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Preparation and evaluation of palmitic acid-conjugated exendin-4 with delayed absorption and prolonged circulation for longer hypoglycemia

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ABSTRACT

Albumin-binding achieved by fatty-acylation to drugs is considered to be an effective means of prolonging the circulation lifetimes of short-lived peptides. Here, exendin-4 was modified with palmitic acid, and the particle size and albumin-binding of palmitic acid-conjugated exendin-4 (Pal-Ex4) purified was investigated and visualized. Additionally, its pharmacokinetics and pharmacodynamics were evaluated in diabetic rodents. Pal-Ex4 had a greater molecular size (~125 nm) and its albumin-binding was 5.6-fold that of Ex4. Molecular imaging showed that the subcutaneous absorption of Pal-Ex4 was delayed until 24 h post-injection, whereas Ex4 was rapidly absorbed and distributed systemically. Pharmacokinetic and pharmacodynamic results confirmed these observations, for example, times to reach peak concentration and to achieve a blood glucose level nadir were greatly delayed *versus* Ex4, and the circulating half-life of Pal-Ex4 was much greater than that of Ex4. Consequently, the hypoglycemic degree of Pal-Ex4 (500 nmol/kg) was 4.2 fold greater than Ex4. Our results show that the extended hypoglycemic efficacy of Pal-Ex4 was due to (i) a delayed absorption due to micelle formation and (ii) an increased *in vivo* circulating half-life due to albumin-binding. We believe that this prototype of exendin-4 has considerable pharmaceutical potential as a systemic type 2 anti-diabetic treatment.

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1. Introduction

Peptides are subjected to rapid renal filtration and aggressive proteolysis by various metabolic enzymes *in vivo* (Kompella and Lee, 1991; Caliceti and Veronese, 2003; Youn et al., 2007). Therefore, peptides have short circulating lifetimes that range from minutes to at most a few hours (Kim et al., 2011c), and thus, peptide-based drugs have unsatisfactory therapeutic efficacies because of their low bioavailabilities. In order to improve their therapeutic values, a variety of pharmaceutical techniques, such as, PEGylation (Uchio et al., 1999; Lee et al., 2005, 2009; Chae et al., 2009), polysialation (Gregoriadis et al., 2005), and albumin-based techniques (Thibaudeau et al., 2005; Kim et al., 2003; Baggio et al., 2004, 2008) have been devised to increase residence times in blood.

Recently, human serum albumin (HSA) has been used as a protein carrier to prolong the *in vivo* half-lives of peptides. HSA has a long *in vivo* half-life (~19 days) and acts as a natural solubilizer of long chain fatty acids (Kratz, 2008). Moreover, it has negligible enzymatic/immunogenic functionality and is not subject to kidney filtration due to its large size and negative surface charge (Chuang et al., 2002; Kratz, 2008). Due to these merits, three different approaches have been used to prolong the lifespans of peptides *in vivo*. The first involves the conjugation of peptides with HSA using various bifunctional cross-linking agents, the second fatty acid-introduction to peptides using the inherent binding capability of long fatty acids to HSA, and the third method involves albumin-fusion to peptide molecules using recombination techniques. These three techniques have been successfully applied to various peptides, such as, human insulin, glucagon-like peptide-1 (GLP-1) analogs, HIV-1 fusion inhibitor C34 peptide, and exendin-4 (Stoddart et al., 2008; Thibaudeau et al., 2005; Kim et al., 2003; Baggio et al., 2008).

Exendin-4 is a 39-amino acid peptide that was originally isolated from Gila monster saliva, and acts as a potent agonist of mammalian GLP-1 receptors (Deacon, 2004; Davidson et al., 2005). In fact, exendin-4 shares 53% homology with GLP-1, and stimulates insulin release, suppresses glucagon secretion in a glucose-dependent manner (Deacon, 2004; Edwards et al., 2005), activates β-cell proliferation, and suppresses appetite (Xu et al.,

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1999; Szayna et al., 2000). However, commercial exendin-4 (Exenatide, Byetta®) must be injected by diabetic patients at least twice a day because of its short *in vivo* lifetime (Davidson et al., 2005). To overcome this problem, we have prepared and examined several versions of albumin-associated exendin-4 (Son et al., 2009; Chae et al., 2010a, b). In the present study, we prepared a palmitic acid-conjugated exendin-4 derivative with the pharmaceutical advantages of a long *in vivo* half-life and an extended anti-hyperglycemic effect. In addition, the reasons for its longer half-life were elucidated by a series of physicochemical and pharmacokinetic data on particle size, albumin-binding characteristics, *in vivo* biodistribution (particularly at injection sites and tissue organs), and hypoglycemic efficacy in type 2 diabetic mice.

2. Materials and methods

2.1. Materials

Exendin-4 and *N*-hydroxysuccinimidyl-activated palmitic acid (Pal-NHS) were purchased from the American Peptide Company (Sunnyvale, CA) and Sigma–Aldrich (St. Louis, MO), respectively. Fluorescein-NHS and rhodamine-NHS were purchased from Pierce (Rockford, IL). Cyanogen-bromide-activated Sepharose 4B resin was purchased from Sigma–Aldrich. All other reagents, unless otherwise specified, were obtained from Sigma–Aldrich.

2.2. Experimental animals

Type 2 diabetic C57BL/6 *db*/*db* mice (males, 5–6 weeks old) were purchased from the Korean Research Institute of Bioscience and Biotechnology (Daejon). ICR mice (males, ~6 weeks old) were purchased from the OrientBio Experimental Animal Laboratory (Busan). Animals were cared for in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 80-23, revised in 1996). Animals were housed in groups of 6–8 under a 12-h light/dark cycle (lights on at 6 A.M.), allowed food and water *ad libitum*, and acclimatized for 2 weeks. This study was approved by the Ethical Committee on Animal Experimentation at Pusan National University.

