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Suppressed Expression of the Urea Cycle Enzyme Genes in the Liver of Carnitine-Deficient Juvenile Visceral Steatosis (JVS) Mice in Infancy and during Starvation in Adulthood[1]

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Systemic carnitine-deficient juvenile visceral steatosis (JVS) mice exhibit decreased expression of some liver-selective genes including those for the urea cycle enzymes during the infantile period. At 25 days, carbamoylphosphate synthetase (CPS) mRNA level was remarkably low in the liver of JVS mice, and the HNF-4 and C/EBP-α mRNA contents were also reduced. HNF-3α and C/EBP-β mRNAs were slightly higher in the liver of JVS mice, and HNF-1 mRNA remained normal. These results, together with the developmental changes of these transcription factor mRNA levels, suggest that HNF-4 and C/EBP-α are involved in the suppression of CPS expression. If JVS mice survived the crisis at 4-5 weeks, their body weight caught up with that of control mice around 7 weeks. The steady-state levels of CPS and argininosuccinate synthetase (ASS) mRNAs in the liver of JVS mice were normalized by no later than 8 weeks. Starvation for 48 h caused an increase of about twofold in CPS and ASS mRNA levels in the liver of control mice, while the same treatment failed to increase their levels in the liver of JVS mice. The starvation similarly caused increases in HNF-4 and C/EBP-β mRNA levels in the liver of both control and JVS mice, but the increases were significantly less in JVS mice than in control mice. Thus, the lack of induction of CPS and ASS mRNAs during development and under starvation in JVS mice correlated with the lower induction of HNF-4 and C/EBP-α mRNAs, and of HNF-4 and C/EBP-β mRNAs, respectively. Furthermore, all these changes seemed to correlate with the presence of fatty liver and the high serum free fatty acid levels, suggesting that disturbance of fatty acid metabolism affects nitrogen metabolism at least in part *via* altered gene expression of transcription factors such as HNF-4, C/EBP-α, and C/EBP-β.

Key words: carnitine deficiency, gene expression, starvation, transcription factor, urea cycle enzymes.

Juvenile visceral steatosis (JVS) mice are autosomal recessive mutants associated with fatty liver, hyperammonemia, and hypoglycemia (*1-3*). About 70% of the mice die in 4-5 weeks. The rest survive the crisis, but die about 1 year later of heart failure associated with cardiac hypertrophy (*4*). JVS mice are carnitine-deficient (*5*), having an impaired renal carnitine reabsorption system (*6*). We found that the expression of genes for the urea cycle enzymes, including carbamoylphosphate synthetase (CPS) and argininosuccinate synthetase (ASS), are suppressed in the liver of JVS mice from 2 to 4 weeks but not in other tissues, except for ASS in the kidney (*7*). Carnitine administration to JVS mice is very effective to ameliorate the symptoms and restores the urea cycle enzyme expression (*8*), indicating that carnitine deficiency affects the gene expression of urea cycle enzymes directly or indirectly.

The five urea cycle enzymes are predominantly and coordinately expressed in the liver during development from the late fetal period to the infantile period (*9, 10*). Feeding a high protein diet and starvation induce the urea cycle enzymes (*11*). Hormones seem to be the factors concerned in these processes. Glucocorticoid and glucagon are positive regulators and insulin is a negative regulator of the urea cycle enzyme gene expression (*12, 13*). Other liver-specific or liver-selective genes, albumin, tyrosine aminotransferase, and serine dehydratase, which are regulated by glucocorticoid as well as the urea cycle enzyme genes, were also suppressed in JVS mice (*7, 14*). The signal transduction system of glucocorticoid from the serum hormone to glucocorticoid receptor in the nucleus in the liver of JVS mice was, however, stimulated (*14*). On the other hand, proto-oncogenes, c-*jun* and c-*fos*, are highly

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Abbreviations: AP-1, activator protein 1; ASS, argininosuccinate synthetase; C/EBP, CCAAT/enhancer binding protein; CPS, carbamoylphosphate synthetase; CRE, cAMP responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid responsive element; HNF, hepatocyte nuclear factor; JVS, juvenile visceral steatosis.

expressed, and the DNA-binding activity of AP-1, their protein product, is stimulated in the liver of JVS mice at 25 days, as we reported previously (*14, 15*). From these results, we reason at present that cross coupling between glucocorticoid receptor and AP-1 (*16-18*) may be a cause of the disordered gene expression in the liver of JVS mice.

