Originals

Receptor-recycling model of clearance and distribution of insulin in the perfused mouse liver

H. Sato, T. Terasaki, H. Mizuguchi, K. Okumura and A. Tsuji

Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa, Japan

Summary. After perfusion of mouse livers with A_{14} - 125 I-insulin for designated intervals, an acid-wash technique was employed to separately measure the surface-bound (X_s) and intracellular (X_i) A_{14} - 125 I-insulin, as well as intracellular degradation products (X_{deg}) of labelled insulin. From the perfusate concentrations (C_p) of A_{14} - 125 I-insulin, the apparent intrinsic hepatic clearance of labelled insulin at a high dose (0.2 nmol/l) was shown to be 60% smaller than that at a low dose (0.018 nmol/l), indicating that the cellular uptake of insulin is remarkably nonlinear at the concentration range examined. From the time courses of C_p , X_s , X_i and X_{deg} , the hepatic insulin disposition was shown to be largely accounted for by the receptor-mediated endocytosis. The observed data at the low dose were analysed to estimate biochemical parameters, (i.e., total receptor number, endocytotic rate constant and intracellular degradation rate constant) according to “receptor-recycling” and “non-receptor-recycling” models, using a computer-aided optimization procedure. The “receptor-recycling” model could not only adequately explain the C_p , X_s , X_i and X_{deg} at the low dose, but also predict the C_p at the high dose. On the other hand, a “non-receptor-re-

cycling” model, in which recycling of receptors was not assumed, could also explain the observed data at the low dose, but failed to predict the C_p at the high dose, indicating that the receptor recycling process is necessary to explain the hepatic insulin clearance at high insulin concentrations, at which hepatic insulin clearance should be limited by the rate of receptor recycling. However, the applicability of our model might be limited within the physiologic insulin concentrations, because of the negative co-operativity of insulin-receptor interaction and a high-capacity, non-degradative and more rapidly recycling pathway for receptors that may occur at high concentrations of insulin. In conclusion, we have developed a mathematical model of hepatic insulin clearance and distribution under physiological conditions, including receptor binding, receptor-mediated endocytosis and receptor recycling, which has been so far demonstrated using isolated hepatocytes.

Key words: Insulin, hepatic clearance, receptor recycling, receptor-mediated endocytosis, physiological model, mouse liver perfusion.

There is a growing body of evidence that various polypeptides are taken up by their target cells *via* receptor-mediated endocytosis [1, 2]. In particular, the uptake of insulin by isolated hepatocytes involves binding to receptors, endocytosis of the insulin-receptor complex, dissociation of the ligand from the receptor in an acidified endosomal compartment, and transport of the ligand to the lysosomes in which the ligand is degraded [3]. On the other hand, the internalized insulin receptors are largely recycled back to the surface of hepatocytes and reutilized [3, 4].

The liver plays a major role in regulating the peripheral insulin concentration due to extraction of insulin from the portal blood before entrance into the systemic circulation. Therefore, there have been a number of reports measuring hepatic extraction of insulin in *in vivo* and in isolated perfused livers, which demonstrate a nonlinear hepatic

clearance at physiological insulin concentrations [5–7]. Moreover, it has been proposed that receptor-mediated endocytosis and its subsequent events such as receptor recycling are the major mechanisms by which receptor down-regulation is mediated [8–10]. However, there have been no explicit approaches to predict the changes in hepatic clearance and distribution of insulin, as well as the changes in surface receptor number in a physiological situation, possibly due to the lack of an adequate kinetic model.

Therefore, in the present study, we performed mouse liver perfusion experiments to examine hepatic clearance and distribution of A_{14} - 125 I-insulin, combined with an acid-wash technique [11, 12] to separately measure the surface-bound and internalized amounts of A_{14} - 125 I-insulin, and intracellular degradation products of A_{14} - 125 I-insulin in

the liver. In order to analyse the observed data, we developed an organ model of hepatic insulin disposition, including receptor binding, receptor-mediated endocytosis and receptor recycling, namely the “receptor-recycling” model. Using this model, we attempted to predict the non-linear hepatic elimination of insulin and the down-regulation of surface insulin receptors in the liver. The importance of receptor recycling on the hepatic insulin clearance was also discussed.

Materials and methods

Materials

Human insulin ^{125}I -labelled at tyrosine- A_{14} (A_{14} - ^{125}I -insulin), with a specific activity of $2000 \text{ Ci} \cdot \text{mmol}^{-1}$, was purchased from Amersham (Amersham, Buckinghamshire, UK). Crystalline porcine insulin and bovine serum albumin (BSA; Fraction V) were obtained from Sigma Chemical Co. (St. Louis, Mo, USA), and trichloroacetic acid (TCA) from Wako Pure Chemical Industries (Osaka, Japan). Bovine erythrocytes were kindly supplied from the Meat Inspection Center of Kanazawa City (Ishikawa, Japan). All other reagents were commercially available and of analytical grade. The labelled insulin was dissolved in phosphate buffered saline (PBS) containing 0.1% BSA and stored at -20°C until study. The labelled insulin used was at least 98% pure as assayed by both TCA-precipitability and HPLC analysis.

Animals

Adult male ddY mice were obtained from Sankyo Laboratory Co. (Toyama, Japan) and allowed free access to standard rodent chow and water. Mice were fasted overnight before use.