2.3. Preparation of palmitic acid-conjugated exendin-4s (Pal-Ex4)

Pal-Ex4 was prepared using a slight modification of a previously described procedure (Kim et al., 2011a, b, c). Briefly, 7 mg of exendin-4 was mixed with 0.9 mg of Pal-NHS (molar ratio 1:1.5) in 3 ml of 0.3% triethylamine/anhydrous dimethylsulfoxide and allowed to react at room temperature for 90 min. The reaction mixture was then subjected to reversed-phase high-performance liquid chromatography (RP-HPLC) on a LiChrospher 100 RP-18 column (250 mm × 4.0 mm, 5 μm, Merck, Germany) at ambient temperature. Gradient elution was carried out at a flow-rate of 1.0 ml/min using solvent A (0.1% trifluoroacetic acid (TFA) in DW) and solvent B (0.1% TFA in acetonitrile), that is, 30–50% B for 10 min and 50–90% B for 20 min. Eluates were monitored at 215 nm, and the fraction corresponding to Pal-Ex4 was collected, dried under nitrogen, and stored in 10 mM phosphate buffer saline (PBS, pH 7.4) at 4 °C until required.

2.4. Particle size characterization of Pal-Ex4

The particle sizes of Ex4 and Pal-Ex4 were measured using a Zetasizer Nano-S90 (Malvern Instruments, USA) and a 633 nm He–Ne laser beam at a fixed scattering angle of 90°. Size analyses were conducted at an Ex4 concentration of 100 μg/ml or a Pal-Ex4 concentration of 30 or 100 μg/ml.

2.5. HSA-binding of Ex4 and Pal-Ex4

The HSA-binding activities of Ex4 and Pal-Ex4 were examined using HSA-immobilized resins, as described previously (Kim et al., 2011a, c). Briefly, cyanogen bromide-activated Sepharose CL-4B (400 mg, Sigma) was mixed with 600 mg of HSA in carbonate buffer (pH 8.5), and reacted for 12 h at 4 °C. Unreacted cyanogen group was then blocked by adding an excess of (15 mg) ethylenediamine. The mixture was then allowed to stand for 3 h at room temperature, and then centrifuged at 3000 rpm. The resin was then washed three times with 10 mM PBS (pH 7.4). The HSA-immobilized Sepharose 4B beads (30 mg/ml) were then treated with rhodamine-NHS (1 mg/ml), and the immobilization degree was checked by examining the beads under a confocal laser scanning microscope (CLSM, Carl Zeiss Meta LSM510, Germany).

To check albumin-binding of Pal-Ex4, fluorescein-NHS (100 μl; 5 mg/ml) in 50 mM phosphate buffer (pH 7.5) was added to Pal-Ex4 (1 ml; 3.0 mg Ex4 equiv.), and reacted at room temperature for 1 h. The reaction mixture was then dialyzed in 10 mM PBS (pH 7.4) using a dialysis kit (*Mw* cutoff 3500, Gene Bio-Application, Israel). Final concentrations of fluorescein-modified Pal-Ex4 were measured using a BCA protein assay kit (Pierce, Rockford, IL), and adjusted to *ca.* 30 μg/ml. Aliquots (400 μl) of fluorescein-modified Pal-Ex4 (30 μg/ml) were then gently shaken with 200 μl of suspended HSA-immobilized Sepharose 4B resins (20 mg/ml HSA equiv.) in 10 mM PBS (pH 7.4) at 37 °C for 3 h. The binding of fluorescein-tagged Pal-Ex4 to resins was visualized by CLSM at excitation and emission wavelengths of 494 and 520 nm, respectively. Separately, 1.0 ml aliquots of Ex4 and Pal-Ex4 (each 30 μg/ml) were mixed with 1.0 ml of HSA (40 mg/ml, pH 7.4) and then gently shaken at 37 °C for 3 h. Each mixture was transferred to Centricon-10 concentrators (Millipore, Beverly, MA, USA), and centrifuged at 3000 rpm until the 1.0 ml filtrate volume. Concentrations of Ex4 or Pal-Ex4 in filtrate fractions were determined using a micro BCA assay kit (Pierce, Rockford, IL).

2.6. Monitoring of injection sites and the tissue distribution of Cy5.5-tagged-Ex4 and -Pal-Ex4

To investigate the biodistributions of Ex4 and Pal-Ex4, 50 μg/100 μl of each sample labeled with Cy5.5 dye (Cy5.5 NHS ester, GE Healthcare, Piscataway, NJ) were administered subcutaneously (s.c.) into the upper backs of ICR mice (one injection/mouse). At predetermined times, NIR fluorescence signals of Ex4 or Pal-Ex4 from injection sites were detected by using a Maestro 2 *in vivo* imaging system (emission filter 690 nm; Cambridge Research and Instrumentation, Inc., Darmstadt, Germany). Separately, at 4 h post-injection, each mice was sacrificed and livers, kidneys, lungs, hearts, and spleens were excised and washed twice with saline. The NIR fluorescence from each organ was visualized using the *in vivo* imaging system.

2.7. Radioiodine labeling of Ex4 and Pal-Ex4

Radioiodine (^{125}I)-labeled samples of Ex4 and Pal-Ex4 were prepared using a modification of the previously described IODOGEN method (Kim et al., 2010, 2011c). In brief, a 100 μl aliquot of water-soluble Bolton–Hunter reagent (*N*-sulfosuccinimidyl-3-4-hydroxyphenyl) propionate (260 μg/ml) in 10 mM PBS (pH 7.0) was mixed with 150 μl of either Ex4 or Pal-Ex4 (1.0 mg/ml) at ambient temperature for 90 min, and then briefly dialyzed *versus* 10 mM PBS to remove the reagent. Separately, 100 μl of IODO-GEN (Pierce, Rockford, IL) solution in methylene chloride (0.5 mg/ml) was dispensed into a fresh tube, and evaporated under a nitrogen stream. To this tube was added a 100 μl aliquot of water-soluble Bolton–Hunter reagent-labeled Ex4 or Pal-Ex4 (approx.