In this report, we first examined developmental changes in mRNA levels of the transcription factors HNF-1 (*19*), HNF-3α (*19, 20*), HNF-4 (*21*), and C/EBP-α (*22*) and -β (*23*), which may be involved in the gene expression of the urea cycle enzymes, to understand the mechanisms by which the carnitine deficiency affects the regulation of genes for urea cycle enzymes. Second, we studied the expression of the urea-cycle enzymes of the surviving JVS mice at 8 weeks under fed and starved conditions.

MATERIALS AND METHODS

Animals—Homozygous mutant mice (JVS) designated as (jvs/jvs) were identified as having swollen fatty liver by observation through the abdominal wall at 2-5 days after birth. Control littermates, designated +/?, including normal homozygous (+/+) and heterozygous (jvs/+) mice, as well as jvs/jvs mice, were obtained from heterozygous parents. Mice were weaned around 1 month and maintained on a regular laboratory chow, CE-2 (CLEA Japan, Tokyo). Liver and blood were taken from mice under ether or pentobarbital anesthesia. Recording of body weight and blood collection were carried out in the morning from 10:00 to 11:00. In the starvation experiments, food was withdrawn at 10:00 and the starved mice were used 48 h after the withdrawal. This study was carried out according to the Guide for Animal Experimentation, Faculty of Medicine, Kagoshima University.

RNA Isolation and RNA Blot Hybridization Analysis—Total cellular RNA was isolated according to Chomczynski and Sacchi (*24*). The amounts of mRNA for CPS, aldolase B, GAPDH, HNF-1, HNF-3α, HNF-4, C/EBP-α, and C/EBP-β were quantitated by Northern blot and slot blot hybridization analyses. Hybridization to appropriate cDNA fragments labeled with a random oligonucleotide primer was carried out under high stringency conditions. The cDNA clones for CPS, ASS, aldolase B, and GAPDH were previously described (*7*). The cDNA fragments of rat HNF-1 (*25*), rat HNF-4 (*26*), mouse C/EBP-α, and mouse C/EBP-β (*27*) were isolated from each clone, and that of rat HNF-3α (*28*) was prepared from reverse transcription-polymerase chain reaction product.

Other Assays—Total lipid was extracted from liver with 20 volumes of 2:1 and 1:2 chloroform/methanol solutions (by volume) (*29*). The residue was weighed after drying. Serum non-esterified (free) fatty acid concentration was measured colorimetrically using a kit from Nippon Shoji (Tokyo). Serum insulin was determined by the double-antibody method with rat insulin as a standard (Incstar, Stillwater, MN). Serum cortisol and plasma glucagon were measured using commercial radioimmunoassay kits from Incstar (Stillwater, MN) and Daiichi Radioisotope (Tokyo), respectively. Plasma was separated from blood collected in tubes containing 500 KIU of trasylol and 1.2 mg of EDTA.

Statistical Analysis—The data are expressed as means ± SD. Statistical analysis was performed by Student's *t*-test.

RESULTS

mRNA Levels Coding for Transcription Factors in the Liver of Control and JVS Mice at 25 Days and during Development—The steady-state mRNA levels of the transcription factors involved in liver-specific gene expression were compared in control and JVS mice by Northern blot analysis at 25 days. As shown in Fig. 1, CPS mRNA was very low in JVS mice. The mRNA contents of HNF-4 and C/EBP-α in the liver of JVS mice were lower than and about half the control, while those of C/EBP-β and HNF-3α were higher in JVS mice. There was no difference in HNF-1 between the two groups of mice. Figure 2 shows the developmental changes of the transcription factor mRNAs by slot blot analysis. CPS and the transcription factor mRNAs tested increased in the control liver during development but aldolase B mRNA did not. HNF-4 and C/EBP-α mRNA contents increased more slowly in JVS mice and were significantly lower in JVS mice than in control at 25 days, while CPS mRNA content in JVS mice was already significantly lower at 15 days. C/EBP-β mRNA content in the liver of JVS mice was higher than but not significantly different from the control, and there was no difference in developmental changes in HNF-1 and aldolase B mRNAs between the two groups.