In situ liver perfusion

The mice were anaesthetized with intraperitoneal pentobarbital ($50 \text{ mg} \cdot \text{kg}^{-1}$), and livers were operated on according to the method of Mortimore and Tietze [13]. The livers were cyclically perfused *in situ* at 37°C in a temperature-controlled cabinet. The perfusate consisted of 10% washed bovine erythrocytes, 3% BSA, and 3.69 mmol/l glucose in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4). Perfusate was continuously oxygenated with 95% O_2 /5% CO_2 by passage through silastic tubing in an oxygenation chamber. The inlet temperature was kept at 37°C . After operation, livers were perfused with erythrocyte-free KRB buffer in a single-pass system for 5 min, and perfused with 12.0 ml erythrocyte-containing KRB buffer in a closed-circuit system for 10 min. Then, a tracer amount of A_{14} - ^{125}I -insulin ($0.44 \mu\text{Ci}$ or 0.018 nmol/l in a final concentration), with and without 2 nmol/l of unlabelled insulin, was added in the reservoir which was constantly stirred. Subsequently, portions ($100 \mu\text{l}$) of the perfusate were sampled at designated times, and centrifuged for 1 min in a microcentrifuge to remove erythrocytes. A_{14} - ^{125}I -insulin in perfusate samples (without erythrocytes) was assayed by either TCA-precipitation or HPLC analysis as described later. Unless otherwise mentioned, the perfusion flow rate (including erythrocytes) was set at $1.85 \text{ ml} \cdot \text{min}^{-1}$ and the perfusion pressure at 15 – 18 mm Hg , using a peristaltic pump (model MP, Tokyo Rikakikai Co, Tokyo, Japan). During the 30-min perfusion the concentrations of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were determined with a commercial test kit (STA-Test Wako, Wako) to assess the viability of the perfused livers.

Acid-wash technique in the perfused liver

At designated times after the addition of A_{14} - ^{125}I -insulin into the reservoir, the acid-wash technique was performed as previously described [12]. After 20 fractions of acid-washout solution (designated as acid samples) containing acid-extractable radioactivity were sequentially collected the livers were quickly removed and a 1 g portion of each liver was homogenized in 4 ml of ice-cold 1 mol/l acetic acid containing 6 mol/l urea. The remaining portion of the liver was weighed and counted for the total radioactivity in a γ -counter (model ARC-300, Aloka Co., Ltd., Tokyo, Japan). The tissue homogenates containing acid-resistant radioactivity were then centrifuged at 3000 rev/min for 15 min in a centrifuge (model RL-500SP, Tomy Seiko Co., Tokyo, Japan) at 4°C , and the obtained supernatants (designated as tissue samples) were transferred to separate tubes for the assay of A_{14} - ^{125}I -insulin. The percentage of radioactivity recovered in the tissue samples was more than 90%.

Analytical methods

For the TCA-precipitation method, perfusate samples ($80 \mu\text{l}$), acid samples (2.5 ml per fraction) or tissue samples (4 ml) were mixed well with 5% TCA solution in a final concentration, kept standing at 4°C for 30 min , and centrifuged at 3000 g for 15 min . For acid samples, $50 \mu\text{l}$ of perfusate (containing 3% BSA) was added to the mixture as a precipitation carrier. The supernatants were transferred into separate tubes by aspiration. The radioactivity in the precipitates and supernatants was measured in a γ -counter.

For the HPLC analysis, each sample was deproteinized by mixing vigorously with an equal volume of ethanol, and centrifuged at 10000 g for 5 min in a microcentrifuge (MR-15A, Tomy Seiko Co.) at 4°C . The ethanol-treated supernatants were filtered through $0.45\text{-}\mu\text{m}$ millipore filters (type HV; Nihon Millipore Kogyo, Yonezawa, Japan) with the recovery of more than 80%. The resultant filtrates ($100 \mu\text{l}$) were loaded onto a reversed-phase HPLC column, μ -Bondapak C_{18} ($30 \text{ cm} \times 3.9 \text{ mm}$ internal diameter, Waters Associates, Inc., Milford, Mass., USA). The eluents were collected automatically and the radioactivity in each fraction (1.0 ml) was measured in a γ -counter. The solvent delivery system, equipments, mobile phase, and elution condition are the same as described previously [12].

“Receptor-recycling” model

Considering a liver perfusion in a recirculation system, we developed an organ model of hepatic insulin clearance and distribution, in which insulin receptors are transferred between the cell surface and intracellular (vesicular) space. The structure of the model is shown in Figure 1. The differential equations describing the system are as follows:

$$\frac{dR_s}{dt} = k_{\text{ret}} \cdot R_i - k_{\text{end}} \cdot X_s - k_{\text{sp}} \cdot (R_s - X_s) \quad (\text{Eq. 1})$$

$$\frac{dR_i}{dt} = k_{\text{end}} \cdot X_s + k_{\text{sp}} \cdot (R_s - X_s) - k_{\text{ret}} \cdot R_i \quad (\text{Eq. 2})$$

