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ARTICLE

Cre Recombinase-Dependent Expression of a Constitutively Active Mutant Allele of the Catalytic Subunit of Protein Kinase A

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Summary: Using the cre-loxP recombination system, we generated a line of mice expressing a constitutively active catalytic subunit of Protein Kinase A (PKA) in a temporally and spatially regulated fashion. In the absence of cre recombinase the modified catalytic subunit allele is functionally silent, but after recombination the mutant allele is expressed, resulting in enhanced PKA effects at basal cAMP levels. Mice expressing the modified protein in hepatocytes using albumin-cre transgenics show defects in glucose homeostasis, glycogen storage, fructose 2,6-bisphosphate levels, and induction of glucokinase mRNA during feeding. Similar to animals lacking glucokinase in the liver (Postic *et al.:* J Biol Chem 274:305–315, 1999), these mice also have defects in glucose-stimulated insulin secretion, a hallmark of Type II diabetes. The widespread expression of PKA and the involvement of this kinase in a myriad of signaling pathways suggest that these animals will provide critical tools for the study of PKA function in vivo. genesis 43:109–119, 2005. **© 2005 Wiley-Liss, Inc.**

Key words: cAMP; mouse genetics; liver; hepatocyte; glucose; insulin; diabetes

INTRODUCTION

The ability to manipulate genes in specific cell types using the cre-*loxP* recombination system, coupled with the ability to place point mutations within the mouse germline, has permitted precise modulation of protein function. Using these strategies, we generated a unique mouse model whereby expression of a constitutively active allele of Protein Kinase A (PKA) can be controlled with regional and temporal specificity.

PKA exists as a holoenzyme composed of combinations of two catalytic (C) and two regulatory (R) subunits. When complexed with R subunits, catalytic activity by C proteins is minimal. When cAMP levels rise, cAMP-binding sites are cooperatively occupied on the R subunits, resulting in a conformational change in the complex. The C subunits are subsequently released, inducing phosphorylation of substrate proteins at the consensus site RRXS/T. In mice, there are two PKA catalytic subunits, termed Cα and Cβ. Four regulatory subunits, termed RIα, RIIα, RIβ, and RIIβ, participate in the formation of the PKA holoenzyme.

Previous studies in our laboratory have identified several mutant Cα proteins that retain full catalytic activity but are partially defective in their ability to bind to R subunits. The functional consequences of these mutations are an enhanced basal level of PKA activity. Two mutations, resulting in the conversion of either histidine 87 to glutamine or tryptophan 196 to arginine, have been shown to be particularly effective in their ability to prevent C subunit interaction with R. Simultaneous mutations at these two positions have a synergistic effect, suggesting that these amino acids provide important structural information for mediating C-R interaction (Orellana and McKnight, 1992).

Further experimentation has defined the functional consequences of these two mutations and their effect on the function of PKA in vitro. These studies showed that the tryptophan to arginine mutant (here called CαR) could bind to R subunits if they were experimentally stripped of cAMP; normal cellular concentrations of cAMP, however, prevented effective inhibition of CαR by either RI or RII proteins (Gibson and Taylor, 1997; Orellana and McKnight, 1992). The mechanism responsible for this phenotype was an enhanced dissociation of

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the CαR protein from R subunits in the presence of cAMP, resulting in a 9-fold lower K_a for cAMP activation. Maximal catalytic activity, however, was unchanged. These studies suggested that expression of the CαR protein could result in enhanced catalytic activity in vivo, especially at low levels of cAMP, making it an attractive and unique method for manipulating PKA activity.

