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Insulin Is a Dominant Suppressor of Sterol 12α-Hydroxylase P450 (CYP8B) Expression in Rat Liver: Possible Role of Insulin in Circadian Rhythm of CYP8B

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Sterol 12α-hydroxylase (CYP8B) is a key enzyme for regulating the cholic acid/chenodeoxycholic acid ratio in bile acid biosynthesis. The hepatic CYP8B level was elevated in streptozotocin-induced diabetic rats, and the elevated CYP8B was suppressed by insulin administration [Ishida, H. *et al.* (1999) *J. Biochem.* 126, 19–25]. The streptozotocin-induced elevation of hepatic CYP8B mRNA concomitantly responded to the decrement of the serum insulin level. The CYP8B mRNA level in the cultivated rat hepatoma H4TG cells was strongly suppressed by insulin, although it was affected by dibutyryl cAMP or thyroxine to lesser extents. These observations demonstrate that *CYP8B* expression is dominantly regulated by the direct action of insulin on hepatocytes. A marked circadian rhythm (maximum at 13:00–16:00 and minimum at 1:00) was observed both on the mRNA level and the activity of CYP8B. This rhythm was shifted from that of cholesterol 7α-hydroxylase, a rate-limiting enzyme of bile acid biosynthesis, showing a maximum at 22:00 and a minimum at 10:00, and this shift might oscillate the cholic acid/chenodeoxycholic acid ratio, which is increased in the late afternoon and decreased at midnight. The rhythm of CYP8B was the inverse of the circadian variation of serum insulin level and was similar to the circadian rhythm of glucose 6-phosphatase. These facts and the potent suppressive effect of insulin on *CYP8B* indicate that the oscillation of the serum insulin may be a factor in producing the circadian rhythm of CYP8B.

Key words: bile acid, cytochrome P450, FXR, insulin, sterol 12α-hydroxylase.

Sterol 12α-hydroxylase (CYP8B) is a liver-specific enzyme catalyzing the monooxygenation at the 12α-position of 7α-hydroxy-4-cholesten-3-one (HCO) in its conversion to cholic acid (CA), a major constituent of the bile acids of most mammals (*1*). This enzyme is considered to be functional in the regulation of the CA/chenodeoxycholic acid (CDCA) ratio in the bile (*2*). A higher CA/CDCA ratio is suggested to be correlated with more efficient absorption of dietary lipids in the intestine (*3–5*). The CA/CDCA ratio also seems to be correlated with gallstone formation, because less gallstone formation was observed when the ratio was low (*6*). Moreover, elevation of the endogenous CDCA level through the suppression of the 12α-hydroxylase activity by competitive inhibitors has been proposed as a therapeutic treatment for dissolving gallstone (*7*). It is also known that diabetes mellitus has been often indicated to be associated with a higher prevalence of gallstones in connection with alteration of CA/CDCA ratio (*8*).

The 12α-hydroxylase activity in rat liver is increased several-fold by cholestyramine feeding and starvation (*9*), and CYP8B mRNA level in rat liver is affected by *in vivo* treatment with such modulators as cholestyramine, starvation, streptozotocin, dexamethasone, and thyroxine (*10*). Among these treatments, elevation of both mRNA level and activity of CYP8B in chronic diabetic rats induced by streptozotocin was outstanding (*9, 10*), and a preliminary result indicated that the enhanced mRNA expression was significantly decreased by insulin administration (*10*). These observations suggested an essential role of insulin in the regulation of *CYP8B*. In this study, we focused on the role of insulin in the regulation of CYP8B expression and found that insulin acts as a potent and direct suppressor of *CYP8B*. We also examined the circadian rhythm of CYP8B and found that this rhythm might evoke marked circadian oscillation of the CDCA/CA ratio. This rhythm may be regulated by the oscillation of serum insulin level.

MATERIALS AND METHODS

Materials—Alpha-[^{32}P]dCTP (111 TBq/mmol) was obtained from Du Pont–New England Nuclear. The Oligolabelling kit was a product of Pharmacia Biotechnology (Uppsala).

Production of Diabetic Rats, Preparation of Liver Microsomes, and Isolation of Liver Poly(A)$^+$ RNA—Two-month-

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Abbreviations: HCO, 7α-hydroxy-4-cholesten-3-one; G6Pase, glucose 6-phosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; bp, base pairs; kbp, kilobase pairs; RT-PCR, reverse transcription-polymerase chain reaction; CA, cholic acid; CDCA, chenode-oxycholic acid.

