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DECREASE IN THE NUMBER OF RECEPTORS FOR EPIDERMAL GROWTH FACTOR IN THE LIVER OF D-GALACTOSAMINE-INTOXICATED RATS

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Abstract—Hepatic transport of epidermal growth factor (EGF) was studied in D-galactosamine-intoxicated rats by the multiple-indicator dilution (MID) method. The extraction ratio of ^{125}I-labeled EGF in the intoxicated rats, obtained from a model-independent analysis of the dilution curves, decreased to 45% of the control values. A distributed two-compartment model was fitted to the dilution data by nonlinear least-squares regression, and the kinetic parameters, $k_{on} \cdot P_T$ (product of on-rate constant and receptor density), k_{off} (off-rate constant) and k_s (sequestration rate constant) were determined. The values of $k_{on} \cdot P_T$ and k_s in the intoxicated rats decreased to approximately one-half and one-third of those in the control rats respectively. Similar decreases in the $k_{on} \cdot P_T$ and k_s values in the intoxicated rats were also observed for the transport of ^{125}I-labeled insulin, a positive control, into the liver. The ^{125}I-labeled EGF binding experiment at equilibrium using liver homogenates revealed that the intoxication reduced the receptor density (P_T) to one-third of the control values, whereas the equilibrium dissociation constant (k_d) did not change significantly. The activities of Na^+, K^+-ATPase, cytochrome P-450 and glutathione *S*-transferase decreased in the intoxicated rats to 70–80% of the control values. The number of nuclei per unit area of tissue slices was also reduced to 70% of the control. Thus, the extent to which the enzyme activities and the number of nuclei decreased in the intoxicated liver was smaller than that of the number of EGF receptors. It is concluded that the reduction of EGF receptors cannot be explained by the "intact hepatocyte hypothesis" but rather by the functional change of hepatocytes induced by the administration of D-galactosamine.

Epidermal growth factor (EGF; mol. wt 6045), isolated first from mouse submaxillary gland [1], is a peptide which has mitogenic and growth-promoting effects on various tissues and organs including hepatocytes [2, 3]. The major organs responsible for the elimination of EGF in systemic circulation are the liver and kidney. Recently, the liver has been considered to play an important role as a "homeostatic regulator" [4, 5]. The specific uptake of EGF by hepatic parenchymal cells was demonstrated when a radiolabeled bolus of EGF was injected into the portal vein of rats [6]. It is well known that EGF is taken up by hepatocytes via receptor-mediated endocytosis (RME) and that the "down-regulation" is caused by a temporal decrease in the number of receptors on the cell surface [7].

Necrosis and regeneration of hepatocytes are thought to occur concomitantly in hepatitis [8]. Therefore, some changes of the numbers and/or properties of EGF receptors may occur in hepatitis [9] for the following reasons: (1) the increase in the multiplication rate may be due to the increase in the number or affinity of EGF receptors, and (2) the increase in the endogenous EGF concentration in hepatitis may cause the down-regulation of receptors in the liver, resulting in the reduction of the receptor density (P_T). In this study, we used D-galactosamine-intoxicated rats as an acute hepatic disease model, in which hepatic logical features are known to be similar to those of human viral hepatitis [10].

We demonstrated previously that the multiple-indicator dilution (MID) method could be a good tool for analyzing the dynamics of interaction between EGF and cell surface receptors in the liver perfusion system, where the spatial architecture between hepatocytes and capillary is maintained [11, 12].

The purpose of the present study was to examine with the MID method the effect of acute hepatic disease on the hepatic transport of EGF, in an attempt to elucidate whether changes in the number or affinity of the receptor occur. We also performed a binding assay using homogenates from intact and intoxicated rat livers to help interpret the MID data. Insulin was used as a positive control, since this peptide is well known to have its specific receptor on the liver cell surface and to be taken up via RME [13].

MATERIALS AND METHODS

Animals. Adult male Wistar rats (Nihon Seibutsu Zairyo, Tokyo, Japan) weighing 250–270 g were used throughout the experiments. The animals were housed under conditions of controlled temperature

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and lighting with access to food and water *ad lib*.

Materials. Human EGF was obtained from the Wakunaga Pharm. Co., Ltd (Osaka, Japan). Porcine insulin and bovine serum albumin (fraction V) were purchased from the Sigma Chemical Co. (St Louis, MO). Sodium iodine-125 (100 mCi/ml) was purchased from the Amersham Corp. (Arlington Heights, IL). [^{14}C]Insulin (2.6 μCi/mmol) and ^{125}I-labeled porcine insulin (99 μCi/μg) were purchased from the New England Nuclear Corp. (Boston, MA). D-Galactosamine HCl was purchased from Wako Pure Chemical Industries, Ltd, Osaka, Japan). All other chemicals were obtained from commercial sources and were of analytical grade. ^{125}I-Labeled EGF (90–110 μCi/μg) was prepared by the chloramine T method [14].

Induction of liver disease. D-Galactosamine HCl, dissolved in isotonic saline, was administered by i.p. injection in a single dose of 1.5 g/kg of body weight. Control rats received equivalent volumes of isotonic saline by a single i.p. injection. Following administration of D-galactosamine or saline, all animals were allowed access to food and water *ad lib*. At 48 hr after the injection, the animals were subjected to the liver perfusion experiments or were killed by exsanguinity from the abdominal aorta. Serum was assayed in duplicate for glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) by the spectrophotometric method of Karmen [15]. Pathophysiological changes after D-galactosamine intoxication are listed in Table 1.

Multiple-indicator dilution (MID) method. Basically, the same method reported previously [16] was used. Control and treated rats were anesthetized with ether. The bile duct was cannulated with PE-10 polyethylene tubing and the portal vein was rapidly catheterized with a polyethylene cannula (2.0 mm O.D.), which was attached to the perfusion system containing Krebs–Ringer bicarbonate buffer and 100 mg/ml glucose, oxygenated with 95% O_2–5% CO_2 to a pH 7.4 at 37°; infusion of the perfusate was started immediately. The portal circulation was interrupted for less than 10 sec. The inferior vena cava was catheterized through the right atrium with PE-240 polyethylene tubing, and then the inferior vena cava was ligated right above the renal vein. The liver was perfused in a temperature controlled cabinet at 37°. The perfusate consisted of 20% (v/v) washed bovine erythrocytes in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 100 mg/ml glucose and 2% (w/v) bovine serum albumin. The perfusate was circulated using a constant rate infusion pump through a polyester filter, and oxygenated with 95% O_2–5% CO_2. The perfusate flow rate was maintained at 12–14 ml/min.

