Hypoglycemia and impaired hepatic glucose production in mice with a deletion of the C/EBPβ gene

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The transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) is enriched in liver and adipose tissue and controls the expression of a wide variety of genes coding for important metabolic pathways, including gluconeogenesis and lipid synthesis. To investigate the role of C/EBPβ on glucose homeostasis, we studied mice with a targeted deletion of the gene for C/EBPβ$^{-/-}$ mice. Adult C/EBPβ$^{-/-}$ mice have hypoglycemia after an 18-hour fast, accompanied by lower hepatic glucose production (40% of that of wild-type mice), with no change in plasma insulin and a lower concentration of plasma free fatty acids (FFA). Glucagon infusion during a pancreatic clamp acutely stimulated hepatic glucose production by 38% in wild-type animals, with no change detected in C/EBPβ$^{-/-}$ mice. Unexpectedly, both the basal and glucagon-stimulated hepatic cyclic adenosine monophosphate (cAMP) levels were lower in C/EBPβ$^{-/-}$ mice, indicating an essential role for C/EBPβ in controlling proximal signal transduction. Fasting hypoglycemia was associated with normal levels of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) gene expression, however net liver glycogenolysis was impaired in C/EBPβ$^{-/-}$ mice. FFA release from isolated adipose tissue in response to epinephrine was 68% lower in C/EBPβ$^{-/-}$ mice than in control animals; however, $N^6,O^{2'}$-dibutyryladenosine (Bt$_2$) cAMP stimulated a twofold increase in FFA release in C/EBPβ$^{-/-}$ compared with no further increase in wild-type mice. Because a deletion in the gene for C/EBPβ reduces blood glucose and circulating FFA, it could be an important therapeutic target for the treatment of non–insulin-dependent diabetes and possibly obesity, based on designing antagonists that decrease C/EBPβ activity.

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Introduction

The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors includes C/EBP α, β, δ, ε, and D-binding protein; these are encoded by separate genes located on different chromosomes (1–3). The earliest finding that C/EBPα was enriched in the liver and adipose tissue suggested that it functioned to control expression of genes important for energy metabolism (4). Later studies showed that several of the C/EBP isoforms could bind and transactivate a wide variety of genes coding for specific enzymes in lipid synthesis and gluconeogenesis (5–8). Recently, gene-targeting methods have been used to generate transgenic mice bearing knockouts in the genes for C/EBPα (9–11), C/EBPβ (12–15), and C/EBPδ (15). The C/EBPα$^{-/-}$ mice are born without lipid or glycogen reserves and die of hypoglycemia within several hours after birth (9, 10), due in part to the absence of hepatic phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), and glycogen synthase in the liver in the first several hours after birth. When glucose was administered repeatedly, the mice could be sustained only 30–36 hours after birth, and the levels of gene expression appeared to recover to near adult levels by 36

hours. Morphological studies revealed immature lung development that may have contributed to their premature death. Because there was no significant increase in either C/EBPβ or C/EBPδ in the livers of these mice, it suggests that C/EBPα is part of a developmental program aimed at preparing the fetus for metabolism during the early prenatal period (15). It also suggests that C/EBPβ or C/EBPδ, expressed primarily after birth (16), are more important in controlling gene expression after the perinatal period.

Mice homozygous for a deletion of the C/EBPβ gene (also known as NF-IL6, IL-6DBP, LAP, CRP2, and NF-M; ref. 18) have a more complex pattern of glucose homeostasis. A subset of the homozygous C/EBPβ$^{-/-}$ pups die within 24 hours after birth (12–16), while the remainder are viable, but appear to have less adipose lipid accumulation, in accordance with previous studies showing that C/EBPβ is required for terminal differentiation of preadipocytes (18–23). In addition, female mice deficient in C/EBPβ are sterile and display impaired bactericidal activity of macrophages (12, 13). C/EBPβ also regulates epithelial cell proliferation and differentiation of the mammary gland (24). However,

the induction of genes coding for proteins involved in the late acute-phase response appears to be abnormal in the livers of C/EBPβ–/– mice.

In this study, we show that surviving adult C/EBPβ–/– mice display fasting hypoglycemia, decreased blood lipids, and impaired hepatic glucose output and adipose tissue lipolysis in response to hormone stimulation. These mice have a critical defect in hormone-induced cyclic adenosine monophosphate (cAMP) production in liver and adipose tissue *in vivo*. Our results also clearly demonstrate the C/EBPβ is not required for the normal induction of PEPCK, G6Pase, and glucokinase mRNA by $N^6,O^{2'}$-dibutyryladenosine (Bt$_2$) cAMP. Thus, while C/EBPβ is not necessary for basal expression of certain genes coding for proteins important for metabolic processes in the liver, it is required to ensure the appropriate concentration of cAMP, which, in turn, is critical for maintaining whole-body lipid and glucose homeostasis and for preventing hypoglycemia.