Employing the cre-*loxP* recombination system, we introduced a "silent" floxed allele encoding the tryptophan to arginine mutation into the Cα gene within the mouse genome. In this study, we examined the physiological consequences of expression of this mutant form of PKA in the liver. Due to its role in the production, storage, and release of glucose, the liver represents an attractive target for therapeutic intervention in Type II diabetes management. Hepatocytes hold a unique role in glucose metabolism, serving to store glucose during times of plenty and release glucose in times of need (Tirone and Brunicardi, 2001). This balance is maintained by the opposing influences of the insulin:cAMP axis; while insulin promotes energy storage, cAMP (and, by extension, PKA) serves as the intracellular signal downstream of glucagon and adrenergic receptors to promote glucose release. We predicted that the consequences of liver-specific expression of CαR would be to promote liver glucose output and result in glucose intolerance and fasting hyperglycemia. If correct, these mice might then serve as a model system to further explore whether primary changes in liver glucose regulation can contribute to the development and progression of Type II diabetes. The results described here demonstrate that mice expressing CαR in the liver do show impairments in glucose regulation that vary depending on the nutritional status of the animal. Surprisingly, the expression of this mutation also results in dramatic changes in insulin levels, suggesting that there exists communication between the liver and pancreatic β-cell that is influenced by liver PKA activity. The widespread expression of the Cα gene as well as the increasing availability of distinct lines of mice expressing cre recombinase in a tissue-specific fashion assures that the CαR mouse will prove useful for the study of PKA-mediated signal transduction in a myriad of normal and pathophysiological processes.

RESULTS

Embryonic Stem Cells Containing the CαR Allele Are Functional Cα Heterozygotes

Embryonic stem (ES) cells that encoded the correctly integrated silent CαR targeting vector were identified using Southern and polymerase chain reaction (PCR) analyses (Fig. 1). Reverse-transcriptase PCR (RT-PCR) analyses of RNA derived from these clones revealed an extremely low level of mutant RNA in clones with the integrated targeting vector, indicating that the presence of the intronic neomycin cassette efficiently interfered with production of full-length mutant CαR mRNA in CαR cells (Fig. 1d). PKA-specific activity analyses of cells encoding the CαR allele indicated that, as predicted, the cells behave as Cα heterozygotes, exhibiting a 50% decrease in kinase as measured by both the basal and cAMP-stimulated levels (Fig. 1e). Western analyses (not shown) revealed that CαR cells express half the level of Cα protein compared to cells lacking the modified allele.

Cre Recombinase-Mediated Recombination of the CαR Allele Results in Increased Basal PKA Activity in ES Cells

Transfection of CαR ES cells with a plasmid encoding cre recombinase resulted in efficient excision of the neomycin cassette, generating the predicted recombined allele (termed CαR/*cre*) and the production of mutant mRNA (Fig. 1d). A comparison of kinase activity between wildtype (WT), CαR, and CαR/*cre* cell lines showed that CαR/*cre* cells exhibited a significant increase in basal kinase levels compared to WT and CαR cell extracts (Fig. 1e). Total, cAMP-dependent kinase activity and Cα protein levels (not shown) derived from extracts of CαR/*cre* cells were intermediate between WT and CαR cells, suggesting rapid proteolysis of the free CαR protein in ES cells. This rapid proteolysis of the CαR protein was expected, since it has been shown that C subunits that are not bound in a holoenzyme with R are turned over rapidly in cells, accounting for the dramatic downregulation of kinase activity when cells are chronically stimulated by cAMP (Amieux *et al.*, 1997).

CαR Allele Is Silent In Vivo in the Absence of Cre Recombinase in All Tissues Examined With the Exception of Testes

Cells encoding the CαR allele were injected into blastocysts to generate chimeras. Mating of male chimeras to C57Bl/6J females produced pups heterozygous for the mutant allele. These mice exhibit no overt phenotype and are fertile. To test the "silence" of the CαR allele, heterozygotes were mated to produce CαR homozygotes and these mice were phenotypically compared to Cα knockout animals previously generated by replacing exons 6–8 with neomycin sequence (Skalhegg *et al.*, 2002). Mice lacking Cα are growth-retarded and exhibit greatly reduced PKA-mediated kinase activity. Mice homozygous for the CαR allele appear outwardly indistinguishable from Cα null animals and show the same growth retardation. Western analyses indicated that there was no detectable Cα protein in tissues examined with the exception of testes, suggesting that, in vivo, CαR functions effectively as a null allele in the absence of cre recombinase in most tissues. Kinase activity assays from diverse tissues revealed a profile similar to that seen in Cα null animals (Skalhegg *et al.*, 2002), with CαR homozygote tissue exhibiting very low kinase activity levels (data not shown) that are consistent with the presence of the Cβ gene that is expressed at low levels in many tissues. The exception was observed when extracts from testes of CαR homozygotes were exam-