$$V_p \frac{dC_p}{dt} = Q_p \cdot (C_e - C_p) \quad (\text{Eq. 3})$$

$$V_c \frac{dC_e}{dt} = Q_p \cdot (C_p - C_e) - k_{\text{on}} \cdot (R_s - X_s) \cdot f_p \cdot C_e + k_{\text{off}} \cdot X_s \quad (\text{Eq. 4})$$

$$\frac{dX_s}{dt} = k_{\text{on}} \cdot (R_s - X_s) \cdot f_p \cdot C_e - k_{\text{off}} \cdot X_s - k_{\text{end}} \cdot X_s \quad (\text{Eq. 5})$$

$$\frac{dX_i}{dt} = k_{\text{end}} \cdot X_s - k_{\text{deg}} \cdot X_i \quad (\text{Eq. 6})$$

$$\frac{dX_{\text{deg}}}{dt} = k_{\text{deg}} \cdot X_i - k_{\text{rel}} \cdot X_{\text{deg}} \quad (\text{Eq. 7})$$

where f_p denotes the fraction of insulin unbound to albumin in the perfusate. During the intracellular degradation products are not yet

Fig. 1. The receptor-recycling model of hepatic insulin clearance and distribution. Solid and broken arrows indicate the movements of insulin and its receptors, respectively. The liver is subdivided into the three compartments, i.e., extracellular fluid, cell surface and intracellular (vesicular) space, which are assumed to be well-mixed. Since the partition of A_{14-125} I-insulin to erythrocytes could be neglected by the method described previously [37], the erythrocyte compartment is not considered. R_s and R_i defines the numbers of surface and internalized receptors, respectively. X_s , X_i and X_{deg} define the amounts of surface-bound insulin, internalized insulin and the intracellular degradation products of insulin, respectively. C_p and C_e define the concentrations in the perfusate and extracellular fluid, respectively. The rate constant k_{rel} defines the release of X_{deg} into the extracellular fluid. The other nomenclature is presented in Table 2. Receptor synthesis, processing and degradation are not involved in this model, because the half-time of receptor turnover, 10 h [8], far exceeds the time interval (30 min) of the present liver perfusion. The non-specific binding of insulin to hepatocytes was neglected, because it is only about 8% of the total binding in isolated rat hepatocytes [38]. Since very little 14 C-sucrose is taken up by perfused rat livers [39], this model confines itself to receptor-mediated endocytosis over and above fluid-phase endocytosis. The total number of mobile receptors, $R_T (= R_s + R_i)$, and receptor affinity, $K_d (= k_{off}/k_{on})$, are assumed to be constant throughout the experiment, according to the previous reports [9, 10].

released substantially (i.e., $k_{deg} \cdot X_i < k_{rel} \cdot X_{deg}$), Eq. 7 can be approximated to:

$$\frac{dX_{deg}}{dt} = k_{deg} \cdot X_i \quad (\text{Eq. 8})$$

Data analysis

The values of X_s , X_i and X_{deg} were expressed as intact A_{14-125} I-insulin (pmol per g liver), and C_p also as intact A_{14-125} I-insulin (pmol per ml perfusate). The hepatic clearance (CL_H) of A_{14-125} I-insulin was calculated as follows:

$$CL_H = k_e \cdot V_p / LW \quad (\text{Eq. 9})$$

where k_e is the exponential slope of the disappearance of A_{14-125} I-insulin in the perfusate, and LW is the liver weight in grammes. Based on the clearance theory [14], the CL_H is a function of Q_p and the apparent intrinsic activity of hepatic elimination, $CL_{int,app}$ [14]. The $CL_{int,app}$ is given by:

$$CL_{int,app} = \frac{Q_p \cdot CL_H}{(Q_p - CL_H) f_p} \quad (\text{Eq. 10})$$

Determination of kinetic parameters

Since our preliminary simulation indicated that k_{end} , k_{deg} and R_T are important parameters that determine the time courses of C_p , X_s , X_i and X_{deg} at a tracer dose, these three parameters were optimized by a procedure as described later. The other parameters (i.e., V_e , V_p , Q_p , f_p , k_{on} , k_{off} , k_{sp} and k_{ret}) were measured experimentally cited or calculated from published references (Table 2). For optimization of kinetic parameters, a nonlinear least-squares regression analysis, NONLIN74 [16], was used throughout this study on a digital computer (FACOM-M360AP) at the Information Processing Center, Kanazawa University. In particular, k_{sp} and k_{ret} were calculated from published data using the methods described as follows:

Calculation of k_{sp} . The apparent endocytotic rate constant ($k_{end,app}$) can be expressed as:

$$k_{end,app} = v \cdot k_{end} + (1 - v) \cdot k_{sp} \quad (\text{Eq. 11})$$

where v is the fraction of surface receptors occupied by insulin and given by:

$$v = \frac{C_e}{K_d + C_e} \quad (\text{Eq. 12})$$

The values of k_{sp} and k_{end} were obtained by a nonlinear least-squares regression analysis according to Eq. 11, based on the concentration-dependent $k_{end,app}$ values observed in isolated rat hepatocytes [11].