Methods

Mice. Adult female C/EBPβ–/– mice, produced as described by Screpanti *et al.* (12), were studied at 10–14 weeks of age. Genotype- and age-matched wild-type mice were used as controls. Female mice heterozygous for the mutation were bred with homozygous C/EBPβ–/– males and maintained in the Case Western Reserve University animal facility. Wild-type and C/EBPβ–/– mice were housed in microisolater cages and were maintained on a fixed 12-h light/12-h dark cycle. Animals had free access to water and were fed regular animal chow (Harlan Teklad Laboratory, Madison, Wisconsin, USA) *ad libitum*. The normal mouse
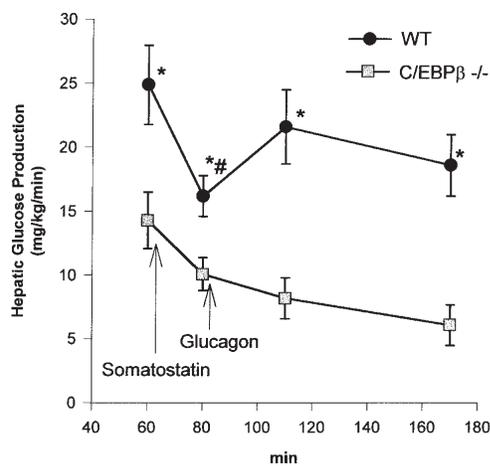

Figure 1
Decreased hepatic glucose production and glucagon resistance in adult mice homozygous for a deletion of the C/EBPβ gene (–/–). Previously catheterized mice were fasted 18 h before undergoing a pancreatic clamp as described in Methods. Somatostatin (0.8 μg/kg/min) was infused at a constant rate after the basal glucose turnover determination at 60 min to suppress endogenous insulin and glucagon secretion. After blood sampling at 80 min, glucagon infusion (0.5 μg/kg/min) was initiated for the next 100 min. Hepatic glucose production was calculated under steady-state conditions (as determined previously by repeated blood sampling every 5 min) at min 60, 80, 110, and 170 by dividing the [³H]glucose infusion rate by the mean plasma glucose specific activity. *Significantly greater than C/EBPβ–/– at each time point; *P* < 0.05. #Significantly less than 60-min or 120-min time point; *P* < 0.05. Graphs represent the mean ± SEM of eight animals in each group. *C/EBPβ,* CCAAT/enhancer-binding protein β.

chow diet used in these studies is Teklad F6 8664, containing 24% protein, 6% fat, and 4.5% crude fiber; the remainder is carbohydrate. Screening for C/EBPβ–/– mice was carried out by Southern blot analysis, as outlined previously (12).

Pancreatic clamp studies. To demonstrate the effects of glucagon on hepatic glucose production, endogenous insulin and glucagon were suppressed, and glucagon was replaced peripherally. Mice were anesthetized by intraperitoneal injection of a solution containing ketamine HCl (65 mg/kg), acepromazine maleate (2 mg/kg), and xylazine HCl (13 mg/kg; all three products from Henry Schein, Port Washington, New York, USA), and a catheter was inserted in the left jugular vein (25). The catheter was advanced to the level of the right atrium. After surgery, the animals were allowed 3–5 days of recovery before the experiment, or until body weight was within 3% of their preoperative weight. Catheterized mice were fasted 18 h before the experiment. To determine hepatic glucose production, [3-³H]glucose (Du Pont NEN Research Products, Boston, Massachusetts, USA) was infused into conscious mice using a constant pump (Harvard Apparatus Inc., South Natick, Massachusetts, USA). The animals were primed with 0.5 μCi [3-³H]glucose in the first 3 min (in 30 μl saline), followed by 0.045 μCi (in 3.2 μl saline) per minute of constant infusion throughout the experiment. For the determination of basal glucose production, 35-μl blood samples were taken from the tip of the tail at 60 min after the initiation of [3-³H]glucose infusion. Somatostatin (0.8 μg/kg/min) was infused at a constant rate after the basal glucose turnover determination at 60 min to suppress endogenous insulin and glucagon secretion. After blood sampling at 80 min, glucagon infusion (0.5 μg/kg/min) was initiated. Steady-state conditions for the calculation of hepatic glucose production was established 20 min after somatostatin infusion, based on previous experiments with repeated blood sampling every 5 min. Blood samples (35 μl each) were taken at 110 and 170 min for glucose turnover determination. The whole-body glucose turnover rate was calculated by dividing the [3-³H]glucose infusion rate by the mean plasma glucose specific activity. The livers were removed at the end of the somatostatin clamp and rapidly freeze-clamped for analysis of glycogen (26).

Glucagon-stimulated cAMP production in liver in vivo. C/EBPβ–/– and control mice were lightly anesthetized, and a liver biopsy was obtained to measure the basal concentration of cAMP. Glucagon was then delivered through the portal vein at 50 μg/kg. One minute after the glucagon injection, a second piece of liver was biopsied to determine the cAMP levels. The liver samples were rapidly frozen using steel tongs cooled to the temperature of liquid nitrogen. Liver samples were stored at –80°C until they were analyzed. For the assay of cAMP, the frozen samples were first homogenized in ice-cold TCA (6% wt/vol), and cAMP was determined using an enzyme immunoassay kit from Amersham Life Sciences Inc. (Arlington Heights, Illinois, USA). These study protocols were reviewed and approved by the Case Western Reserve University Animal Care and Use Committee.