After a stabilization period by preperfusion for 10 min, 250 μl of [^{14}C]inulin (2.0 μCi), as an extracellular reference substance, and ^{125}I-labeled peptide (3.0 μCi of ^{125}I-EGF or 2.0 μCi of ^{125}I-insulin) were injected rapidly (within 0.5 sec) as a bolus into the portal vein. Subsequently, the total hepatic vein effluent was collected at 1-sec intervals for 30–60 sec. After a stabilization period of 10 min, the second run was performed using the other labeled peptide; that is, if the first run was performed using ^{125}I-EGF as a test substance, the second one was done using ^{125}I-insulin. After a 10-min stabilization period, the final run was performed using the same ^{125}I-labeled peptide as used in the first run.

Radioactivity in the collected samples and the aliquot of injection solution was determined as follows. The samples and the aliquot were centrifuged immediately for 1 min in a table-top microfuge (Beckman Instrument, Fullerton, CA, USA). Supernatant fractions of samples (50 μl) were added to 1 ml of chilled 15% trichloroacetic acid containing 0.25% bovine serum albumin. After agitation using a Vortex mixer and standing at 4° for 30 min, the samples were centrifuged for 2 min in a table-top microfuge. ^{125}I-Radioactivities in the pellet (intact peptide) and the supernatant fraction (degraded product) were determined in a gamma-counter (model ARC-300, Aloka Co., Tokyo, Japan). The ^{14}C-radioactivity was determined in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Corp., model 3255, Downers Grove, IL, USA) and corrected for ^{125}I-radioactivity.

Analysis of dilution curve. To permit comparison between [^{14}C]inulin and ^{125}I-labeled peptide, the outflow radioactivity of each sample was normalized by dividing it by that of the injectate. The concentrations in the effluent were thus expressed as the outflow fraction of dose per milliliter. The data obtained were analyzed in both model-dependent and -independent fashions.

In the model-independent analysis, we calculated the extraction ratio (E) and the distribution volume (Vd) from the zero moment (AUC) and the first moment ($AUMC$) of the dilution curve [17, 18]. The recovery in the outflow of the injected peptide hormone decreases if the peptide is degraded or taken up into the cell during transit through the liver, and the decrease of the recovery lessens the AUC ratio of the peptide to the reference substance. Thus, the extraction ratio (E) is calculated by the following equations:

$$AUC_{PT} = \int_0^\infty C_{PT} \cdot dt \tag{1}$$

$$AUC_{ref} = \int_0^\infty C_{ref} \cdot dt \tag{2}$$

$$F = AUC_{PT}/AUC_{ref} \tag{3}$$

$$E = 1 - F \tag{4}$$

where C_{PT} and C_{ref} are the normalized concentrations of the peptide hormone and the reference substance (inulin) in the effluent, respectively, and F is the hepatic availability of the peptide hormone.

The volume of extracellular space, Vd_{ext} was calculated by the following equations:

$$AUMC_{ref} = \int_0^\infty t \cdot C_{ref} \cdot dt \tag{5}$$

$$\bar{t}_{ref} = AUMC_{ref}/AUC_{ref} \tag{6}$$

$$Vd_{ext} = Q \cdot \bar{t}_{ref} \tag{7}$$

where Q is the plasma flow rate of the perfusate and $\bar{t}$ is the mean transit time.

When first-order kinetics hold, the dilution curve

of the tracer dose of the test substance (^{125}I-EGF or ^{125}I-insulin) could also be analyzed according to the flow-limited distributed model proposed by Goresky and Bach [19] with some modifications. The model is expressed by

$$C_{PT}(t') = \exp(-k_1 \cdot t') \times C_{ref}(t') + \exp\{-(k_2 + k_3)\cdot t'\} \times \int_0^{t'} \{\exp[-(k_1 - k_2 - k_3)\cdot \tau']\} \times C_{ref}(\tau') \times \left[k_1 \cdot k_2 \cdot \frac{\tau'}{t' - \tau'}\right]^{1/2} \times I_1\{2[k_1 \cdot k_2 \cdot \tau' \cdot (t' - \tau')]^{1/2}\} d\tau' \quad (8)$$

where $C_{PT}(t')$ is the concentration of a peptide hormone in the effluent at time t', t' is the elapsed time (t) minus the larger vessel transit time (t_0), τ' is a dummy variable representing time, $C_{ref}(t')$ is the concentration of [^{14}C]inulin in the effluent at time t', and k_1, k_2 and k_3 are the apparent influx, efflux and sequestration rate constants for a peptide, respectively. Equation 8 was fitted to the experimental data to obtain the parameters (k_1, k_2 and k_3) by iterative nonlinear least-squares regression [20–22]. When the RME system plays a major role in the hepatic removal of a peptide hormone, the three parameters thus obtained may represent the corresponding kinetic steps in the RME system as shown in Eqns 9–11:

$$k_1 = k_{on} \cdot P_T / V d_{ext} \quad (9)$$

$$k_2 = k_{off} \quad (10)$$

$$k_3 = k_s \quad (11)$$

where k_{on} is the association rate constant of a peptide from the extracellular space to the cell surface receptor, k_{off} is the dissociation rate constant from the cell surface receptor to the extracellular space, k_s is the sequestration rate constant from the cell surface receptor to the interior of the cell, and P_T is the density of the available receptor on the cell surface.

