Mice with a Deletion in the Gene for CCAAT/Enhancer-binding Protein β Have an Attenuated Response to cAMP and Impaired Carbohydrate Metabolism*

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Fifty percent of the mice homozygous for a deletion in the gene for CCAAT/enhancer-binding protein β (C/EBPβ−/− mice; B phenotype) die within 1 to 2 h after birth of hypoglycemia. They do not mobilize their hepatic glycogen or induce the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK). Administration of cAMP resulted in mobilization of glycogen, induction of PEPCK mRNA, and a normal blood glucose; these mice survived beyond 2 h postpartum. Adult C/EBPβ−/− mice (A phenotype) also had difficulty in maintaining blood glucose levels during starvation. Fasting these mice for 16 or 30 h resulted in lower levels of hepatic PEPCK mRNA, blood glucose, β-hydroxybutyrate, blood urea nitrogen, and gluconeogenesis when compared with control mice. The concentration of hepatic cAMP in these mice was 50% of controls, but injection of theophylline, together with glucagon, resulted in a normal cAMP levels. Agonists (glucagon, epinephrine, and isoproterenol) and other effectors of activation of adenylyl cyclase were the same in liver membranes isolated from C/EBPβ−/− mice and littermates. The hepatic activity of cAMP-dependent protein kinase was 80% of wild type mice. There was a 75% increase in the concentration of RIAs and 27% increase in RIIa in the particular fraction of the livers of C/EBPβ−/− mice relative to wild type mice, with no change in the catalytic subunit (Co). Thus, a 45% increase in hepatic cAMP (relative to the wild type) would be required in C/EBPβ−/− mice to activate protein kinase A by 50%. In addition, the total activity of phosphodiesterase in the livers of C/EBPβ−/− mice, as well as the concentration of mRNA for phosphodiesterase 3A (PDE3A) and PDE3B was approximately 25% higher than in control animals, suggesting accelerated degradation of cAMP. C/EBPβ influences the regulation of carbohydrate metabolism by altering the level of hepatic cAMP and the activity of protein kinase A.

The CCAAT/enhancer-binding protein (C/EBP)1 family of transcription factors has been implicated in the coordinated expression of genes involved in glucose homeostasis (1–5). For example the genes for phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phatase, glycogen synthase, and albumin are not expressed at birth in the livers of mice that are homozygous for a deletion in the gene for C/EBPα (C/EBPα−/− mice) (1). This results in an absence of hepatic glycogen and a delay in the initiation of gluconeogenesis. These animals also have lower levels of plasma glucose than their wild type counterparts and die within 2 h after birth (1–3).

Another member of the C/EBP family of transcription factors, C/EBPβ, also plays an important role in the regulation of glucose homeostasis (2, 6–8). In mice with a deletion in the gene for this transcription factor (C/EBPβ−/− mice), two distinct phenotypes have been observed (phenotypes A and B) (2). Mice with phenotype A live to be adults but have hypoglycemia as well as a lower than normal concentration of plasma free fatty acids, triglycerides, and ketone bodies in the blood after an overnight fast (4). These mice also have a compromised immune system and an impaired ability to activate macrophages (9, 10). Newborn C/EBPβ−/− mice with the phenotype B are unable to maintain normal blood glucose levels and die within 2 h after birth (2).

In this study we further characterize the metabolism of C/EBPβ−/− mice during the perinatal period. Our goal is to identify the metabolic defect(s) responsible for the death of C/EBPβ−/− mice (B phenotype) and to determine the metabolic alterations that contribute to the hypoglycemia noted in mice of the A phenotype that survive until adulthood.

EXPERIMENTAL PROCEDURES

Materials—Theophylline, dexamethasone, streptozotocin, guanidine thiocyanate, and protease inhibitors for mammalian cell and tissue extracts were purchased from Sigma. Anti-chicken IgG peroxidase was from Upstate Biotechnology (Lake Placid, NY). The following kits were used in these studies: RETROscript® first strand synthesis kit and the Stip-EZ® DNA probe synthesis kit from Ambion Inc. (Austin, TX), the QuickPrep® Total RNA extraction kit and the BIOTRAK® cAMP enzyme immunoassay system from Amersham Pharmacia Biotech, and the RNeasy® Mini kit from Qiagen Inc. (Valencia, CA). The insulin radioimmunoassay kit was from Linco Research (St. Charles, MO); the cAMP radioimmunoassay kit was from Amersham Life Sciences. Kits for the determination of blood ammonia and β-hydroxybutyrate were purchased from Sigma.

1 The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; Bt,cAMP, dibutyryl cyclic AMP; PEPCK, phosphoenolpyruvate carboxykinase; BUN, blood urea nitrogen; Glc-6-P, glucose-6-phosphatase; WT, wild type; PCR, polymerase chain reaction; bp, base pair; PKA, cAMP-dependent protein kinase A, PDE, phosphodiesterase.

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NaCl. Blood samples (25 of scintillation fluid was added, and the samples were counted in a glucose was measured using a Beckman glucose analyzer. Statistical technology (Santa Cruz, CA), and against C (CBA into C57Bl6 blastocysts and were transplanted into the uteri of F1 KOH, precipitated in ethanol, and hydrolyzed by boiling in 1N HCl, and Glycogen was extracted from frozen livers by homogenization in 6% a remainder carbohydrate). The composition of the high carbohydrate animals were given free access to water and standard chow (Tekland F6 systems were transferred into the ury of F1 (CBA×C57Bl6) foster mothers. Male chimeras were mated to MF1 females, and offspring heterozygous for the mutant allele were intercrossed to obtain homozygous mice. Adult male and female mice were 8–12 weeks of age at the time of their use. Straining for C/EBPβ−/− mice was carried out by Southern analysis as described previously (9). The animals were given free access to water and standard chow (Tekland F6 8664 containing 24% protein, 6% fat, and 4.5% crude fiber and the remainder carbohydrate). The composition of the high carbohydrate diet used in this study was described previously (11). The mice were killed between 9 and 11 a.m., and where indicated, they were injected with Br2CAMP (35 mg/kg of body weight) and theophylline (30 mg/kg of body weight).

