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Serum release of hepatic calcium-binding protein regucalcin by liver injury with galactosamine administration in rats

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Abstract

Whether calcium-binding protein regucalcin, which mainly localizes in liver, is released into the serum by liver injury was investigated in rats administered galactosamine. Galactosamine (25 mg/100 g body weight) was intraperitoneally administered 3 times at 2 h intervals in rats, and the animals were sacrificed at 10, 24 and 48 h after the first administration of galactosamine. Liver regucalcin mRNA levels were clearly reduced at 24 and 48 h after galactosamine administration with estimating for Northern blotting assay. When hepatic regucalcin concentration was estimated by enzyme-linked immunoadsorbent assay (ELISA) with rabbit-anti-regucalcin IgG, liver regucalcin concentration was not significantly altered by galactosamine administration. Serum regucalcin concentration was markedly elevated at 10 and 24 h after the first administration of galactosamine. Serum transaminases (GOT and GPT) activities were significantly increased by galactosamine administration, indicating that liver injury was induced. The present study demonstrates that liver regucalcin is released into the serum by liver injury with galactosamine administration in rats. (Mol Cell Biochem **136**: 85–90, 1994)

Key words: regucalcin, calcium-binding protein, liver injury, galactosamine

Introduction

It is known that liver metabolism is regulated by the increase of calcium ion (Ca^{2+}) in the cytosol of liver cells due to hormonal stimulation [1–3]. Recently, it has been reported that a novel Ca^{2+}-binding protein regucalcin, which differs from calmodulin, is distributed in the hepatic cytosol of rats [4, 5]. Regucalcin has a reversible effect on the activation and inhibition of various enzymes by Ca^{2+} in liver cells [6–9]. More recently, it has been demonstrated that regucalcin is mainly localized in liver by Northern blotting analysis with a regucalcin complementary deoxyribonucleic acid (cDNA) probe [10] and by enzyme-linked immunoadsorbent assay (ELISA) with rabbit-anti-regucalcin IgG [11]. Regucalcin was predominantly in the liver of rats.

Whether regucalcin in liver is altered by liver injury is not clarified so far. This may be an important to understand a pathophysiological role of regucalcin in liver cells. Meanwhile, it is well known that serum transaminases (GOT and GPT) are routinely used as the diagnosis of liver injury, although the serum elevation is also seen by heart disease. Since regucalcin is synthesized in liver with tissue specific [10, 11], the serum release of hepatic regucalcin with liver injury may be useful as a specific diagnosis of the injury.

The present study, therefore, was undertaken to clarify the alteration of regucalcin in the liver and serum of rats administered galactosamine, which is known as a chemical inducer of liver injury [12, 13]. It was found that hepatic regucalcin

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is released into the serum by liver injury, suggesting a role in biochemical diagnosis of the injury.

Materials and methods

Chemicals

Deoxycytidine 5′-[α-^{32}P]triphosphate ([^{32}P]dCTP); 110 Tbq/mmol and nylon membranes (Hybond N^{+}) for Northern hybridization were obtained from Amersham (Buckinghamshire, UK). A human β-actin gene fragment (1.4 kb) as an internal standard was obtained from Wako Pure Chemical Company (Osaka, Japan). Molecular-size standards (0.24–9.5 kb RNA Ladder) for electrophoresis of RNA was purchased from Bethesda Research Laboratories (Gaithersburg, MD, USA). NHS-LC-Biotin was obtained from Pierce (Rockford, IL, USA). Streptavidin-peroxidase conjugate was obtained from Tago, Inc. (Burlingame, CA, USA). o-Phenylenediamine was purchased from Nakarai tesque (Kyoto, Japan). D-Galactosamine and other reagents were obtained from Wako Pure Chemical Company and Sigma Chemical Company (St. Louis, MO, USA). Any water and solutions used for RNA preparation were treated with chemical diethylpyrocarbonate (DEPC, Sigma) to inhibit RNase activity.