old male Wistar rats were kept under natural lighting conditions (6:00–18:00) and fed *ad libitum* normal laboratory rat chow and tap water. To produce diabetes mellitus, rats were once intraperitoneally injected with streptozotocin (65 mg/kg body weight) dissolved in 0.1 M sodium citrate buffer, pH 4.5. Onset of diabetes was monitored by urinary glucose using a test paper (Pretest 3a, Wako Pure Chemical, Osaka), and it was usually evident 1 week after the streptozotocin treatment. All animals were sacrificed within 30 min at 16:00 to avoid experimental deviation due to the time of sample preparation, unless otherwise stated. Liver of individual rats was removed quickly and separated into two portions to prepare microsomes and poly(A)$^+$ RNA. Liver microsomes were prepared as described previously (*11*) and homogenized in 50 mM potassium phosphate buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, and 1 μg/ml leupeptin. Total liver RNA was extracted by the guanidine HCl method (*12*) and subjected to oligo(dT)-cellulose column chromatography to enrich poly(A)$^+$ RNA (*13*).

Cell Cultures—H4TG cells, derived from a rat hepatoma cell line (*14*), were seeded at a density of 5×10^5 cells/100-mm dish (Corning Coastar, Tokyo) and grown in Minimum Essential alpha Medium (Sanko Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (Dainippon Pharmaceutical, Osaka), 32 U/ml of penicillin, 60 μg/ml of kanamycin (Meiji Seika, Tokyo), and 250 ng/ml of amphotericin B (Dainippon Pharmaceutical) at 37°C under 5% CO_2 in air. When cultures became confluent, they were treated with bovine insulin (Sigma-Aldorich, Tokyo, 1×10^{-6} M), $(Bu)_2$cAMP (1×10^{-6} M), dexamethasone (1×10^{-6} M), or thyroxine (1×10^{-8} M) in a serum-free medium. Total RNA was extracted by the guanidine thiocyanate/cesium trifluoroacetate method (*15*) from the cells.

Preparation of cDNA Probes—Rat cDNA clones used as hybridization probes for CYP8B (*10*) and CYP7A (*16*) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (*10*) were prepared as described previously. Rat cDNAs for G6Pase and β-actin were generated by the reverse transcription-polymerase chain reaction (RT-PCR) from mRNA of rat liver by using a suitable pair of primers: (5′-GAACGCCTTCTATGTCCTCT-3′) and (5′-AAGGGTGTAGTGTCAAGGTG-3′) for G6Pase, and (5′-TTCAACACCCCAGCCATGTA-3′) and (5′-ATCTCCTTCTGCATCCTGTC-3′) for β-actin. The primers were synthesized based on the sequences of rat G6Pase (EMBL/Gen Bank/DDBJ No. D78592) and rat β-actin (EMBL/Gen Bank/DDBJ No. J00691). The PCR products were subcloned into the pGEM T-Easy vector (Promega, Madison, WI) and subjected to sequencing for their identification.

Northern Blot Analysis—Poly(A)$^+$ RNA preparations (1–2 μg/lane) were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde as described by Thomas (*17*) and transferred to Nytran membranes (Schleicher & Schuell GmbH, Dassel). The membranes were hybridized with the ^{32}P-labeled cDNA probes in the hybridization solution containing 6 × SSC, 5 × Denhardt's, 10 mM EDTA, 1% SDS, and 0.5 mg/ml sonicated salmon sperm DNA at 68°C for overnight. The membranes were washed with 0.1 × SSC containing 0.5% SDS at 50°C and exposed to Kodak BMX films at –80°C. The radioactivities of the hybridized areas were quantified using Bio-imaging Analyzer System BAS2000 (Fuji Photo Film, Tokyo).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)—The first strand cDNA was synthesized from 100 ng of poly(A)$^+$ RNA of livers or from 1 μg of total RNA of H4TG hepatoma cells using the Superscript preamplification system (Life Technologies, Rockville, MD) and oligo dT primer. PCR was carried out with denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and primer extension at 68°C for 3 min in a total of 18 cycles using the following pairs of primers: P3 (5′-ATGATGCTCTGGGCCTCCCA-3′) and P10 (5′-TGAGATGTCTAGGAGGCAAG-3′) for CYP8B; 5′-GGCATCTCAAGCAAACACCA-3′ and 5′-GTCAAAGGGTCTGGGTAGAT-3′ for CYP7A; and the pairs of primers for G6Pase and β-actin described above to generate 790-, 346-, 654-, and 572-bp products, respectively. The PCR products