Growth of JVS Mice Surviving the Crisis at 4-5 Weeks—JVS mice show growth retardation which starts from 2 weeks and becomes remarkable at 4 weeks, when the CPS and ASS mRNA contents in the liver were very low compared with those of control mice (*8*). Although about

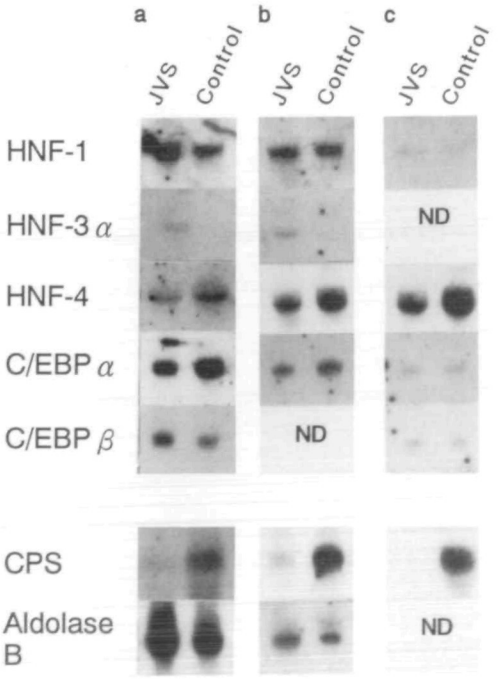


Fig. 1. **Northern blot analysis of liver-selective transcription factors in the livers of JVS and control mice at 25 days.** Total RNA (20 μg/lane) isolated from the livers of JVS (jvs/jvs) and control (+/?) mice at 25 days of age was analyzed by Northern blot hybridization with cDNA probes described under "MATERIALS AND METHODS." a, b, and c denote three representative experiments using littermates. ND, not determined.

70% of JVS mice die by 5 weeks, the rest survive the crisis. Figure 3 displays the time course of body weight from 4 weeks to 8 weeks of mice which survived. They began to gain weight rapidly from 32 days and almost reached the control level at 46 days.

Effect of Starvation for 48 h on Body Weight, Liver Weight, Hepatic Lipid Content, and Serum or Plasma Concentrations of Hormones at 8 Weeks—To examine whether JVS mice suffer from disorders other than cardiac hypertrophy at 8 weeks, we compared several parameters between fed and starved conditions (Table I). As mentioned above, there was no difference in body weight between fed control and JVS mice. Starvation for 48 h caused loss of body weight in both mice to the same extent. The liver of fed JVS mice looked less fatty at 8 weeks than at 4 weeks and was a little heavier than the control. On the other hand, the 48-h starvation caused about 50% loss of liver weight in control mice and almost no change in JVS mice. The color of the liver of JVS mice changed from reddish in fed mice to whitish in starved mice. The hepatic lipid concentration of control mice was not altered by the starvation. On the other hand, that of JVS mice was twice that of the control mice under fed conditions, and remarkably increased by the starvation. The level was comparable to that at 15-29 days (*8*). There were no significant differences in the concentrations of serum insulin, plasma glucagon, and serum cortisol

