Sexual Difference of Hepatic Calcium-binding Protein Regucalcin mRNA Expression in Rats with Different Ages: Effect of Ovarian Hormone

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The sexual difference of calcium-binding protein regucalcin mRNA expression in the liver of rats was investigated by Northern blot analysis. Liver regucalcin cDNA (0.9 kb of open reading frame) was used as a probe. The analysis of total RNAs extracted from various tissues of rats indicated that regucalcin mRNA was present primarily in the liver with a size of 1.8 kb. Hepatic regucalcin mRNA was expressed in male rats more than in females. This sexual difference was also seen in aged rats (50 weeks old), although the expression was decreased with increasing age. Ovariectomy did not cause a significant alteration in hepatic regucalcin mRNA levels. The subcutaneous administration of 17β -estradiol (0.2 mg/100 g body weight) in ovariectomized rats did not cause an appreciable increase in hepatic mRNA levels. The results demonstrate that regucalcin mRNA expression in rat liver is based on sex, and that this difference may not be related to estrogen.

Key words regucalcin; calcium-binding protein; sexual difference; estrogen; rat liver; aging

Regucalcin, a calcium-binding protein, has a reversible effect on the activation and inhibition of various enzymes by Ca^{2+} in liver cells.¹⁾ The physiological role of regucalcin may differ from that of calmodulin which can amplify the Ca^{2+} effect on liver metabolism.^{2,3)} The gene of regucalcin is localized on rat chromosome Xq11.1–12 proximal end, and consists of seven exons and six introns.^{4–6)} Regucalcin mRNA is mainly expressed in liver but slightly in kidney with a size of 1.8 kb.⁷⁾ It has been shown that the expression of regucalcin mRNA in rat liver is stimulated by various factors including calcium or insulin administration.^{7,8)} The characterization of regucalcin mRNA expression in rat liver may be important in determining understanding the physiologic role of regucalcin, but this has not yet been completely done. The present study was undertaken to learn whether regucalcin mRNA expression in the liver is altered by sexual difference or increasing age in rats. The expression was found to be lowered in female species of young and older rats, but this reduction may not be involved with the ovarian hormone.

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 (0.43 kb) as an internal standard was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Molecular-size standards (0.24–9.5 kb RNA Ladder) for electrophoresis of RNA was purchased from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). 17β -Estradiol was obtained from Sigma Chemical Company (St. Louis, Mo, U.S.A.). Other reagents were obtained from Wako Pure Chemical Industries, Ltd. Any water and solutions used for RNA preparation were treated with chemical diethylpyrocarbonate (DEPC, Sigma) to inhibit RNase activity.

Animals and Administration Procedures Male and female Wistar rats (5 and 50 weeks old) purchased from Japan SLC Inc. (Hamamatsu, Japan) were fed commercial laboratory chow (solid, Oriental Yeast Company, Ltd., Tokyo, Japan) containing 57.5% carbohydrate, 1.1% calcium and 1.1%

phosphorus, and distilled water *ad libitum*. Females at 6 weeks of age were given a bilateral ovariectomy under ether anesthesia, then fed for 7 more days (to 7 weeks of age). 17β -Estradiol was diluted in ethanol solution (99.5%) at a concentration of 0.4 mg/ml. The solution (0.5 ml/100 g body weight) was subcutaneously administered to ovariectomized rats (5 weeks old). The animals were sacrificed by bleeding at 3, 6, and 24 h after a single administration. The livers were perfused with ice-cold 0.25 M sucrose solution and immediately removed and frozen at -80°C . Control rats received vehicle solution (99.5% ethanol) or sterile distilled water.

Isolation of RNA Hepatic total RNAs were prepared as described previously.⁹⁾ 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 $10000\times g$ 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 micrograms of total RNAs extracted from liver was electrophoresed in 1.2% agarose denaturing gels containing 2.2 M formaldehyde in MOPS buffer (pH 7, containing 20 mM 3-*N*-morpholinopropansulfonic 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. Total regucalcin cDNA (the 0.9 kb, *KpnI*–*PstI* insert) was labeled with [^{32}P]dCTP by random primers with the DNA polymerase Klenow fragment. This radioactive probe was used to detect hybridization of RNAs on blots. The membranes were prehybridized, and hybridized in buffer solution containing 50% formamide, 5xSSPE (1xSSPE; 1.15 M NaCl, 10 mM NaH_2PO_4 , 1 mM EDTA), 5xDenhardt's reagent (1xDenhardt's reagent; 0.02% (w/v) each of bovine serum albumin, Ficoll, polyvinylpyrrolidone) and 0.5% SDS with ^{32}P -labeled regucalcin cDNA in a sealed plastic bag at 42°C for 16 h. After hybridization the mem-

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Fig. 1. Alteration in Regucalcin mRNA Levels in the Liver of Male and Female Rats of Different Ages

Livers were obtained from rats of both sexes of 5 or 50 weeks old. Total RNA (10 μ g) isolated from the liver was subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. Arrowhead (1.8 kb) indicates hybridizing bands corresponding to mRNA encoding regucalcin. The figure shows one of four experiments with separate rats. The densitometric data (% of value from 5 week-old male rats; mean $\pm$ S.E.M. of 4 rats) were 72.3 ± 3.5 , 55.1 ± 3.9 , and 35.8 ± 2.0 , respectively. β -Actin mRNA levels in the same samples were not altered significantly (data not shown).

branes were washed as follows: 2xSSPE and 0.1% SDS at 42 $^{\circ}$ C (twice, each for 15 min), followed by 0.1xSSPE and 0.1% SDS at room temperature (twice, each for 15 min), and then 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 β -actin gene fragment under identical conditions. No noticeable change in the level of RNA hybridized with the β -actin probe was observed throughout the present experiments. 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 densitometric scanning (Dual-wavelength Flying-spot Scanner, CS-9000, Shimadzu Company, Japan).

