. SC200: $P = 0.031$; control vs. INS200: $P = 0.006$; control vs. INS600: $P = 0.018$). There was no difference in escape latency between INS200 and SC200 on that day ($P = 0.471$). On the fifth day of MWM test, most animals learned to escape irrespective of GH administration, and there was no difference in the escape latency.

When the animals were killed after completion of MMW test, the transcription levels of NMDA receptor subunits increased significantly in all animals receiving GH, particularly in the INS600 groups (Fig. 3B). However, there was no statistically significant difference between SC200 and INS200 in transcription levels of NMDA receptor subunits (SC200 vs. INS200: NR1, $P = 0.437$).

**Pharmacokinetics**

The time course of growth hormone concentrations in plasma was evaluated with normal Sprague Dawley rats (Fig. 4). The area under the curve (AUC) ($0 \to \infty$) was $9319 \pm 481 \text{ ng-min/ml}$ following inhalation and $10732 \pm 1570 \text{ ng-min/ml}$ following sc administration. As can be seen, the time courses were not significantly different, consistent with the similar effects on bone growth, learning/memory, and receptor expression.

**Escape latency and level of NR1 and NR2B expression in cohort 2**

The escape latency on the second day of the MWM test (fourth day of the treatment) was significantly lower in the INS200 compared with the IV200 ($P = 0.004$). However, there was no statistically significant difference in the second day escape latency between SC200 and IV200 ($P = 0.16$) or between INS200 and SC200 ($P = 0.078$) (Fig. 5A). The expression levels of NR1 and NR2B were significantly higher after insufflations compared with intravenous administration at both time points (Fig. 5B), consistent with the improvement of the escape latency of GH treatment.

**Discussion**

This study shows that the insufflated GH was as effective as sc GH injection in promoting the weight gain and growth of the tibial growth plate, consistent with the pharmacokinetics results. Except for the modest increase in the water content in the lungs, the GH inhalation showed comparable histological responses and lung functions to the sc injection. The increase of water content is worth mentioning. Previously, it was reported that inhaled substances can affect the epithelial barrier by opening tight junctions or increasing cell membrane permeability in animal models for drug delivery across the lung mucosal barrier (14). Moreover, GH itself can increase the water content in body. However, judging from the extent of water content in the lung, it is unlikely that this amount of increase of water content would be clinically significant in human. Therefore, this study can be interpreted that insufflated GH was as effective and safe as sc GH injection if inhalable GH is properly administered.
It was intriguing to observe that inhaled GH was equivalent to injectable GH in bioactivity, because inhalation has largely been considered as a convenient yet relatively less potent alternative to the injection routes. For example, in a recent clinical study the inhalable formulation was administered at a dose 16.8 times higher than that for sc injection to achieve comparable pharmacokinetics (20). The main reason for the difference between our observation and the existing study may be the method of delivering inhalable GH powder to the lungs. We used an insufflator that directly delivered the desired dose to the carina, whereas the mentioned clinical study was performed with a portable dry powder inhaler, which is prone to misuse and dose loss to the mouth and upper airways during the delivery. The results of this study highlight that inhaled drugs, if delivered effectively, could achieve similar bioactivities to other dosage forms. While the insufflator is invasive and not appropriate for human use, it bears mentioning that insufflation generates higher pressure than a portable dry powder inhaler commonly used for asthmatics and has a shorter distance between outlet of insufflator and the destination, most importantly, bypassing the oral cavity. These characteristics of insufflation should be considered in designing the device for inhalable GH.

Interestingly, the inhaled GH significantly improved escape latency consistent with the increased expression of NR1 and NR2B to a greater extent than IV admin-

**FIG. 5.** Improvement of escape latency at d 4 of GH treatment, and the elevated level of expression of NR1 and NR2B in INS200 were compared with IV200 at d 3 and 6 from the start of GH treatment. A, Effects of GH delivered by different routes on the performance of HYPOX rats in the MWM test. At d 2 (d 4 of GH treatment), escape latency of inhalation group was the shortest. *, $P = 0.031$ among groups; SC200 vs. INS200: $P = 0.078$; IV200 vs. INS200: $P = 0.004$; SC200 vs. IV200: $P = 0.16$ (using ANOVA and LSD post hoc test). B and C, Expression of NMDA receptors. Expression of NR1 and NR2B was elevated in the INS200 group compared with the IV200 group at d 3 (B) and d 6 (C) (*, $P = 0.030$; **, $P = 0.027$; ***, $P = 0.027$; ****, $P = 0.027$; Student’s t test).
istration. The increase of NR2B in hippocampus may result in positive influence onto the memory acquisition (7). Although the reason is not clear at present, this result suggests that the extent of certain pharmacological effects may depend on the route of administration, which should be clinically exploited if confirmed by future studies.

In conclusion, the inhalable form of GH was as effective and safe as sc injectable form of an equivalent dose in growth promotion, memory/learning acquisition, and transcription of NMDA receptor subunits in the brain. Provided that an efficient inhaler device is available, inhalation would be a promising route of administration of GH not only for the promotion of growth but also for clinical applications in several disorders, where the expression of NMDA receptor is decreased. To take advantage of the potential benefit of the inhaled GH, development of an efficient inhaler device is most important.

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