As can be expected from Eqn 8, the outflow response for the peptide consists of two parts: (1) the throughout material, which consists of the progressively damped [^{14}C]inulin profile, and (2) the returning material, which has been bound to the cell surface receptors, to dissociate later. Initially the throughout component dominates the output, and a ratio plot of $\ln[C_{ref}(t)/C_{PT}(t)]$ versus time gives a straight line with a slope of k_1. Therefore, the initial slope of the ratio plot yields the hybrid parameter consisting of k_{on} and P_T as expected from Eqn. 9.

Binding assay of ^{125}I-EGF to the liver homogenate. The livers were excised from control and intoxicated rats, and a 4% (w/v) homogenate of each liver was prepared by a Teflon homogenizer. The buffer used in the preparation of homogenates and the binding assay contained 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*^{1}-2-ethanesulfonic acid (Hepes)-Tris (pH 7.4) and 0.1% (w/v) bovine serum albumin (BSA). The protein concentration was determined with a Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, CA) with BSA as the standard.

Binding of ^{125}I-EGF was measured by a rapid filtration technique [23]. The binding reaction was initiated by addition of 800 μl of the 4% liver homogenate to 200 μl of the buffer containing 0.03 μCi of ^{125}I-EGF and various concentrations of unlabeled EGF (0.6 to 100 nM). After incubation for 60 min at 4°, 200 μl of the reaction mixture was immediately filtered through a glass microfiber filter (Whatman GF/F; Whatman Ltd., Maidstone, England), which was quickly washed once with 5 ml of ice-cold buffer to separate the tissue-associated ligands from the unbound ligands. Since the maximal EGF binding was observed within 40 min at 4° [4], these assay conditions represent the steady state. Radioactivity on the filter was determined in a gamma-counter. The binding was corrected for nonspecific adsorption on the filter. The nonspecific adsorption was 1 to 1.5% of the total count.

The data of EGF binding to the liver homogenates were analyzed on the assumption of a single class of binding site and nonspecific binding by the following equation:

$$C_b = \frac{P_T \cdot C_f}{K_d + C_f} + \alpha \cdot C_f \quad (12)$$

where C_b and C_f are the bound and unbound concentrations of EGF, respectively. P_T and K_d denote the binding capacity and the dissociation constant, respectively, and α is the proportional constant related to the nonspecific binding. The binding parameters were obtained by an iterative non-linear least-squares method [24] based on Eqn 12. The initial estimations of the binding parameters were obtained from Scatchard plots.

Enzyme assay of liver homogenates. To examine the damage to the liver plasma membrane and cellular organella after intoxication with D-galactosamine, the enzyme activities of Na^+,K^+-ATPase, Mg^{2+}-ATPase, alkaline phosphatase, cytochrome P-450 and glutathione *S*-transferase were determined as follows: Na^+,K^+-ATPase and Mg^{2+}-ATPase activities were determined by the method of Schoner *et al.* [25]. Alkaline phosphatase activities were determined in 5 mM *p*-nitrophenylphosphate/5 mM $MgCl_2$/50 mM 2-amino-2-methylpropanol-HCl (pH 10.0). After 20 or 30 min of incubation at 37°, the reaction was terminated by addition of 10% trichloroacetic acid. The denatured protein was removed by centrifugation, the clear supernatant fraction was neutralized by addition of 0.1 M NaOH, and the amount of *p*-nitrophenol released was determined by the absorbancy at 420 nm [26]. The amount of cytochrome P-450 was determined with the method of Matsubara *et al.* (CO-difference spectrophotometry of dithionite-reduced samples) [27]. Glutathione-*S*-transferase activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate [28].

Light microscopic photography. For light microscopy, liver specimens from control and intoxicated rats were fixed in 3% formaldehyde for 24 hr at room temperature. After fixation, the tissue was embedded by conventional techniques. Thin sections were examined with a light microscope. To examine the change of the hepatocyte number, we counted the number of nuclei per 0.038 mm^3 of the liver slice.

Table 1. Pathophysiological changes after D-galactosamine intoxication*

	Control (N = 4)	D-Galactosamine intoxicated (N = 5)
Body weight (g)	250 ± 7.5	222 ± 8.9
Liver weight (g)	10.79 ± 0.38	8.66 ± 0.77
GOT (Karmen unit)	56.3 ± 9.1	4322 ± 977†
GPT (Karmen unit)	16.3 ± 1.5	3056 ± 619†
Bile flow (μl/min/g liver)	0.780 ± 1.5	0.074 ± 0.047†

* Results are means ± SE.
† $P < 0.05$.

Table 2. Extraction ratio and distribution volume of ^{125}I-EGF and ^{125}I-insulin in control and intoxicated rat liver*

	Control (N = 4)	D-Galactosamine intoxicated (N = 5)
$\bar{t}_{inulin}$ (sec)	10.0 ± 0.30	12.0 ± 0.60†
Vd_{ext} (ml/g liver)	0.15 ± 0.01	0.23 ± 0.03†
E_{EGF}	0.83 ± 0.01	0.31 ± 0.06†
$E_{insulin}$	0.46 ± 0.06	0.24 ± 0.08

* Results are means ± SE.
† $P < 0.05$.

The infiltration area percentage was determined by means of weighing the piece corresponding to the infiltrated area which was excised from the sample of the whole liver slice.

RESULTS

Pathophysiological consequences after D-galactosamine intoxication. Pronounced increases in the serum levels of GOT and GPT were evident only in the animals intoxicated with D-galactosamine 48 hr after treatment with saline or D-galactosamine (Table 1). There was no significant decrease in the body weight or the liver weight of D-galactosamine intoxicated rats compared to the control animals, whereas the bile flow rates during the liver perfusion decreased to 10% of those of the control rats (Table 1).

Figure 1 shows the representative dilution curves for [^{14}C]inulin, ^{125}I-EGF, and ^{125}I-insulin in the control (panels a and b) and D-galactosamine-intoxicated rats (panels c and d). In the control animals, the AUCs of ^{125}I-EGF (panel a) and ^{125}I-insulin (panel b) were approximately 20 and 50% of that of [^{14}C]inulin respectively. In the D-galactosamine-intoxicated rats, the recoveries of ^{125}I-EGF and ^{125}I-insulin into the outflow increased, and the curves of both peptides became close to that of [^{14}C]inulin (panels c and d).