Perinatal Studies—Mice were delivered at day 19 of gestation by cesarean section, and where indicated, they were injected with 125 mg of Br2cAMP/kg of body weight (12). All pups were maintained in a humidicrib at 37 °C from birth to the completion of the experiment (up to 4 b). When the pups of phenotype B became lethargic due to a low blood glucose concentration, they were killed together with their littermates. Blood was collected by decapitation, blood glucose was measured using a Glucometer (Ames Products, Indianapolis, IN), and insulin was measured by radioimmunoassay. The liver was freeze-clamped, and total RNA was isolated as described below, glycogen was measured (13), and cAMP was determined using an enzyme immunoassay procedure. Glycogen was determined from frozen kits. Glycogen was measured using a Beckman glucose analyzer. Statistical comparison between groups was performed using Student’s t test.

Metabolic Measurements—Adult C/EBPβ−/− mice (A phenotype) were given 5 mg of glucose/kg of body weight orally by gavage and then fasted for 16 and 30 h. Blood was taken from the tails of mice at 30 min, and 0.1 ml of fasting, and after the injection of 0.2 mg of proteinase K. The DNA was digested with DNA fragmentation, and the diabetic mice were injected into the tail vein 100 μg of 3H-glucose in 0.9% NaCl. Blood samples (25 μl) were obtained via the tail vein at 5, 15, and 30 min, and serum was used for the determination of glucose (glucose oxidase method). For the determination of radioactivity, 10 μl of blood was deproteinized with 200 μl of 20% trichloroacetic acid. Samples were centrifuged, and the supernatants were evaporated to dryness overnight at 65 °C. The residues were reconstituted in 200 μl of water, 5 ml of scintillation fluid was added, and the samples were counted in a β-scintillation spectrometer. The rate of systemic glucose production was calculated using steady-state equations. (15) Statistical comparison between groups was made using Student’s t test.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted from the liver and kidney of mice using a Quick Prep total RNA kit (Amersham Pharmacia Biotech) by a modified acid-phenol guanidinium thiocyanate procedure that has been described in detail previously (16). Northern blot analysis was carried out as described previously (17) using 20 μg of total RNA. After electrophoresis of the RNA, the gels was transferred to Gene Screen Plus® membrane and hybridized with a probe. The probe for PEPCK was a 1.1-kilobase Psll fragment from the 3′-end of the PEPCK cDNA that was isolated as described previously (16). The probe for glucose-6-phosphatase (Glc-6-P) mRNA was a 1.1-kilobase BglII probe from Glc-6-P mRNA. The C/EBPβ cDNA probe was a 0.7-kilobase BamHI fragment of the mouse cDNA (9). The concentration of 18 S RNA was determined by Northern blotting using a 752-nucleotide Sac1 fragment of a cDNA made from mouse 18 S RNA. The signal from this hybridization was used to standardize the concentration of RNA on the Northern blots. All probes were labeled by Labeling Kit and was used for RNA & DNA probe synthesis and removal kit (Ambion Inc., Austin, TX).

DNA Analysis—DNA was isolated from the tail of mice by lysis overnight at 55 °C in a buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl2, 0.1% gelatin, 0.45% Nonidet P-10, 0.45% Tween 20, and 24 μg of proteinase K. The DNA was digested with EcoRI, and the resulting fragments were separated by electrophoresis on 1% agarose gel, transferred to Gene Screen Plus® and hybridized to a cDNA probe for C/EBPβ.

Glucagon-stimulated cAMP Production in Liver in Vivo—Fed C/EBPβ−/− mice and control littermates (WT) (8–12 weeks of age) were anesthetized with avertin, the liver was clamped, and a biopsy was taken for the measurement of basal cAMP. The mice were then injected with glucagon (50 μg/diole of body weight) via the tail vein, and liver was removed 1 min later for the assay of glucagon-induced cAMP. C/EBPβ−/− and wild type mice were given an intraperitoneal injection of either theophylline (30 mg/kg of body weight) or RO 20-1724 (15 mg/kg of body weight). Thirty min later, the mice were anesthetized with avertin, the liver was clamped to prevent bleeding, and a liver biopsy was obtained. This was used for the basal concentration of cAMP. Glucagon (50 μg/kg of body weight) was injected into the portal vein, and one min later, another piece of liver was removed for the quantitation of cAMP. The liver samples were quickly frozen and assayed for cAMP using an enzyme immunoassay (Amersham Pharmacia Biotech).

Quantitative Reverse Transcription-PCR—Quantitative competitive reverse transcription-PCR was used to measure the relative concentrations of phosphodiesterase mRNA in the livers of C/EBPβ−/− and control littermates. This procedure involves four steps: first, total RNA was isolated from the liver; second, reverse transcription was performed to create cDNAs; third, competitive PCR was performed; and fourth, the DNA bands were quantitated. The RNA was isolated from the livers of mice using the Amersham Pharmacia Biotech QuickPrep® RNA isolation kit. The cDNA was synthesized from the sample by treatment with 10 units of RNase-free DNase I, and the RNA was further purified by using a Qiagen RNeasy Mini Kit®. Reverse transcription was performed at 42 °C for 2 h using an Ambion RETROscript kit®. Briefly, 2 μg of total RNA was reverse-transcribed in a reaction mixture containing 0.5 mM dNTP, 5 μM random primer, 1× buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 30 mM MgCl2, and 50 mM creatine phosphate), 1× reverse transcriptase, and 0.5 units of SuperTaq DNA polymerase. The competitive PCR reaction was carried out in a volume of 25 μl that contained 2 μl of the solution from the reverse-transcribed reaction with varied concentrations of an internal DNA control fragment as well as 0.2 mM dNTP mix, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 3 mM MgCl2, 0.5 μM mixed primer, and 0.5 units of SuperTaq DNA polymerase. The competitive PCR reaction was performed at 94 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 1 min for 30 cycles. For each RNA sample, one reverse transcription reaction and eight PCR reactions were performed. The internal DNA control fragments were constructed as follows. The internal control for PDE3A was obtained from a 482-bp segment of the mouse PDE3A by deleting a 178-bp StyI fragment. The internal control for PDE6B was generated by deleting a 124-bp SacII segment of the 682-bp XhoI-StuI cDNA fragment, and for the PDE4B internal control, the fragment was obtained by deleting a 152-bp NsiI fragment from the 1968-bp EcoRI cDNA.