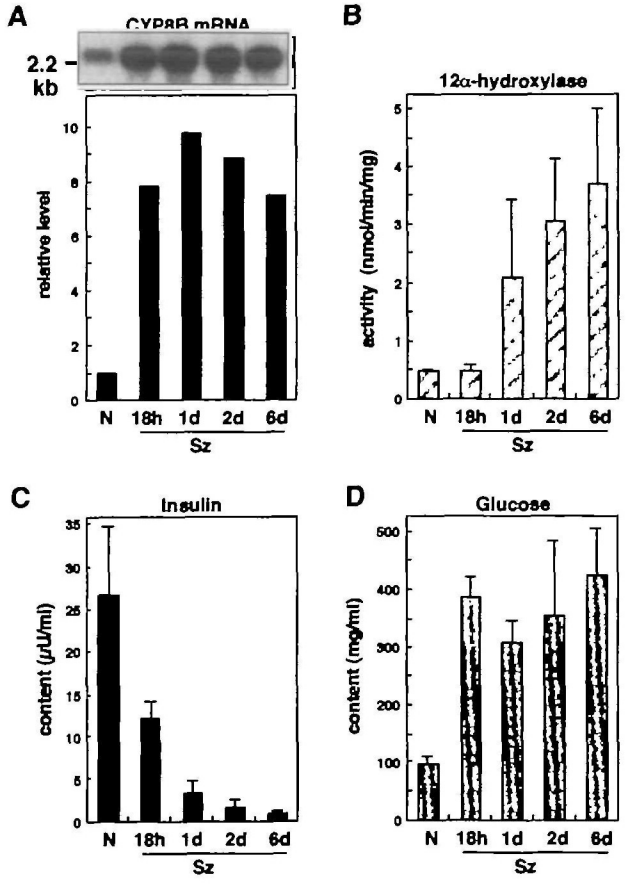

Fig. 1. **Time course of the increment of CYP8B expression by streptozotocin.** Streptozotocin (65 mg/kg body weight) dissolved in 0.1 M sodium citrate buffer pH 4.5 was administered subcutaneously to the rats. Rats were sacrificed at indicated time points after the administration of streptozotocin. Poly(A)$^+$ RNA and microsomes were prepared from the livers as described in "MATERIALS AND METHODS." Determination of CYP8B mRNA level (A) by Northern blotting and microsomal HCO 12α-hydroxylase activity assay (B) were performed as described in "MATERIALS AND METHODS." Insulin (C) and glucose (D) contents of the serum were determined as described in "MATERIALS AND METHODS." For Northern blotting, three to six poly(A)$^+$ RNA samples were mixed for each group and loaded to an agarose gel. Methylene blue staining of the blotted membrane confirmed the equal loading of RNA sample on each lane (not shown). Results were shown both in the exposed film image (upper panel) and corresponding quantified values (lower panel). All other values were mean of three to six rats for each group. N, normal; Sz, streptozotocin; h, hour; d, day.

were electrophoresed and subjected to Southern hybridization with respective cDNA probes to detect and quantify the specific products using a Bio-imaging Analyzer System BAS2000. Preliminary experiments confirmed that the RT-PCR under these conditions allowed semi-quantitative estimation of the expression levels of mRNA.

Other Assays—Microsomal 7α-hydroxy-4-ene-3-one (HCO) 12α-hydroxylase and cholesterol 7α-hydroxylase activities were assayed as described previously (*18*, *19*).

Concentrations of insulin and glucose in serum were assayed by using Glazyme insulin-EIA test kit and Glucose CII-test kit (Wako Pure Chemical, Osaka), respectively.

RESULTS

Effects of Insulin on the In Vivo and In Vitro Expression of CYP8B—Chronic diabetic rats induced by 6-week treatment with streptozotocin showed elevated expression of