Calculation of k_{ret} . Using v , Eq. 1 can be written as follows [17]:

$$\frac{dR_s}{dt} = k_{ret} \cdot (R_T - R_s) - [v \cdot k_{end} + (1 - v) \cdot k_{sp}] \cdot R_s \quad (\text{Eq. 13})$$

Since most of the mobile receptors are considered to be located at the surface of hepatocytes in fasted rats (i.e., $v \div 0$), R_T can be expressed as follows, under the steady-state basal condition (i.e., $dR_s/dt = 0$ in Eq. 13):

$$R_T = \frac{k_{sp} + k_{ret}}{k_{ret}} R_{s,0} \quad (\text{Eq. 14})$$

where $R_{s,0}$ represents the R_s under the steady-state basal condition in fasted rats. On the other hand, when non-fasted rats received an intraportal injection of a large excess of unlabelled insulin (i.e., $v \div 1$), Eq. 13 can be transformed to:

$$\frac{dR_s}{dt} = k_{ret} \cdot R_T - (k_{ret} + k_{end}) \cdot R_s \quad (\text{Eq. 15})$$

Integration of Eq. 15 from time 0 to t and rearrangement gives:

$$\ln \frac{R_{s,0} - R_{s,eq}}{R_s - R_{s,eq}} = (k_{ret} + k_{end}) \cdot t \quad (\text{Eq. 16})$$

where $R_{s,0}$ and $R_{s,eq}$ are the surface receptor numbers at the initial and equilibrium states, respectively, in non-fasted rats injected with intraportal dose of unlabelled insulin; and $R_{s,eq}$ is expressed as:

$$R_{s,eq} = \frac{k_{ret}}{k_{end} + k_{ret}} R_T \quad (\text{Eq. 17})$$

Substitution for R_T in Eq. 17 according to Eq. 14 gives:

$$R_{s,eq} = \frac{k_{sp} + k_{ret}}{k_{end} + k_{ret}} R_{s,0} \quad (\text{Eq. 18})$$

Solving Eq. 16 for $R_s/R_{s,0}$ and rearrangement using Eq. 18 yields:

$$\frac{R_s}{R_{s,0}} = (1 - \alpha) \cdot \exp[-(k_{ret} + k_{end}) \cdot t] + \alpha \quad (\text{Eq. 19})$$

where α is expressed as:

$$\alpha = \frac{k_{ret} + k_{sp}}{k_{ret} + k_{end}} \cdot \frac{R_{s,0}}{R_{s,eq}} \quad (\text{Eq. 20})$$

Table 1. Characteristics of the perfused mouse liver^a

Characteristics	Value
Body weight (g)	31.26 ± 0.49 (10)
Liver weight (g)	1.43 ± 0.04 (10)
Endogenous insulin	ND ^b (10)
Perfusion pressure (mm Hg)	15–18
GOT releasing rate constant (min ⁻¹)	0.0108 ± 0.0095 ^c (4)
GPT releasing rate constant (min ⁻¹)	0.0112 ± 0.0102 ^c (4)
CL _H (ml · min ⁻¹ per liver) ^d	
Low dose (0.018 nmol/l)	0.89
High dose (2.0 nmol/l)	0.52
CL _{int,app} (ml · min ⁻¹ per g liver) ^e	
Low dose (0.018 nmol/l)	1.33
High dose (2.0 nmol/l)	0.53

^a Numbers in parentheses indicate the number of perfused liver preparations from mice; ^b Not detected by radioimmunoassay (< 100 pmol/l); ^c Expressed as the mean ± SEM, as assessed by a linear least-squares regression of the glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activity (milliunits per milliliter) vs. time curves; ^d Determined by Eq. 9, using the perfusate volume (V_p) and exponential slope (k_e) of the disappearance of A₁₄-¹²⁵I-insulin from the reservoir perfusate; ^e Determined by Eq. 10, using the f_p (= 1.0), CL_H and Q_p values

According to the report by López and Desbuquois [10], the R_{s,0}/R_{s,0} value is 1.1, indicating that the R_s in fasted rats is slightly (10%) higher than that in fed rats. Therefore, one can estimate the k_{ret} and k_{end} values by fitting the reported R_s/R_{s,0} vs time curve (i. e., *in vivo* down-regulation) to Eq. 19, using a nonlinear least-squares regression analysis.

In order to optimize the R_T, k_{end} and k_{deg}, the observed data on C_p (0–30 min), X_s, X_i (0–20 min) and X_{deg} (0–5 min) at the tracer dose (0.018 nmol/l) were fitted to the simultaneous differential equations (Eqs. 1–6 and 8) by a nonlinear least-squares regression analysis. Moreover, in order to examine the effect of receptor recycling on hepatic insulin clearance, the observed data were also fitted to the simultaneous differential equations of a “non-receptor-recycling” model, in which k_{ret} is set at zero.

Since the initial values of k_{end}, k_{deg} and R_T are required for the above-mentioned optimization procedure, the following methods were employed for the estimation of respective initial values.

Initial estimation of k_{end}. Summation of both hand sides of Eqs. 6 and 8 gives:

$$\frac{d(X_i + X_{deg})}{dt} = k_{end} \cdot X_s \quad (\text{Eq. 21})$$

Therefore, the integration of Eq. 21 from time 0 to t gives:

$$X_i + X_{deg} = k_{end} \cdot \int_0^t X_s dt \quad (\text{Eq. 22})$$

where $\int_0^t X_s dt$ was calculated numerically by a trapezoidal rule. Then, a plot of (X_i + X_{deg}) vs. $\int_0^t X_s dt$ should give a straight line passing through the origin, and the slope of the plot should yield an initial estimate of k_{end}.