After the PCR reaction, 8 μl of the reaction solution was subjected to electrophoresis in a 1.6% agarose gel. The DNA bands were recorded using an IS-500 digital imaging system (Alpha Innotech Corp. San Leandro, CA), and the bands were quantitated using Molecular Dynamics (Sunnyvale, CA). The concentration of mRNA was determined as the point where the internal control band is equal in intensity to that of the test sample. To find that point, the ratios of the intensities of test cDNA and internal control bands were calculated and fitted to a regression curve using GraphPad Prism® software (GraphPad Software, San Diego, CA).
Adenylyl Cyclase Activity—Partially purified liver plasma membranes were prepared from the livers of C/EBPβ and control mice as described previously (19), with slight modifications. Briefly, the livers were homogenized (Dounce homogenizer) in 5 ml of Buffer A (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 3 mM β-mercaptoethanol, and 1 mM leupeptin) containing 500 mM sucrose and homogenates were centrifuged at 17,000 × g for 10 min, and the pellet was suspended in 4 ml of the homogenization buffer. This suspension was layered onto 42.3% (w/w) sucrose in Buffer A and centrifuged at 100,000 g for 45 min. The final pellet was re-suspended in Buffer A and stored at −80 °C until used. The yield of membrane protein, as determined by the method of Lowry (20), was about 15 mg of protein/g of liver; there was no difference in the yield of liver membranes of C/EBPβ and control mice. Adenylyl cyclase activity in the membrane preparations was assayed for 10 min at 30 °C using 300 μM [m32P]ATP as a substrate, as described previously (19).

Protein Isolation for Western Blotting—Proteins were isolated from the livers of C/EBPβ/−/− and wild type mice that were fasted overnight. A piece of frozen liver was homogenized in 15 ml of tissue of ice-cold homogenizing buffer consisting of 20 mM Tris, pH 7.6, 0.1 mM EDTA, 0.5 mM EGTA, 0.1% Triton-D, 250 mM sucrose, and 50 μl of protease inhibitor cocktail. Homogenates were centrifuged at 19,500 × g for 30 min at 4 °C to separate cellular debris, mitochondria, microsomes, and plasma membranes. The pellet was suspended in 4 ml of homogenizing buffer (N fraction). The supernatant was then centrifuged at 100,000 × g for 30 min at 4 °C. The resulting supernatant (the cytosolic fraction) was removed, and the remaining pellet (particulate fraction) was re-suspended in 4 ml of homogenizing buffer. The particulate fraction contained membranes of the endoplasmic reticulum and the Golgi complex. The concentration of protein was measured with the Bio-Rad protein assay using bovine serum albumin as a standard.

Electrophoresis and Western Blotting—The N fraction as well as the cytosolic and particulate fractions were sonicated for 20 s, and 20 μg of protein was diurnated in 2× loading buffer containing 100 mm/liter Tris-Cl, pH 6.8, 20% β-mercaptoethanol, 4% SDS, 0.2% bromphenol blue, 20% glycerol and separated by 10% SDS-PAGE. Proteins were electrophoretically transferred to Immobilon-P® polyvinylidene difluoro membranes and stained with Coomassie stain (45% methanol, 10% acetic acid, 2.5% Coomassie Blue R250) to ensure even loading. For electrophoretic transfer of Immobilon-P® polyvinylidene difluoro membranes to Immobilon-P® polyvinylidene difluoro membranes and stained with Coomassie stain (45% methanol, 10% acetic acid, 2.5% Coomassie Blue R250) to ensure even loading. For detection of the subunits of protein kinase A, RIIα, RIIβ, and Ca2+ polyvinylidene difluoro membranes were incubated in blocking buffer containing 5% nonfat dried milk in 10 mM/liter Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were then incubated with primary antibody diluted in blocking buffer for 1 h as follows: RIA (1:250), RIIα (1:1000), Ca2+ (1:250), washed 3 times for 5 min each in TBS-T. The membranes were then incubated with secondary antibody diluted in blocking buffer for 1 h as follows: rabbit anti-human IgG peroxidase (1:1000) for RIIα and Ca2+ and RIIβ and Ca2+. Membranes were washed 3 times for 5 min each in TBS-T, and the incubations were run in a humidicrib. C/EBPβ/−/− mice identified as being of the B phenotype were typically lethargic, had difficulty breathing, and remained cyanotic until they died within 2 h after delivery. The C/EBPβ/−/−/− mice that were viable and breathing well were classified as the phenotype A. The plasma glucose levels of the B phenotype were about 50% that of control pups (wild type) (p < 0.01) (Fig. 1). The C/EBPβ/−/−− mice (B phenotype) also had difficulty in mobilizing hepatic glycogen compared with wild type littersmates, as was evident from the higher level (p < 0.03) of hepatic glycogen in these animals (70 mg/g of liver glycogen in C/EBPβ/−/− versus 40 mg/g of liver glycogen in the wild type mice). Because maintenance of normal blood glucose in the neonate depends on the capacity to mobilize hepatic glycogen as well as to initiate hepatic gluconeogenesis, we measured the ability of these animals to initiate gluconeogenesis by measuring the level of mRNA for PEPCCK, the last of the gluconeogenic enzymes to develop in newborn mammals (23). As shown in Fig. 1, the level of PEPCCK mRNA in C/EBPβ/−/− mice (B phenotype) was only 30% that of either the A phenotype or the wild type control animals. PEPCCK gene expression is down-regulated by insulin (24). We therefore measured the concentration of insulin in the plasma of C/EBPβ/−/− mice and control mice; the insulin levels were found to be the same (data not shown).

