Hypoglycemia-associated Hyperammonemia Caused by Impaired Expression of Ornithine Cycle Enzyme Genes in C/EBPα Knockout Mice*

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Ammonia produced by amino acid metabolism is detoxified through conversion into urea by the ornithine cycle in the liver, whereas carbon skeletons of amino acids are converted to glucose by gluconeogenic enzymes. Promoter and enhancer sequences of several genes for ornithine cycle enzymes interact with members of the CCAAT/ enhancer-binding protein (C/EBP) transcription factor family. Disruption of the C/EBPα gene in mice causes hypoglycemia associated with the impaired expression of gluconeogenic enzymes. Here we examined the expression of ornithine cycle enzyme genes in the livers of C/EBPα-deficient mice. mRNA levels for the first, third, fourth, and fifth enzymes of five enzymes in the cycle were decreased in C/EBPα-deficient mice. Protein levels for the first, second, fourth, and fifth enzymes were also decreased. In situ hybridization analysis revealed that the enzyme mRNAs were distributed normally in the perportal region but were disordered in C/EBPα-deficient mice with relatively higher mRNA levels in the midlobular region. Blood ammonia concentrations in the mutant mice were severalfold higher than in wild-type mice. Thus, C/EBPα is crucial for ammonia detoxification by ornithine cycle enzymes and for coordination of gluconeogenesis and urea synthesis.

Urea synthesis and gluconeogenesis, representative traits of the liver, are closely related to each other with regard to amino acid metabolism. Deamination of the α-amino group of amino acids produces ammonia, a toxic metabolite that is detoxified by conversion into urea via the ornithine cycle in the liver. On the other hand, carbon skeletons derived from deaminated amino acids are primarily converted into glucose by gluconeogenic enzymes. Besides this relationship, the ornithine cycle and the gluconeogenic pathway are correlated by sharing common steps in two enzymatic reactions (1). Reflecting these close relationships, genes for both pathways are regulated in a coordinate manner. Urea synthesis and gluconeogenesis are induced in the perinatal period and postnatally are up- or down-regulated by dietary changes, generally in the same direction (reviewed in Refs. 2–5). These responses are mediated mainly by hormones. Glucagon and glucocorticoids stimulate, whereas insulin represses, the expression of genes for both pathways (2–5).

Studies of transcriptional regulation of genes for five enzymes of the ornithine cycle revealed the architecture of regulatory regions such as the promoter and enhancer (reviewed in Ref. 3). In the promoter region of the first enzyme, carbamylphosphate synthetase (CPS)1 (6), the enhancer region of the second enzyme, ornithine transcarbamylase (OTC) (7–9), and the promoter and enhancer regions of the last enzyme, arginase (10–13), there are binding sites for members of the CCAAT/ enhancer-binding protein (C/EBP) family (14, 15). C/EBPα (16) is the original member of this family and is the prototype of basic leucine zipper transcription factors (17). C/EBPα is most enriched in the liver and adipose tissues (18) and has been implicated in the expression of traits characteristic of terminally differentiated cells (18, 19).

Targeted disruption of the C/EBPα gene in mice leads to neonatal death because of hypoglycemia associated with impaired expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the liver (20–22). Here we found that in C/EBPα-deficient mice mRNA and protein levels for ornithine cycle enzymes are decreased and the distribution of these mRNAs in liver lobules is disordered. Hyperammonemia as well as hypoglycemia were evident in the mutant mice. Therefore, C/EBPα functions as a regulator for coordination of multiple steps of the ornithine cycle and further for coordination of gluconeogenesis and urea synthesis.

**EXPERIMENTAL PROCEDURES**

**Mice—**C/EBPα heterozygous mutant mice (20, 23) were mated, and genotypes of their offspring were determined by Southern blotting as described (20). Homozygous mutant mice and, as controls, their wild-type and heterozygous littermates were examined.

**Northern Blot Analysis—**Total RNA was isolated from the liver using the acid guanidinium thiocyanate/phenol/chloroform extraction procedure (24). RNA (2 μg/lane) was electrophoresed in denaturing formaldehyde-agarose (1%) gels and blotted onto nylon membranes. Digoxigenin-labeled antisense RNA probes were synthesized from cDNAs under the control of the T7 or SP6 promoter using a transcription kit (Boehringer Mannheim). cDNAs of the following enzymes were used for

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1 The abbreviations used are: CPS, carbamylphosphate synthetase; OTC, ornithine transcarbamylase; C/EBP, CCAAT/enhancer-binding protein; AS, argininosuccinate synthetase; AL, argininosuccinate lyase.
probes: CPS at nucleotide positions 2799–3237 (25), OTC (26), argininosuccinate synthetase (AS) at nucleotide positions 326–775 (27), argininosuccinate lyase (AL) (28), and arginase (29). Hybridization, washing, and chemiluminescent detection on x-ray films were done as recommended by Boehringer Mannheim. Densitometric quantification was done using MacBas software (Fuji Photo Film Co., Tokyo, Japan).

