IN VIVO CONVERSION OF PEPTIDE DRUGS INTO HIGH MOLECULAR WEIGHT FORMS

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Following injection of $^{125}$I-porcine insulin, $^{125}$I-aprotinin, $^{125}$I-salmon calcitonin, or $^{125}$I-(Asu$^{1,7}$)-eel calcitonin into rats, high molecular weight (HMW) forms of these peptides were detected in serum or plasma when analyzed by gel chromatography. The conversion into HMW forms occurred after 1) intravenous bolus injection of insulin, aprotinin, or calcitonins, 2) intravenous infusion of insulin or aprotinin, and 3) subcutaneous injection of insulin, indicating that HMW forms were produced in the general circulation not in the subcutaneous tissue. Rechromatography of HMW forms produced in vivo from insulin or aprotinin showed the release of lower molecular weight component which was eluted at the same position of parent peptide, the immunoreactivity of the released component derived from insulin was almost the same as for insulin. These results suggest that the conversion of peptide drugs into HMW forms is generally occurred in vivo and they play a role as a depot in circulation.

Keywords — peptide high molecular weight form; peptide metabolism; insulin; aprotinin; calcitonin; gel chromatography; immunoassay

INTRODUCTION

With progress of techniques in peptide synthesis, large numbers of peptide hormones have become available for therapeutic use. However many remaining problems need to be elucidated for their effective and safe clinical application. One important aspect is the clarification of their disposition, such research being hindered by their short half-lives and lack of rapid and specific assay method.

There is some evidence supporting conversion of peptide drugs into high molecular weight (HMW) forms in circulation after their intravenous injection.$^{1-8}$ However, little is known about the mechanism of their production, composition, or physiological roles. Thus, studies on these problems are important for a better understanding of the in vivo behavior of peptide drugs.

On the other hand, the immunological heterogeneity of endogenous peptide hormones in blood and tissue has been extensively studied in relation to their biosynthesis. But the relationship between HMW forms produced after the administration of exogenous peptides and the heterogeneity of endogenous peptides has not yet been demonstrated. In this study, porcine insulin, aprotinin, salmon calcitonin, and (Asu$^{1,7}$)-eel calcitonin were used to investigate whether the conversion of peptide drugs into HMW forms is a general phenomenon following administration of exogenous peptide drugs. In addition, an attempt to clarify the physiological roles of the HMW forms was made.

MATERIALS AND METHODS

Materials — Monocomponent porcine insulin and aprotinin (from beef lung) were pur-
Conversion of Peptides into HMW Forms

chased from Novo Industri A/S, Copenhagen and C. F. Boehringer & Soehne, Mannheim, respectively. Salmon calcitonin was a gift from Sandoz AG, Basel and (Asu\(^1-7\))-eel calcitonin was a gift from Toyo Jozo Ltd., Shizuoka. Sodium iodide (Na\(^{125}\)) was purchased from The Green Cross Corp., Osaka. Anti-porcine insulin guinea pig serum and anti-guinea pig immunoglobulin G (IgG) rabbit serum were purchased from Miles Laboratories, Inc., Elkhart, Indiana and Medical and Biological Laboratories Ltd., Osaka, respectively. Other chemicals were of reagent grade.

Each peptide was labeled with \(^{125}\)I\(^+\) and purified by gel filtration of Bio-Gel P-150 (0.9 \(\times\) 50 cm, 0.1 M borate buffer, pH 8, containing 1\% bovine serum albumin as an eluant). In some cases peptides were dialyzed against saline. Specific activities were about 100 mC/mg for insulin and aprotinin, and about 50 mC/mg for calcitonins.

**Animal Studies** — Male rats of Wistar strain weighing 120 - 160 g were injected with peptide drugs under anesthesia with pentobarbital. The agents for injection were a mixture of tracer amount \(^{125}\)I-peptides and conventional doses of unlabeled peptides. The agents were administered by intravenous bolus injection (femoral vein), intravenous infusion (femoral vein), or subcutaneous injection (back skin). Blood samples were obtained from aorta or cervical artery, and serum or plasma was separated by centrifugation for analysis. For the characterization of isolated HMW forms produced in vivo, insulin or aprotinin was administered intravenously (jugular vein) and blood was obtained from the aorta at 27 min after start of injection. Serum separated by centrifugation was analyzed.