The high levels of glycogen in the livers of animals of the B phenotype suggested a defect in the ability of these mice to mobilize their hepatic glycogen. C/EBPβ/−/− mice at 19 to 20 days of fetal life were delivered by cesarean section and given an intraperitoneal injection of Bt-cAMP (125 mg/kg) immediately after delivery. These mice mobilized their hepatic glycogen to the same extent as control littersmates (to a level of 35 mg/g of liver) (data not shown). Surprisingly, mice with the B phenotype responded immediately to the Bt-cAMP by breathing normally and becoming less lethargic. All of the C/EBPβ/−/−− mice injected with Bt-cAMP survived for up to 4 h, the duration of the experiment (Fig. 2). Since the administration of Bt-cAMP rescued the C/EBPβ/−/−− mice (B phenotype) from death within the first 2 h after birth, we considered it important to determine whether endogenous levels of cAMP in the livers of B-phenotype mice were different from the normal littersmates. For this experiment, the animals were delivered at 19–20 days of fetal life. The newborn animals were treated as one group representing a mixture of both A and B phenotypes. The C/EBP β−/− mice had the same level of cAMP as control mice (data not shown), suggesting that there is no defect up-stream of cAMP production. The biochemical basis for the decreased viability of C/EBP β−/− mice of the B phenotype remains enigmatic, but it seems likely that their inability to respond to the normal concentration of cAMP may be due to an alteration in downstream target(s) required to activate critical
metabolic processes during the perinatal period.

**Metabolic Characteristics of C/EBP**β−/− mice and Wild Type Mice—Based on the blunted response of the C/EBPβ−/− mice (B phenotype) to the endogenous concentrations of hepatic cAMP and the resulting aberrations in carbohydrate metabolism, we extended this study to adult animals (the A phenotype) to determine the differences in metabolic response from adult wild type control mice. To simulate the fed state, adult mice were given glucose orally (5 g/kg), and blood was taken from the tail vein 30 min later. The mice were then fasted for 30 h, blood was collected from the tail vein at 16 and 30 h, and the concentrations of glucose, β-OH butyrate, and BUN were determined. The concentration of glucose in the blood of the C/EBPβ−/− mice was 25% lower than that of wild type control animals, whereas the level of β-hydroxybutyrate was 50% lower than the wild type animals (Fig. 3). The lowered concentration of β-hydroxybutyrate probably reflects a decreased oxidation of fatty acids in the C/EBPβ−/− mice because these animals exhibit lower fasting free fatty acids levels than wild type counterparts (4).

The adult C/EBPβ−/− mice resemble “sparse fur mice” (25) that have a defect in the urea cycle enzyme, ornithine transcarbamylase, and exhibit lower than normal levels of BUN, elevated blood ammonia, and premature hair loss. For this reason, we also investigated alterations in ammonia metabolism in the C/EBPβ−/− mice. In 16-h fasted animals, the concentration of BUN was 40% lower in C/EBPβ−/− mice compared with control littermates, suggesting derangement in ammonia metabolism or urea production; however, this value returned to control levels after 30 h of fasting (Fig. 4). To confirm a lower rate of flux through the urea cycle, the concentration of ammonia was determined in the plasma of C/EBPβ−/− mice that had been fasted for 16 h (Fig. 4, inset). C/EBPβ−/− mice had blood ammonia levels of 425 μg/dl as compared with 200 μg/dl in wild type mice. This suggests that ammonia metabolism in the C/EBPβ−/− mice is compromised, an abnormality that may contribute to the premature death of these animals, as noted earlier by Screpanti et al. (9).

**Systemic Glucose Production**—Because C/EBPβ−/− mice have difficulty maintaining normal levels of blood glucose during fasting, systemic glucose production was determined in vivo. In these experiments [1H]glucose was injected into the tail vein of conscious C/EBPβ−/− mice that had been fasted overnight, and the rate of dilution of the [1H]glucose was then measured. As shown in Fig. 5, glucose production in the C/EBPβ−/− mice was half that of control littermates, suggesting a defect in gluconeogenesis. The levels of insulin were the same in both the wild type and C/EBPβ−/− mice (data not shown). The levels of mRNA for two gluconeogenic enzymes, PEPCK and Glc-6-P, were then measured in the livers of C/EBPβ−/− mice and control littermates fed a high carbohydrate diet for 1 week and then injected with Bt2cAMP (Fig. 6). In livers of normal animals, both hepatic PEPCK and Glc-6-P mRNA levels were repressed by a high carbohydrate diet were induced by the administration of Bt2cAMP. The inhibitory response of the PEPCK gene to a high carbohydrate diet and normal induction by Bt2cAMP was blunted in the livers of C/EBPβ−/− mice in comparison to control animals. In addition, a high carbohydrate diet, rather than repressing Glc-6-P mRNA in control animals, induced its levels in the livers of the C/EBPβ−/− mice. One possible explanation for this finding
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Fig. 3. The effect of starvation on the concentration of glucose and β-hydroxybutyrate in the blood plasma of C/EBPβ−/− and wild type mice. C/EBPβ−/− mice and control littersmates (WT) (8–12 weeks of age) were fed 5 g of glucose by oral gavage and then starved for 16 and 30 h, at which time the concentration of glucose and β-hydroxybutyrate were determined. The values are expressed as the mean ± S.E. of six mice for the blood glucose and four-five mice for β-hydroxybutyrate. (*, p < 0.05; **, p < 0.002).

wild type mice.

C/EBP and response of the gene for Glc-6-P to dietary carbohydrate. 6 S.E. of six mice for the blood glucose and four-five mice for b weeks of age) were fed5go f glucose by oral gavage and then starved for

C/EBP relatively lower concentration (50%) of hepatic cAMP in the marked difference from that noted in wild type animals. The response of the gene for Glc-6-P to dietary carbohydrate is involved in controlling the Glc-6-P gene transcription by carbohydrate but not in the repression of PEPCK gene transcription. Although PEPCK and Glc-6-P share a common set of regulatory signals, they respond in a different manner to high concentrations of glucose (18). Our findings suggest that C/EBPβ is involved in controlling the response of the gene for Glc-6-P to dietary carbohydrate.