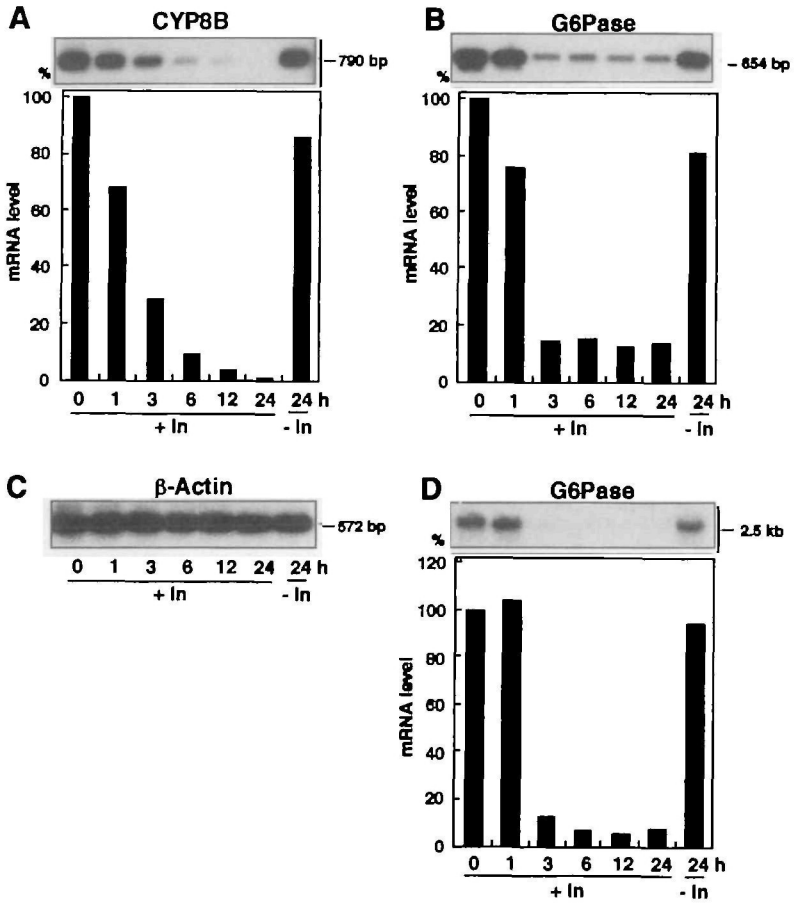

Fig. 2. **Effects of insulin on the expression of CYP8B and glucose 6-phosphatase mRNAs in the cultured rat hepatoma H4TG cells.** Insulin (1×10^{-6} M) was added to the culture medium when the cells became confluent. RNA was extracted at the indicated times after the addition of insulin. RNA preparations were subjected to RT-PCR as described in "MATERIALS AND METHODS." PCR was repeated for 18 cycles. The PCR products of CYP8B (A), G6Pase (B), and β-actin (C) were electrophoresed, hybridized with respective ^{32}P-labeled cDNA probes, visualized by exposure to Kodak BMX film (upper panels), and quantified with Bio-imaging Analyzer System BAS2000 (lower panels). Northern blotting was carried out for glucose 6-phosphatase (D) for the same RNA preparations as used in RT-PCR. Sizes of the PCR products and mRNA are shown in the right side of respective panels. In, insulin.

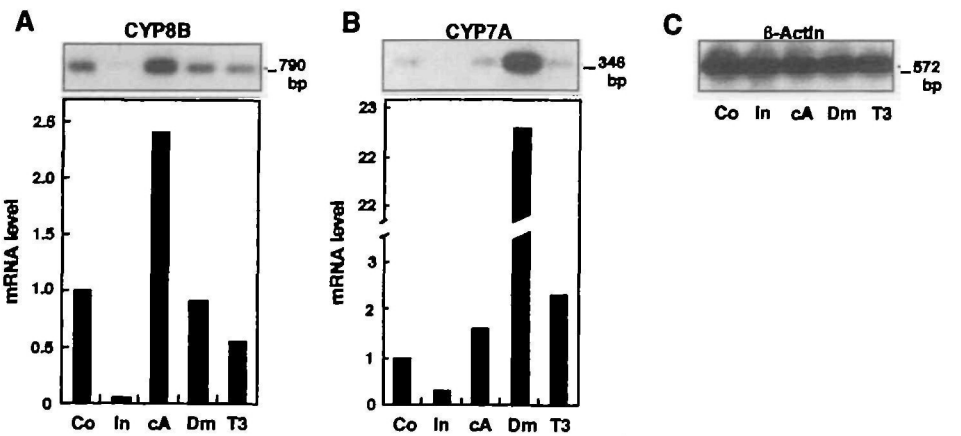

Fig. 3. **Effects of various factors on the expression of CYP8B (A), CYP7A (B), and β-actin (C) mRNA in H4TG rat hepatoma cells.** When cells became confluent, cultures were treated with insulin (1×10^{-6} M), $(Bu)_2$cAMP (1×10^{-6} M), dexamethasone (1×10^{-6} M), or thyroxine (1×10^{-6} M) in a serum-free medium. RNA was extracted 24 h after the addition of the factors. The RNA preparations were subjected to RT-PCR as described in "MATERIALS AND METHODS." Other experimental conditions were the same as those described in the legend to Fig. 2. Co, control; In, insulin; cA, $(Bu)_2$cAMP; Dm, dexamethasone; T3, thyroxine.

CYP8B mRNA and HCO 12α-hydroxylase activity, and the elevated mRNA level was decreased by insulin administration (*9*, *10*). To elucidate whether the elevation of *CYP8B* expression in the streptozotocin-induced diabetic rats was simply due to the removal of insulin, correlation between the serum insulin level and hepatic CYP8B levels observed soon after the administration of streptozotocin was analyzed. It is known that serum insulin level is transiently increased about 12 h after streptozotocin treatment by the release of pooled insulin due to the degeneration of pancreatic β-cells (*20*), and then reduced rapidly within the next 12 h. Actually, serum insulin level was significantly decreased at 18 h after the streptozotocin administration and reached nearly minimum in 1 day after the treatment (Fig. 1C). As shown in Fig. 1A, hepatic CYP8B mRNA level quickly responded to the disappearance of serum insulin by streptozotocin, and this response of *CYP8B* expression to insulin was as rapid as that of the elevation of blood glucose (Fig. 1D). These observations indicate that high CYP8B expression observed in the streptozotocin-induced diabetic rats is due to the removal of insulin suppression, and *CYP8B* gene quickly responds to serum insulin level in the removal of suppression. However, induction of hepatic HCO 12α-hydroxylase activity was slower than that of the mRNA level (Fig. 1B).