Initial estimation of k_{deg}. The integration of Eq. 8. from time 0 to t gives:

$$X_{deg} = k_{deg} \cdot \int_0^t X_i dt \quad (\text{Eq. 23})$$

where $\int_0^t X_i dt$ was calculated numerically by a trapezoidal rule. Then, a plot of X_{deg} vs. $\int_0^t X_i dt$ should give a straight line passing through the origin, and the slope of the plot should yield an initial estimate of k_{deg}.

Initial estimation of R_T. When a ligand is taken up *via* receptor-mediated endocytosis, the CL_{int,app} of the ligand is expressed as follows, under a linear condition (i. e., at a tracer dose) [18]:

$$CL_{int,app} = \frac{k_{on} \cdot R_s \cdot k_{end}}{k_{end} + k_{off}} \quad (\text{Eq. 24})$$

Eq. 24 gives an initial estimate of R_s, by substituting the reported values of k_{on} and k_{off} (Table 2), the graphical estimate of k_{end} calculated by Eq. 22, and the CL_{int,app} calculated by Eq. 10. The initial estimate of R_s was considered as that of R_T, because most of the mobile receptors should be located at the cell surface when a tracer dose of insulin was added in the perfused liver.

Prediction of the time course of C_p at a high dose (2 nmol/l)

Using the values of R_T, k_{end} and k_{deg} estimated above at the low dose, both the “receptor-recycling” and “non-receptor-recycling” models were employed to predict the disappearance of A₁₄-¹²⁵I-insulin from the perfusate at a high dose (2 nmol/l) to test the feasibility of these models. Two nanomolar was chosen as a high concentration of in-

Fig. 2A–E. High performance liquid chromatography profiles of A₁₄-¹²⁵I-insulin in the perfusate after 0 min (A), 10 min (B), 15 min (C), 20 min (D) and 30 min (E) perfusion of the mouse livers. Open circles represent ¹²⁵I-radioactivity (cpm) in 0.8-ml fractions eluting from μ-Bondapak C₁₈ column (30 cm × 3.9 mm), which had been standardized with 3-I-L-tyrosine, A₁₄-¹²⁵I-insulin and unlabelled porcine insulin, with the acetonitrile gradient shown by a broken line. The flow rate was set at 1.0 ml · min⁻¹

Fig. 3. Relationship of percentage of intact A₁₄-¹²⁵I-insulin between trichloroacetic acid (TCA)-precipitation and high-performance liquid chromatography (HPLC) methods in perfusates obtained 0 (●), 10 (○), 15 (■), 20 (□), 30 (▲) and 45 (△) min after the administration of A₁₄-¹²⁵I-insulin into the reservoir of cyclically perfused mouse liver. The percentage of radioactivity precipitable by TCA was calculated as (cpm in precipitate)/[(cpm in precipitate) + (cpm in supernatant)] × 100, whereas the percentage of intact A₁₄-¹²⁵I-insulin on HPLC was calculated by comparing the peak areas of radioactivity eluted from the column

sulin, since portal insulin concentrations in the post-absorptive state were reported to be 0.4–1.1 nmol/l [5].

Statistical analysis

Data were analysed by Student's *t*-test.

Results

In situ liver perfusion

Livers were perfused via the portal vein at a constant physiological pressure with a perfusate containing 10% erythrocytes as an oxygen carrier, and there was no evidence of oedema during the experiment. The characteristics of the perfused mouse livers are summarized in Table 1. The perfusion flow rate and perfusion pressure were constant during the experiments. The releasing rates of GOT and GPT are close to that of GOP reported in normal rat livers [19]. Taken together, the viability of the livers was judged to be normal and constant.

Figures 2A–E show typical HPLC profiles of ¹²⁵I-radioactivity collected from the reservoir perfusate at 0, 10, 15, 20, and 30 min. The total radioactivity eluted from the column was almost 100% of the radioactivity loaded onto the column. A small peak eluting at 3–4 min probably represents a mixture of ¹²⁵I- and monoiodotyrosine, because a standard 3-I-L-tyrosine eluted as early as 3.9 min and very close to the salt peak (3.1 min) under the present analytical condition. The retention time of intact A₁₄-¹²⁵I-insulin was 20 min, and it was clearly seen that A₁₄-¹²⁵I-insulin was extensively metabolized by the mouse liver during the perfusion. The identity of a small peak eluting at 16 min (seen in the profiles of the perfusate samples at 10–20 min) is not clear from the present study,

Fig. 4. The disappearance of A₁₄-¹²⁵I-insulin from the perfusate (○) and the appearance of its degradation products in the perfusate (●), after bolus administration of a tracer amount of A₁₄-¹²⁵I-insulin into the reservoir (0.018 nmol/l in a final concentration) in the perfused mouse liver. Each point and vertical bar represents the mean ± SEM from 3–13 rats (for labelled insulin) or 4 rats (for degradation products). When A₁₄-¹²⁵I-insulin was perfused in the perfusion apparatus without the liver, no loss of the ¹²⁵I-radioactivity from the reservoir was observed. Thus, the disappearance of A₁₄-¹²⁵I-insulin from the reservoir was not due to adsorption of labelled peptide to the perfusion apparatus, but to hepatic elimination. Moreover, when portions of perfusate were occasionally obtained from the perfused livers to examine *in vitro* degradation of A₁₄-¹²⁵I-insulin at 37 °C, there was essentially no change in insulin concentration for up to 60 min. Therefore, the release of proteases from the perfused livers was negligible

but it possibly represents intermediate degradation products of labelled insulin, because previous HPLC studies have demonstrated TCA-precipitable, insulin-sized intermediate products derived from enzymatic interactions of insulin with hepatocytes [20].