Animals

Male Wistar rats, weighing 80–100 g, purchased from Japan SLC Inc. (Hamamatsu, Japan), were fed commercial laboratory chow (solid, Orriental Yeast Company Limited, Tokyo, Japan) containing 57.5% carbohydrate, 1.1% calcium and 1.1% phosphorus, and distilled water, *ad libitum*.

Administration procedure

D-Galactosamine was diluted in 0.9% sodium chloride solution at a concentration of 50 mg/ml. The solution (0.5 ml/100 g body weight) was intraperitoneally administered 3 times at 2 h intervals in rats. At 10, 24 and 48 h after the first administration, the rats were sacrificed by bleeding. The livers were perfused with ice-cold 0.25 M sucrose solution and immediately removed and frozen at –80°C. The serum obtained after centrifugation of blood was stored at –80°C. Control animals received vehicle solution.

Isolation of RNA

Hepatic total RNAs were prepared as described previously [10]. Liver was rinsed with ice-cold 0.25 M sucrose solution, and homogenized in buffer solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol and 2 M sodium acetate. Total RNAs were extracted by vigorous shaking in a mixture of phenol, chloroform and isoamyl alcohol, and the phases were separated by centrifugation at 10,000 × g for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isopropanol at –20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in 50 μl of DEPC-treated 0.5% sodium dodecyl sulfate (SDS).

Northern blotting

Ten μg of total RNAs extracted from liver were electrophoresed in 1.2% agarose denaturing gels containing 2.2 M formaldehyde in MOPS buffer (pH 7, containing 20 mM 3-N-morpholino-propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA) with 3 V/cm for 3 h [10]. The electrophoresed gels were transferred to nylon membranes by blotting. Part of regucalcin cDNA (the 0.6 kb, *Kpn*I-*Pst*I insert) was labeled with [^{32}P]dCTP by random primers with the DNA polymerase Klenow fragment. This radioactive probe was used for hybridization detection of RNAs on blots. The membranes were prehybridized, and hybridized in buffer solution containing 50% formamide, 5 × SSPE (1 × SSPE; 1.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5 × Denhardt's reagent (1 × Denhardt's reagent; 0.02% w/v each of bovine serum albumin, Ficoll, polyvinylpyrrolidine) and 0.5% SDS with ^{32}P-labeled regucalcin cDNA in a sealed plastic bag at 42°C for 16 h. After hybridization the membranes were washed as follows: 2 × SSPE and 0.1% SDS at 42°C (twice, each for 15 min), followed by 0.1 × SSPE and 0.1% SDS at room temperature (twice, each for 15 min), and then the membranes were exposed to X-ray film for 12 h.

Quantity and integrity of mRNA were monitored by rehybridizing with a radioactive cDNA probe from human α-actin gene fragment under identical conditions. No noticeable change in the level of RNA hybridized with the β-actin probe was observed throughout present experiments (data not shown). The size of the hybridizing RNA was determined by running the standard RNA molecules of known sizes in parallel. The density of the autoradiographic data was quantified by densitometer scanning (Dual-wavelength Flying-spot Scanner, CS-9000, Shimadzu Company, Japan).

Isolation of regucalcin

Livers were perfused with 100 mM Tris-HCl buffer (pH 7.4, containing 120 mM NaCl, 4 mM KCl, cooled to 4°C), suspended 1:4 in Tris-HCl buffer (pH 7.4) and homogenized

in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 550 × g in a refrigerated centrifuge for 10 min and the supernatant was spun at 105,000 × g for 60 min (Hitachi SCP-85H2). The supernatant from the latter was collected and heated at 60°C for 10 min. The solution was then cooled and recentrifuged at 38,000 × g for 20 min. Ca^{2+}-binding protein, regucalcin, in the 38,000 × g supernatant was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously [4]. Protein concentration was determined by the method of Lowry *et al.* [14].

Anti-regucalcin IgG

Rabbits (from Japan SLC Inc., 2.5–3.0 kg), used to raise anti-(rat liver regucalcin) antibodies, were subcutaneously injected with 1.0 mg per animal of antigen emulsified with Freunds' complete adjuvant and then four more times, at 2-week intervals, with Freunds' incomplete adjuvant. Animals were killed by bleeding 10 days after the last injection to obtain antiserum. The antiserum was purified by Protein A column (Nygen Company, Yonkers, NY, USA) to obtain anti-regucalcin IgG.