CAMP Metabolism in the C/EBPβ−/− A-phenotype and Wild Type Mice—We previously noted that the basal level of cAMP in the livers of C/EBPβ−/− mice was about half that of littermates (4). The concentration of hepatic cAMP was 296.68 ± 32.98 pmol/g as compared with 581.63 ± 92.98 pmol/g of liver in control littersmates (Fig. 7A). After the administration of glucagon into the portal vein, the concentration of hepatic cAMP increased to 495.32 ± 84.17 pmol/g of liver in C/EBPβ−/− mice and to 1162.96 ± 171.11 pmol/g of liver in the wild type mice (Fig. 7A). Although the fold change in the concentration of cAMP was about the same, the absolute level of the cyclic nucleotide in the livers of C/EBPβ−/− mice was markedly different from that noted in wild type animals. The relatively lower concentration (50%) of hepatic cAMP in the C/EBPβ−/− mice might be due to the inability of the liver to synthesize cAMP at the appropriate rate or might have resulted from an increase in cAMP degradation. To test these possibilities, basal and activated adenyl cyclase activities were measured in liver plasma membranes of C/EBPβ−/− and wild type mice (Table I). In response to glucagon, cholera toxin, or forskolin, and isoproterenol, membranes from the C/EBPβ−/− mice synthesized cAMP at the same rate as those from control littersmates. This indicates that the capacity to produce cAMP was intact in the livers of these mice and was not altered by a deletion of the gene for C/EBPβ. However, there appeared to be an accelerated rate of degradation of cAMP in levels of these mice.

The degradation of cAMP was tested by first administering the phosphodiesterase (PDE) inhibitors theophylline (a non-selective inhibitor of PDE) or RO 20-1724 (a specific inhibitor of PDE 4) to fed C/EBPβ−/− and control mice. The concentration of cAMP was then measured in liver biopsies taken before and after glucagon injection into the portal vein (Fig. 7, panels B and C). After the administration of theophylline or RO 20-1724, the basal levels of cAMP in the livers of C/EBPβ−/− mice were the same as control animals, and there was no significant difference in the response of cAMP to glucagon injection.

Because these data suggest an accelerated rate of degradation of cAMP in the livers of C/EBPβ−/− mice, we determined the total activity of PDE in liver homogenates of fed mice using 1 μm cAMP as substrate. The specific activity of PDE was 56.7 pmol/min/mg of protein in C/EBPβ−/− mice as compared with 44.1 in the livers of control animals (Fig. 8). The level of mRNA for PDE 3A, 3B, and 4B was also measured using a liver biopsy taken 1 min after glucagon injection. The results show that the concentrations of PDE 3A and PDE 3B mRNA were 25% higher in C/EBPβ−/− mice (p < 0.01 for PDE 3B); no difference in the levels of PDE 4 was detectable. It is important to note that PDE 3B is the major phosphodiesterase isoform in the liver, and its mRNA was 100-fold higher that of PDE3A. In agreement with these findings, the rate of cAMP degradation in vitro was determined from parallel experiments in which diluted liver homogenates were incubated with 1 μm [3H]cAMP. As shown in Fig. 9, the disappearance (degradation) of [3H]cAMP by liver extracts from C/EBPβ−/− mice was more rapid than noted for controls.

Since cAMP levels can affect the activity of PKA, we next determined the levels of both the regulatory (RIα and RIα) and the catalytic (Ca) subunits of PKA in the nuclear (N), cytosolic (C) and particulate (P) fractions of livers from adult C/EBPβ−/− and wild type mice by Western blotting (Fig. 10). We found significant changes in the regulatory subunits of PKA in the livers of C/EBPβ−/− mice. The concentration of RIα was 79% higher in the particulate fraction and 17% higher in the cytosolic fraction as compared with wild type mice, whereas the concentration of RIα in the livers of C/EBPβ−/− mice was increased by 27% in particular fraction and 5% in the cytosolic fraction as compared with wild type littersmates. In contrast, the concentration of the catalytic subunit of PKA in C/EBPβ−/− mice was the same as wild type (Fig. 10). RIα antibody reacted with two protein bands, one at 56 kDa and another at 52 kDa; the 56-kDa band is characteristic of a phosphorylated form of RIα (28). The concentration of the 52-kDa band (non-phosphorylated RIα) was higher in the P and C fractions as compared with wild type, whereas the phosphorylated band was similar to wild type mice in the P and C fractions. The potential physiological significance of this result is not clear.

A change in the ratio of regulatory to catalytic subunits of PKA has a profound effect on the total activity of the enzyme at a given concentration of cAMP. In Table II the levels of RIα, RIα, and Ca were measured by scanning the Western blots in Fig. 10. The values for the particulate and cytosolic fractions are represented as percent of wild type. For RIα, the phospho-
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**DISCUSSION**

The deletion of the gene for C/EBPβ markedly alters the normal initiation of glucose homeostasis in the immediate perinatal period. Only 50% of the C/EBPβ−/− mice (animals with the A phenotype) survive the first hours after birth. C/EBPβ−/− mice (B phenotype) display profound hypoglycemia despite the fact that they have higher than normal levels of hepatic glycogen (Fig. 1). Kawai and Arinze (46) demonstrate that in newborn guinea pigs the response of hepatic glycogenolysis to administered glucagon in the first 3–4 h after birth is blunted when compared with the response beyond 4 h. A similar age-dependent response was observed with epinephrine and isoproterenol. In contrast, cAMP itself induced glycogenolysis independent of age, suggesting that the retarded rate of hepatic glycogen mobilization might be due to a delayed responsiveness of the receptor-coupling system in the livers of newborn guinea pigs. The results of the present studies suggest that C/EBPβ−/− mice (B phenotype) do not respond appropriately to the normal stimuli that occur at birth and that these can be by-passed by an injection of Bt2cAMP immediately after birth.

One possible downstream target is PKA. We show that in the livers of C/EBPβ−/− mice (A phenotype) there is a 79% increase in the concentration of regulatory subunits RIα and a 27% increase in the concentration of RIα in the particulate fractions of the liver as compared with wild type. There was no difference in the levels of the Cα subunit of PKA. The observed increase in the level of regulatory subunits of PKA, the concentration of cAMP in the livers of C/EBPβ−/− mice is critical in determining the total activity of the enzyme. The result could be a failure to fully stimulate many of the cAMP-dependent processes vital to the metabolic function of the liver.
constant. This leads to a disproportion between the R and C concentration of cAMP in the livers of adult C/EBPβ mice. The activity of adenylyl cyclase was determined as outlined under “Experimental Procedures.” Values are the mean ± S.E. for six mice in each group.