Bt$_2$cAMP administration and Northern blot analysis in livers of C/EBPβ–/– and control mice. Mice were injected intraperitoneally with 35 mg Bt2cAMP/kg of body weight and 30 mg/kg of theophylline in 150 μl of saline, sacrificed 2 h later (27), and their livers frozen at –80°C until they were analyzed. RNA was isolated as described previously (16), and 20 μg of total RNA was electrophoresed through a 1.5% denaturing formaldehyde agarose gel and transferred to Gene Screen (Du Pont NEN Research Products) using the method of Chirgwin *et al.* (28). The cDNA probes were labeled by random priming using a labeling kit (Boehringer Mannheim, Indianapolis, Indiana, USA), according to the manufacturer's instructions. The PEPCK cDNA probe was a 1.6-kb *Pst*I fragment (29). The G6Pase probe was a 1.1-kb *Xba*I–*Pst*I fragment of human cDNA used as described previously (30). The glucokinase cDNA probe was a 3.3-kb fragment obtained from Mark Magnuson (Van-

derbilt University, Nashville, Tennessee, USA). For quantitative analysis, the intensity of the hybridized band was scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA), and densitometric values were normalized to mouse β-actin as described previously (31).

Effect of epinephrine and cAMP on lipolysis by adipose tissue in vitro. Periovarian fat-pads weighing 50–70 mg were isolated from fed C/EBPβ$^{-/-}$ and control mice and incubated in 1 ml of Krebs-Henseleit buffer with 3% porcine albumin (essentially fatty acid free; Sigma Chemical Co., St. Louis, Missouri, USA) and 5 mM glucose at 37°C in a shaking water bath (30 cycles/min) for 3 h. The medium was gassed with 95% O_2/5% CO_2. Of the two fat-pads from each animal, one was incubated with epinephrine (5 μg/ml; Sigma Chemical Co.) and 0.01% ascorbic acid (Sigma Chemical Co.), while the other was incubated with a maximal dose of Bt_2 cAMP (0.43 mg/ml) and theophylline (Sigma Chemical Co.). At the end of the 3-h incubation, the medium was frozen at –20°C for free fatty acid (FFA) and glycerol analysis. DNA was measured in adipose tissue samples taken from the animal and frozen in liquid nitrogen, and was quantified in crude homogenates by a spectrofluorometric assay (32).

Analytical procedures. The concentration of unlabeled plasma glucose was determined by the glucose oxidase method using a glucose diagnostic kit from Sigma Chemical Co. For the determination of [3-^{3}H]glucose, 10 μl of plasma was first mixed with an equal volume of 20% TCA to precipitate protein. The supernatant was then heated at 65°C to dryness to evaporate [^{3}H]H_2O. The samples were then reconstituted in 100 μl H_2O before 5 ml of scintillation fluid (Fisher Scientific Co., Pittsburgh, Pennsylvania, USA) was added. Radioactivity was detected using a β scintillation counter. The concentration of FFA, lactate, and β-hydroxybutyrate was measured using an automated chemistry analyzer (COBAS; Roche Diagnostic, Nutley, New Jersey, USA), or with diagnostic reagent kits from Sigma Chemical Co. (lactate, triglycerides) or Wako Pure Chemical Industries (nonesterified FFA). Glycerol was measured by enzymatic determination using glycerol kinase coupled with glycerophosphate dehydrogenase, according to the conditions supplied by the manufacturer (Boehringer Mannheim). Insulin and corticosterone levels in plasma were determined using radioimmunoassay kits from Linco Research Inc. (St. Charles, Missouri, USA) and ICN Pharmaceuticals Inc. (Costa Mesa, California, USA). Glucose turnover was calculated by dividing the infusion rate of [3-^{3}H]glucose (dpm/kg/min) by the specific radioactivity (dpm/mg glucose) in the plasma. Glycogen was extracted from frozen livers by homogenization in 6% perchloric acid, precipitated in ethanol, hydrolysed by boiling in 1 N HCL, and glucose measured as for plasmas (33). Statistical comparisons between groups were made using Student's *t* test.