**Analysis** — Serum or plasma sample was analyzed by gel filtration on Bio-Gel P-150 (0.9 \(\times\) 50 cm, 0.1 M borate buffer, pH 8, containing 1\% bovine serum albumin, or 2\% sodium dodecyl sulfate as an eluant), or Toyopearl HW-40 (1.5 \(\times\) 73 cm, 0.1 M borate buffer, pH 8, containing 1\% bovine serum albumin as an eluant). Each column was calibrated with dextran blue (void volume), \(^{125}\)I-peptide, and free \(^{125}\)I (column volume). Fractions of 25 drops each (Bio-Gel P-150) or 89 drops each (Toyopearl HW-40) were collected. Radioactivity was measured by an automatic well-type scintillation counter.

Immunoprecipitation with the double antibody technique was used to estimate the immunoreactivity of samples. After rechromatography of HMW forms produced in vivo on Bio-Gel

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**FIG. 1. Gel Filtration Pattern on Bio-Gel P-150 of Rat Serum after Administration of \(^{125}\)I-Insulin**

a) Intravenous bolus injection (1 U/kg, 30 min). b) Intravenous infusion (0.010 U/kg/min. for 98 min). c) Subcutaneous injection (0.35 U/kg, 30 min).
P-150 (2% sodium dodecyl sulfate as an eluant), two fractions of each peak (high molecular weight, intact peptide, or low molecular weight region) and control insulin solution (in 2% sodium dodecyl sulfate) were refrigerated at 4 °C and centrifuged to partially remove sodium dodecyl sulfate. Supernatant was transferred to another tube and the volume of each sample adjusted to the initial volume (about 1 ml) by addition of 0.1 M borate buffer, pH 8, containing 1% bovine serum albumin. Then 0.1 ml of the first antibody, anti-porcine insulin guinea pig serum (10-fold dilution with 0.1 M borate buffer, pH 8, containing 1% bovine serum albumin) was added to each tube and incubated at 4 °C. After 24 h, 0.1 ml of the second antibody, undiluted anti-guinea pig IgG rabbit serum, was added. After 16–24 h incubation at 4 °C, each incubation mixture was centrifuged at 3000 rpm for 30 min in a refrigerated centrifuge. Supernatant was decanted and radioactivity of the precipitate was measured.

RESULTS

Production of HMW Forms Following the Injection of Peptide Drugs

Serum or plasma separated and analyzed by gel filtration exhibited peaks at a HMW region 30 min after bolus injection of insulin (Fig. 1a). Similar results were obtained for intravenous infusion (Fig. 1b) and subcutaneous injection (Fig. 1c). Using the same analytical method HMW peaks were observed in serum 30 min after intravenous bolus injection and intravenous infusion of aprotinin (Fig. 2a and b). High molecular weight peak also appeared 30 min after bolus injection of salmon calcitonin (Fig. 3a) or (Asu₁₋₇)-eel calcitonin (Fig. 3b).

**FIG. 2. Gel Filtration Pattern on Bio-Gel P-150 of Rat Serum or Plasma after Administration of ¹²⁵I-Aprotinin**  
a) Intravenous bolus injection (6000 U/kg, 30 min). b) Intravenous infusion (52 U/kg/min for 96 min).

**FIG. 3. Gel Filtration Pattern on Bio-Gel P-150 of Rat Serum after Intravenous Bolus Injection of ¹²⁵I-Calcitonins**  
a) Salmon calcitonin (0.7 U/kg, 30 min). b) (Asu₁₋₇)-eel calcitonin (0.7 U/kg, 30 min).
Rechromatography of HMW Forms Produced in Vivo

The appearance of a peak corresponding to insulin was observed by rechromatography of HMW form produced after intravenous injection of insulin (Fig. 4). Elution with borate buffer gave the insulin peak 9% of total radioactivity and with sodium dodecyl sulfate 14%. Repeated rechromatography with 2% sodium dodecyl sulfate showed that HMW form released insulin corresponding to 30% of initial radioactivity.

Rechromatography of HMW form produced after intravenous injection of aprotinin resembled the results obtained from insulin (Fig. 5). In the case of aprotinin, 25—30% of radioactivity corresponding to aprotinin was released from HMW form by elution with borate buffer or 2% sodium dodecyl sulfate. Repeated rechromatography with sodium dodecyl sulfate showed that the HMW form releases aprotinin at over 50% of initial radioactivity.

Immunoreactivity of Released Insulin from HMW Form Produced in Vivo

High molecular weight form produced in vivo included insulin as shown in Fig. 4. For the fractions obtained by rechromatography of HMW form with sodium dodecyl sulfate, their immunoreactivity was estimated by immunoprecipitation and was compared to the value for

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**FIG. 4. Rechromatography of High Molecular Weight Form Produced in Vivo from Insulin**

- a) Isolation of high molecular weight form by gel filtration on Bio-Gel P-150 using 0.1 M borate buffer (pH 8, containing 1% bovine serum albumin) as an eluant. Hatched area represents the fractions of high molecular weight form.
- b) Rechromatography on Bio-Gel P-150 column with 0.1 M borate buffer (pH 8, containing 1% bovine serum albumin) as an eluant.
- c) Rechromatography on Bio-Gel P-150 column with 2% sodium dodecyl sulfate as an eluant.
intact insulin. Figure 6 shows the rechromatographic pattern of HMW form and the immunoreactivity of each peak fraction. As shown here, released insulin had almost the same immunoreactivity as control insulin, and HMW form retained the immunoreactivity but to a lesser extent than intact insulin. Low molecular weight component had little immunoreactivity.