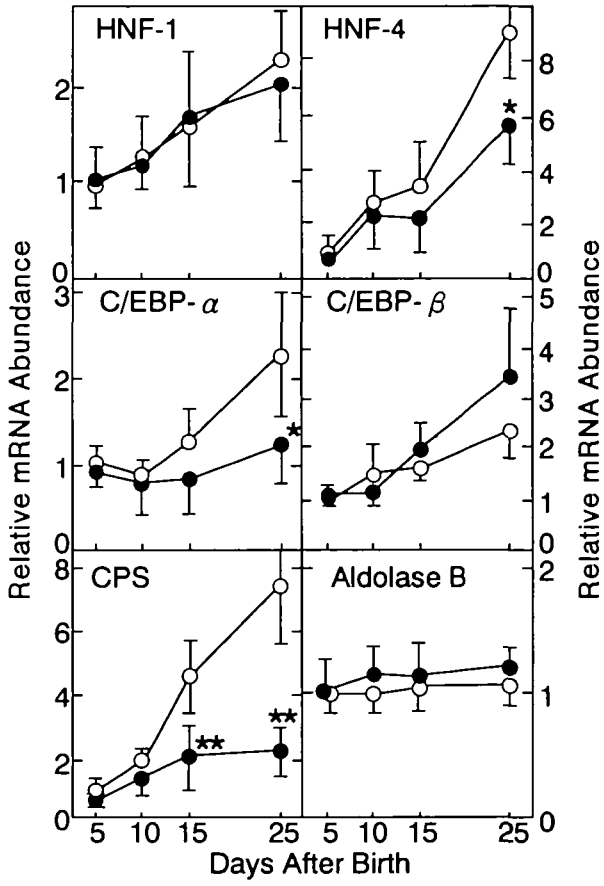


Fig. 2. **Developmental changes in the content of CPS, aldolase B, and liver-selective transcription factor mRNAs.** Total RNA was isolated from livers of control (○) and JVS (●) mice at the ages indicated. The mRNA content was quantitated by slot hybridization analysis. Values are presented as means±SD ($n=6$) in relative abundance units taking the control at 5 days as 1. $^{*}p<0.05$ and $^{**}p<0.01$, compared with control mice at corresponding age.

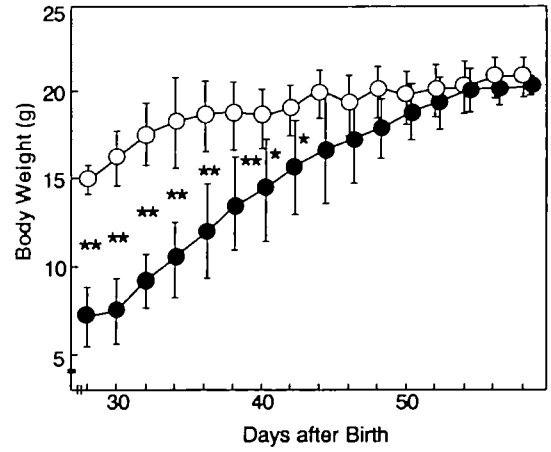


Fig. 3. **Growth curves of control and JVS mice.** Body weights of control (○) and JVS (●) mice were measured every 2 days from 28 days to 58 days after birth. Values are presented as means±SD ($n=8$). $^{*}p<0.05$, $^{**}p<0.01$.

TABLE I. **Effects of 48-h starvation on body and liver weights, hepatic lipid content and blood hormone levels in control and JVS mice at 8 weeks.**

	Control		JVS	
	Fed	Starved	Fed	Starved
Body weight (g)	21.4±1.8	16.3±1.8[a]	19.5±2.4	16.2±1.9[b]
(number of mice)	(7)	(8)	(6)	(8)
Liver weight (g)	1.31±0.17	0.69±0.09[a]	1.50±0.11[d]	1.72±0.26[e]
	(7)	(8)	(6)	(8)
Hepatic lipid concentration (mg/g)	46.1±5.2	42.1±2.9	107.9±35.3[d]	309.6±71.7[c,e]
	(5)	(5)	(5)	(5)
Insulin (ng/ml)	1.38±0.37	<0.38	1.05±0.65	<0.38
	(5)	(4)	(4)	(4)
Glucagon (pg/ml)	81.5±22.8	92.3±29.7	93.3±29.1	159.5±71.4
	(6)	(6)	(4)	(4)
Cortisol (μg/dl)	1.24±0.32	1.49±0.14	1.34±0.24	1.95±0.48[b]
	(6)	(4)	(4)	(5)

Concentrations of serum insulin and cortisol and plasma glucagon were assayed as described in "MATERIALS AND METHODS." Mice were starved for 48 h. Data are expressed as the mean±SD. $^{a}p<0.01$ compared with fed control mice; $^{b}p<0.05$ and $^{c}p<0.01$ compared with fed JVS mice; $^{d}p<0.05$ compared with fed control mice; $^{e}p<0.01$ compared with starved control mice.

under fed conditions between control and JVS mice. The starvation caused a large decrease in the insulin concentration and no significant change in the glucagon concentration in both groups of mice. The cortisol concentration was significantly increased in JVS mice but did not change in control mice.