Results

Metabolic characteristics of C/EBPβ$^{-/-}$ and wild-type mice. Adult C/EBPβ$^{-/-}$ mice weigh approximately 10% less than their wild-type counterparts after fasting overnight (Table 1), but this difference is not statistically significant. The weight of the periuterine fat-pad from overnight-fasted mice is approximately 50% of that found in control mice. The amount of DNA per gram of adipose tissue was 40% greater in C/EBPβ$^{-/-}$ mice than wild-type controls, suggesting reduced lipid content per cell. The concentration of glucose in the plasma of C/EBPβ$^{-/-}$ mice was similar in the fed state. After 18-h fasting, plasma glucose is about 30% lower in C/EBPβ$^{-/-}$ mice than in control animals, while insulin levels were comparable between the two groups. The initial concentration of glycogen in the livers of C/EBPβ$^{-/-}$ mice was

similar to that noted in control animals. The levels of circulating triglyceride in overnight-fasted C/EBPβ$^{-/-}$ mice were reduced by 31% compared with controls. The concentration of corticosterone in the blood of C/EBPβ$^{-/-}$ was slightly increased in C/EBPβ$^{-/-}$ compared with wild-type mice, a situation that normally favors increased glucose output from the liver (30, 31).

Pancreatic clamp studies. A lower concentration of blood glucose after fasting could be caused by either lower hepatic glucose production or increased peripheral glucose uptake. To delineate the mechanism for the lowered circulating level of glucose, hepatic glucose production (HGP) was determined under basal (no hormone infusion) and maximum-effective glucagon-infusion stages. Basal HGP was about 40% lower ($P < 0.05$) for the C/EBPβ$^{-/-}$ mice compared with normal littermates (Fig. 1). Infusion of somatostatin was used to suppress endogenous insulin and glucagon secretion. Under these conditions, HGP decreased to 16.2 ± 1.6 mg/kg/min (35%) in control mice and to 10.1 ± 1.3 mg/kg/min (29%) in C/EBPβ$^{-/-}$ mice; however, only the decrease in control animals was statistically significant ($P < 0.05$). Thirty minutes after the initiation of glucagon infusion, HGP increased to 21.6 ± 2.9 mg/kg/min (33%; $P < 0.05$) in control mice. HGP was decreased to 8.2 mg/kg/min (not statistically significant) in C/EBPβ$^{-/-}$ mice in response to glucagon infusion. HGP was lower in C/EBPβ$^{-/-}$ mice, compared with wild-type mice, throughout the pancreatic clamp ($P < 0.05$).

To determine the factor(s) contributing to the lowered basal HGP and impaired response to glucagon stimulation, selected plasma metabolites, as well as hepatic glycogen content, were determined at the end of the pancreatic clamp (Table 2). Both FFA and 3-hydroxybutyrate were 50% lower in C/EBPβ$^{-/-}$ mice than in control animals ($P < 0.05$). There was also a 43% decrease in the concentration of lactate in the plasma of C/EBPβ$^{-/-}$ mice ($P < 0.05$). The liver glycogen remaining at the end of the pancreatic clamp was greater in C/EBPβ$^{-/-}$ mice ($P < 0.05$). The net hepatic glycogen depletion, calculated based on the ini-
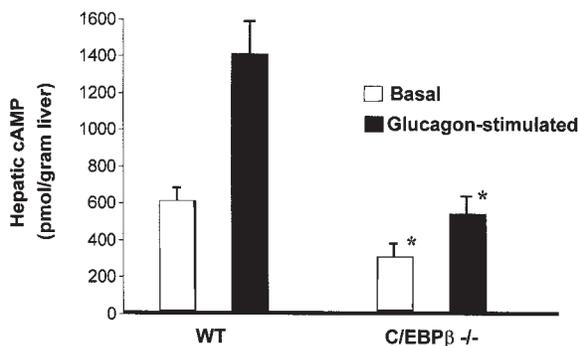