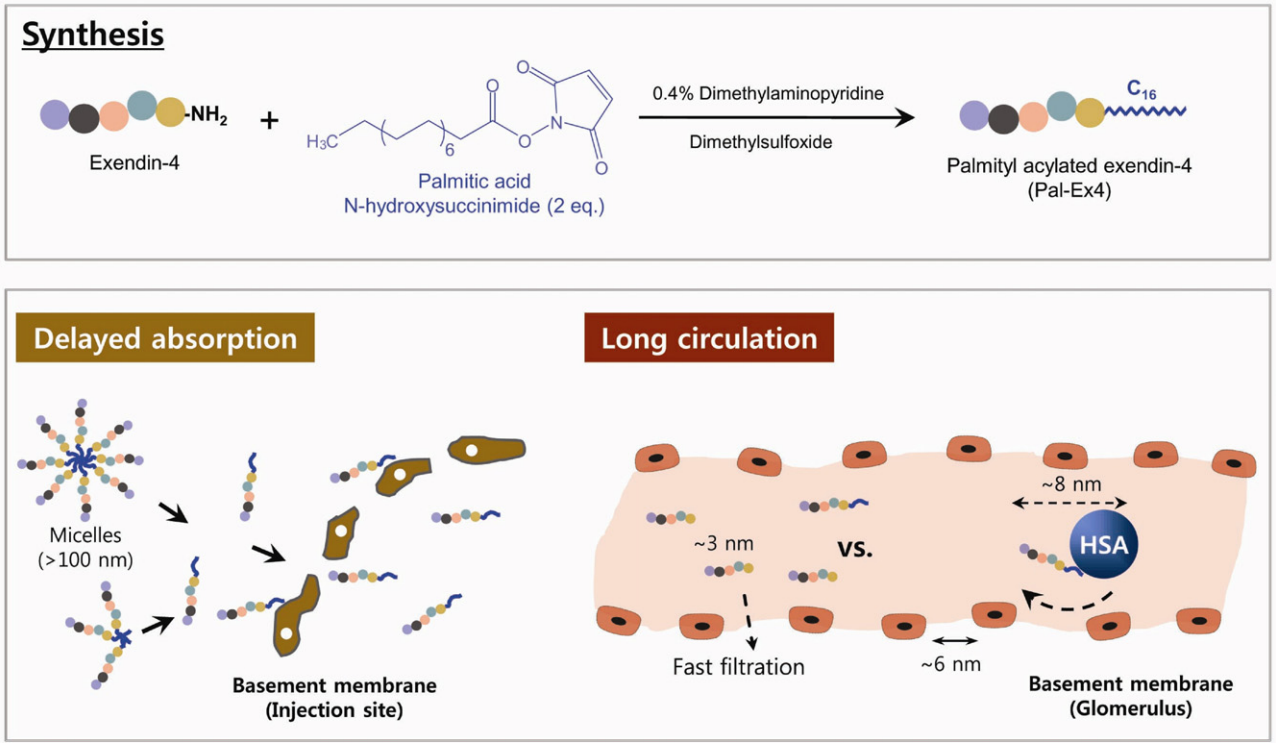

Fig. 1. Synthesis scheme for Pal-Ex4 and proposed model explaining the delayed absorption and the persistence of Pal-Ex4 *in vivo*.

100 μg/ml) and 50 μCi of $Na^{125}I$ (Perkin-Elmer, Boston, MA) in 10 mM PBS (pH 7.4). The reaction was allowed to proceed for 5 min, and then supernatants were loaded onto a LiChrospher 100 RP-8 column (250 mm × 4.0 mm, 5 μm, Merck, Germany) connected to a flow-through radioisotope detector (Ramona 200, Raytest, Straubenhardt, Germany). ^{125}I-labeled fractions were collected, dried under nitrogen, and stored in 10 mM PBS (pH 7.4) at 4 °C until required.

2.8. Pharmacokinetics of Pal-Ex4 in mice

The pharmacokinetics of Ex4 and Pal-Ex4 were evaluated using a modification of a previously described method (Chae et al., 2009; Kim et al., 2010, 2011c). Briefly, radioiodine (^{125}I)-labeled samples were administered s.c. to mice (approximately 0.8 μg/mouse; 7.0×10^4 to 7.3×10^4 cpm). Mice were sacrificed by heart puncture for blood collection at predetermined times and the radioactivities of 0.2 ml blood samples were determined by gamma counting (Cobra™ Series Auto-Gamma Counting System, Packard Instruments Co., Groningen, The Netherlands). These radioactivities were then converted to Ex-4 and Pal-Ex4 concentrations in blood. All pharmacokinetic parameters described were calculated by non-compartmental analysis using WinNolin Ver. 1.1 (Scientific Consulting, Inc., Cary, NC). In particular, AUC_{inf} values were obtained by computing areas under the curve from $t = 0$ to ∞ using the trapezoidal rule. Circulating half-lives ($t_{1/2}$) in the elimination phase were calculated using the same method.

2.9. Anti-hyperglycemic efficacy of Pal-Ex4 in fasted db/db mice

Oral glucose tolerance testing (OGTT) was performed to evaluate the anti-hyperglycemic efficacy of Pal-Ex4 after an overnight fast (18 h), as previously described (Chae et al., 2009, 2010a, b). Briefly, one hour prior to the first oral glucose challenge (0.2 ml, 1.0 g/kg body wt) (0 h), male type 2 diabetic *db/db* mice ($n = 6$/group, 7–8 weeks old) were administered saline, Ex4, or Pal-Ex4 (s.c., 10 nmol/kg body wt). At 3 and 6 h after the first challenge, two additional glucose similar doses were administered. A drop of blood was drawn from a tail vein of each animal at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 3.25, 3.5, 3.75, 4, 4.5, 5, 6, 6.25, 6.5, 6.75, 7, 7.5, 8, and 9 h post-first challenge. Blood glucose levels (BGL) were determined using a one-touch blood glucose meter (ACCU-CHEK® Sensor, Roche Diagnostics, USA). Anti-hyperglycemic efficacies were expressed as total hypoglycemic degrees (HD_{total} *vs.* saline group) and calculated using: $[(AUC_{saline,0\text{-last time}} - AUC_{test,0\text{-last time}})]$.