The extraction ratio (E) of ^{125}I-EGF, calculated from the AUC ratio of the dilution curves, decreased significantly ($P < 0.05$) with the D-galactosamine intoxication to 37% of the control values (Table 2).

Figure 2 shows the natural logarithm of the ratios of ([^{14}C]inulin/^{125}I-EGF) (panel a) and ([^{14}C]inulin/^{125}I-insulin) (panel b) respectively. As mentioned above (see Materials and Methods), an estimate of the influx rate constant ($k_1 = k_{on} \cdot P_T/Vd_{ext}$) can be obtained from the initial slope of this plot. In the intoxicated rats, both the peak and initial slope became smaller for either ^{125}I-EGF or ^{125}I-insulin. Parameters (k_1, k_2, and k_3) calculated from these dilution curves are listed in Table 3. The values of k_1 and k_3 for ^{125}I-EGF decreased significantly ($P < 0.05$) with the D-galactosamine intoxication to 47 and 36% of the controls values, respectively, whereas k_2 did not change significantly. The change of the parameters for ^{125}I-insulin was similar to that for ^{125}I-EGF. The values of k_1 and k_3 decreased significantly ($P < 0.05$) with the intoxication to 42 and 57% of the controls values, respectively, whereas k_2 did not change. It is evident that the D-galactosamine intoxication resulted in an approximately 50% loss of the apparent influx rate constant (k_1) of ^{125}I-EGF.

Analysis of the equilibrium binding of EGF to liver homogenate. The k_1 value calculated from the analysis of the dilution curve is a hybrid parameter consisting of k_{on}, P_T, and Vd_{ext}. Therefore, the binding assay of ^{125}I-EGF to the liver homogenate was performed to examine whether the binding capacity (P_T) and/or the binding affinity ($1/K_d = k_{on}/k_{off}$) were changed with D-galactosamine intoxication.

The representative results of the binding study are shown in Fig. 3 as a Scatchard plot. It is evident that the D-galactosamine intoxication reduced the binding capacity to 33% (per g tissue) and 40% (per mg protein) of the control values. The 60–70% loss of P_T is comparable to the 50% reduction in k_1 ($= k_{on} \cdot P_T/Vd_{ext}$) obtained from the liver perfusion study. The dissociation constant appeared to increase to 160% of the control values, but this value is not statistically significant (Table 4).

Determination of enzyme activities and the numbers of hepatocytes. To evaluate to what extent the reduction of the liver cell mass contributes to the 60% loss of the P_T value, we assayed the enzyme contents and activities in the hepatocytes (Table 5). The activity of Na^+,K^+-ATPase, a marker enzyme of the sinusoidal liver plasma membrane, was decreased by D-galactosamine intoxication to 64% (per g tissue) of the control values. The activities of two marker enzymes for the canalicular portion of the surface membrane changed in different ways, i.e. the Mg^{2+}-ATPase activity did not change with the

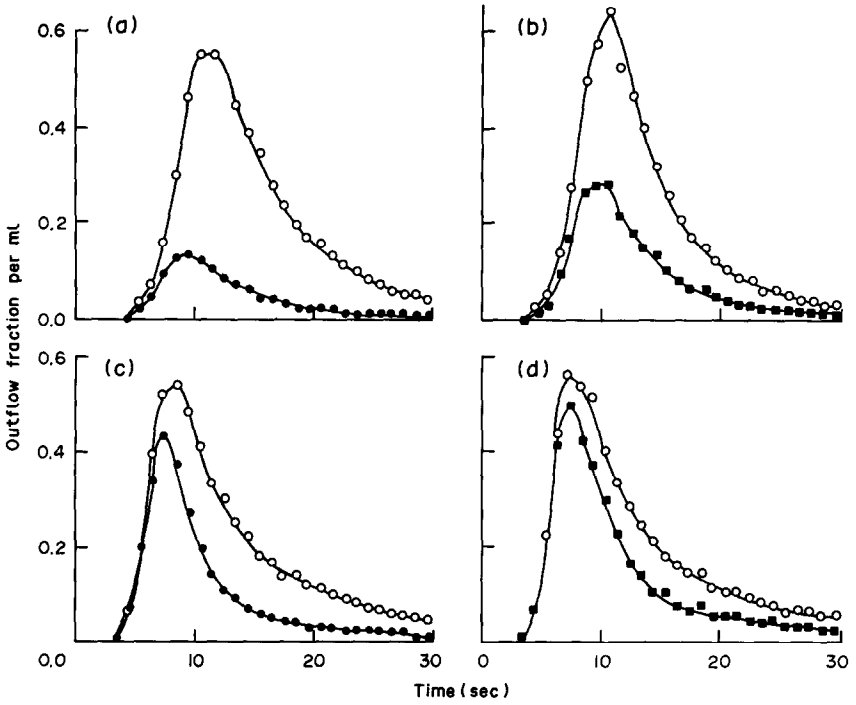

Fig. 1. Representative dilution curves for [^{14}C]inulin, ^{125}I-EGF and ^{125}I-insulin. Control (panels a and b); D-galactosamine intoxicated rats (panels c and d). Animals were subjected to the liver perfusion study at 48 hr after i.p. injection of 1.5 g/kg of D-galactosamine dissolved in isotonic saline or injection of an equivalent volume of isotonic saline respectively. The signal on the time scale represents the sum of the input and collection catheter transit times (the same as in Fig. 2). Key: (○) [^{14}C]inulin; (●) ^{125}I-EGF; and (■) ^{125}I-insulin.