Figure 2

Reduced hepatic cAMP production in mice homozygous for a deletion of the C/EBPβ gene (–/–). C/EBPβ$^{-/-}$ and WT control mice were lightly anesthetized, and a liver biopsy was freeze-clamped to measure the basal concentration of cAMP. Glucagon was then delivered through the portal vein at 50 μg/kg. One minute after the glucagon injection, a second piece of liver was biopsied for the determination of cAMP levels. The data are mean ± SEM for seven to eight animals per group. *Significantly reduced compared with WT mice; $P < 0.05$. *WT*, wild-type.
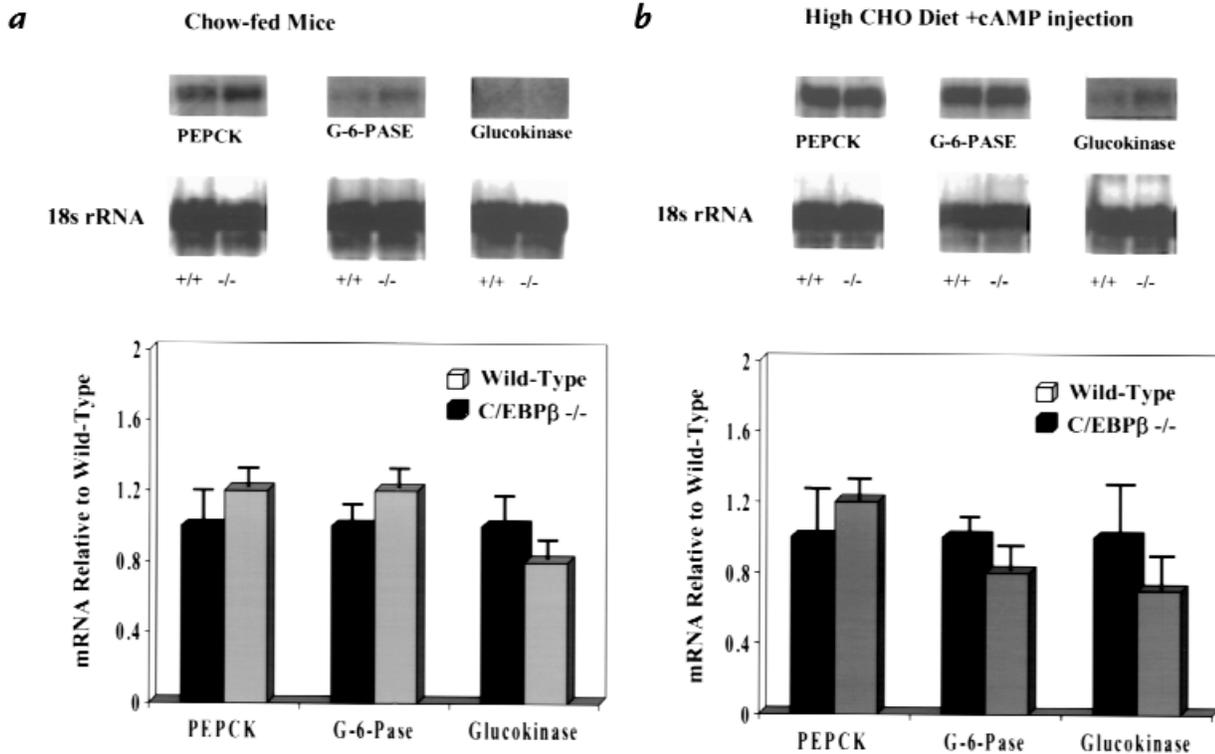
Figure 3

Liver gene expression in adult WT mice and mice homozygous for a deletion of the C/EBPβ gene (–/–). (*a*) Ten-week-old female C/EBPβ$^{-/-}$ and WT mice were fasted overnight before being anesthetized and sacrificed, and the total liver RNA was isolated. The RNA was subjected to Northern blot analysis, and the blot was hybridized sequentially with probes for PEPCK, G6Pase, and glucokinase. Results were normalized to the level of actin mRNA and expressed relative to WT controls. (*b*) Effect of Bt$_2$cAMP on the expression of genes for PEPCK, G6Pase, and glucokinase. Mice were fed a high-carbohydrate diet (81% sucrose) for 5 days, followed by intraperitoneal injection with 60 mg Bt$_2$cAMP/kg of body weight and 25 μg of theophylline in 150 μl of saline, and were sacrificed 2 h later (27). The livers were frozen at –80°C until they were analyzed. RNA was isolated and Northern blot analysis carried out as described in *a*. The bars represent the mean ± SEM of four to six animals per group. *PEPCK,* phosphoenolpyruvate carboxykinase; *G6Pase,* glucose-6-phosphatase; *Bt$_2$, N^6,O$^{2'}$*-dibutyryladenosine.

tial fed glycogen levels, was 22% lower in C/EBPβ$^{-/-}$ mice compared with wild-type littermates (*P* < 0.05), indicating an impairment in either breakdown or mobilization in response to fasting and glucagon infusion**.**

Glucagon-stimulated cAMP production in liver in vivo. The higher residual glycogen, and yet lower HGP in response to glucagon stimulation, suggests there might be a defect(s) in hepatic glycogen mobilization in the C/EBPβ$^{-/-}$ mice. To locate the potential defect(s), a liver biopsy was taken before and after glucagon injection, and cAMP levels in liver were determined for both fed control and C/EBPβ$^{-/-}$ mice (Fig. 2). The basal concentration of cAMP in the livers of C/EBPβ$^{-/-}$ mice was 47% of that noted in the livers of normal littermates (0.28 and 0.59 μmol/g tissue, respectively; *P* < 0.05). After glucagon injection, the concentration of hepatic cAMP increased to 1.38 (μmol/g tissue) in control mice compared with 0.50 in C/EBPβ$^{-/-}$ mice (*P* < 0.05). These results suggest that a defect(s) exists in coupling between the glucagon receptor and cAMP production in C/EBPβ$^{-/-}$ mice.

Bt$_2$cAMP administration and gene expression in livers of C/EBPβ$^{-/-}$ and control mice. To determine the role of C/EBPβ in the expression of genes involved in hepatic glucose homeostasis, we determined the level of PEPCK, G6Pase, and glucokinase mRNA in the livers of adult

C/EBPβ$^{-/-}$ and control mice in response to cAMP administration. The expression of PEPCK, G6Pase, and glucokinase mRNAs were similar in C/EBPβ$^{-/-}$ and control mice after an overnight fast (Fig. 3*a*). Two hours after injection of Bt$_2$cAMP, the relative levels of PEPCK and G6Pase mRNA in the livers of C/EBPβ$^{-/-}$ mice increased to the same extent in both C/EBPβ$^{-/-}$ and control mice (Fig. 3*b*), indicating that cAMP treatment can induce a normal transcriptional response in these two genes, even in the absence of C/EBPβ. There was no statistically significant acute effect of cAMP injection on expression of glucokinase mRNA. The concentration of glycogen was also analyzed and was similar in wild-type and C/EBPβ$^{-/-}$ mice after Bt$_2$cAMP administration (wild-type 1.3 ± 0.3 vs. C/EBPβ$^{-/-}$ 1.8 ± 0.5 mg glucose/g liver).