2.10. Hypoglycemic efficacies of Pal-Ex4 in non-fasted db/db mice

Male *db/db* mice (7–8 weeks old) were used for hypoglycemia testing. Under non-fasting conditions, that is, with free access to water and food, animals were administered a single injection of saline, Ex4, or Pal-Ex4 (50, 100, or 500 nmol/kg body wt, s.c.). Blood glucose levels were monitored using the procedure mentioned above. In addition, a drop of blood was drawn from a tail vein of each animal at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, and 96 h after sample injection, and blood glucose levels (BGL) were determined using a one-touch blood glucose meter. Hypoglycemic efficacies are presented as total hypoglycemic degrees as described above. In addition, hypoglycemic durations (defined as the duration of a blood glucose level of <150 mg/dl after the first injection) were noted.

2.11. Data analysis

Data are presented as means ± SDs. Statistical significances were determined using the One-Way ANOVA test, and *P*-values of <0.05 were considered significant.

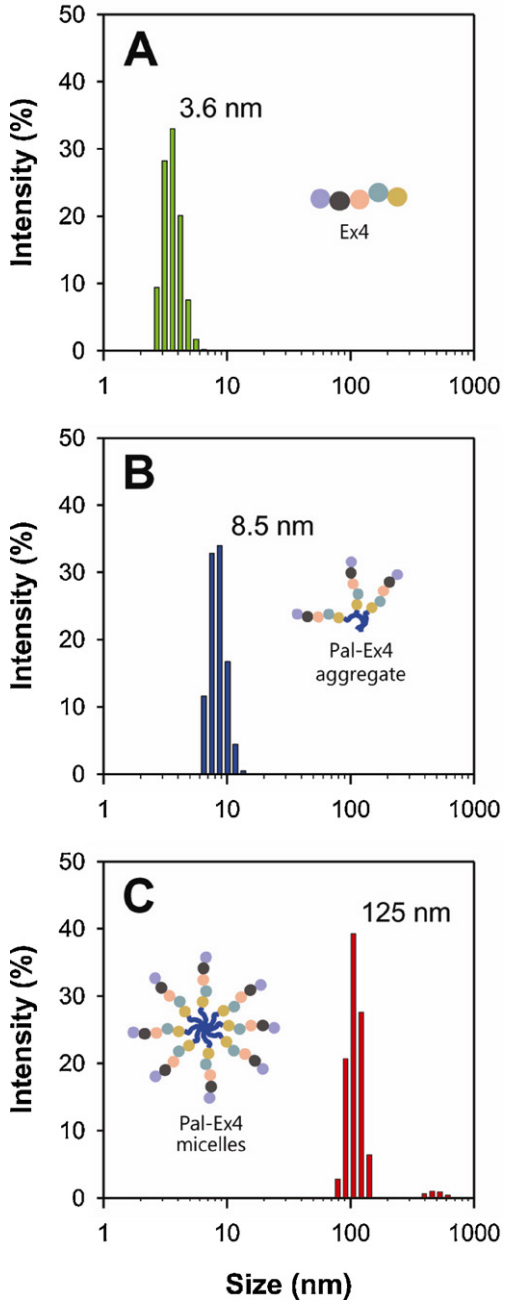

Fig. 2. Particle size histograms of native Ex4 and Pal-Ex4. (A) Ex4 (100 μg/ml); (B) Pal-Ex4 (30 μg/ml); (C) Pal-Ex4 (100 μg/ml).

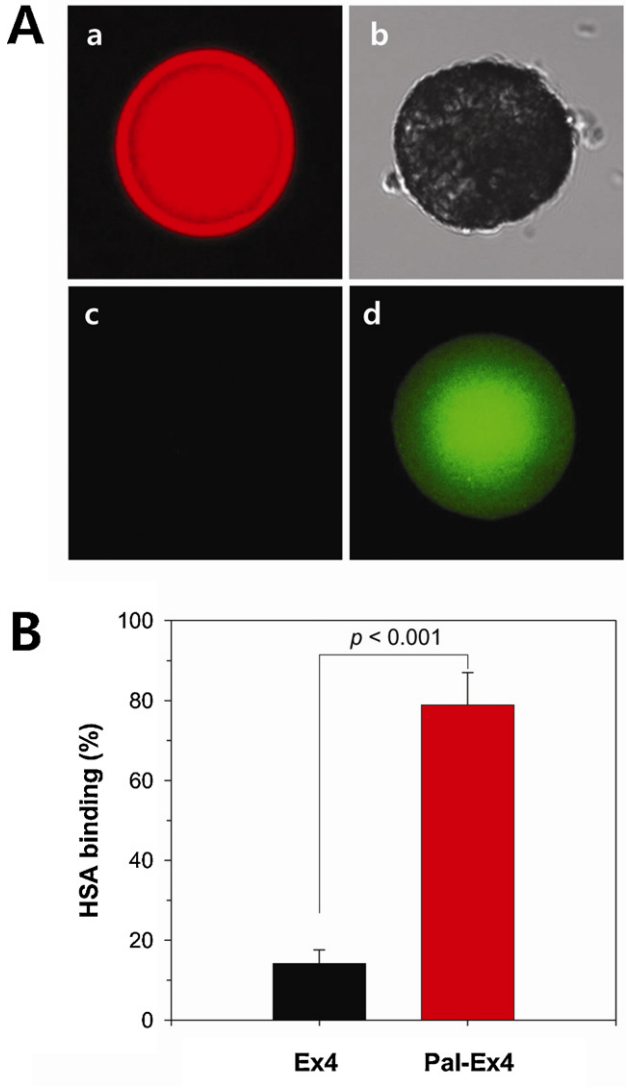

Fig. 3. Confocal laser scanning microscopic images of HSA-immobilized Sepharose CL-4B. (A) a – rhodamine-NHS conjugated; b – morphology of a; c – fluorescein-Ex4 adsorbed; d – fluorescein-Pal-Ex4 adsorbed; (B) HSA-binding percentages of Ex4 and Pal-Ex4.