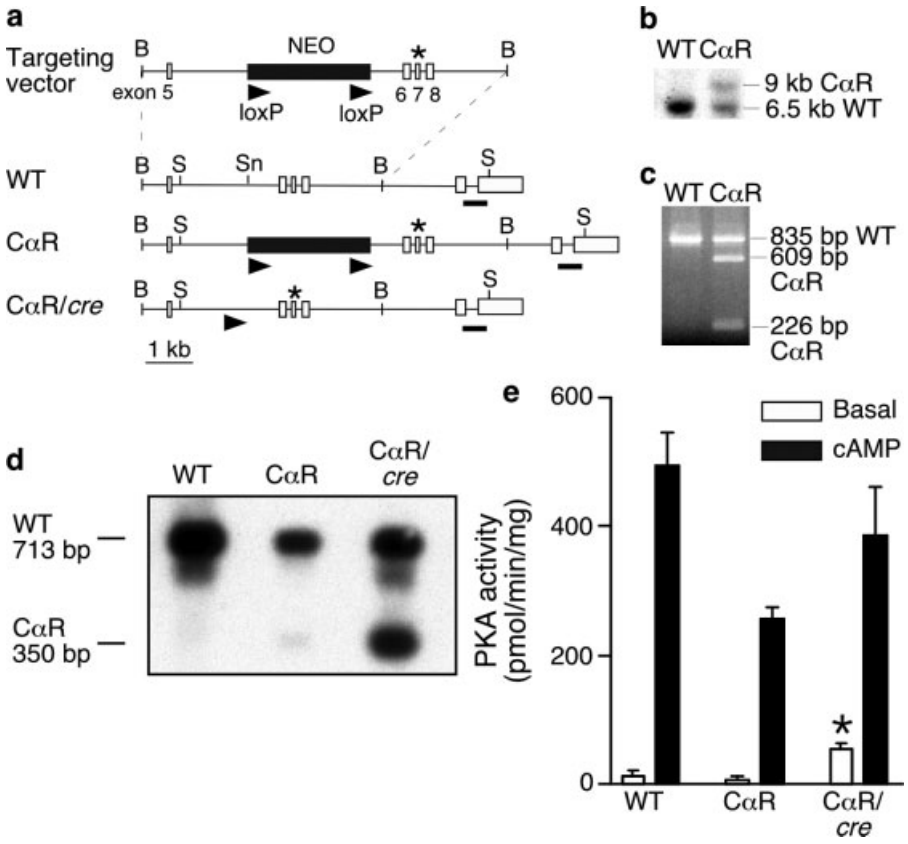

FIG. 1. Targeting strategy and the development of CαR and CαR/*cre* ES cells. **a:** Top: schematic depiction of the targeting vector used for these studies is shown. Exons 5–8 are represented as white boxes, the SV40 neomycin resistance cassette (NEO) is depicted as a black box, and the *loxP* sequences are indicated by wedges. Middle: the WT Cα gene is shown and the hybridization probe used for screening in **b** is indicated by a black bar. Bottom: after exposure of the allele to Cre recombinase, the neomycin cassette is removed and one *loxP* site is retained. This converts the allele from CαR (silent) to CαR/*cre* (expressing). **b:** Southern screening of heterozygous CαR ES cells. The probe depicted in **a** was used to screen Sac I-digested ES cell DNA; a 6.5 kb band represents the WT allele and a 9-kb band indicates correct integration of the targeting vector within the Cα locus. **c:** PCR analysis to confirm the presence of the W196R mutation. PCR fragments were amplified as described in Materials and Methods, digested with *Mlu I*, and resolved on a 2% agarose gel; the WT product is 835 bp and the digested CαR allele is represented by bands of 609 and 226 bp. B = *BamHI*, S = *SacI*, Sn = *SnaBI*. **d:** RT-PCR and restriction enzyme analysis of WT, CαR, and CαR/*cre* ES cells was performed; the ratio of bands derived from WT (713 bp) and CαR (350 bp) mRNA was quantified using Southern blotting. **e:** PKA-specific activity assays were performed in triplicate on extracts of ES cells genotyped as WT, CαR, or CαR/*cre* (*$P = 0.0121$, one-way ANOVA, basal PKA activity, n = 3–6 cell lines per genotype).

ined; these samples showed a significant elevation of basal PKA activity levels compared to WT mice and indicate that the tandem poly A signal at the 3′ end of the neomycin resistance cassette was not completely effective in preventing continued transcription of the Cα allele and splicing around the neo cassette in testis.