Serum Free Fatty Acid Concentration in Control and JVS Mice—As shown in Fig. 4, serum free fatty acid concentration in JVS mice increased during development and reached three times the control level at 4 weeks. At 8 weeks, however, it was reduced to the control level under fed conditions. The 48-h starvation caused a great elevation of the free fatty acid concentration in JVS mice, almost to the level at 4 weeks, but no change in control mice.

Steady-State Levels of CPS, ASS, and GAPDH mRNAs and Effect of the 48-h Starvation on Them in Control and JVS Mice at 8 Weeks—CPS, ASS, and GAPDH mRNA contents in the liver of fed JVS mice were comparable to

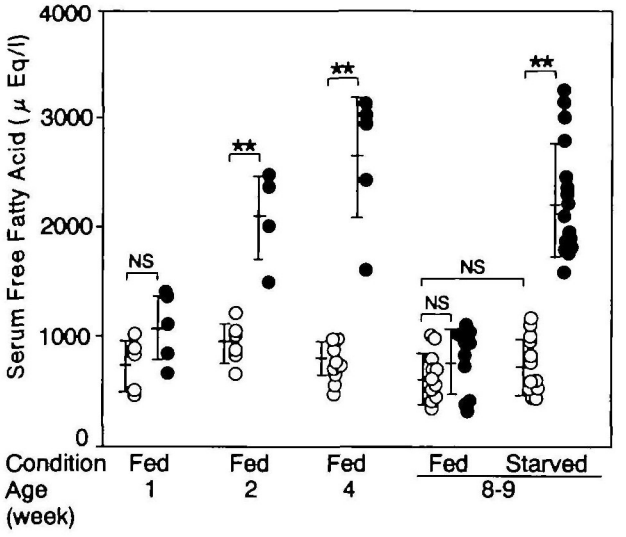
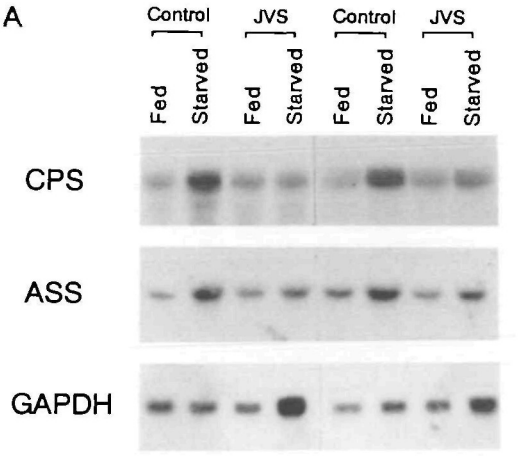


Fig. 4. **Serum free fatty acid concentrations of control and JVS mice during development and effect of starvation.** Serum was taken from control (○) and JVS (●) mice at various ages under fed or 48-h starved conditions. Serum free fatty acid concentrations were measured as described in "MATERIALS AND METHODS." $^{**}p<0.01$. NS, no significant difference.