3. Results

3.1. Preparation and particle size of Pal-Ex4

Pal-Ex4 was prepared by the brief synthesis scheme as seen Fig. 1. The particle size of free Ex4 at 100 μg/ml in 10 mM PBS (pH 7.4) was 3.6 ± 0.4 nm, and that of Pal-Ex4 size at 30–35 μg/ml was 8.5 ± 2.3 nm. However, the particle size of Pal-Ex4 increased to >120 nm at 50 μg/ml (Fig. 2).

3.2. Albumin-binding of Pal-Ex4

Fig. 3A-a shows the rhodamine fluorescence of HSA immobilized on the surface of Sepharose 4B resin; approximately 150 μg of HSA was found to be immobilized by 1 mg of the resin. While fluorescein-derivatized native Ex4 did not show a significant fluorescence (Fig. 3A-c), indicating negligible adsorption, Pal-Ex4 was highly adsorbed by the resin. Consequently, the albumin-bindings of Ex4 and Pal-Ex4 were found to be 14.2 ± 3.4 and 78.9 ± 8.1%, respectively, and this difference was significant ($P < 0.001$; $n = 3$).

3.3. Biodistribution monitoring of Pal-Ex4

As shown in Fig. 4, native Ex4 was rapidly absorbed from the s.c. injection site, distributed systemically, and considerable portion was cleared within 8 h post-injection. On the other hand, Pal-Ex4 was gradually absorbed until nearly 12 h post-injection, and then disappeared over 2 days. Differences between Ex4 and Pal-Ex4 in the distributions into liver, heart, lung, and spleen were insignificant, but kidney concentrations of Pal-Ex4 at 4 h post-injection demonstrated that it was much more resistant to glomerular filtration than native Ex4 (Fig. 5).

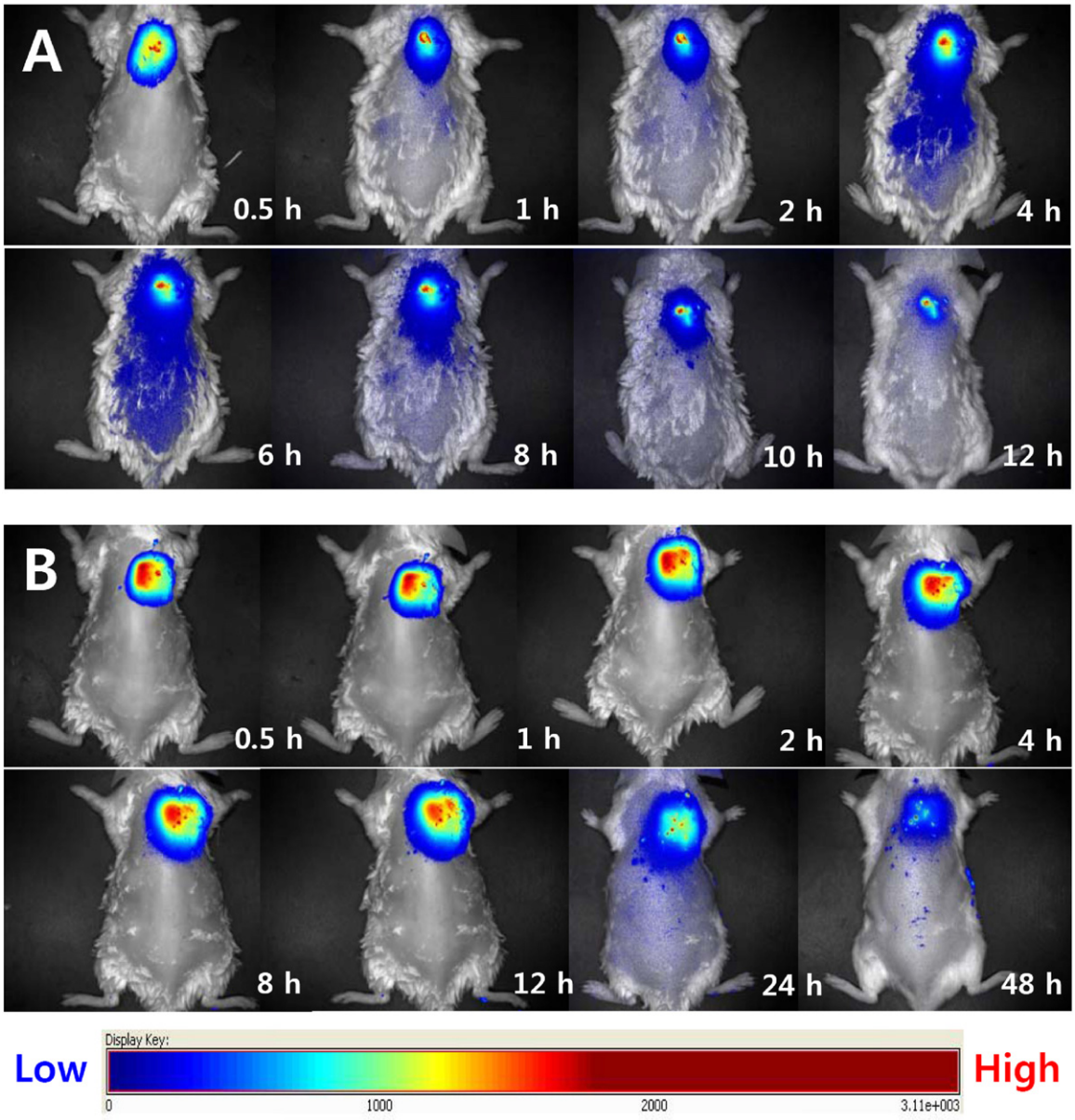

Fig. 4. Monitoring of Cy5.5-labeled Ex4 (A) and of Pal-Ex4 (B) at predetermined times after s.c. administration by NIR imaging.