The relationship of the intactness of A₁₄-¹²⁵I-insulin in perfusate samples between the TCA-precipitation and HPLC-separation methods (Fig. 3) indicates that the TCA-precipitation overestimates the intactness of A₁₄-¹²⁵I-insulin, as shown in a previous *in vivo* study [21], and further underestimates the hepatic insulin clearance. Moreover, correlation of intactness of A₁₄-¹²⁵I-insulin in the acid samples and tissue samples between the two methods was almost superimposed with the curve shown in Figure 3 (not shown). Although HPLC analysis has a greater resolving power to differentiate between the intact peptide and its metabolites compared with other methods, it is laborious to analyse many samples by HPLC. Therefore, we routinely employed the TCA-precipitation method, and the measured TCA-precipitability (%) was converted to the percentage of intact A₁₄-¹²⁵I-insulin on HPLC, using the correlation between these two methods.

Figure 4 shows the disappearance of A₁₄-¹²⁵I-insulin from the perfusate and the appearance of its degradation products in the perfusate, after the addition of a tracer amount of A₁₄-¹²⁵I-insulin in the reservoir.

Acid-wash technique in the perfused liver

When a liver was washed with ice-cold 154 mmol/l NaCl for 5 min and then with ice-cold acidic buffer for 20 min, two sharp peaks of ¹²⁵I-radioactivity representing the acid-ex-

Fig. 5. A representative elution profile of ^{125}I -radioactivity through the outflow cannula during 5-min wash with 154 mmol/l NaCl (saline) and 20-min wash with acidic buffer (pH 3.5) at 4°C , after $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin was perfused for 30 min in a cyclically perfused mouse liver. Briefly, the livers were replaced in an open-circuit system and washed for 5 min with ice-cold 154 mmol/l NaCl at the flow rate of $2.5\text{ ml} \cdot \text{min}^{-1}$, to remove unbound ^{125}I -radioactivity in the extracellular fluid and within the cannula. The livers were then perfused for 20 min with acidic buffer (acetic acid 100 mmol/l; mannitol 100 mmol/l; NaCl 45 mmol/l; pH 3.5) at a flow rate of $2.5\text{ ml} \cdot \text{min}^{-1}$

Fig. 6A, B. The clearance of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin by cyclically perfused mouse livers at low (0.018 nmol/l ; $\bullet$) and high (2 nmol/l ; $\circ$) doses (in panel A). The perfusate concentrations of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin at a low dose in Figure 4 are presented again in a logarithmic plot. Each point and bar represents the mean $\pm$ SEM from 3–13 rats (for a low dose) or 4 rats (for a high dose). In panel B, the time-courses of acid-extractable ($\circ$) and acid-resistant ($\bullet$) $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin, as well as that of acid-resistant degradation products ($\blacktriangle$) of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin are presented. Each point and vertical bar represents the mean $\pm$ SEM from 4 rats. In panels A and B, solid and dashed lines show the computer-generated simulation curves using “receptor-recycling” ($k_{\text{ret}} \neq 0$) and “non-receptor-recycling” ($k_{\text{ret}} = 0$) models of hepatic insulin clearance. The curves indicated by arrows especially provide prediction of the disappearance of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin at a high dose (2 nmol/l), using the set of parameters determined at the low dose (0.018 nmol/l)

tractable and acid-resistant $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin were sequentially eluted from the liver through the outflow cannula (Fig. 5). We analyzed some acid-extractable samples by HPLC and confirmed that more than 90% of the radioactivity represents intact $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin (result not shown).

Hepatic clearance and distribution of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin

The time courses of C_p at low and high doses are shown in Figure 6A. According to a linear regression analysis, the k_e values were determined to be 0.0739 ± 0.0017 and

$0.0436 \pm 0.0015\text{ min}^{-1}$ (mean $\pm$ SD) with the correlation coefficients (r) of -0.998 ($p < 0.001$) and -0.995 ($p < 0.001$) at low and high doses, respectively. These k_e values are significantly different from each other, as assessed by Student's t -test ($p < 0.001$). In a separate *in vitro* experiment, protein binding of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin in the perfusate (KRB buffer containing 3% BSA) was examined using the polyethylene glycol precipitation method [22], and f_p was determined to be 1.0. Thus, the CL_{H} and $\text{CL}_{\text{int,app}}$ of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin at these doses were calculated by Eqs. 9 and 10, respectively, and are listed in Table 1. The $\text{CL}_{\text{int,app}}$ at the high dose was shown to be 60% smaller than that at the low dose, indicating that the cellular uptake of insulin is remarkably nonlinear at the concentration range examined.

Moreover, the time courses of acid-extractable and acid-resistant $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin, as well as that of acid-resistant degradation products of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin at the tracer dose are shown in Figure 6B.