Determination of regucalcin by ELISA

Livers were homogenized in 4 ml volume of 10 mM phosphate buffered saline (PBS; pH 7.4 containing 0.1% sodium dodecyl sulphate, 0.1 mM phenylmethylsulfonyl fluoride, cooled) to 4°C with the Teflon homogenizer. The homogenate was centrifuged at 105,000 × g for 1 h and the resulting supernatant cytosol fractions were used for ELISA assays.

Detection of regucalcin in the liver cytossol and serum was performed by using an enzyme-linked immunoadsorbent assay (ELISA) [11]. This assay system was specific for regucalcin [11]. 96-Well assay plates (Nunc-Imuno, USA) were coated with 50 µl of anti-regucalcin IgG diluted in 0.1 M carbonate buffer (pH 9.7) to a final concentration of 10 µg/ml for 2 h at 37°C. Wells were then blocked with 5% BSA/PBS for 1 h and washed three times in PBS + 0.05% Tween 20. The plates were incubated with standard amounts of regucalcin (0.5–10 ng/ml, 50 µl) or samples (50 µl) for 18 h at 4°C. After wash, biotinylated anti-regucalcin IgG with NHS-LC-Biotin were added. Plates were then incubated for 2 h at 37°C and washed. Plates were incubated with streptavidin-peroxidase conjugate (1/40000) for 2 h at 37°C, washed and finally incubated with o-phenylenediamine (3 mg/ml, 100 µl) for 15 min at room temperature. The reaction was stopped with 100 µl 4N H_2SO_4 and the plate read at 450 nm. Regucalcin concentration was calculated from the standard curve for the determination of regucalcin. The concentration of regucalcin was expressed as µg/g wet liver tissue or ng/ml of serum.

Assay of GOT and GPT

Glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) activities in the serum were determined by using the enzyme assay KIT (Wako Pure Chemical Company). The enzyme activity was expressed as K.U.

Statistical methods

The significance of diferences between values was estimated by using Student's *t*-test; P values of less than 0.05 were considered to indicate statistically significant differences.

Results

The alteration of regucalcin mRNA levels in the liver of rats administered intraperitoneally galactosamine is shown in Fig. 1. The solution of galactosamine (25 mg/100 g body weight) was intraperitoneally administered 3 times at 2 h intervals in rats, and then the animals were sacrificed at 10, 24 and 48 h after the first administration. Liver regucalcin mRNA levels were clearly reduced at 24 and 48 h after galactosamine administration.

The alteration of regucalcin concentration in the liver of rats administered galactosamine is shown in Fig. 2. Liver regucalcin concentration was not significantly altered by galactosamine administration, although the regucalcin mRNA levels were decreased.

The alteration in serum regucalcin concentration of rats after galactosamine administration is shown in Fig. 3. The serum regucalcin concentration was significantly increased at 10 h after the first administration of galactosamine (25 mg/100 g). This increase was about 20-fold of the control value. Further increase was seen at 24 h after the first administration of galactosamine. At 48 h after the first administration, the serum regucalcin concentration was markedly decreased in comparison with that at the 24 h, although it was significantly increased yet.

On the other hand, the administration of galactosamine (25 mg/100 g) caused a remarkable increase of transaminase activity (GOT and GPT) in the serum of rats, as shown in Table 1. At 10 and 24 h after the first administration of galactosamine, the serum GOT and GPT activities were clearly elevated. At 48 h, a significant increase in serum GOT activity was not seen, while serum GPT activity was elevated appreciably.