Recombination of the CαR Allele in the Presence of Albumin-Cre Results in High Levels of CαR mRNA in Hepatocytes

The cAMP/PKA system is of critical importance in regulating glucose output by the liver by modulating aspects of gluconeogenesis, glycolysis, and glycogen metabolism. It was, therefore, of interest to determine the consequences of expressing the CαR protein in hepatocytes. The albumin promoter has been used by several groups to induce specific recombination in the liver (Postic and Magnuson, 2000; Postic *et al.*, 1999). When offspring expressing a cre-activatable *lacZ* transgene (Postic and Magnuson, 2000) or a floxed glucokinase allele (Postic *et al.*, 1999) were mated with the *alb-cre* line, low levels of recombination were observed at birth and these levels gradually increased to a maximal point by postnatal week 8. For this reason, the experiments described below were performed using animals 12 weeks of age or older. All of the experiments described below involve mice that are either CαR heterozygotes, *alb-cre* heterozygotes, or the combined double heterozygote, CαR/*alb-cre*.

Offspring that are double-positive for both CαR and *alb-cre* were born at the predicted Mendelian ratio from the initial cross and were ~10–15% smaller than littermate controls at 12 weeks of age (WT, 30.62 ± 0.57 g; CαR, 29.89 ± 0.58 g; CαR/*alb-cre*, 26.61 ± 0.74 g, $P < 0.0002$ by one-way ANOVA, n = 4–6 per genotype). This

phenotype was apparent from the age of weaning and appears to stem from an overall growth defect, as CαR/*alb-cre* animals have a shorter nose–anus length compared to littermate controls (WT, 9.97 ± 0.08 cm; CαR, 9.88 ± 0.07; CαR/*alb-cre*, 9.48 ± 0.08; $P = 0.0027$ by one-way ANOVA, n = 4–6 per genotype). Percent adiposity, measured by dividing the combined weight of the retroperitoneal, reproductive, and inguinal fat pads by total body weight, was indistinguishable between genotypes.

PCR analyses of specific tissues derived from CαR/*alb-cre* animals revealed recombination of the mutant allele only in the liver, consistent with previous reports (Postic and Magnuson, 2000). Using RT-PCR analyses, we compared the amount of CαR-encoding mRNA with the amount expressed from the WT allele (Fig. 2a,b). Because only one allele can undergo recombination with this breeding scheme, the maximal level of CαR-encoding mRNA in hepatocytes is 50%; due to the presence of 20–30% nonhepatic cells within the liver, the predicted maximal level is 35–40% of the total Cα mRNA pool. As shown in Figure 2b, expression from the mutant allele accounted for ~30% of the total liver Cα mRNA.