3.4. Blood clearances of Ex4 and Pal-Ex4

Blood concentration *vs.* time curves plotted for Ex4 and Pal-Ex4 in ICR mice after administration. As shown in Fig. 6, Ex4 was rapidly removed from the circulation with a circulating $t_{1/2}$ of 1.8 ± 0.5 h, whereas Pal-Ex4 had a substantially larger $t_{1/2}$ of Pal-Ex4 20.1 ± 2.2 h, and an AUC_{inf} value (1457.4 ± 84.6 ng/ml h) was 18.3 times greater than that of Ex4 (79.3 ± 5.3 ng/ml h). The C_{max} values of the two agents were not significantly different 26.9 ± 1.2 *vs.* 33.2 ± 1.4), but T_{max} of Pal-Ex4 occurred much later (8.0 ± 0.5 h *versus* 1.0 h; Table 1).

3.5. Anti-hyperglycemic activities of Ex4 and Pal-Ex4 in fasted db/db mice

The anti-hyperglycemic efficacies of Ex4 and Pal-Ex4 were compared in fasted type 2 diabetic *db*/*db* mice by OGTT testing 1 h post-injection. As shown in Fig. 7A, the total hypoglycemic degree (HD_{total}: 657.9 ± 49.3) of Pal-Ex4 over three consecutive OGTTs until 9 h was significantly greater than that of Ex4 (364.6 ± 98.3, $P = 0.03$). The first peak glucose level (215.3 ± 56.4 mg/dl) of Pal-Ex4 was not significantly different from that of Ex4 (204.3 ± 55.7 mg/dl, $P > 0.6$) at 0.25 h after glucose administration. However, its second and third peak glucose levels at 3.25 and 6.25 h (124.3 ± 13.7 and 115.8 ± 19.6 mg/dl, respectively) were much lower than those of native Ex4 (200.0 ± 34.0 and 246.3 ± 48.9 mg/dl, respectively, $P < 0.04$).

3.6. Hypoglycemic activities of Ex4 or Pal-Ex4 in non-fasted db/db mice

The effects of Pal-Ex4 on hypoglycemia were examined at three doses (50, 100, and 500 nmol/kg, i.p.) in *db*/*db* mice. As shown in Fig. 7, the lowest glucose levels (43.1–87.3 mg/dl) reached in Pal-Ex4-treated mice were slightly lower than in Ex4-treated mice (50.3–94.8 mg/dl) at each dose, but this difference was not significant ($P > 0.5$). However, the time taken to reach a glucose nadir in Pal-Ex4-treated mice (6.1 ± 1.1 h) was much