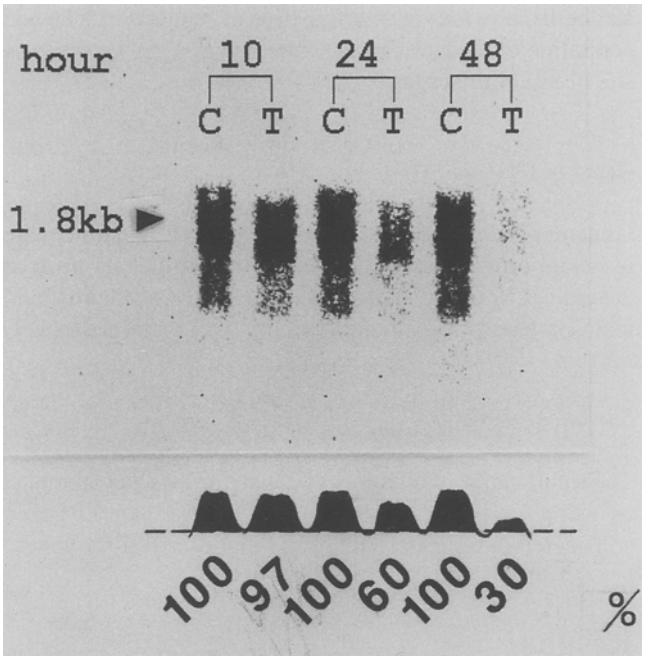

Fig. 1. The alteration of regucalcin mRNA levels in the liver of rats administered galactosamine. Galactosamine (25 mg/100 g body weight) was intraperitoneally administered 3 times at 2 h intervals in rats, and the animals were sacrificed at 10, 24 and 48 h after the first administration of the chemical. Control rats (C) received an equivalent volume of 0.9% sodium chloride solution (vehicle). Total RNA (10 μg) isolated from the liver were subjected to Northern blotting analysis. The pattern of hybridization obtained with the rat liver regucalcin cDNA is shown. The arrowhead indicates hybridization bands corresponding to mRNA encoding the regucalcin. The result shows one of three experiments with separate rats.

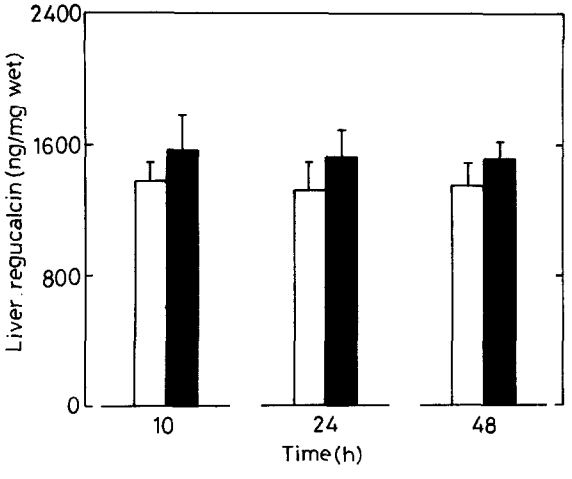

Fig. 2. The alteration of regucalcin concentration in the liver of rats administered galactosamine. Galactosamine (25 mg/100 g) was intraperitoneally administered 3 times at 2 h intervals in rats, and the animals were sacrificed at 10, 24 and 48 h after the first administration of the chemical. Control rats received an equivalent volume of the vehicle solution. Each value represents the mean ± SEM of 6 animals. Data were not significant. □, control; ■, galactosamine administration.

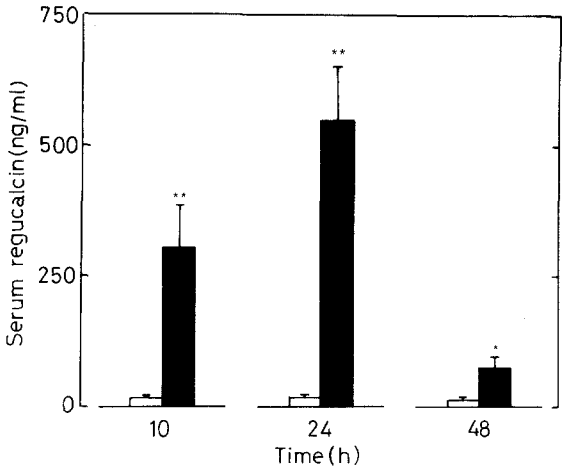

Fig. 3. The alteration of regucalcin concentration in the serum of rats administered galactosamine. Galactosamine (25 mg/100 g) was intraperitoneally administered 3 times at 2 h intervals in rats, and the animals were sacrificed at 10, 24 and 48 h after the first administration of the chemical. Control rats received a vehicle solution. Each value represents the mean ± SEM of 6 animals. * $P < 0.05$ and ** $P < 0.01$, as compared with the control value. □, control; ■, galactosamine administration.