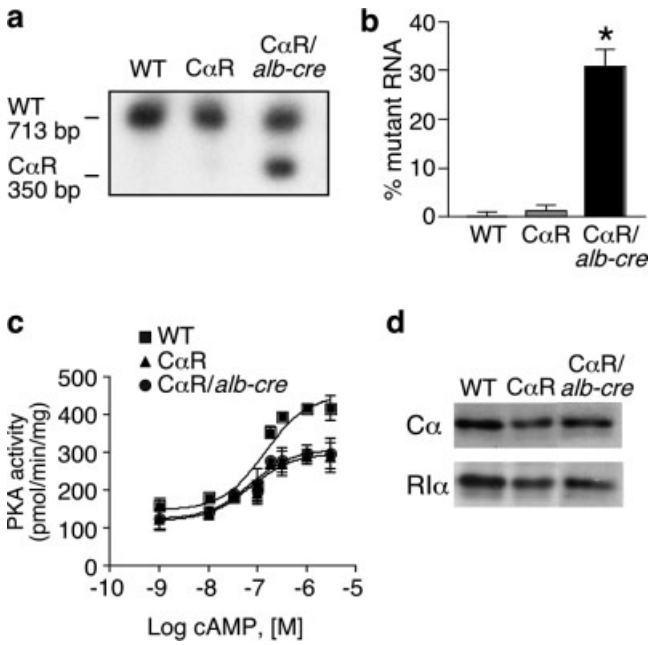

FIG. 2. Recombination of the CαR allele in the liver using *alb-cre* results in high levels of CαR mRNA but no detectable CαR protein activity in in vitro extracts. **a:** RT-PCR analyses were performed from liver extracts of 12–16-week-old male littermates derived from CαR × *alb-cre* matings. PCR products were digested with *Mlu I* and analyzed by Southern analysis; 713 bp = WT Cα and 350 bp = CαR. **b:** Summary of the amount of CαR RNA in liver (*$P < 0.0001$ by one-way ANOVA, 5–8 animals per genotype). **c:** Representative cAMP dose–response curves from liver extracts from fed WT (squares), CαR (triangles), and CαR/*alb-cre* (circles) mice (representative of 2–3 animals per genotype). **d:** Representative Western analyses of Cα and RIα protein levels in liver extracts from fed WT, CαR, and CαR/*alb-cre* animals (representative of 3–4 animals per genotype).

Despite the expected level of CαR mRNA being expressed in liver, kinase assays performed on liver extracts from CαR/*alb-cre* animals did not detect a difference in either basal or total PKA activity between CαR and CαR/*alb-cre* samples (Fig. 2c). Similar levels of Cα protein were also observed in liver extracts from CαR and CαR/*alb-cre* mice and these levels were approximately half that of WT (Fig. 2d). It is well known that C subunit protein turns over rapidly when not complexed in a holoenzyme (Hemmings, 1986; Richardson *et al.*, 1990), and this degradation has been shown to be mediated by the proteasome (Boundy *et al.*, 1998). We have used several other transgenic Cre recombinase lines to activate CαR and despite robust phenotypes, we find that the mutant protein, as evidenced by constitutive PKA activity, is either at very low or undetectable levels. In ES cells where we know that the recombination is complete and that there are equal levels of Cα WT and CαR mRNA, the level of constitutive activity is less than 20% of that expected (Fig. 1). The levels of mRNA for CαR in the liver suggest that hepatocytes are synthesizing equal amounts of WT and CαR mutant protein, but the inability of the CαR mutant protein to bind to R and become stabilized in an inactive holoenzyme targets it for rapid degradation. We suggest that hepatocytes may be more efficient at degrading free C subunit than are ES cells.

Expression of CαR in the Liver Decreases Both Glycogen Stores and the Level of Fructose-2,6-bisphosphate

Despite our inability to observe a change in PKA activity in liver extracts, we predicted that several metabolic pathways should serve as "readouts" for even transient expression of the constitutively active CαR protein in vivo. We examined potential changes in three pathways thought to involve PKA in the liver: regulation of glycogen levels, control of the amount of the important metabolic regulator, fructose-2,6-bisphosphate (F-2,6-P_2), and amounts of mRNA levels encoding gluconeogenic and glycolytic enzyme mRNA. We also predicted that changes in these pathways stemming from activity of the CαR protein might be modulated by differences in the level of cAMP that occur in fed versus fasted animals.

PKA is known to phosphorylate both glycogen synthase and phosphorylase kinase, key enzymes involved in the synthesis and storage of glycogen, respectively (reviewed in Brushia and Walsh, 1999; Exton, 1987). Measurement of glycogen levels from 24-h fasted, 6-h refed mice revealed that CαR/*alb-cre* livers had significantly lower levels of glycogen compared to WT and CαR littermates (Fig. 3a). Similar results were obtained from mice that were fed ad libitum (not shown). As predicted, during fasting the levels of glycogen declined in all groups of mice, but CαR/*alb-cre* samples were now equivalent in glycogen content compared to their littermate controls. A similar result was observed when levels of F-2,6-P_2 were measured; levels of this important