Effect of epinephrine and cAMP on lipolysis from adipose tissue. Gluconeogenesis is stimulated, in part, by increased supply of lactic acid and by increased circulating FFA and glycerol, released from adipose tissue in response to hormonal stimulation. The expression of adipocyte-specific genes and adipogenic transcription factors appears to be nearly indistinguishable in interscapular fat of C/EBPβ$^{-/-}$ mice and normal littermates (13). However, the lower circulating FFA levels observed in C/EBPβ$^{-/-}$ mice could be caused by a defect(s) in the generation of cAMP in

response to stress hormone stimulation in adipose tissue. To investigate the effect of epinephrine and Bt$_2$cAMP on the release of FFA and glycerol from adipose tissue, we obtained adipose tissue from fed control and C/EBPβ$^{-/-}$ mice (Fig. 4). In response to epinephrine, FFA release was stimulated more than 20-fold in wild-type mice, while the FFA released from fat-pads of C/EBPβ$^{-/-}$ mice was reduced by 68% compared with wild-type mice ($P < 0.05$). However, in the presence of Bt$_2$cAMP, FFA released into the incubation medium increased twofold above epinephrine-stimulated FFA levels in C/EBPβ$^{-/-}$ mice ($P < 0.05$), compared with no further change in wild-type mice ($P < 0.05$). These results may reflect a decreased ability of adipose tissue from C/EBPβ$^{-/-}$ mice to generate cAMP; the same result was observed in the blunted response of these animals in mobilizing hepatic glycogen in response to administered glucagon. We also analyzed the cAMP levels in adipose tissue from wild-type and C/EBPβ$^{-/-}$ mice. The levels of cAMP were 42% greater in the wild-type mice compared with C/EBPβ$^{-/-}$ mice (wild-type 24.4 ± 3.9 vs. 14.6 ± 2.18 pmol/mg DNA; $P < 0.05$). These differences in cAMP levels observed in adipose tissue correlate well with the differences observed in the liver of C/EBPβ knockout mice. The ratio of FFA to glycerol (an indication of FFA reesterification) was 2:1 in both wild-type and knockout mice. Thus, the impaired FFA release in C/EBPβ$^{-/-}$ mice is most likely due to an impairment in the ability to generate cAMP in response to epinephrine.

Discussion

Mice homozygous for a deletion of the gene for C/EBPβ were initially generated to study the effects of C/EBPβ on the interleukin-6 (IL-6) signaling pathway. Screpanti *et al.* (12) reported that the mice developed a pathology similar to animals that overexpress the cytokine IL-6; beginning at 16 weeks of age, they display splenomegaly, peripheral lymphadenopathy, enhanced hemopoiesis, and an altered T-helper function. Despite these problems with the immune system, the mice had no overt disruption of glucose homeostasis. However, both Screpanti *et al.* (12) and Tanaka *et al.* (13) noted a failure to obtain the expected mendelian ratio of adult mice homozygous for a deletion in the gene for C/EBPβ, although the appropriate number of C/EBPβ$^{-/-}$ mice were present at 20 days of fetal life. Recently, Croniger *et al.* (16) reported that there are two

phenotypes noted with the C/EBPβ$^{-/-}$ mice. Under pathogen-free conditions, approximately one-half to two-thirds of the mice (phenotype A) appear to survive normally, while the remaining one-half to one-third of the C/EBPβ$^{-/-}$ mice (phenotype B) die within 24 hours after birth of profound hypoglycemia (our unpublished results). Animals with the B phenotype have normal levels of hepatic glycogen but do not initiate the transcription of the gene for PEPCK in the liver, which is characteristic of the neonatal period. The lack of a full response of the liver to administered glucagon observed in this study provides a potential mechanism to explain the inability of the C/EBPβ$^{-/-}$ mice (B phenotype) to survive the perinatal period. Their death is likely due to a failure of the liver to respond to the elevated concentration of glucagon and epinephrine with an increased intracellular level of cAMP.