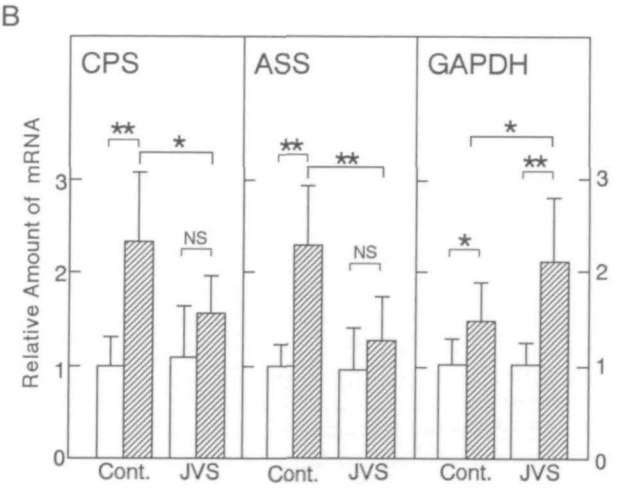


Fig. 5. **Effects of 48-h starvation on the content of CPS, ASS, and GAPDH mRNAs in the liver of control and JVS mice at 8 weeks.** A: Northern blot of total RNA from livers of control and JVS mice (two separate litters) at 8 weeks. Livers were isolated from fed or 48-h starved mice. B: The mRNA contents in fed (open boxes) and starved (hatched boxes) mice quantitated by slot blot hybridization analysis. Vertical axis indicates relative amount of mRNA calculated from abundance units taking the control under fed conditions as 1. Values are expressed as means±SD ($n=8$ or 9). $^{*}p<0.05$, $^{**}p<0.01$. NS, no significant difference.

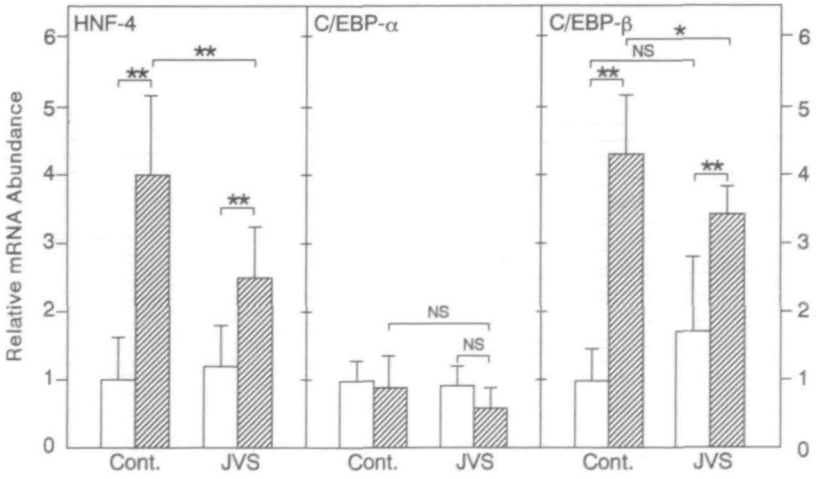


Fig. 6. **Effect of 48-h starvation on the content of HNF-4, and C/EBP-α and -β mRNAs in the liver of control and JVS mice.** The mRNA contents in fed (open boxes) and starved (hatched boxes) mice were quantitated by slot blot hybridization analysis. Values are expressed as means±SD ($n=8$ or 9) in relative abundance units taking the control under fed conditions as 1. $^{*}p<0.05$, $^{**}p<0.01$. NS, no significant difference.

those of the control mice at 8 weeks (Fig. 5). The 48-h starvation doubled CPS and ASS mRNA contents in the liver of the control mice. Those in the liver of JVS mice were not significantly changed by the treatment. On the other hand, GAPDH mRNA content was increased under the 48-h starved conditions in both mice, but more markedly in JVS mice.

Effect of the Starvation on the HNF-4, and C/EBP-α and -β mRNA Contents—The HNF-4 mRNA content under fed conditions was the same in control and JVS mice, and increased about fourfold in control mice and twofold in JVS mice after the 48-h starvation (Fig. 6). The content of starved JVS mice was significantly lower than that of the control. Very similar results were observed for the C/EBP-β mRNA contents of control and JVS mice. On the other hand, there was no significant difference in C/EBP-α mRNA content between control and JVS mice and no significant change after the starvation.