Then, the correlation between regucalcin concentration and transaminase activity in the serum of rats administered galactosamine was examined. Galactosamine (25 mg/100 g) was intraperitoneally administered 3 times at 2 h intervals in rats. The serum values obtained at 10, 24 and 48 h after the first administration of galactosamine were plotted with a correlation between regucalcin level and GOT or GPT activitiy. There was a correlation between regucalcin and transaminase activity in the serum of rats after galactosamine administration (Fig. 4).

Discussion

A novel Ca^{2+}-binding protein regucalcin is distributed in the hepatic cytoplasm of rats [4, 5]. Regucalcin can regulate the

Table 1. The alteration of transaminase (GOT and GPT) activity in the serum of rats administered galactosamine

Time (h)	GOT (K.U.)		GPT (K.U.)	
	Control	Galactosamine	Control	Galactosamine
10	118 ± 4	301 ± 36**	26.6 ± 1.4	125.2 ± 23.4**
24	119 ± 5	674 ± 185**	30.4 ± 1.8	404.1 ± 140.5**
48	108 ± 3	145 ± 23	32.4 ± 1.1	64.6 ± 15.5*

Rats received intraperitoneal administration of galactosamine (25 mg/100 g) 3 times at 2 h intervals, and they were sacrificed at 10, 24 and 48 h after the first administration of the chemical. Each value represents the mean ± SEM of 6 animals. * $P < 0.05$ and ** $P < 0.01$, as compared with the control value.

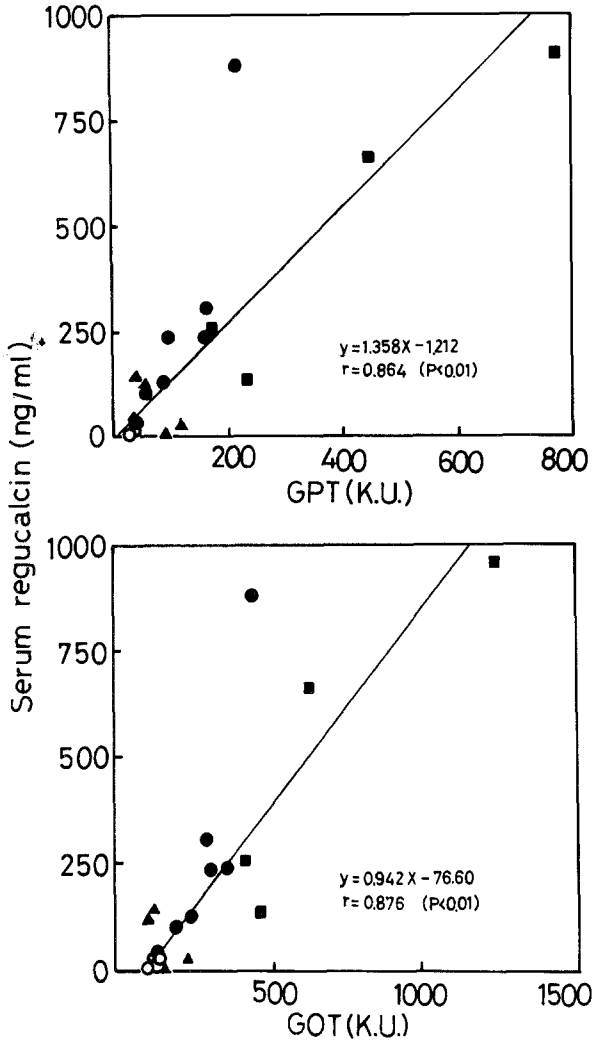

Fig. 4. Correlation between regucalcin concentration and transaminase (GOT and GPT) activity in the serum of rats administered galactosamine. Rats received intraperitoneal administration of galactosamine (25 mg/100 g) 3 times at 2 h intervals. Each value was obtained from the serum of rats sacrificed at 10 (•), 24 (■) and 48 (▲) h after the first administration of galactosamine. Control value (o) was obtained from rats received vehicle solution.