In this study, we find that C/EBPβ is required for liver and adipose tissue to respond to the normal signals that increase glucose production and adipose tissue lipolysis. This suggests that the absence of C/EBPβ may cause down-regulation of genes coding for proteins responsible for cAMP generation, resulting in a lower concentration of glucose and FFA in the blood. Because the metabolic response to both epinephrine and glucagon is impaired in the C/EBPβ$^{-/-}$ mice, it is likely that the defect is not in the receptors for these two hormones, but rather in the G-pro-

Table 1

Metabolic characteristics of wild-type and C/EBPβ$^{-/-}$ mice

	Wild-type	C/EBPβ$^{-/-}$
Body weight (g)	24.8 ± 1.4	22.4 ± 1.7
Fat-pad weight (mg)	360 ± 47	168 ± 37[A]
DNA content (μg/mg)	5.9 ± 1.6	9.6 ± 2.6[A]
Plasma glucose, fed (mg/dl)	169 ± 11	152 ± 4
Plasma glucose, fasted (mg/dl)	142 ± 7	104 ± 10[A]
Liver glycogen (mg/g liver)	11.6 ± 5.6	14.0 ± 2.6
Triglyceride (mg/dl)	144 ± 63	99 ± 15
Insulin (ng/ml)	0.35 ± 0.06	0.32 ± 0.03
Corticosterone (ng/ml)	28.6 ± 10.7	32.2 ± 10.6

The average values for body weight, plasma glucose, triglycerides, and hormone levels were measured in wild-type and C/EBPβ$^{-/-}$ mice after an 18-h overnight fast. Plasma glucose and glycogen levels were also measured in the fed state. Values are mean ± SEM with 6–12 observations. [A]$P < 0.05$ vs. wild-type mice. *C/EBPβ*, CCAAT/enhancer-binding protein β.

Figure 4

Impaired lipolysis in fat-pads from mice homozygous for a deletion of the C/EBPβ gene (−/−). Periovarian fat-pads weighing 50–70 mg were isolated from fed C/EBPβ$^{-/-}$ and WT control mice and incubated in 1 ml of Krebs-Henseleit buffer with 3% porcine albumin and 5 mM glucose at 37°C in a shaking water bath (30 cycles/min) for 3 h. The medium was gassed with 95% O$_2$/5% CO$_2$. Of the two fat-pads from each animal, one was incubated with epinephrine (5 μg/ml) and 0.01% ascorbic acid, while the other was incubated with a maximal dose of Bt$_2$cAMP (0.43 mg/ml) and theophylline (25 μg/ml). At the end of the 3-h incubation, the medium was frozen at −20°C for FFA and glycerol analysis as described in Methods. The DNA content was quantified in adipose tissue crude homogenates by a spectrofluorometric assay (31). The bars represent the mean ± SEM of 8–10 animals per group. *Significantly reduced compared with WT; $P < 0.05$. +Significantly increased compared with epinephrine-treated; $P < 0.05$. *FFA*, free fatty acids
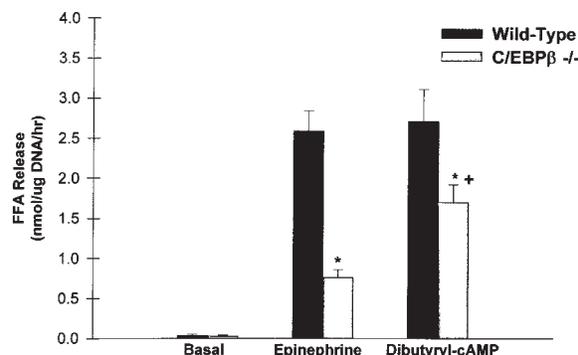
Table 2

Metabolite levels in wild-type and C/EBPβ⁻/⁻ mice at the end of the pancreatic clamp

	Wild-type	C/EBPβ⁻/⁻
Nonesterified FFA (mM)	0.68 ± 0.08	0.33 ± 0.05[A]
3-hydroxybutyrate (mg/dl)	16.0 ± 2.1	7.72 ± 2.4[A]
Lactate (mg/dl)	26.3 ± 3.3	15.0 ± 0.95[A]
Liver glycogen (mg/g liver)	4.0 ± 0.23	7.9 ± 2.14[A]
Net liver glycogen depletion(Δmg/g liver)	7.8 ± 0.23	6.1 ± 0.14[A]

Plasma metabolites and the concentration of liver glycogen were determined at the end of 3 h of the pancreatic clamp as outlined in Methods and in Fig. 1. The calculation of net glycogenolytic rates was based upon subtracting the liver glycogen content at the end of the clamp from average fed liver glycogen levels in a separate set of mice. Values are mean ± SEM with 5–12 observations. [A]$P < 0.05$ vs wild-type mice. *FFA*, free fatty acids.

tein coupling mechanism or in adenylate cyclase. The mechanism for decreased cAMP production could be a decrease in the quantity of Gs and/or an increase in Gi at the plasma membrane. The levels of G proteins were measured in enriched liver plasma membranes from C/EBPβ⁻/⁻ mice by Western blotting, using specific antibodies (data not shown). Our results indicated there were no changes in the levels of these proteins in the C/EBPβ knockout mice (Arinze, K., and Croniger, C., unpublished observations). In addition, the maximal activity of phosphodiesterase in total membranes and supernatants prepared from the livers C/EBPβ⁻/⁻ mice did not reveal any significant differences between wild-type and knockout mice (Arinze, K., and Croniger, C., unpublished data). Thus, the molecular mechanisms responsible for reduced cAMP remain unclear at the moment. Further studies are underway to determine the exact nature of the defect in stimulation of cAMP by glucagon and epinephrine C/EBPβ⁻/⁻ mice.