Western Blot Analysis—Mouse livers were homogenized in 9 volumes of 20 mM potassium HEPES buffer, pH 7.4, containing 0.5% Triton X-100, 1 mM dithiothreitol, 50 μM antipain, 50 μM leupeptin, 50 μM chymostatin, and 50 μM pepstatin. The homogenates were centrifuged at 25,000 × g for 30 min at 4 °C, and the supernatants were used as tissue extracts. The extracts (20 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. Antibodies against the following enzymes were used: CPS (30), OTC (31), AS (32), AL (32), and arginase (33). Densitometric quantification was done using the MacBas software.

In Situ Hybridization Analysis—Detection of mRNA molecules in liver sections was done as described (34). Briefly, freshly isolated livers were fixed in 4% formaldehyde and embedded in paraffin. Serial sections 7 μm thick were probed for ornithine cycle enzyme mRNAs with the respective 35S-labeled antisense RNAs (35). Hybridization was followed by exposure to an autoradiographic emulsion G-5 (Ilford Nuclear Research, Cheshire, UK) for 7 days and development for 4 min.

Measurement of Blood Ammonia and Glucose—Blood ammonia con-
RESULTS

Decreased mRNA and Protein Levels of the Ornithine Cycle Enzymes in Livers of C/EBPα-deficient Mice—Because mice homozygous for the disrupted C/EBPα allele die within 8 h of birth (20, 21), litters were investigated within 2 h (designated as newborn) and at 5–7 h postnatally. Total RNA was extracted from the liver and subjected to blot analysis for measuring mRNA levels for the five enzymes of the ornithine cycle. Representative chemiluminograms and quantified results are shown in Fig. 1. mRNA levels for β-actin were apparently higher in mice of newborn homozygous –/– litters as previously reported (21). In both newborn and 5–7-hour-old animals, the mRNA level for the first enzyme, CPS, was dramatically decreased in –/– mice, although no apparent difference was observed between wild-type +/– and heterozygous +/– mice. The mRNA encoding the second enzyme, OTC, was variable especially at 5–7 h from one littermate to another irrespective of the genotype, and no significant difference was observed. Fluctuation of the OTC mRNA levels may reflect sharp increases of OTC mRNA levels at earlier embryonic stages leading to variations in accumulated protein in the perinatal period and/or because of indirect effects of C/EBPα deficiency on synthesis or degradation of the OTC protein. On the other hand, differences of AS protein levels between genotypes were less distinct than those of AS mRNA levels, presumably from causes contrary to those for OTC. AL protein levels in –/– mice were reduced to 50% of those in +/– mice. Arginase was barely detectable in –/– mice. Coomassie staining of total proteins separated by gel electrophoresis revealed that several bands were specifically diminished in –/– mice. One of these bands corresponded to the protein empirically known to be CPS. In summary, protein levels of four ornithine cycle enzymes were decreased in –/– mice.

Disordered Zonation in Liver Lobules—In situ hybridization analysis was done to examine the lobular distribution of the mRNAs in livers of newborn mice (Fig. 3). Similar to the previous results for the normal rat liver (35), in +/– mice, mRNAs for the enzymes, except for CPS, were most abundant in the perportal region and gradually decreased toward the central vein exhibiting a distribution pattern called zonation (35, 38). Indistinguishably similar patterns were observed in +/– mice (data not shown), CPS mRNA in +/– mice, as well as in +/– mice (data not shown), exhibited a homogeneous distribution over the liver lobule. It was reported that the portal-to-central gradient of CPS mRNA becomes apparent at 1.5 days after birth (35). In –/– mice, aberrant patterns were evident especially for OTC and AS mRNAs, which showed high level expressions in midlobular regions. Residual mRNAs for CPS and arginase in –/– mice also exhibited relatively higher levels in midlobular regions, whereas residual AL mRNA showed a uniform distribution in all lobular compartments. Therefore, zonation characterized by portal-to-central gradients in gene expression for the ornithine cycle enzymes was disordered in –/– mice.
Hypoglycemia-associated Hyperammonemia—Because a decrease in the expression of the ornithine cycle enzyme genes or a difference in the localization of cells expressing these genes may cause impaired ammonia detoxification, we measured blood ammonia concentrations in mice of each genotype from the same litter (Table I). Ammonia levels in +/+ and +/− mice exhibited considerable variability, presumably reflecting rapid increases in ammonia levels during the first postnatal day (39). In −/− mice, ammonia concentrations were elevated compared with +/+ and +/− mice. We also measured blood glucose concentrations. Concordant with previous reports (20, 21), −/− mice exhibited hypoglycemia. Therefore, hyperammonemia was associated to hypoglycemia in −/− mice.