effect of Ca^{2+} on various enzymes in liver cells [6–9]. Recently, it has been demonstrated that regucalcin mRNA in liver is increased by a single intraperitoneal administration of calcium chloride in rats [15]. Furthermore, it is shown that regucalcin mRNA and its protein concentration are mainly localized in the liver but only slightly in kidney, while it was not detected in the heart, lung, brain, skeletal muscle and smooth muscle [10, 11]. Thus, regucalcin is unique in the liver of rats.

The present study was undertaken to clarify whether regucalcin concentration in liver and serum is altered by liver injury. We used a galactosamine as a chemical inducer of liver injury [12, 13], although the effect of galactosamine is weaker than carbon tetrachloride which can cause a remarkable elevation of serum regucalcin level with liver injury [16]. It was found that liver regucalcin mRNA levels were clearly reduced at 24 and 48 h after galactosamin administration, suggesting that galactosamine-induced liver injury may cause a decrease of regucalcin synthesis in the liver. However, regucalcin concentration in the liver was not decreased by galactosamine administration, indicating that regucalcin is not decomposed in the liver of rats administered galactosamine. The 3′ untranslated region of the mRNA for regucalcin contains a rapid mRNA degradation signal, ATTTA [17]. This may partly explain the observations that the mRNA levels for regucalcin in liver were dramatically reduced by the administration of galactosamine, whereas the protein contents were not. In addition, it is possible that the chemical treatment may cause an inhibition of protease in liver cells. This may lead to the result that the decrease in hepatic regucalcin mRNA level does not cause the reduction of liver regucalcin concentration.

Galactosamine administration caused a remarkable increase of serum regucalcin concentration at 10 h after the first administration, although hepatic regucalcin mRNA level and regucalcin concentration were not significantly altered by the administration. Regucalcin content in the liver was much more in comparison with serum regucalcin concentration. Presumably, the damage of hepatic plasma membranes with galactosamine administration induces a release of regucalcin from liver cells into serum. However, hepatic regucalcin concentration may be not influenced appreciably by regucalcin release into serum, because liver contains a large amount of regucalcin.

Galactosamine administration, which the dose of 25 mg/100 g can induce liver injury [12, 13], caused a remarkable increase of regucalcin concentration and a corresponding elevation of transaminase (GOT and GPT) activity in the serum of rats. Also, the alteration of both serum findings was associated by the restoration of liver injury with time course after the galactosamine administration. The good correlation between regucalcin concentration and transaminase (GOT and GPT) activity in the serum was observed in galactosamine-administered rats, although more data points located along a continuum would allow for a more convincing argument that regucalcin would be useful as a diagnostic tool. The increase of regucalcin concentration in the serum of galactosamine-administered rats was in the range of 40 to 950 ng/ml. This variation may be due to the extent of liver damage after galactosamine administration. Meanwhile, serum regucalcin concentration in normal rats was in the range of 5 to 15 ng/ml. Thus, an increase in the serum regu-

calcin concentration may be significant as an index for liver injury. Presumably, the estimation of serum regucalcin has a role as diagnostic tool in developing of liver injury, because regucalcin is mainly localized in liver. This, however, remains to be demonstrated in human with liver injury, although regucalcin is detected in the serum of subject with liver disease (unpublished observation). Furthermore, the implication that this serum regucalcin can be used to detect liver injury requires many more trials with a variety of subjects with liver disease.

In conclusion, it has been demonstrated that galactosamine administration produces a remarkable elevation of regucalcin concentration in the serum of rats, supporting the view that hepatic regucalcin is released into the serum by liver injury. The estimation of serum regucalcin concentration may be a role as the biochemical diagnostic tool for liver injury.

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