DISCUSSION

Fat is supplied from milk and constitutes the major energy source in the suckling period. Carnitine-deficient JVS mice can hardly use long-chain fatty acids as an energy source because carnitine is an essential factor for the transport of fatty acyl-CoA into the mitochondria where β-oxidation occurs. This is why severe fatty liver is observed during this period and is probably closely connected with such severe symptoms as hyperammonemia and hypoglycemia at 4 weeks. About 70% of the mice die in 5 weeks. The rest survive this crisis and grow rapidly to reach the control level of body weight around 7 weeks (Fig. 3). The CPS and ASS mRNA contents in the liver were also normalized at this age under the fed condition (Fig. 5). Starvation for 48 h caused a twofold increase in these mRNA levels in control mice, but no significant increase in JVS mice. As in the suckling period, the starvation induced lipid accumulation in the liver of JVS mice, which was correlated with the increase of serum free fatty acid concentration (Fig. 4). These findings suggest that expression of the urea cycle enzyme genes is suppressed when the energy metabolism shifts to lipid utilization.

Many factors are involved in the regulation of gene expression of the urea cycle enzymes in the liver. Glucagon and glucocorticoid induce and insulin suppresses the urea cycle enzymes (*12, 13*). Some liver-selected genes, albumin, tyrosine aminotransferase, and serine dehydratase, which are known to be stimulated by glucocorticoid, were also suppressed in the liver of JVS mice, as we reported previously (*7, 14*). The serum cortisol concentration was higher in JVS mice at 4-5 weeks and the glucocorticoid receptor was found to be accumulated in the nuclear fraction of the liver of JVS mice, suggesting that the glucocorticoid pathway is activated (*14*). We also reported that proto-oncogenes, *c-jun* and *c-fos* mRNAs, were highly expressed in the liver of JVS mice at 25-27 days (*15*) and the DNA-binding activity of AP-1, their protein product, also increased during development (*14*). The cross coupling between glucocorticoid receptor and AP-1, suggested by Yang-Yen *et al.* (*16*), Schüle *et al.* (*17*), and Schüle and Evans (*18*), appears to be one of the most likely mechanisms for the suppression of the glucocorticoid-responsive gene expression. AP-1 appears to be activated by free fatty acids or their metabolites, because serum free fatty acid concentration changed coordinately with the disordered gene expression at 4 weeks and during starvation at 8 weeks (Fig. 4). Triacylglycerol, the major accumulation in the liver of JVS mice, is not a candidate, because the amount of carnitine which caused complete normalization of CPS mRNA content in the liver was insufficient for its removal (*8*). Recently we obtained data showing the suppressive effect of long-chain unsaturated fatty acids on the induction of the urea cycle enzyme mRNAs by glucocorticoid in primary cultured hepatocytes (manuscript in preparation).

Binding sites for the liver-selected transcription factors have been sought to explain the liver-selective expression of the urea cycle enzymes: HNF-4 binding sites were found in OTC (*30, 31*) and CPS (*32*) genes; and C/EBP binding sites in OTC (*30*), CPS (*33*), and arginase (*34, 35*) genes. Nishiyori *et al.* (*27*) demonstrated that HNF-4 and C/EBP-β are important for the activation of the OTC gene. An imperfect CRE and half-sites of GRE have been found in the enhancer region of the CPS gene, and substitutional mutations in these sites strongly affected hormone-induced expression (*32*). The urea cycle enzymes, however, are induced by glucocorticoid with delayed onset which depends on *de novo* protein synthesis (*36*), suggesting that the glucocorticoid receptor does not act directly on the genes. Gotoh *et al.* (*34*) described that C/EBP binding sites are present in the delayed glucocorticoid-responsive enhancer of the arginase gene. Matsuno *et al.* (*37*) showed that C/EBP-β is induced by glucocorticoid and glucagon, and suggested that the accumulated C/EBP-β protein is involved in secondary activation of target genes such as the urea cycle enzyme genes in response to the hormones in the liver.

During development, the amounts of C/EBP-α and HNF-4 mRNAs were lower in the liver of JVS mice than in the control (Figs. 1 and 2), which may at least in part account for the decreased expression of the genes for urea cycle enzymes including CPS and ASS. On the other hand, in the starved adult JVS mice (Figs. 5 and 6), repression of C/EBP-β and HNF-4 genes is likely to be involved in impaired induction of CPS and ASS genes. The repression of genes for HNF-4 and C/EBPs appears to be caused, at least in part, by AP-1, which is activated as described above. Investigation into this hypothesis is now under way.

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