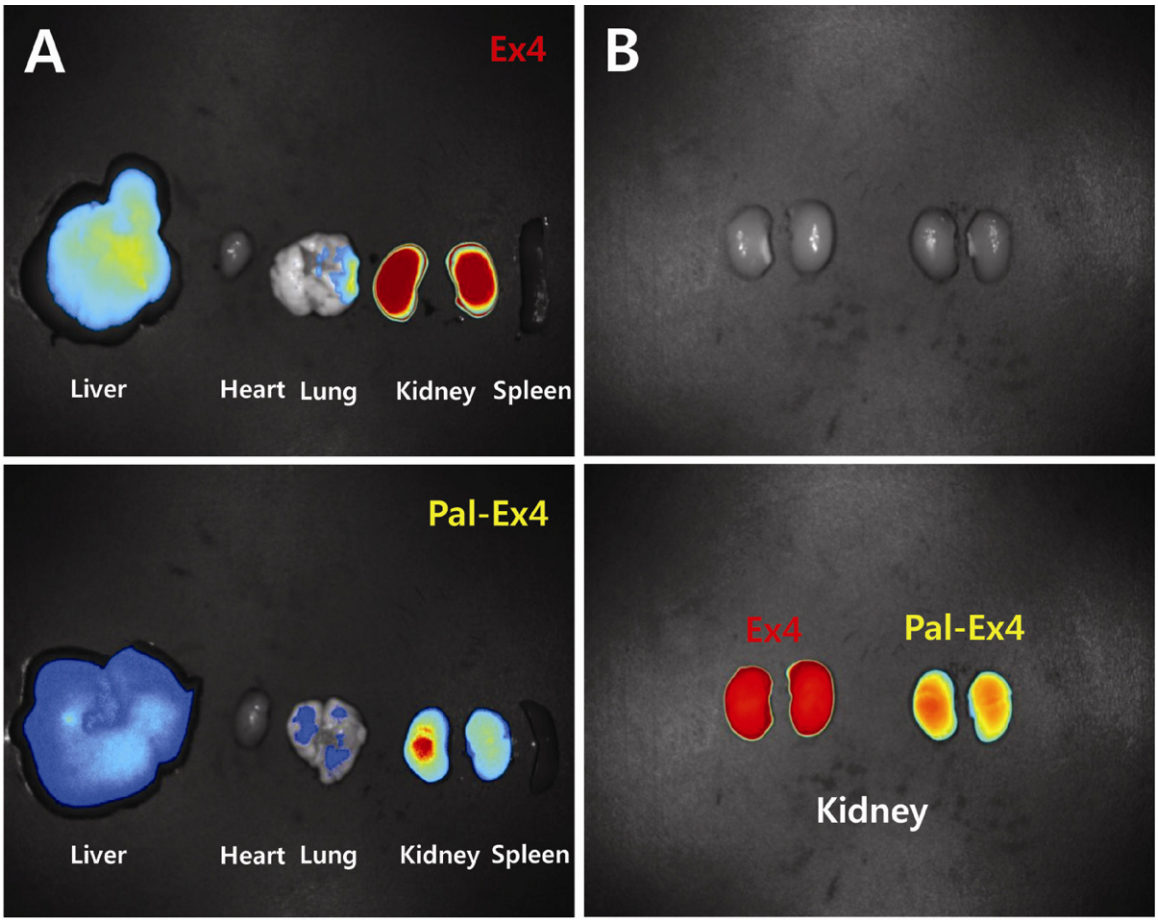

Fig. 5. Images of various organs excised from ICR mice at 4 h after the s.c. administration of Cy5.5-labeled Ex4 or Pal-Ex4. (A) Various tissue organs; (B) Kidney.

greater than in Ex4-treated mice (2.1 ± 0.5 h) ($P < 0.01$). Moreover, the HD_{total} values of Pal-Ex4 (3583.5–17595.8) were remarkably greater by about 4 fold at all doses than those of Ex4 (960.1–4154.5). In addition, the hypoglycemic durations of Pal-Ex4 treated mice were much greater and times to rebound ~150 mg/dl for Pal-Ex4 (12, 36, and 78 h) were 2.2-, 5.1-, and 4.3-times longer than those for Ex4 treated mice (5.5, 7.0, 7.0 h) at doses of 50, 100, and 500 nmol/kg, respectively ($P < 0.01$).

4. Discussion

Fatty acid-conjugation caused hydrophilic peptides to bind to albumin, because albumin possesses approximately five binding positions for long fatty acids. Therefore, fatty acid-modified drugs can bind tightly but reversibly to circulating albumin (Kratz, 2008; Kim et al., 2011c). This method has clear advantages in terms of preserving receptor-binding activity over other albumin-based techniques, such as, albumin-conjugation or albumin-fusion, which result in permanent covalent cross-linking between albumin and peptides. Actually, human serum albumin molecule (~67 kDa) is much larger than most peptides, which are usually a few thousands Daltons, and hence, albumin is likely to interfere with the binding of peptides to their action-receptors (Kim et al., 2003, 2011c; Subramanian et al., 2007). For this reason, albumin-linked peptides exhibit considerable losses in intrinsic bioactivity *in vitro* and low therapeutic efficacy, whereas, fatty-acid conjugated peptides have additional few hundred Dalton-hydrocarbons, and can reversibly interact with circulating albumin with minimum loss of bioactivity. This is one of the reasons why a myristic acid-insulin (DesB30-Insulin detemir: Levemir®, Novo Nordisk) and a palmitic acid-glucagon-like peptide-1 analog (Victoza®: previous Liraglutide, Novo Nordisk) were approved by the FDA in 2005 and 2010, respectively, earlier than the other albumin-based peptide drugs.

Palmitic acid-conjugated exendin-4 (Pal-Ex4) was prepared and characterized as previously described (Kim et al., 2011a, c). The palmitic hydrocarbon chain is so hydrophobic that Pal-Ex4 assembles in solution to form micelles. Actually, Pal-Ex4 had a significant particle size (8.5 nm) even at a concentration of 30 μg/ml,

Table 1
Pharmacokinetic parameters and pharmacological scores of Ex4 and Pal-Ex4 in ICR and *db*/*db* mice.

PK parameters	Ex4	Pal-Ex4	Pharmacological scores	Ex4	Pal-Ex4
AUC_{inf} (ng/ml h)	79.3 ± 5.3	1457.4 ± 84.6	HD_{total} at 10 nmol/kg (OGTT)	364.6 ± 98.3	657.9 ± 49.3
C_{max} (ng/ml)	26.9 ± 1.2	33.2 ± 1.4	HD_{total} at 50 nmol/kg	960.1 ± 145.7	3583.5 ± 532.2
T_{max} (h)	1.0 ± 0.0	8.0 ± 0.5	HD_{total} at 100 nmol/kg	1955.1 ± 554.3	9325.6 ± 1056.2
$t_{1/2}$ (h, β)	1.8 ± 0.5	20.1 ± 2.2	HD_{total} at 500 nmol/kg	4154.5 ± 344.9	17595.8 ± 2562.7
			Time to reach glucose level nadir (h)	2.1 ± 0.5	6.1 ± 1.1

Data are means ± SDs. AUC_{inf}, area under the curve from zero to infinity; C_{max}, maximum concentration after intraperitoneal administration; T_{max}, required time to reach C_{max}; $t_{1/2}$, elimination half-life.

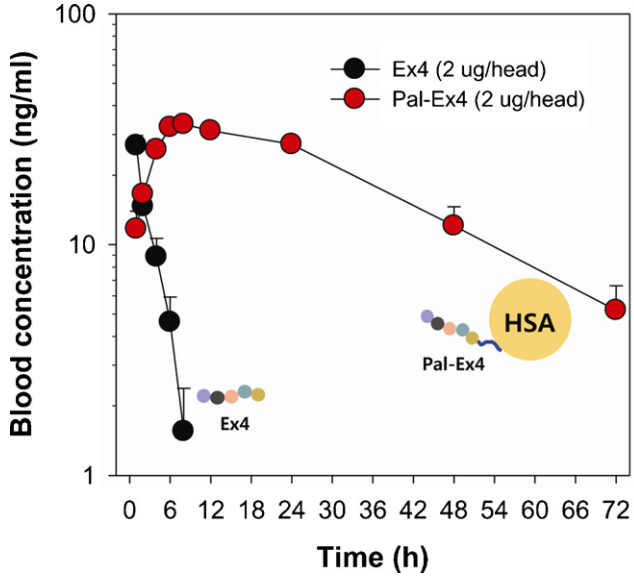

Fig. 6. Blood clearance profiles of Ex4 and Pal-Ex4 in ICR mice after s.c. administration (approximately 0.8 μg). Data represent four individual animals and are presented as means ± SDs.