Table 3. Kinetic parameters derived from inulin and peptide dilution curves*

		Control (N = 4)	D-Galactosamine intoxicated (N = 5)
EGF	k_1 (/sec)	0.252 ± 0.013	0.119 ± 0.015†
	k_2 (/sec)	0.040 ± 0.006	0.054 ± 0.011
	k_3 (/sec)	0.128 ± 0.009	0.046 ± 0.009†
Insulin	k_1 (/sec)	0.127 ± 0.013	0.053 ± 0.011†
	k_2 (/sec)	0.066 ± 0.005	0.047 ± 0.012
	k_3 (/sec)	0.093 ± 0.011	0.053 ± 0.019†

* Results are means ± SE. All the data were derived from the same animals listed in Table 1.
† $P < 0.05$.

intoxication, whereas the alkaline phosphatase activity increased significantly ($P < 0.05$) to 177% (per g tissue) of the control values. The contents and activities of enzymes in microsomes (cytochrome P-450) and cytosol (glutathione *S*-transferase) were reduced by D-galactosamine intoxication. The cytochrome P-450 content decreased significantly ($P < 0.05$) to 61% (per g tissue) of the control values. The intoxication also resulted in a 30% (per g tissue) loss of the glutathione *S*-transferase activity (Table 5).

The number of nuclei, which can be approximated to those of hepatocytes, decreased with D-galactosamine intoxication to 71% of the control values [but not significantly ($P > 0.05$)] (Table 6). The infiltration area was found to occupy only 4% of the liver slice.

Thus, the contents and activities of enzymes, and the number of hepatocytes, decreased at most by 30–40% with D-galactosamine intoxication, which cannot fully explain the greater (60–70%) loss of the EGF binding capacity (P_T).

DISCUSSION

The use of the MID method for the analysis of the hepatic transport of peptide hormones has made it possible to calculate the individual kinetic parameters ($k_{on} \cdot P_T$, k_{off}, and k_s) and to determine, thereby, the factor(s) causing those changes in the pathological conditions.

The present results indicate that D-galactosamine intoxication, a model acute hepatitis, reduced the elimination ability (clearance) of the liver to EGF and insulin in the same degree (Table 2). However, such a model-independent analysis cannot reveal which step of the elimination is affected. Therefore, we also analyzed the indicator–dilution curves based on a distributed model [19].

As shown in Fig. 2, the initial slopes of the ratio plots of both EGF and insulin decreased markedly in the intoxicated rats, suggesting a decrease in the

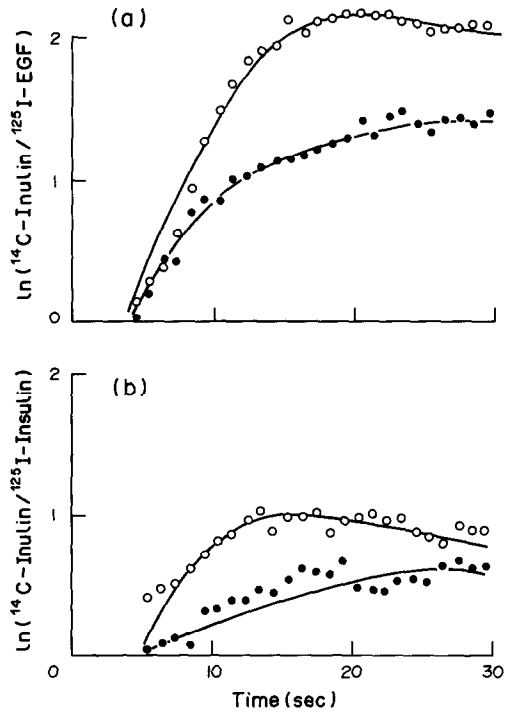

Fig. 2. Representative plots of the natural logarithm of the ratio [^{14}C]inulin outflow fraction per ml)/(^{125}I-EGF (a) or ^{125}I-insulin (b) outflow fraction per ml) versus time. ^{14}C-Labeled inulin was used as the extracellular reference substance. The solid lines were calculated by iterative nonlinear least-squares regression. Key: (○) control; and (●) D-galactosamine-intoxicated.

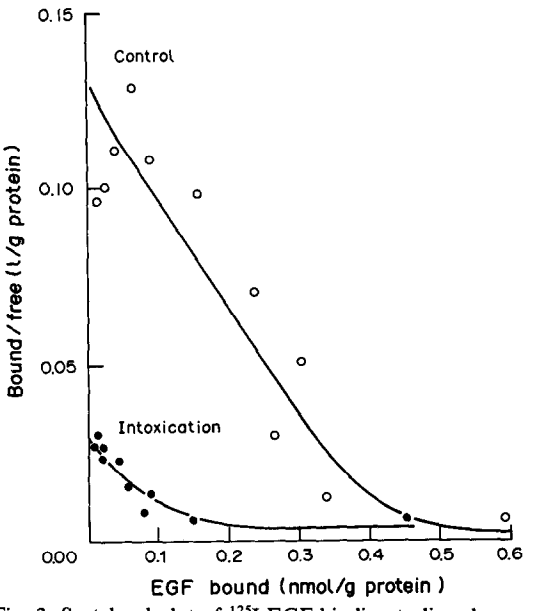

Fig. 3. Scatchard plot of ^{125}I-EGF binding to liver homogenates. The liver homogenates (3.2%, w/v) were incubated with 0.03 μCi of ^{125}I-EGF and 0.6–100 nM unlabeled EGF, and the amount of EGF binding was determined (see Materials and Methods). Resulting K_d and P_T values were derived by an iterative nonlinear least-squares method. The solid lines were calculated by a nonlinear iterative least-squares method. Key: (○) control; and (●) D-galactosamine-intoxicated.

Table 4. Binding parameters of ^{125}I-EGF to liver homogenates*

		Control (N = 6)	D-Galactosamine intoxicated (N = 6)
Protein	(mg/g tissue)	121.0 ± 3.37	106.4 ± 5.18†
P_T	(pmol/g tissue)	43.9 ± 9.06	14.6 ± 3.47†
	(pmol/mg protein)	0.379 ± 0.070	0.151 ± 0.034†
K_d	(nM)	2.58 ± 0.63	4.10 ± 0.69

* Results are means ± SE.
† $P < 0.05$.

k_1 value. The computer-calculated k_1 values, as well as the k_3 values, indeed were decreased to some extent (by 40–60%) in the intoxicated rats. The MID experiment revealed that D-galactosamine intoxication resulted in a 53% loss of k_1 ($= k_{on} \cdot P_T/V_E$) (Table 3). The binding assay of EGF to liver homogenates proved that the intoxication resulted in a 76% (per g tissue) loss of the binding capacity "P_T", which directly corresponds to the receptor density "P_T" (Table 4).