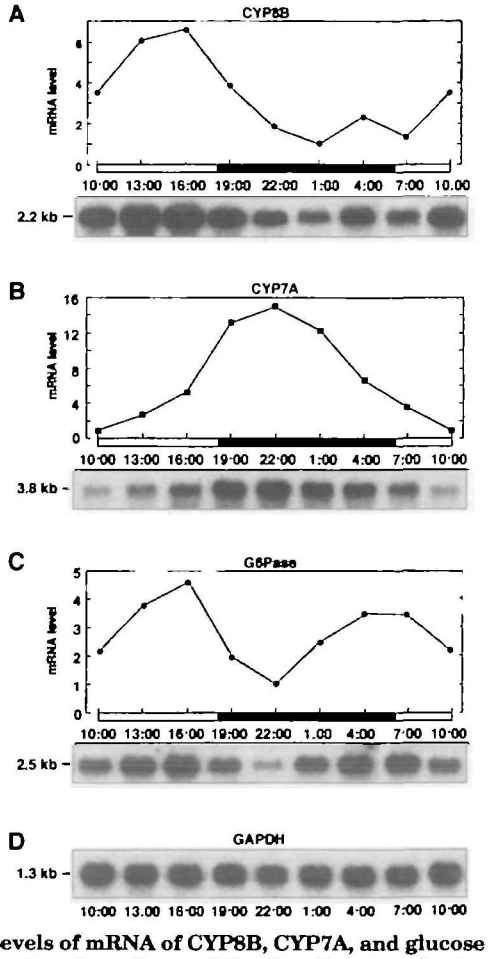

Fig. 4. **Levels of mRNA of CYP8B, CYP7A, and glucose 6-phosphatase at various times of the day.** Rats were kept under the standard conditions and sacrificed at the indicated clock times. RNA preparations obtained from the livers of three rats for each time point were mixed and poly(A)$^+$ RNA samples were prepared as described in "MATERIALS AND METHODS." mRNAs for CYP8A (A), CYP7A (B), and glucose 6-phosphatase (C) were determined by Northern blot analysis as described in "MATERIALS AND METHODS." Methylene blue staining of the blotted membrane confirmed the equal loading of RNA sample on each lane (not shown). Results were shown both in the exposed film images (lower panels) and corresponding quantified values (upper panels). The quantified values are expressed relative to the lowest one of each group. The same poly(A)$^+$ RNA preparations were also hybridized to cDNA probe for glyceraldehyde 3-phosphate dehydrogenase (D). Sizes of the hybridized mRNA are shown on the left side of respective panels.

To confirm the direct effect of insulin to *CYP8B*, effects of insulin on the CYP8B mRNA level of H4TG rat hepatoma cells were examined *in vitro* by RT-PCR method. As shown in Fig. 2A, addition of insulin to the culture medium rapidly decreased the CYP8B mRNA to an undetectable level in 24 h, whereas that of the control cells (minus insulin) did not change significantly within 24 h. This change in the level of CYP8B mRNA paralleled that of G6Pase mRNA (Fig. 2B), which is directly regulated by the insulin-signaling pathway at a transcriptional level (*21–23*). The change in the G6Pase mRNA level estimated by RT-PCR essentially paralleled that determined by Northern blotting (Fig. 2D), and no significant change was observed on the β-actin mRNA level determined by the RT-PCR (Fig. 2C). These facts strongly support the semi-quantitative accuracy of the mRNA determination by RT-PCR under the conditions employed in this study. It can thus be concluded that insulin acts as a direct suppressor of the expression of *CYP8B*, probably by a similar mechanism to that of suppression of G6Pase.