**DISCUSSION**

*In vivo* conversion into HMW forms has been previously described for insulin, human calcitonin, and human growth hormone.1–6) However, *in vivo* conversion into HMW form was not demonstrated as a general phenomenon for exogenously injected peptides.

This study demonstrates that *in vivo* conversion into HMW form following insulin administration occurs independent of administration routes, that is intravenous bolus injection, intravenous infusion, or subcutaneous injection. This is the first report on conversion into HMW form *in vivo* after intravenous bolus injection or intravenous infusion of aprotinin. Studies on two species of calcitonin also demonstrated *in vivo* conversion into HMW forms after their intravenous injection.

There are two similarities between the molecular structures of aprotinin and insulin: 1) Molecular weight of aprotinin is 6513 and that of insulin is 5777, and 2) both have three intramolecular disulfide bonds. Though it is not clear what properties are needed for the conversion into HMW forms *in vivo*, the above similarities were a reason to expect the conversion of aprotinin, as was confirmed in this study. Further in-

**FIG. 5. Rechromatography of High Molecular Weight Form Derived from Aprotinin**

*High molecular weight form was isolated and processed in the same manner as shown in Fig. 4.*
vestigations on two calcitonins, with a molecular weight nearly half that of insulin or aprotinin, also demonstrated the conversion into HMW form in vivo. Taking into consideration reports on other peptides, such as growth hormone,\textsuperscript{5,6} it is suggested that conversion into HMW forms following administration of peptide drugs might be a common phenomenon.

Though physiological roles of HMW forms produced in vivo has not yet been demonstrated, studies on the composition of HMW forms may give a clue to this question. Rechromatography of HMW forms derived from insulin or aprotinin indicates that intact peptides are included in HMW forms which can also be released from them. This is supported by the finding that released insulin from HMW form retained almost the same immunoreactivity as intact insulin. Such data suggest that HMW forms, produced in vivo after the injection of peptide drugs, may act as their depot.

There are several possibilities for the composition of HMW forms produced in vivo: 1) Aggregated peptides, 2) macromolecule-bound peptides, 3) macromolecule-bound fragments of degraded peptides, and 4) newly biosynthesized products from degraded peptides. Studying the in vivo behavior of isolated HMW form produced after intravenous injection of insulin, Antoniades \textit{et al.} suggested that it contained insulin or modified insulin and was not derived from the incorporation of the radioactive fragment of insulin or iodine into other serum macromolecules.\textsuperscript{5,8} On the other hand, Halban \textit{et al.} used \textsuperscript{3}H-insulin and showed that some portions of the HMW form may be produced by reincorporation of \textsuperscript{3}H-Phe, which is liberated by the degradation of \textsuperscript{3}H-insulin, into newly synthesized protein.\textsuperscript{41} Though we observed a small radioactive HMW peak after intravenous injection of \textsuperscript{125}I-Tyr (data not shown), this material probably comprised only a small fraction of the HMW form, this is deduced from lack of immunoreactivity in the material and the appearance of HMW form in circulation in a few minutes after administration. Gjedde \textit{et al.}, using immunological techniques, demonstrated that insulin was associated with one serum protein to produce HMW form after intravenous injection.\textsuperscript{1,49} Although there are contradictory estimations for the composition of HMW form derived from insulin, all these reports are from data obtained under limited conditions. It is most likely that the total composition of HMW form from insulin is a mixture of all these components and is dependent on experimental conditions including time from injection to analysis, this may be true for other peptides. Data shown in this study indicate that HMW forms, derived from peptide drugs, are partly composed of dissociable intact peptides retaining their biological activity. Therefore HMW forms may act as a depot for these peptides in the circulation.

**FIG. 6. Immunoreactivity of Released Components from High Molecular Weight Form Derived from Insulin**

\textit{a) Rechromatography of high molecular weight form with 2% sodium dodecyl sulfate as an eluant. Solid circles represent the fractions examined. b) Immunoreactivity of the peak fractions shown in a) and intact insulin (control) assessed by immunoprecipitation with double antibody technique. Control insulin was treated in the same manner as other samples.}
REFERENCES