### Treatments

|          | Wild Type | C/EBPβ−/− |
|----------|-----------|------------|
| Basal + MgCl₂ | 7.94 ± 1.35 | 8.89 ± 1.60 |
| Isoproterenol + GTP | 14.18 ± 1.90 | 15.43 ± 1.68 |
| Glucagon + GTP | 131.64 ± 11.20 | 134.46 ± 17.76 |
| NaF + AlCl₃ | 143.82 ± 21.30 | 140.32 ± 34.84 |
| GTP₇S | 52.12 ± 4.40 | 56.92 ± 4.24 |
| Choleratoxin + NAD | 29.51 ± 4.48 | 30.45 ± 2.81 |
| Forskolin | 212.50 ± 26.83 | 233.27 ± 12.97 |
| Forskolin + MnCl₂ | 470.11 ± 36.52 | 508.86 ± 32.20 |

*GTP₇S, guanosine 5′-3′-O(thiotriphosphate).

FIG. 7. Effect of glucagon, theophylline, and RO-20-1724 on the concentration of cAMP in the livers of adult C/EBPβ−/− mice and wild type mice. Panel A, fed C/EBP β−/− mice and control littersmates (WT) (8–12 weeks of age) were anesthetized with avertin, the liver was clamped, and a biopsy was taken for the measurement of basal cAMP. Glucagon (50 μg/kg of body weight) was then injected via the portal vein, and the liver was biopsied 1 min later for the assay of glucagon-induced cAMP. The data in this panel were redrawn from Liu et al. (4). *, p < 0.05. Panel B, fed C/EBP β−/− mice and control littersmates (WT) (8–12 weeks of age) were administered theophylline (30 mg/kg of body weight) by intraperitoneal injection. Thirty min later, the animals were anesthetized with avertin, the liver was clamped, and a biopsy was taken for the measurement of basal cAMP. Glucagon (50 μg/kg of body weight) was then injected via the portal vein, and the liver was biopsied 1 min later for the assay of glucagon-induced cAMP. Panel C, the protocol was the same as in B except that the RO 20-1724 (15 mg/kg of body weight) was injected instead of theophylline. The values are expressed as the mean ± S.E. of the mean for 6 mice in each group.

subunits of PKA that diminishes the concentration of Ca during the cAMP burst that occurs with liver regeneration (27). The increase in the R subunit during liver regeneration has been interpreted as a response to the increase in cAMP, since the elevation of the R subunit of PKA may be a method of down-regulating PKA activity (hysteresis).

The observed alterations on the relative location of the isoforms of the R subunit of PKA in the livers of C/EBPβ−/− mice may also be of significance. RIIα is the predominant regulatory subunit of PKA in the cytosol of hepatocytes, whereas RIIα predominates in the cytoskeleton, the Golgi apparatus, microtubules, and nucleus (28). We have noted an increase in the concentration of both of the R subunits of PKA in the particulate fraction of liver cell and a decrease in the presence of these proteins in the cytosolic fraction (Fig. 10). In contrast to our results, Ekanger et al. (27), in their studies of regenerating liver, note that the change in the increased concentrations of the RIIα and RIIα (relative to the Ca) was constant in the cytosol and particulate fractions of the liver. The reason for the selective increase that we observe in the particulate fraction of C/EBPβ−/− mice is not clear, but it could be related to the concentration of PKA-anchoring protein in the liver. Anchoring proteins bind specifically to RII and control the movement of PKA to the particulate fraction of the liver, thus partitioning its activity in the cell (29). The concentration of anchoring protein in the livers of C/EBPβ−/− mice has not been determined.

Alterations of the concentration of the regulatory subunits in various fractions of the liver have profound implications in the response of PKA to cAMP. For example, O’Brien et al. (30) demonstrate that dietary protein restriction or reduction of the caloric content of the diet resulted in a loss of RIIα in the cytosol of rat livers and an increase in the amount of the RIIα subunit. This was accompanied by a sharp reduction in the level of the catalytic subunit of PKA in the particulate fraction of the liver cell. In addition, the activation of glycogen phosphorylase and the phosphorylation of the cAMP regulatory element-binding protein (CREB) by glucagon was lower in hepatocytes isolated from rats fed a 0.5% protein diet as compared with control animals that had been fed a standard diet containing 15% protein (30). This dietary-induced shift in the ratio of the RIIa and RIIα subunits of PKA in the liver would explain the blunted response of these animals to glucagon, despite the fact that the concentration of hepatic cAMP is the same as wild type mice, since the two subunits of PKA have different affinities for cAMP (31).

Although the C/EBPβ−/− mice (A phenotype) survive to adulthood, they display critical metabolic abnormalities. They have pronounced hypoglycemia associated with fasting and an
impaired hepatic glucose production (4). There was a blunted rate of hepatic glucose production caused by glucagon injection into 18-h fasted mice that were infused with somatostatin to clamp the insulin and glucagon output from the pancreas (4). The level of cAMP in the livers of C/EBPβ−/− mice (A phenotype) was about half that noted in control littersmates, and the response to glucagon was also less robust. In addition, these mice have a lower rate of epinephrine-induced release of free fatty acids from epididymal adipose tissue in vitro (4). This may explain the lower concentration of blood ketone bodies noted in the blood of C/EBPβ−/− mice after fasting (Fig. 3). In addition to alterations in the rate of hepatic glucose output, insulin sensitivity in the C/EBPβ−/− mice was greater, resulting in a rate of whole body glucose disposal that was 77% higher than noted in control littermates (32). This is in part due to an increased response of muscle from the C/EBPβ−/− mice to insulin stimulation; the insulin-stimulated phosphorylation of the insulin receptor and phosphatidylinositol 3-kinase activities as well as insulin receptor kinase substrate-1 and Akt-Ser473 were all about 2-fold greater in the skeletal muscle of the C/EBP β−/− mice as compared with littermates (32). This suggests that the marked drop in the concentration of blood glucose in the C/EBP β−/− mice (A phenotype) during fasting is due in part to an accelerated rate of removal of glucose by muscle as well as a diminished rate of gluconeogenesis. This may also contribute to the profound hypoglycemia noted in C/EBPβ−/− mice (B phenotype) in the immediate perinatal period.