By using this *in vitro* system, effects of $(Bu)_2$cAMP, dexamethasone, and thyroid hormone on *CYP8B* expression were examined (Fig. 3A). In contrast to the marked suppression by insulin, cAMP, the second messenger of glucagon antagonizing insulin action in hepatocytes, induced the CYP8B mRNA level by 2.5 times. Dexamethasone did not affect the CYP8B mRNA *in vitro*. This is probably not attributable to the insensitivity of H4TG cells to the hormone, since CYP7A mRNA was markedly increased in the same cells (Fig. 3B). This finding suggested that the repressive effect of dexamethasone on *CYP8B* in the *in vivo* experiment (*10*) is an indirect effect of this hormone. Thyroid hormone slightly decreased the level of CYP8B mRNA,

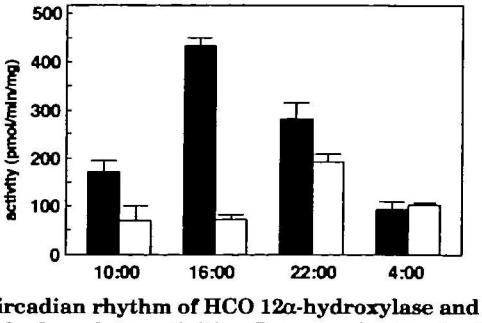

Fig. 5. **Circadian rhythm of HCO 12α-hydroxylase and cholesterol 7α-hydroxylase activities.** Rats were kept under the standard conditions and sacrificed at the indicated clock times. HCO 12α-hydroxylase (closed bar) and cholesterol 7α-hydroxylase (open bar) activities were assayed using HCO and endogenous cholesterol as the substrates, respectively (*18*, *19*). All values were the mean of three rats for each time point.

which was also observed in the *in vivo* experiment reported previously (*10*). The level of CYP7A mRNA was significantly decreased by insulin, whereas it was slightly increased by cAMP and thyroid hormone (Fig. 3B), as reported previously (*24*). The level of β-actin mRNA in H4TG cells was not significantly affected by these treatments (Fig. 3C).

Circadian Rhythm of CYP8B and Serum Insulin Level— A marked circadian rhythm was observed both in the mRNA level and the activity of CYP8B as well as those of CYP7A (Figs. 4 and 5). As shown in Fig. 4A, the level of CYP8B mRNA was maximum at 13:00–16:00 and minimum at 1:00, and a small but significant peak was observed at 4:00, which was reproducible in another experiment (see Fig. 6A). This rhythm was similar to that of G6Pase mRNA (Fig. 4C), although the peak at 4:00 was small in CYP8B. In contrast, the level of CYP7A mRNA reached a maximum at 22:00 and a minimum at 10:00 (Fig. 4B), in agreement with the previous observation (*16*). No significant variation was observed in the level of glyceraldehyde 3-phosphate dehydrogenase mRNA (Fig. 4D). The circadian rhythms of the catalytic activities of CYP8B and CYP7A (Fig. 5) qualitatively paralleled those of the mRNA levels (Fig. 4). The amplitude of HCO 12α-hydroxylase activity (maximum/minimum ratio, *ca.* 5, Fig. 5) was not so different from that of the mRNA level (*ca.* 7, Fig. 4A), whereas that of cholesterol 7α-hydroxylase activity (*ca.* 3, Fig. 5) was one-fifth of the mRNA level (*ca.* 15, Fig. 4B). It is noteworthy that the amplitude of the 12α-hydroxylase activity (*ca.* 5) was larger than that of the 7α-hydroxylsae activity (*ca.* 3), and the former activity was always higher than the latter activity (Fig. 5). This fact suggested that the rhythm of the 12α-hydroxylase activity does not limit the formation of the total amount of bile acids but modifies the ratio of cholic acid to chenodeoxycholic acid formed in liver.

As described in the preceding section, CYP8B was sensitive to the serum insulin level, and serum insulin in rats has been reported to exhibit a circadian rhythm showing the maximum in the night and the minimum in the daytime (*25, 26*), although phase is shifted by such factors as feeding time (*25, 27*) and seasons (*28*). Both CYP8B mRNA and serum insulin levels were determined at 6-h intervals by using hepatic mRNA and serum prepared from the same rats. In this experiment, CYP8B mRNA level was maximum at 16:00, coincident with the result in Fig. 4A, while the minimum level was observed at 10:00 (Fig. 6A). In contrast, serum insulin exhibited a circadian variation with a minimum at 16:00 and a maximum at 10:00 (Fig. 6B), which was qualitatively the inverse of that of CYP8B. This fact suggests a possible contribution of the serum insulin level to the circadian rhythm of CYP8B.

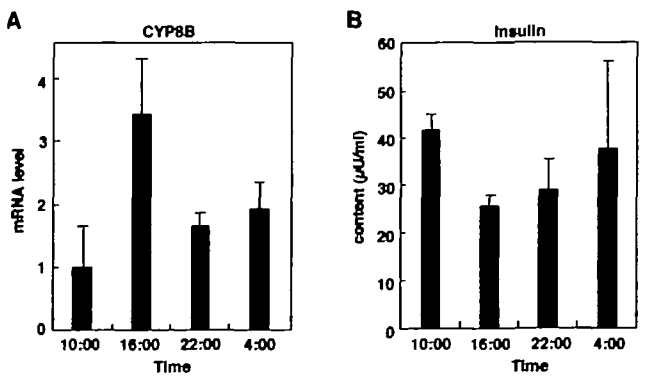

Fig. 6. **Liver CYP8B mRNA and serum insulin levels of rats at various times of the day.** Rats were kept under the standard conditions and sacrificed at the indicated clock times. CYP8B mRNA and serum insulin were assayed as described in Fig. 1. All values were the mean of three to six rats for each time point.