DISCUSSION

In higher eukaryotes such as mammals, there have been few genetic demonstrations of the presence of transcriptional regulators that control multiple steps in a metabolic pathway and even fewer for regulators that coordinate multiple related metabolic pathways. We found that C/EBPα is critical for the expression of ornithine cycle enzyme genes. Taken together with previous reports for a crucial role in gluconeogenesis (20–22), C/EBPα is considered to be a transcriptional regulator involved in the coordination of the two amino acid-metabolizing pathways (Fig. 4). Jungas et al. (1) postulated that the gluconeogenic pathway and the ornithine cycle are closely interrelated because the reactions catalyzed by AS and AL are common to both pathways. Therefore, these two pathways constitute a “metabolic block.” Control of this block by a common transcription factor C/EBPα represents a paradigm for a highly coordinated form of gene regulation.

Studies with in vitro assays and transfections into cultured cells have shown that members of the C/EBP family interact with regulatory regions of the genes for CPS (6), OTC (7–9), and arginase (10–13). The present findings of a dramatic fall in the expression of CPS and arginase genes in the livers of C/EBPα-deficient mice are consistent with these previous observations and underline the importance of C/EBPα in the regulation of the expression of these two genes. However, our previous studies have shown that C/EBPβ rather than C/EBPα activates the arginase promoter more strongly (12) and is more crucial for stimulation of the arginase enhancer in response to glucocorticoids (13, 40). Thus, it is likely that C/EBPα is involved in basal level expression of the gene, whereas C/EBPβ is important for high level expression of the arginase gene induced by glucocorticoids in response to dietary changes, etc. Because no apparent compensatory change in mRNA (20) and protein (21) levels of C/EBPβ and C/EBPδ was detected, it can be expected that relatively direct effects of the C/EBPα deficiency were manifested in this knockout system.

OTC mRNA levels were not affected in the C/EBPα-deficient mice; however, the lobular distribution of the OTC mRNA was disordered. OTC protein levels were decreased in neonatal −/− mice, and it remains to be examined whether, in the embryonic stages, OTC mRNA levels were lowered. In cell transfection studies (9), the OTC enhancer was activated almost exclusively by C/EBPβ in combination with another liver-enriched transcription factor, hepatocyte nuclear factor-4. Further investigations are required to clarify the differential roles, if any, of C/EBP family members in regulation of the OTC gene. As for the AS and AL genes, no C/EBP-binding site has been detected in the promoter regions of these genes (41, 42). The present results underscore the importance of re-examination of promoter regions for binding of C/EBP family members and/or searches for remote C/EBP-responsive enhancer-like regions of...
C/EBPα region in –/– mice, whereas in control mice the majority of highest mRNA levels were detected around the midlobular underlined ornithine cycle and the gluconeogenic pathway converge at AS and AL of two nitrogen atoms of urea comes from aspartate. The view that the enzymes for which mRNA and/or protein levels were seen to be dephosphorylation, and interaction with other factors, might particularly in neonatal mice.

Underlined

These genes, although the possibility of indirect effects of C/EBPα deficiency on AS and AL gene expression cannot be formally ruled out.

C/EBPα−/− mice exhibited disordered lobular zonation in the distribution of mRNAs for the ornithine cycle enzymes. The highest mRNA levels were detected around the midlobular region in −/− mice, whereas in control mice the majority of mRNAs were localized to the periporal region. Moorman et al. (43) reported that C/EBPα mRNA was distributed homogeneously in a lobule of the normal adult rat liver and that dexamethasone treatment caused elevation of the mRNA levels in the pericentral region. Therefore, the distribution pattern of C/EBPα mRNA by itself cannot explain the zonations of mRNAs for the enzymes or their disrupted patterns in −/− mice. Regulation of C/EBPα function by translational and post-translational events, such as nuclear transport, phosphorylation/dephosphorylation, and interaction with other factors, might bring about activation of target genes preferentially in the periporal region. This possibility remains to be examined especially in neonatal mice.

Previous analyses of C/EBPs −/− mice showed decreases in mRNA levels for glycogen synthase (20) and serum albumin (20, 21), as well as in mRNA levels for gluconeogenic enzymes. Recently, a conditional knockout study using targeted insertion of loxP sequences followed by the delivery of a Cre recombinase ade

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Fig. 4. Coordination of gluconeogenesis and urea synthesis by C/EBPα. The ornithine cycle pathway for conversion of amino acid-derived ammonia into urea is shown by black arrows, whereas the gluconeogenic pathway for conversion of carbon skeletons into glucose with representative regulatory enzymes is shown by gray arrows. One of two nitrogen atoms of urea comes from aspartate. The view that the ornithine cycle and the gluconeogenic pathway converge at AS and AL reaction steps was reported by Jungas et al. (1). Circles on the mitochondrial membrane represent substrate transporters. Underlined are enzymes for which mRNA and/or protein levels were seen to be decreased in livers of C/EBPα−/− mice for the gluconeogenic pathway (20) and for the ornithine cycle in the present study. G6Pase, glucose-6-phosphatase; FBPase, fructose-1,6-bisphosphatase; PEPCk, phosphoenolpyruvate carboxykinase.
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