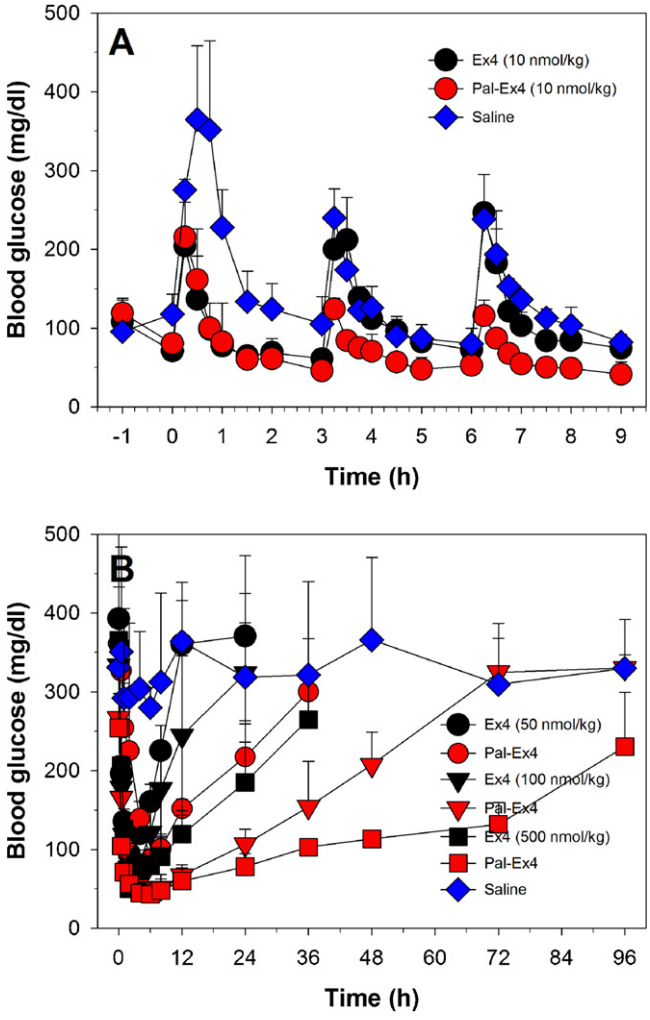

Fig. 7. (A) Anti-hyperglycemic activities of Ex4 and Pal-Ex4 as determined by three consecutive OGTTs in type 2 diabetic *db*/*db* mice fasted for 18 h after each s.c. administration (10 nmol/kg); (B) hypoglycemic efficacies of Ex4 and Pal-Ex4 in non-fasted *db*/*db* mice at doses of 50, 100, and 500 nmol/kg. Data represent six individual mice and are presented as means ± SDs.

and formed larger micelles (~125 nm) at higher concentrations. Micelles probably do not enter circulation, but rather dissociated first into monomers, which considerably delays absorption. In the present study, Pal-Ex4 was found at injection sites 12 h post-injection, whereas native Ex4 was absorbed within minutes and was undetectable in blood at 6 h. In addition, Pal-Ex4 had much larger T_{max} values (8.0 *vs.* 1.0 h).

The albumin-binding ability of palmitic acid-conjugated exendin-4 was confirmed by two different binding experiments. First, the binding of Pal-Ex4 onto immobilized HSA was visualized by CLSM, and fluorescein-tagged Pal-Ex4 exhibited much strong adsorption. Although this kind of binding study was useful in terms of fluorescence visualization, immobilized-HSA might not have full the molecular integrity of free HSA. Therefore, we also investigated the binding activity of Pal-Ex4 in HSA solution at a high free HSA concentration (35–50 g/L), mimicking actual plasma *in vivo* and at a low concentration (~30 μg/ml). It was found that Ex4 and Pal-Ex4 were able to bind freely to HSA without forming micelles, suggesting tight binding between Pal-Ex4 and albumin.

Furthermore, the kidney localization of Pal-Ex4 were dramatically less than those of native Ex4, and its circulating half-life was substantially greater (~20 *vs.* 1.8 h), which supports the notion that Pal-Ex4 was bound to circulating HSA *in vivo*. Actually, almost all peptides and proteins below 70 kDa are believed to be removed by glomeruli, but HSA is clearly resistant to the process because of its size (67 kDa) and negative charge (Caliceti and Veronese, 2003).

In the present study, the *in vivo* efficacies of Ex4 and Pal-Ex4 were evaluated using an oral glucose tolerance test (OGTT) in the fasted states, which mimic the postprandial and interprandial states, respectively. At the first OGTT, Pal-Ex4 was found to be as anti-hyperglycemic as Ex4, and have a much longer anti-hyperglycemic effect and to maintain a normal glucose level (<150 mg/dl) longer than Ex-4 at the second and third OGTTs. This observation demonstrates that Pal-Ex4 is able to stabilize glucose levels for a longer time than Ex4.

Summarizing, palmitic-acid conjugated exendin-4 was found to have greatly improved *in vivo* pharmacokinetic and pharmacodynamic characteristics than exendin-4 in a diabetic rodent model. We believe that this enhanced *in vivo* performance of Pal-Ex4 is due to albumin-binding and micelle formation as described in Fig. 1. Based on these results, we believe that the described palmitic acid-conjugated exendin-4 derivative has considerable pharmaceutical potential as a treatment for type 2 diabetes.

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