DISCUSSION

This study revealed that hepatic CYP8B level was increased as rapidly as blood glucose in response to the depletion of serum insulin caused by the disruption of pancreatic β-cells by streptozotocin (Fig. 1). Furthermore, the suppressive effect of insulin on CYP8B expression was also observed *in vitro* in cultured hepatoma H4TG cells (Fig. 2). These results clearly demonstrate that the expression of hepatic *CYP8B* is directly suppressed by serum insulin. Suppression of the CYP8B mRNA level by insulin *in vitro* was observed in parallel with that of the G6Pase mRNA (Fig. 2). The G6Pase gene is a typical example of the genes down-regulated by insulin (*22, 29*). The consensus element responsible for insulin, insulin response sequence (IRS), which has commonly been identified in the 5′-upstream region of the insulin-suppressive genes, such as insulin-like growth factor binding protein-1 (*30*), and phosphoenolpyruvate carboxykinase (*31*), has also been identified in the promoter region of G6Pase gene (*22, 23, 32*). By examining published sequences of human and mouse *CYP8B* genes (GenBank/EMBL/DDBJ; AF090319 and AF090320 for mouse and human, respectively) (*33*), we found consensus or similar sequences to the core motif of IRS, T(A/G)-TTTTG, at around 130 and 530 bp upstream from the transcriptional initiation sites of human and mouse *CYP8B* genes, respectively. Mouse *Cyp8b* gene has two copies of the motif arranged as an inverted palindrome. These facts suggest that the expressions of CYP8B and G6Pase genes are regulated by the insulin-signaling pathway (*34*) with closely similar mechanisms. These considerations strongly support the above conclusion that insulin is the potent regulatory factor of *CYP8B* expression.

Insulin is known to be an important regulatory factor of bile acid formation. In diabetic patients and experimental diabetic animals, the bile acid pool and its secretion are increased (*35*), and these parameters return to normal levels following insulin administration (*36–38*). CYP7A mRNA level is known to be decreased by insulin in primary rat hepatocytes (*39–41*). The same effect of insulin was also observed on the cultured rat hepatoma cells (Fig. 3B), and CYP7A expression was increased in the diabetic rats (*10*). These observations suggest that insulin reduces the overall bile acid formation through the suppression of *CYP7A*. Thus, the correlation between CYP8B and CYP7A in the insulin-dependent regulation of bile acids formation is worth considering, because the CA/CDCA ratio in bile is dependent on the CYP8B activity. As described in the previous paper, the induction of CYP8B by insulin depletion in diabetes was much higher than that of CYP7A (*10*), and the CA/CDCA ratio in bile is reported to be increased in diabetic animals (*3, 37*). Increment of total amount of bile acids and elevation of CA/CDCA ratio in diabetic animals enhance lipid absorption in the intestine, since cholic acid

absorbs dietary lipids much more efficiently than do the other bile acids (*3–5*). Enhancement of intestinal lipid absorption in diabetes by a higher amount of cholic acid in the bile (*3*, *35*) is physiologically significant, because cells starved of glucose in diabetic animals begin to use lipids to supplement their energy source. On the other hand, such enhanced lipid absorption may cause hyperlipidemia in diabetes mellitus (*3*). A higher prevalence of gallstones (*8*) in diabetes mellitus can be explained by the elevation of the 12α-hydroxylase activity, and the elevation of *CYP8B* expression in starved animals (*9*, *42*) may be due to the reduction of plasma insulin by starvation (*43–45*).

CYP8B mRNA of the cultured rat hepatoma cells was significantly induced by the cAMP analogue (Fig. 3A). The consensus sequences of cAMP responsive element (CRE) were found in the human and mouse *CYP8B* genes (*33*) at 1,054 bp (human) and 1,708 bp (mouse) upstream from the transcriptional initiation sites, respectively. These facts suggest a possible role of cAMP as a positive regulator in the expression of *CYP8B*. This is an interesting subject for study in relation to the competitive effect of glucagon on insulin suppression of CYP8B.

Expression of *CYP8B* in rat liver was also affected by dexamethasone (*10*). However, the role of glucocorticoid in *CYP8B* regulation must be indirect, because this hormone did not affect the expression level of CYP8B mRNA *in vitro* (Fig. 3A). Since the plasma concentrations of glucocorticoid and insulin are correlated with each other (*27*, *46*), the effect of dexamethasone on *CYP8B* expression *in vivo* might appear through the influence of this hormone on plasma insulin concentration. The weak suppressive effect of thyroxine on *CYP8B* expression was observed both *in vivo* (*10*). Since thyroxine suppressed CYP8B in H4TG cells *in vitro* (Fig. 3A), this hormone may directly affect *CYP8B* expression in hepatocytes to a low extent, as described by Andersson *et al.* (*47*).