The concentration of cAMP in tissues is regulated not only by adenylyl cyclase but also by the activity of the various isoforms of PDE isozymes (33). The PDE families of enzymes are comprised of multiple isoforms within each family generated from alternative splicing of their precursor RNA. For example, the PDE3 family consists of PDE3A and PDE3B (34). PDE3A has been identified in smooth muscle, platelets, and cardiac tissue, whereas PDE3B is most abundant in adipocytes and liver. PDE4 is the largest member of the PDE families and is derived from at least four different gene PDE4 products (35). However, there is little information available concerning the tissue specificity of the members in this family (36). Our data show a 25% increase in both mRNA levels for PDE3A and PDE3B and a 25% increase in PDE activity in the livers of fasted C/EBPβ−/− mice. It is known that interleukin-3 and -4 activate PDE3 in FDCP2 myeloid cells (37), and the concentration of interleukin-6 increased in fasted C/EBPβ−/− mice (9). It is intriguing to speculate that the increased PDE activity observed in the C/EBPβ mice is accomplished through the increase in interleukin-6, which could in turn cause a cascade effect through the insulin receptor substrate 2 (IRS-2), phosphatidylinositol 3-kinase, protein kinase B pathway and ultimately affect PDE3B activity. Another possibility would be a direct effect of C/EBPβ on the promoter for the PDE3A and PDE3B genes. Little is known about the transcriptional regulation of PDE genes. It is known that cAMP down-regulates the expression of the gene for PDE3 (38). It is thus possible that in the absence of C/EBPβ there is decreased inhibition of gene transcription, leading to
an accumulation of PDE3 mRNA. This would require that C/EBPβ be involved as a negative regulator of PDE gene transcription, for which there is no direct information to date. However, a cAMP regulatory binding protein (CREB)-binding site is present in the PDE3B gene promoter; this might serve as a binding site for C/EBPβ, as occurs with the PEPCK gene promoter (39). A third possibility is that C/EBPβ may directly regulate one of the G proteins in the adenyl cyclase pathway. This is unlikely since we found no changes in the relative levels of $G_\alpha 1$, $G_\alpha 2$, $G_\alpha 3$, $G_\beta 2$, and $G_\beta 1$ in membranes of livers of C/EBPβ−/− mice and control littersmates, as determined by Western blotting (data not shown). This result agrees with the

\begin{table}
\centering
\caption{The effect of the relative concentration of the various isoforms of PKA on the relative concentration of cAMP required for a 50% activation of PKA in the livers of C/EBPβ−/− mice.}
\begin{tabular}{ |c|c|c|c|c| }
\hline
PKA subunit & Protein level & Percent increase of [cAMP] 50% activity required for PKA \\
& P & C & P & C \\
\hline
RIα & 179 & 117 & 33 & 8 \\
RIα total (56 + 52 kDa) & 127 & 105 & 12 & 2 \\
Ca & 101 & 98 & & \\
\hline
\end{tabular}
\end{table}

\[ R_{c+2cAMP} = R_{2cAMP} + C \]
\[ K = [RC][cAMP]^2[R-2cAMP][C] \]
\[ [\text{cAMP}]_{50\% \text{ activity}} = ([R-2cAMP]K_{50\% \text{ activity}})^{0.5} \]

\[ \text{data on adenylyl cyclase activation in the same liver membranes (Table I).} \]

Abnormalities in the C/EBPβ−/− mice are not limited to carbohydrate and lipid metabolism; they also extend to amino acid metabolism. The concentration of BUN in C/EBPβ−/− mice after 16 h of fasting is half that of control littersmates, a value that is reflected in the 2-fold increase in blood ammonia in the C/EBPβ−/− mice. After 30 h of fasting, the concentration of BUN decreases to near normal levels, indicating a sparing of amino nitrogen characteristic of prolonged starvation. The levels of BUN have been shown to increase in humans during the first few days of fasting and to return to normal levels by the end of 2 weeks (40). Rats also spare body protein; this is reflected in a decreased concentration of BUN at 24 h of fasting (41–43). We have determined the concentration of 20 amino acids in the blood of C/EBPβ−/− mice and control littersmates that were fasted for 30 h. The most notable difference was a 3-fold increase in the concentration of taurine relative to control littersmates (1035 μmol/liter versus 333 μmol/liter), a 2-fold increase in ornithine (127 μmol/liter versus 64 μmol/liter), and a 30% increase in glutamine (647 μmol/liter versus 452 μmol/liter). The higher levels of ammonia, ornithine, and glutamine in the blood of the C/EBPβ−/− mice may reflect a decreased rate of urea cycle activity in the livers of these mice. C/EBPβ is required for the glucocorticoid induction of transcription of the gene for arginase, a critical urea cycle enzyme (44). It has been proposed that C/EBPα is involved in the regulation of basal expression of the gene for arginase and that C/EBPβ controls the high level of expression of the gene in the adult mouse in response to dietary protein (45). C/EBPβ−/− mice have the appearance of sparse fur, a phenotype associated with a defect in the gene for ornithine transcarbamylase caused in part by elevated levels of blood ammonia.

The results of the present paper clearly demonstrate the far-reaching metabolic consequences caused by the absence C/EBPβ. A series of relatively small changes in the rate of hepatic cAMP degradation and a shift in the pattern of expression of the genes for the isoforms of the regulatory and catalytic subunits of PKA result in a lower concentration of cAMP in the
liver. When combined with the increased requirement for cAMP to attain activation of PKA in the livers of C/EBPβ−/− mice, the result is a failure to respond to glucagon in a normal fashion. Thus, a 25% increase in the activity of PDE3 in the liver ensures a rapid enough removal of hepatic cAMP to cause the animals to have lowered rate of hepatic glucose output in response to fasting and to glucagon administration.