Kinetic analysis

The k_{sp} and k_{ret} values were determined to be 0.022 and 0.11 min^{-1} by Eq. 11 and Eq. 19, respectively (Table 2). Using Eqs. 21 and 23, the initial estimates of k_{end} and k_{deg} were given as 0.655 ± 0.046 and $0.693 \pm 0.024\text{ min}^{-1}$ (mean $\pm$ SD) with the r values of 0.998 ($p < 0.05$) and 0.999 ($p < 0.05$), respectively. Using Eq. 24, the initial estimate of R_T was given as $11.1\text{ pmol per liver}$. The R_T , k_{end} and k_{deg} were then optimized by fitting the observed data at the low dose (Fig. 6) to Eqs. 1–6 and 8 using a nonlinear least-squares regression analysis, and are listed in Table 2.

The “receptor-recycling” model (Fig. 1) could adequately explain the hepatic clearance (Fig. 6A) and distribution (Fig. 6B) of $\text{A}_{14}\text{-}^{125}\text{I}$ -insulin at the low dose. The “non-receptor-recycling” model, in which no recycling of receptors ($k_{\text{ret}} = 0$) was assumed, could also simulate the observed data at the tracer dose, using another set of optimized parameters (i.e., k_{end} ; $0.600 \pm 0.036\text{ min}^{-1}$, k_{deg} ; $0.866 \pm 0.058\text{ min}^{-1}$, R_T ; $9.93 \pm 0.57\text{ pmol per g liver}$, mean $\pm$ SD). On the other hand, a good prediction of the hepatic insulin clearance at the high dose was accomplished by the receptor-recycling model, but not by the non-receptor-recycling model (Fig. 6A).

Moreover, as can be seen from Figure 7, a computer-aided prediction using the receptor-recycling model at varying initial concentrations of insulin (C_p) suggests that the elimination rate constant (k_e) reduces dramatically at $C_p > 1\text{ nmol/l}$, and that a marked reduction of surface receptors (i.e., “down-regulation”) occurs also at $C_p > 1\text{ nmol/l}$.

Discussion

Our kinetic model of hepatic insulin disposition includes several assumptions and approximations as described in the legend to Figure 1. Unique to this model is the ability to predict the changes in surface receptor number in relation to extracellular insulin as well. By use of this model, moreover, one could also examine quantitatively how the

Table 2. Parameters used in model prediction of hepatic elimination and distribution of insulin in the perfused mouse liver

Parameters	Symbol	Value	Units	Source
Extracellular volume	V_e	0.55	ml	experiment ^a
Perfusate volume	V_p	12.0	ml	experiment
Perfusion flow rate (without erythrocytes)	Q_p	1.67	ml · min ⁻¹	experiment
Fraction unbound to proteins	f_p	1.0		experiment
Insulin-receptor association rate constant	k_{on}	2.4×10^8	min ⁻¹ · mol ⁻¹ · l	ref. 31
Insulin-receptor dissociation rate constant	k_{off}	0.12	min ⁻¹	ref. 31
Receptor total number	R_T	8.66 ± 0.48	pmol per g liver	estimation ^b
Endocytotic rate constant of occupied receptor	k_{end}	0.62 ± 0.03	min ⁻¹	estimation ^b
Endocytotic rate constant of unoccupied receptor	k_{sp}	0.022 ± 0.010	min ⁻¹	calculation ^c
Receptor returning rate constant	k_{ret}	0.11 ± 0.02	min ⁻¹	calculation ^d
Intracellular degradation rate constant	k_{deg}	0.907 ± 0.057	min ⁻¹	estimation ^b

^a Obtained as the sum of interstitial volume (V_{is}) and capillary volume (V_{CB}). The V_{is} was determined to be 0.55 ml using ¹⁴C-inulin in mice (unpublished observation), and the V_{CB} was extrapolated from the reference value in the rat liver [36] to be 0.14 ml in the mouse liver; ^b Mean $\pm$ SD, estimated from the observed data on X_s , X_i , X_{deg} and C_p (Fig. 6) according to Eqs. 1–6 and 8, using a nonlinear

least-squares regression analysis [16]; ^c Mean $\pm$ SD, estimated from the reported data [11] according to Eq. 11 using a nonlinear least-squares regression analysis [16]; ^d Mean $\pm$ SD, estimated from the reported data [10] according to Eq. 19 using a nonlinear least-squares regression analysis [16]

Fig. 7A,B. Computer simulation of the time-dependent changes in the perfusate concentration of $A_{14-125}I$ -insulin (panel A) and surface receptor number (panel B) at varying initial concentrations of insulin in the reservoir, using the “receptor-recycling” model of hepatic insulin clearance and distribution. The numbers of 1–10 accompanying the lines correspond to the initial insulin concentrations of 0.02, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 10.0 and 20.0 nmol/l, respectively, used in the simulation

hepatic insulin clearance would be affected by variations in the affinity of insulin-receptor interaction, which may result from mutation of insulin receptor, abnormalities of insulin processing or mutation of insulin itself. However, the applicability of our model might be limited within the physiologic insulin concentrations, because of the negative co-operativity of insulin-receptor interaction [23] and a high-capacity, non-degradative and more rapidly recycling pathway for receptors that occurs at high (supraphysiologic) concentrations of insulin [24].