On the other hand, the k_2 ($= k_{off}$) value determined by the MID analysis was little altered by D-galactosamine intoxication (Table 3), and the K_d ($= k_{off}/k_{on}$) value determined by *in vitro* binding experiments did not show significant change, either (Table 4). Thus, it is suggested that the reduced binding capacity of the EGF-receptor contributes to the decrease in the extraction ratio of EGF in the intoxicated rat liver.

The extracellular volume (V_{ext}) in the intoxicated rats, calculated from the mean transit time of [^{14}C]inulin, increased to 153% of the control value (Table 2). The decrease in the k_1 value which can be explained only from the increase in the V_{ext} value, was 35% of the total decrease. The calculated value cannot explain the actual decrease in k_1 (53%) adequately. Thus, both the decrease in the receptor density and the increase in the V_{ext} value must be

Table 5. Enzyme activities in liver homogenate derived from control and intoxicated rats*

	Control (N = 6)	D-Galactosamine intoxicated (N = 6)
Na^+,K^+-ATPase (μmol P_i/hr/g tissue)	171.8 ± 19.2	110.4 ± 14.2†
(μmol P_i/hr/mg protein)	1.57 + 0.22	1.16 + 0.18
Mg^{2+}-ATPase (μmol P_i/hr/g tissue)	707.8 ± 41.2	617.5 ± 40.0
(μmol P_i/hr/mg protein)	6.34 + 0.22	6.35 + 0.47
Alkaline phosphatase (μmol/hr/g tissue)	10.2 ± 2.1	18.1 ± 1.7†
(μmol/hr/mg protein)	0.089 + 0.015	0.187 + 0.018†
Cytochrome P-450 (nmol/g tissue)	53.5 ± 6.0	32.7 ± 2.6†
(nmol/mg protein)	0.444 + 0.027	0.306 + 0.015†
Glutathione *S*-transferase (μmol/min/g tissue)	79.2 ± 4.7	55.2 ± 3.3†
(μmol/min/mg protein)	0.652 + 0.024	0.523 + 0.034†

* Results are means ± SE. All the data are derived from the same animals listed in Table 3.
† $P < 0.05$.

Table 6. Numbers of nuclei and infiltration area in the liver slices derived from control and intoxicated rats*

	Control (N = 4)	D-Galactosamine intoxicated (N = 5)
Number of nuclei (per 0.038 mm^2)	49.8 ± 3.6	35.3 ± 4.2
Infiltration area (% of total area)	None	4.29 ± 0.50

* Results are means ± SE. All the data were derived from the same rats listed in Table 3.

taken into account to explain the decrease of the k_1 value.

The third parameter, k_3, obtained from the MID experiment corresponds to the elimination rate constant of peptides from the receptor site, and was decreased by the intoxication to 36% (^{125}I-EGF) and 47% (^{125}I-insulin) of the respective control value. Since the k_3 value obtained from the MID method (0.05–0.1 sec^{-1}) is far larger than the reported rate constants (0.002–0.01 sec^{-1}) that reflect the internalization process [29, 30], k_3 may correspond to the following steps: (1) the conformational change of the EGF–receptor complex which makes its binding tighter [31], and (2) the irreversible binding of the complex to the coated pit protein, clathrin [32]. Determination of which of these steps was perturbed by the intoxication requires further investigation.

By the binding assay of ^{125}I-EGF to the liver homogenates, the binding capacity (P_T) and the dissociation constant (K_d) of the control rats were determined to be 0.044 nmol/g tissue and 2.6 nM respectively. The reported value of P_T was 0.025–0.033 nmol/g tissue for the liver homogenates at 4° [4, 23], and that of K_d was 1–4 nM for the liver homogenates or isolated hepatocytes at 4° [4], 1.5 nM for the liver plasma membrane at 24° [33], and 1.8 nM at 37° [34]. The binding assay of EGF to the intact hepatocytes and the homogenates prepared from the same hepatocytes gave comparable binding parameters [4].

The application of the MID method is limited to the analysis of the interaction between the EGF and its receptor on the cell surface, whereas these reports show that the affinity and density of the EGF receptor obtained from isolated hepatocytes are not different from the parameters with liver homogenates. These reports thus support that the binding assay we performed using the liver homogenates was the proper experiment for comparing the change of the binding property of ^{125}I-EGF between the control and the intoxicated rats.

It is evident that the D-galactosamine intoxication resulted in a pronounced loss of cytochrome P-450 content and glutathione *S*-transferase activity (Table 5). The alkaline phosphatase activity, on the contrary, increased to approximately 200% of the control value. It has also been reported that the hepatic alkaline phosphatase activity in bile duct ligated rats increases to four times that of the control value [35]. However, the physiological function of this enzyme is still unclear. In our case, the following mechanism to rescue the hepatocytes from the D-galactosamine-induced acute liver disease may explain the doubled activity of alkaline phosphatase; D-galactosamine caused the decrease in the UDP-glucose content in the hepatocytes, which occurs from the fact that the D-galactosamine-1-phosphate antagonizes the glucose-1-phosphate in the UDGP-pyrophosphorylase action [36]. It is likely, therefore, that the hepatocytes would enhance the alkaline phosphatase activity to accelerate the dephosphorylation of the galactose-1-phosphate and to recover the UDP-glucose level in the cell. Further enzymatic investigation will be required to examine this hypothesis.