CYP8B showed a marked circadian rhythm, which was the inverse of that of CYP7A (Figs. 4–6), and this fact is worth discussing in relation to the regulation of bile acids production through CYP7A, the key enzyme of bile acid synthesis. The circadian rhythm of rat CYP7A has been reported to exhibit a maximum at night and a minimum in the daytime (*16*, *48–51*). Two independent regulatory mechanisms are considered for the circadian rhythm of *CYP7A*. One is the well-known regulation through DBP, which is a basic leucine zipper protein acting as a liver-enriched transcriptional activator (*49*, *50*). This regulation is inferred to be an essential factor producing the synchronized circadian rhythm of *CYP7A* and HMG-CoA reductase genes (*52*). Another is the recently demonstrated suppressive effect of bile acids on the *CYP7A* expression through a nuclear receptor FXR/BAR (farnesoid X receptor/bile acid–binding receptor) (*53*), which interacts with CDCA as the most efficient ligand (*53–55*). The circadian rhythm of CYP8B possibly influences the latter regulatory mechanism, since CDCA production is dependent on the activity of CYP8B. As shown in Fig. 5, the 12α-hydroxylase activity was always higher than the 7α-hydroxylase activity in spite of their inverted circadian rhythms. This fact indicates that the rhythm of CYP8B oscillates the CA/CDCA ratio, which is high in the late afternoon and low at the end of night. This estimation is coincident with the observation by Vonk *et al.* (*56*) that CA/CDCA ratio in the bile of rats was *ca.* 1 at 4:00–5:00 and increased to 2.5 at 16:00–17:00. The reduced hepatic CDCA level in the late afternoon may trigger the removal of the suppression of CYP7A gene by the active FXR/BAR-CDCA complex to elevate the CYP7A level at night. This hypothesis concerning the significance of CYP8B in the regulation of bile acid formation *via* FXR/BAR is an interesting problem to be examined.

There arises the problem of the mechanism producing the circadian rhythm of CYP8B. As shown in Fig. 6, the circadian rhythms of CYP8B and serum insulin were apparently inverted. Since the suppressive effect of serum insulin on CYP8B was evident, this finding seemed to suggest a possible contribution of the circadian variation of serum insulin level to producing the circadian rhythm of CYP8B. Another candidate for producing the circadian rhythm of CYP8B is hepatic cAMP level, since CYP8B mRNA of the cultured rat hepatoma cells was induced by the cAMP analogue (Fig. 3A). However, plasma glucagon and hepatic cAMP levels of murines were reported to exhibit circadian rhythms with a maximum at the end of the dark period (4:00–6:00) (*25*, *44*, *57*), which might coincide with the small elevation of CYP8B mRNA level at 4:00 (Figs. 4A and 6A). Consequently, it is obvious that cAMP does not contribute to the appearance of the marked peak of *CYP8B* expression observed in the daytime (Figs. 4A, 5, and 6A). The expression of G6Pase gene is enhanced by the glucagon-cAMP pathway (*58*, *59*) and repressed by insulin (*22*, *29*) to regulate blood glucose. As shown in Fig. 4C, G6Pase mRNA exhibits two peaks of almost equal amplitude: one at 4:00–7:00, which may be due to the inductive effect of the glucagon-cAMP pathway, and one at 16:00, which may be caused by the reduction of insulin repression owing to the minimum plasma insulin level at this time point (Fig. 6B). This fact indicates that the inductive effect of the glucagon-cAMP pathway can overwhelm the repressive effect of insulin in the regulation of G6Pase gene expression. In contrast, the elevation of CYP8B mRNA at 4:00 was far smaller than that at 16:00 (Fig. 4A). Together, these observations suggest that the most probable driving force of the circadian rhythm of CYP8B is oscillation of serum insulin level, and the repressive effect of insulin on *CYP8B* gene seems to overwhelm the inductive effect of glucagon-cAMP pathway on this gene. The glucagon–cyclic AMP pathway and the insulin-signaling pathway antagonize each other in hepatic glucose metabolism, and similar antagonism was observed in the expression of CYP8B. This is an interesting and important problem for considering the regulatory mechanism of *CYP8B* expression, because both IRS and CRE were found in the promoter region of mouse and human *CYP8B* genes. However, questions remain, such as the critical levels of serum insulin and glucagon for regulating *CYP8B* expression. We are examining such problems together with the confirmation of the role of IRS and CRE in the regulation of *CYP8B* expression.

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