Since about 50% of liver cells are not hepatocytes [25], there is a possibility that the observed clearance and distribution of $A_{14-125}I$ -insulin in the perfused livers are not due to specific interactions with hepatocytes. However, we could exclude this possibility, because the initial distribution of ¹²⁵I-insulin was limited to the hepatocyte plasmalemma after its intraportal injection [26, 27], and its localization was diminished with simultaneous injection of excess unlabelled insulin [27].

In this study, an effective separation between the acid-extractable and acid-resistant $A_{14-125}I$ -insulin was per-

formed by the acid-wash technique. Moreover, our preliminary experiments show that ¹²⁵I-radioactivity in both the acid-extractable and acid-resistant samples considerably decreased in the presence of 1 μ mol/l unlabelled insulin in the perfusate, and ¹²⁵I-radioactivity in the acid-resistant samples markedly decreased in the presence of 250 μ mol/l of an endocytosis inhibitor, phenylarsine oxide (results not shown). These lines of evidence suggest that the observed acid-resistant $A_{14-125}I$ -insulin largely represents the intracellular $A_{14-125}I$ -insulin, and that the acid-extractable ¹²⁵I-radioactivity was bound to surface receptors.

As shown in Figure 6B, the peak of the intracellular $A_{14-125}I$ -insulin was several minutes later than that of surface-bound $A_{14-125}I$ -insulin, suggesting that the surface-bound ligand may be regarded as the substrate of endocytosis. Moreover, the increase of degradation products of $A_{14-125}I$ -insulin in the reservoir was small for the first 5 min, and rapidly increased thereafter. Since the lag time for $A_{14-125}I$ -insulin in the reservoir to reach the inlet of the organ was subtracted from the sampling time, this is not

due to an access delay of $A_{14-125}\text{I}$ -insulin to the liver. Therefore, that the major site of insulin degradation is in an intracellular compartment but not in an extracellular compartment is suggested in this study, as reported by other workers [28, 29]. Taken altogether, the results obtained indicate that the hepatic insulin clearance is largely accounted for by the receptor-mediated endocytosis in the perfused mouse livers, and supports the feasibility of the present model of hepatic insulin clearance and distribution.

As listed in Table 2, the value of R_T was estimated to be 8.66 pmol per g liver, that is 44 000 sites per cell, assuming that the number of hepatocytes per liver in mice is the same as that in rats, 1.3×10^8 cells per g liver [30]. This R_T value is in good agreement with the R_s for isolated rat hepatocytes *in vitro*, e. g., 45 000 [31] and 50 100 sites per cell [32], in which almost all mobile receptors may be located on the surface of isolated cells due to the absence of extracellular insulin during preparation. On the other hand, the estimated k_{end} is somewhat greater than that reported for isolated rat hepatocytes, 0.38 min^{-1} [24].

A good agreement between the observed and simulated values of C_p , X_s , X_i and X_{deg} at the low dose by both the receptor-recycling and non-receptor-recycling models suggests that internalization process, not receptor recycling, is important for the hepatic insulin clearance at low insulin concentrations. On the other hand, the predictions of C_p at the high dose by these models suggest an important role of receptor recycling in the hepatic insulin clearance at high insulin concentrations, at which surface receptors are considered to be largely occupied. It probably, therefore, follows that when the R_s is considerably decreased at high insulin doses, the hepatic insulin clearance should be limited by the rate of receptor recycling. We propose, therefore, that the rate-limiting step of hepatic intrinsic clearance of insulin is the internalization process at low insulin concentrations, but moved to the receptor recycling process at relatively high concentrations. This is consistent with the fact that chloroquine, which blocks receptor recycling *via* preventing intravesicular acidification [2], did not alter the disappearance of $A_{14-125}\text{I}$ -insulin (tracer dose) in the perfused rat livers [33]. Thus, a dynamic "receptor-recycling" model could be advantageous to a static model of insulin clearance [34] that assumes a constant surface receptor number, because the latter model cannot evaluate the significance of receptor recycling on hepatic insulin clearance. From the computer simulation, it was predicted that the changes in the k_e (as well as CL_{H}) and R_s are not so sensitive to the initial concentrations of less than 1 nmol/l and more than 10 nmol/l, while very sensitive to those of 1–10 nmol/l. This is in agreement with the report that the hepatic extraction of insulin is reduced with increasing endogenous insulin secretion after pancreatic stimulation with oral glucose [35], when the portal vein insulin is reported to be 4.5–7.0 nmol/l [5]. However, further study should be focussed on the validation of the model at a wider concentration range of insulin, and, if necessary, a more detailed modeling of hepatic insulin disposition should be performed to accommodate a full dose response curve as shown in Figure 7A.

In conclusion, we have developed a "receptor-recycling" model of hepatic insulin clearance, based on a physiological and biochemical approach. The model could afford to predict not only the nonlinear clearance and distribution of insulin in the perfused liver, but also the change in surface receptor number, in a quantitative manner. The mathematical methods described here should be useful to analyse the receptor-mediated endocytosis of other ligands in an intact organ.

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Dr. A. Tsuji
Faculty of Pharmaceutical Sciences
Kanazawa University
Takara-machi 13-1
Kanazawa 920
Japan