The enzyme activities and the number of hepatocytes decreased at the most by 30–40% in the intoxicated rats (Tables 5 and 6). On the other hand, the receptor densities (P_T) and the k_1 value of EGF decreased by 67 and 53%, respectively. Thus, the conventional "intact hepatocytes hypothesis" cannot fully explain the loss of receptor densities or the decrease in the k_1 value. A decrease in cellular function with regard to the interaction of EGF with the cell surface receptors is required for the explanation of this phenomenon. Such a "sick cell hypothesis" has been presented with several clinical and

experimental observations. [37–40]. The most unequivocal evidence for the "sick cell hypothesis" was obtained in hepatocytes isolated from experimental cirrhotic rat liver, where function may be related to the hepatocytes only, excluding the altered microcirculation [39, 40]. A simple explanation of the mechanism of the reduction of EGF receptor is that the D-galactosamine-induced intoxication increases the plasma concentrations of EGF, which leads to "down-regulation" (it corresponds to the second hypothesis mentioned in the introduction).

To examine this possibility, we measured with a radio-receptor assay the endogenous concentration of EGF in the serum collected from both control and intoxicated rats (data not shown). The results showed that the endogenous concentration of EGF was not detectable (<0.3 nM) in either the control or the intoxicated rats. This suggests only a slim possibility that the above-mentioned hypothesis, is valid, although it is not excluded completely.

According to Earp and O'Keefe [41], several alternative explanations must be taken into account: (1) some endogenous factors, which may not bind to the EGF receptor, modulate the number of EGF receptors. It is reported that platelet-derived growth factor modulates the epidermal growth factors on 3T3 cells [42]; and (2) an intracellular process caused the loss of the EGF receptors. The elucidation of the mechanism for receptor loss, however, requires further investigation.

A 60% decrease in the number of EGF receptors, without any change of the affinity, has been reported in the plasma membrane from rat liver at 48 hr after a 70% partial hepatectomy [41]. Taken together with our data, the loss of the EGF receptor number may be a common phenomenon in the regenerating liver.

The decrease in the number of EGF receptors in the liver is also caused by fasting [43]; the specific binding of EGF to the liver membrane, prepared from rats fasted for 72 hr, was reduced to 50–60% of that of sham-operated rats. Scatchard analysis revealed that the decrease is due only to a decrease in the binding sites, not in the affinity. Thus, it seems possible that the decrease in food intake causes the reduction of the EGF receptors in the intoxicated rats.

To examine this possibility, the food intake for 48 hr was determined in the intoxicated and control rats. The food intake of the intoxicated and control rats was 16.0 ± 2.1 (g/48 hr) and 44.3 ± 2.4 (mean $\pm$ SE; $N = 6$), respectively. The food intake was decreased significantly (to 36% of the control value) by the intoxication. These results suggest that the decrease in the food intake in the intoxicated rats could be one of the causes for the decrease in the density of liver cell surface receptors.

In conclusion, the present study revealed that the density of EGF receptors on the liver cell surface was reduced by D-galactosamine intoxication, which caused a reduction of hepatic extraction of EGF. This decrease in receptor density cannot be fully explained only by the intact-hepatocyte hypothesis.

REFERENCES

1. Carpenter G and Cohen G, Epidermal growth factor. *Annu Rev Biochem* **48**: 193–216, 1979.
2. Shimizu N, Cell genetic analysis of the receptor systems for bioactive polypeptides. In: *Receptors and Recognition* B-16, pp. 109–142. Chapman and Hall, London, 1984.
3. Draghi E, Armoto U, Andreis PG and Mengato L, The stimulation by epidermal growth factor (urogastrone) of the growth of neonatal rat hepatocytes in primary tissue culture and its modulaton by serum and associated pancreatic hormones. *J Cell Physiol* **103**: 129–147, 1980.
4. Yanai S, Sugiyama Y, Kim DC, Sato H, Fuwa T, Iga T and Hanano M, Binding of human epidermal growth factor to tissue homogenates of the rat. *Chem Pharm Bull (Tokyo)* **35**: 4891–4897, 1988.
5. Kim DC, Sugiyama S, Sato H, Fuwa T, Iga T and Hanano M, Kinetic analysis of *in vivo* receptor-dependent binding of human growth factor by rat tissues. *J Pharm Sci* **77**: 200–206, 1988.
6. St. Hilaire RJ, Hradek GT and Jones AL, Hepatic sequestration and biliary secretion of epidermal growth factor: evidence for a high-capacity uptake system. *Proc Natl Acad Sci USA* **30**: 3797–3801, 1983.
7. Moriarity DM and Savage CR Jr, Interaction of epidermal growth factor with adult rat liver parenchymal cells in primary culture. *Arch Biochem Biophys* **203**: 506–518, 1980.
8. Piazza M, *Experimental Viral Hepatitis*. Charles C. Thomas, Springfield, IL, 1968.
9. St. Hilaire RJ and Jones AL, Epidermal growth factor: its biologic and metabolic effects with emphasis on the hepatocyte. *Hepatology* **2**: 601, 1982.
10. Keppler D, Lesch R, Reutler W and Decker K, Experimental hepatitis induced by D-galactosamine *Exp Mol Pathol* **9**: 279–290, 1968.
11. Sato H, Sugiyama Y, Kim DC, Sawada Y, Iga T, Fuwa T and Hanano M, Kinetic analysis of hepatic transport of epidermal growth factor (EGF) in the perfused rat liver. *J Pharmacobiodyn* **10**: s-75, 1987.
12. Sato H, Sugiyama Y, Kim DC, Sawada Y, Iga T, Fuwa T and Hanano M, Kinetic analysis of hepatic transport of epidermal growth factor (EGF) in the perfused rat liver. In: *18th Symposium on Drug Metabolism and Action*, Toyama, Japan, 20–21 October 1986, pp. 67–70. Pharmaceutical Society of Japan, Tokyo, 1986.
13. Izzo JL, Roncone AM, Helton DL and Izzo MJ, Subcellular distribution of intraportally injected ^{125}I-labeled insulin in rat liver. *Arch Biochem Biophys* **198**: 97–109, 1979.
14. Hunter WM and Greenwood FC, Preparation of iodine-131 labelled growth hormone of high specificity. *Nature* **194**: 495–496, 1962.
15. Karmen A, A note on the spectrophotometric assay of glutamic-oxaloacetic transaminase in human blood serum. *J Clin Invest* **34**: 131, 1955.
16. Tsao SC, Sugiyama Y, Sawada Y, Nagase S, Iga T and Hanano M, Effect of albumin on hepatic uptake of warfarin in normal and analbuminemic mutant rats: analysis by multiple indicator dilution method. *J Pharmacokinet Biopharm* **14**: 51–64, 1986.
17. Kakutani T, Yamaoka K, Hashida M and Sezaki H, A new method for assessment of drug disposition in muscle: application of statistical moment theory of local perfusion systems. *J Pharmacokinet Biopharm* **13**: 609–631, 1985.
18. Benet LZ and Galeazzi RL, Noncompartmental determination of the steady-state volume of distribution. *J Pharm Sci* **68**: 1071–1074, 1979.
19. Goresky CA and Bach GG, Membrane transport and the hepatic circulation. *Ann NY Acad Sci* **170**: 18–45, 1970.
20. Miyauchi S, Sugiyama Y, Sawada Y, Morita K, Iga T