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REFERENCES
1. Wang, N. D., Finegold, M., Bradley, A., Ou, C. N., Abeldsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) Science 269, 1108–1112
2. Croniger, C., Trus, M., Lysek-Stupp, K., Cohen, H., Liu, Y., Darlington, G. J., Poli, V., Hanson, R. W., and Seshadri, L. (1997) J. Biol. Chem. 272, 26306–26312
3. Flodby, P., Barlow, C., Kylefjord, H., Ahrlund-Richter, L., and Xanthopoulos, K. G. (1996) J. Biol. Chem. 271, 24753–24760
4. Liu, S., Croniger, C., Ren, J., Shiha, M., Poli, V., Hanson, R. W., and Freedman, J. E. (1999) J. Clin. Invest. 103, 207–213
5. Arizmendi, K., Liu, S., Croniger, C., Poli, V., and Friedman, J. E. (1999) J. Biol. Chem. 274, 13033–13040
6. Park, E. A., Song, S., Vinson, C., and Roseler, W. J. (1999) J. Biol. Chem. 274, 211–217
7. Park, E. A., Gurney, A. L., Nizielski, S. E., Hakimi, P., Cao, Z., Moorman, A., and Hanson, R. W. (1993) J. Biol. Chem. 268, 613–619
8. Yamada, K., Duong, D. T., Scott, D. R., Wang, J.-C., and Graner, D. K. (1999) J. Biol. Chem. 274, 5880–5887
9. Sprea, F., Romani, L., Musiani, P., Modesti, A., Fattor, E., Lazzaro, D., Selliott, C., Scarpa, S., Bellavia, D., Lattanzio, G., Bistoni, F., Prati, L., Cortese, R., Gulino, A., Liberto, G., Costantino, F., and Poli, V. (1995) EMBO J. 14, 1932–1941
10. Tanaka, T., Akira, S., Yoshida, K., Uemoto, M., Yoneda, N., Shirufuji, N, Fujihara, H., Suehama, T., Yoshida, N., and Kishimoto, A. (1995) Cell 80, 333–361
11. McGrane, M. M., deVente, J., Yun, J., Bloom, J., Park, E. A., Wynshaw-Boris, A., Wagner, T., Rottman, F. M., and Hanson, R. W. (1998) J. Biol. Chem. 266, 11443–11451
12. Loose, D., Shav, P., Krauter, K. S., Robinson, C., Englund, S., Hanson, R. W., and Gluecksohn-Waelsch, S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5184–5188
13. Lo, S., Russell, J. C., and Taylor, W. A. (1970) J. Appl. Physiol. 28, 234–236
14. Kuo, E., and Kearney, E. B. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., Ed) 2nd Ed., pp. 1801–1806, Academic Press, Inc., New York
15. Hetenyi, G. Jr., and norwich, K. H. (1974) Fed. Proc. 33, 1841–1848
16. Yee-Warren, H., cimbal, M. A., Felz, K., Monahan, J. E., Leis, J. P., and Hanson, R. W. (1981) J. Biol. Chem. 256, 10224–10229
17. Liu, J., Park, E. A., Gurney, A. L., Roesler, W. J., and Hanson, R. W. (1991) J. Biol. Chem. 266, 19095–19102
18. Masillo, D., Chen, W., Barzilai, N., Prus-Wortheimer, D., Hawkins, M., Liu, R., Taub, R., and Rossetti, L. (1998) J. Biol. Chem. 273, 228–234
19. Kawai, Y., and Arinze, I. J. (1993) Mol. Cell. Endocrinol. 90, 203–209
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21. Kemp, B. E., Cheng, H. C., and Walsh, D. A. (1988) Methods Enzymol. 159, 173–183
22. Shahid, M., and Nicholson, C. D. (1995) Methods Mol. Biol. 41, 129–150
23. Ballard, F. J., and Hanson, R. W. (1987) Biochem. J. 104, 866–871
24. Graner, D. K., Andreen, T., Sasaki, K., and Beale, E. (1983) Nature 305, 549–551
25. Veres, G., Gibbs, R. A., Scherer, S. E., and Caskey, C. T. (1987) Science 237, 415–417
26. Houge, H., Vintermyr, O. K., and Dorschel, S. O. (1990) Mol. Endocrinol. 4, 481–488
27. Ecken, R., Vintermyr, O. K., Houge, G., Sand, T. E., Scott, J. D., Krebs, E. G., Eltkom, T. S., Christoffersen, T., Ogred, D., and Dorschel, S. O. (1989) J. Biol. Chem. 264, 4374–4382
28. Nigg, E. A., Schafer, G., Hille, H., and Epplenberger, H. M. (1985) Cell 41, 1039–1051
29. Dell’Acqua, M. L., and Scott, J. D. (1997) J. Biol. Chem. 272, 12881–12884
30. O’Malley, L. J., Levac, K. D., and Nagy, L. E. (1988) J. Nutr. 128, 927–933
31. Cadd, G. G., Uhler, M. D., and McKnight, G. S. (1990) J. Biol. Chem. 265, 19502–19506
32. Wang, L., Shao, J., Muhlenkamp, P., Liu, S., Klepecyk, P., Ren, J., and Friedman, J. E. (2000) J. Biol. Chem. 275, 14175–14181
33. Conti, M., and Jint, S. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5325–5329
34. Beavo, J. A., and Reifsnyder, D. H. (1990) Trends Pharmacol. Sci. 11, 150–155
35. Swinnen, J. V., Joseph, D. R., and Conti, M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5325–5329
36. Beavo, J. (1995) Physiol. Rev. 75, 725–748
37. Ahmad, F., Gao, G., Wang, L. M., Landstrom, T., Degerman, E., Pierce, J. H., and Manganuillo, V. C. (1999) J. Immunol. 162, 4864–4875
38. Degerman, E., Belfrage, P., and Manganuillo, V. C. (1997) J. Biol. Chem. 272, 6823–6826
39. Roesler, W. J., Vandenbark, G. R., and Hanson, R. W. (1989) J. Biol. Chem. 264, 9657–9664
40. Owen, O. E., Patel, M. S., Block, B. B. S., Kreunen, T. H., Reichle, F. A., and Mozzoli, M. A. (1976) in Glucagonogenesis, Its Regulation in Mammalian Species (Hanson, R. W., and Mehlman, M. A., eds) pp. 533–558, Wiley Interences, New York
41. Lopez, A. H., Moundras, C., Morand, C., Demigne, C., and Remesy, C. (1998) J. Nutr. 128, 1487–1494
42. Parrilla, R. (1978) Pfluegers Arch. Eur. J. Physiol. 374, 9–14
43. Parrilla, R. (1978) Pfluegers Arch. Eur. J. Physiol. 374, 3–7
44. Takiguchi, M., and Mori, M. (1991) J. Biol. Chem. 266, 725–748
45. Kimura, T., Christoffersen, T., Oggred, D., and Dorschel, S. O. (1990) J. Biol. Chem. 265, 2639–2643
46. Kawai, Y., and Arinze, I. J. (1981) JBC 26, 853–859