The decreased rate of hepatic glucose production in response to glucagon noted in the pancreatic clamp studies presented in Fig. 4 may be due to decreased cAMP production. However, it has not been established whether replacing normal levels of circulating fatty acids and lactate, which have a stimulatory effect on gluconeogenesis (34), would be sufficient to induce an increase in hepatic glucose production to restore normal blood glucose in mice missing C/EBPβ. The mice also demonstrated an equivalent level of PEPCK gene expression after an overnight fast, suggesting the failure of C/EBPβ⁻/⁻ mice to acutely respond to glucagon stimulation may be attributable to defects in other rate-limiting steps in the gluconeogenic pathway, such as the bifunctional enzyme 6-PFK-2/Fru-2,6-P2ase or pyruvate kinase, both of which undergo cAMP-dependent phosphorylation to increase gluconeogenic flux in the liver. In agreement with this study, Greenbaum *et al.* (35) recently demonstrated that in C/EBPβ⁻/⁻ mice, PEPCK and G6Pase mRNA were induced normally in response to partial hepatectomy, but the animals remained hypoglycemic. These results suggest that C/EBPβ affects metabolic processes in the liver that are independent of activation of gluconeogenic genes but are critical for preventing hypoglycemia.

The impaired lipolysis observed in adipocytes from C/EBPβ⁻/⁻ mice is consistent with reduced circulating FFA levels. A decreased ability to mobilize FFA would be expected, in the long term, to increase fatty acid deposition within the adipose tissue. However, mice lacking the C/EBPβ gene exhibit approximately 35% less epididymal white adipose tissue mass at eight weeks of age, despite normal expression pattern of adipogenic marker genes (13). The adipocyte size in differentiated adipocytes from C/EBPβ⁻/⁻ mice appears smaller compared with wild-type mice (13), suggesting that loss of C/EBPβ gene expression is associated with reduced lipid accumulation. Indeed, our estimates of cell number, based on the amount of DNA per gram of tissue, suggest that C/EBPβ⁻/⁻ mice have reduced lipid content per cell. Tanaka *et al.* (13) also reported that mice homozygous for a deletion in the genes for both C/EBPβ and C/EBPδ had a more severe reduction in size of the white adipose tissue than was noted with mice where the genes for either C/EBPβ or C/EBPδ were deleted individually. Interestingly, there was no upregulation of expression of the gene for C/EBPα, which is known to be required for the normal development of white adipose tissue (20). The overall effect exerted by the C/EBPβ knockout on adipose tissue mass may depend on C/EBPβ-controlled genes involved in esterification, lipolysis, or fatty acid oxidation, in addition to differentiation. The decreased plasma FFA in the C/EBPβ⁻/⁻ mice may be due to a reduced size of the adipose tissue mass. However, even accounting for cell number, there is a reduced β-adrenergic stimulation of FFA release. Thus, a major reason for hypoglycemia in the C/EBPβ⁻/⁻ mice appears to be a lack of counterregulation by stress hormones.

To our knowledge, the finding that C/EBPβ is a critical component for regulating cAMP levels *in vivo* represents the first demonstration that a transcription factor participates in regulation of proximal signal transduction pathways from the epinephrine and glucagon receptor. The glucagon and β-adrenergic receptors share a similar signal transduction pathway with the gonadotropin hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to generate cAMP. This may explain why C/EBPβ⁻/⁻ females are infertile (12–13) as well as hypoglycemic, and have reduced FFA as reported here. Because we have identified a lesion associated with the generation of cAMP, the C/EBPβ⁻/⁻ mice should provide a unique model system to define the essential genes involved in a common signal transduction pathway encoded by C/EBPβ. C/EBPβ has also been shown to interact cooperatively in steroid hormone regulation of gene expression, including signaling from the glucocorticoid (36–38) and estrogen receptor (39). We have found that inactivation of C/EBPβ attenuates the normal induction of liver PEPCK mRNA during streptozotocin diabetes (Arizmendi, C., and Friedman, J.E., manuscript submitted for publication), a pathway that requires activation by glucocorticoids (30, 31). Thus, it is likely that C/EBPβ represents a funnel through which several extracellular signaling pathways are potentially channeled to give rise to transcriptional activation of many different genes. The observation that C/EBPβ deficiency decreases cAMP production and reduces circulating lipid and glucose levels adds a new dimension to our understanding of the role of C/EBPβ in energy metabolism and could lead to potential new therapeutic opportunities for anti-diabetes therapy. Further

studies will be required to determine whether transcriptional regulation by other hormones such as insulin and glucocorticoids, which regulate the various C/EBP isoforms, are preserved in C/EBPβ⁻/⁻ mice.

Acknowledgments

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