and Hanano M, Kinetics of hepatic transport of 4-methylumbelliferone in rats: analysis by multiple indicator dilution method. *J Pharmacokinet Biopharm* **15**: 25–38, 1987.
21. Itoh N, Sawada Y, Sugiyama Y, Iga T and Hanano M, Kinetic analysis of rat renal tubular transport based on multiple-indicator dilution method. *Am J Physiol* **251**: F103–F114, 1986.
22. Sawada Y, Itoh N, Sugiyama Y, Iga T and Hanano M, Analysis of multiple indicator dilution curves for estimation of renal tubular transport parameters. *Comput Methods Programs Biomed* **20**: 51–61, 1985.
23. Dunn WA and Hubbard AL, Receptor-mediated endocytosis of epidermal growth factor by hepatocyte in the perfused rat liver: ligand and receptor dynamics. *J Cell Biol* **98**: 2148–2159, 1984.
24. Yamaoka K, Tanigawara Y, Nakagawa T and Uno T, A pharmacokinetic analysis program (MULTI) for microcomputer. *J Pharmacobiodyn* **4**: 879–885, 1981.
25. Schoner W, von Ilberg C, Kramer R and Seubert W, On the mechanism of Na^+- and K^+-stimulated hydrolysis of adenosine triphosphate: 1. Purification and properties of a Na^+- and K^+-activated ATPase from ox brain. *Eur J Biochem* **1**: 334–343, 1967.
26. Yachi K, Sugiyama Y, Iga T, Ikeda Y, Toda G and Hanano M, Comparison of bile acid binding to sinusoidal and bile canalicular membranes isolated from rat liver. *Biochim Biophys Acta* **91**: 15–22, 1987.
27. Matsubara T, Koike M, Touchi A, Tochino Y and Sugeno K, Quantitative determination of cytochrome P-450 in rat liver homogenate. *Anal Biochem* **75**: 596–603, 1976.
28. Sugiyama Y, Yamada T and Kaplowitz N, Glutathione *S*-transferase in elasmobranch liver: molecular heterogeneity, catalytic and binding properties and purification. *Biochem J* **199**: 749–756, 1981.
29. Wiley HS and Cunningham DD, The endocytotic rate constant. *J Biol Chem* **257**: 4222–4229, 1982.
30. Gex-Fabry M and Delisi C, Receptor-mediated endocytosis: a model and its implications for experimental analysis. *Am J Physiol* **247**: R768–R779, 1984.
31. Pastan IH and Willingham MC, Journey to the center of the cell. *Science* **214**: 504–509, 1981.
32. Adamson ED and Rees AR, Epidermal growth factor receptors. *Mol Cell Biochem* **34**: 129–152, 1981.
33. O'Keefe E, Hollenberg MD and Cuatrecasas P, Epidermal growth factor: characteristics of specific binding in membranes from liver, placenta, and other target tissues. *Arch Biochem Biophys* **164**: 518–526, 1974.
34. Yachi K, Sugiyama Y, Sato H, Kim DC, Fuwa T, Iga T and Hanano M, Comparison of specific binding of human epidermal growth factor (EGF) to sinusoidal and bile canalicular membranes isolated from rat liver. *J Biochem (Tokyo)* **103**: 448–451, 1988.
35. Komoda T, Koyama I, Nagata A, Sakagishi Y, Kurata M and Kumegawa M, A possible mechanism of induction and translocation into blood stream of rat alkaline phosphatase activity by bile duct ligation. *Arch Biochem Biophys* **251**: 323–335, 1986.
36. Keppler D and Decker K, Studies on the mechanism of galactosamine hepatitis: accumulation of galactosamine-1-phosphate and its inhibition of UDP-glucose pyrophosphorylase. *Eur J Biochem* **10**: 219–225, 1969.
37. Pessayre D, Lebrec D, Descatoire V, Peignoux M and Benhamou JP, Mechanism for reduced drug clearance in patients with cirrhosis. *Gastroenterology* **74**: 566–571, 1978.
38. Huet PM and Villeneuve JP, Determinations of drug disposition in patients with cirrhosis. *Hepatology* **3**: 913, 1983.
39. Villeneuve J-P, Wood AJJ, Shand DG, Rogers L and Branch RA, Impaired drug metabolism in experimental cirrhosis in the rat. *Biochem Pharmacol* **27**: 2577–2581, 1978.
40. Reichen J, Hoilien C, Le M and Jones RH, Decreased uptake of taurocholate and ouabain by hepatocytes isolated from cirrhotic rat liver. *Hepatology* **7**: 67–70, 1987.
41. Earp HS and O'Keefe EJ, Epidermal growth number decreases during rat liver regeneration. *J Clin Invest* **67**: 1580–1583, 1981.
42. Wrann M, Fox CF and Ross R, Modulation of epidermal growth factor receptors on 3T3 cells by platelet-derived growth factor. *Science* **210**: 1363–1365, 1980.
43. Freidenberg GR, Klein HH, Kladde MP, Cordena R and Olefsky M, Regulation of epidermal growth factor number and phosphorylation by fasting in rat liver. *J Biol Chem* **261**: 752–757, 1986.