Statistical Methods The significance of differences between values was estimated 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 livers of male and female rats of different ages is shown in Fig. 1. The results showed the existence of two distinct mRNA isoforms of 1.8 kb and 1.6 kb, although the 1.6 kb mRNA was present only slightly. These isoforms are thought to be generated by alternative splicing of common RNA precursor molecules; the 1.6 kb mRNA may be an intermediate molecule of the mature mRNA of regucalcin.^{4,7)} In 5-week old rats, regucalcin mRNA levels in the liver were significantly reduced ($p < 0.01$) in the female animals in comparison with that in the males. Such reduction was also seen in older rats (50 weeks old), although hepatic regucalcin mRNA levels were significantly ($p < 0.01$) decreased with increasing age. Thus, sexual difference was found in the expression of regucalcin

Fig. 2. Effect of Ovariectomy (OVX) on Regucalcin mRNA Levels in the Liver of Female Rats

Control livers were obtained from female rats (7 weeks old). Rats were ovariectomized at 6 weeks of age, and 7 d later were sacrificed. Total RNA (10 μ g) isolated from the liver was subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. Arrowheads indicate hybridizing bands corresponding to mRNA encoding regucalcin. The figure shows one of four experiments with separate rats. The densitometric data (% of value from control rats; mean $\pm$ S.E.M. of rats) were 108.2 ± 3.9 (not significant). β -Actin mRNA levels in the same samples were not altered significantly (data not shown).

Fig. 3. Effect of 17 β -Estradiol on Regucalcin mRNA Levels in the Liver of Ovariectomized Rats

Rats were ovariectomized at 6 weeks of age and were used 7 d later. 17 β -estradiol (0.2 mg/100 g body weight) was subcutaneously administered, and 3, 6 or 24 h later the animals were sacrificed. Total RNA (10 μ g) isolated from the liver was subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. Arrowhead (1.8 kb) indicates hybridizing bands corresponding to mRNA encoding regucalcin. The figure shows one of four experiments with separate rats. The densitometric data (% of value from the zero time point; mean $\pm$ S.E.M. of four rats) were 73.1 ± 6.5 , 86.3 ± 7.9 , and 75.5 ± 6.0 , respectively. β -Actin mRNA levels in the same samples were not altered significantly (data not shown).

mRNA in the liver of rats.

The effect of sexual hormones on hepatic regucalcin mRNA expression of females are shown in Fig. 2. Rats were ovariectomized and 7 d later were sacrificed. Ovariectomy can lead to a deficiency of sexual hormones including estrogen, however, hepatic regucalcin mRNA expression was not appreciably altered by this procedure.

The effect of 17 β -estradiol on regucalcin mRNA expression in the liver of ovariectomized rats is shown in Fig. 3. Seven-week old ovariectomized rats received a single subcutaneous administration of 17 β -estradiol (0.2 mg/100 g body weight) of a comparatively higher dose, and 3, 6 or 24 h later were sacrificed. Hepatic regucalcin mRNA levels were not significantly altered by this administration.

DISCUSSION

Regucalcin mRNA expression in the liver was reduced in female rats of different ages, indicating that there is a sexual difference in the gene expression. Hepatic regucalcin mRNA expression in females was not appreciably altered by ovariectomy. Ovarian hormones may not be related to the reduction of hepatic regucalcin mRNA expression in females. The estrogen receptors may be decreased by ovariectomy. However, hepatic regucalcin mRNA expression in ovariectomized rats was not appreciably stimulated by the administration of 17β -estradiol, suggesting that the endogenous ovarian hormones play no part in the regulation of the regucalcin gene expression. Presumably, the sexual difference of hepatic regucalcin mRNA expression is related to other factors; for instance, the transcriptional factor may be important. This, however, may not be related to the transcriptional factors of ovarian hormones. Regucalcin protein concentration in the liver of female rats has been shown to be lower than that of male rats.¹¹⁾

Hepatic regucalcin mRNA expression in male and female rats decreased with increasing age. The sexual difference of the gene expression was also seen in aged rats. This suggests that senescence factors are not involved in the sexual difference of hepatic regucalcin mRNA expression. The transcriptional factors for the regucalcin gene are thought to be influenced by senescence. The physiologic role of regucalcin in aged rats is unknown and remains to be elucidated.

In conclusion, it has been demonstrated that regucalcin mRNA expression is determined by sexual difference in the liver of rats of different ages. The regucalcin gene may be a useful tool to study the gene transcriptional factors involved in the sexual difference.

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