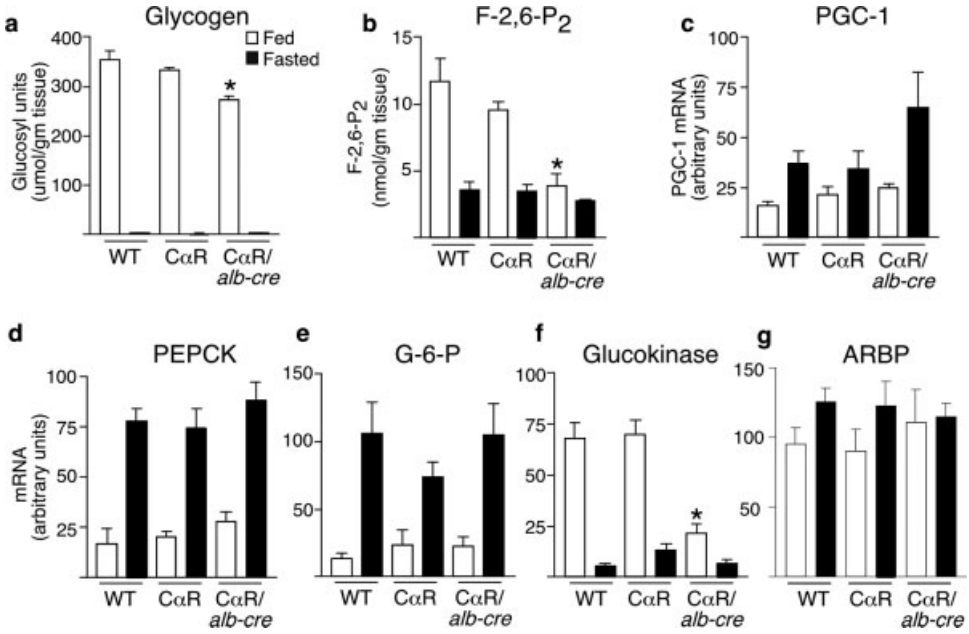

FIG. 3. CαR/*alb-cre* livers show decreased levels of glycogen, F-2,6-P_2, and glucokinase mRNA compared to controls. Glycogen (**a**) and F-2,6-P_2 (**b**) levels were measured as described in Materials and Methods from fed (white bars) and fasted (black bars) WT, CαR, and CαR/*alb-cre* mice. PGC-1 (**c**), PEPCK (**d**), glucose-6-phosphatase (**e**), glucokinase (**f**), and ARBP (**g**) (used as a control for equal RNA input) mRNA levels were assessed in the fed and fasted state using quantitative real-time PCR. (**a:** $^*P < 0.0006$ fed CαR/*alb-cre* versus fed WT and CαR by one-way ANOVA; **b:** $^*P < 0.0027$ fed CαR/*alb-cre* versus fed WT and CαR by one-way ANOVA; **f:** $^*P < 0.0001$ CαR/*alb-cre* versus fed WT and CαR by one-way ANOVA. Each bar represents 4–9 animals.)

metabolite are regulated in the liver by the activity of the bifunctional enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase 2 (PFK-2/FBPase-2) (reviewed in Okar *et al.*, 2001). In addition to other cellular regulators, PKA plays a key role in the control of the kinase:phosphatase activity ratio of the liver isoform of the enzyme. PKA-mediated phosphorylation of serine 32 of PFK-2/FBPase-2 shifts the enzyme towards phosphatase activity, lowering the level of F-2,6-P_2 (Okar *et al.*, 2001). F-2,6-P_2 is the most powerful allosteric activator of phosphofructokinase-1 and inhibitor of fructose-1,6-bisphosphatase (Pilkis *et al.*, 1981a,b); high levels of F-2,6-P_2, therefore, promote glycolysis rather than gluconeogenesis (Okar *et al.*, 2001). Consistent with the predicted role of PKA in promoting the phosphatase activity of PFK-2/FBPase-2, we observed a large decrease in F-2,6-P_2 levels in CαR/*alb-cre* livers in the fed state (Fig. 3b). These results indicate that there are decreases in glycogen and F-2,6-P_2 levels in CαR/*alb-cre* livers that are consistent with unregulated activity of the CαR protein.

Expression of CαR Does Not Affect the Expression of CREB-Dependent Gluconeogenic Genes But Impairs the Induction of Glucokinase mRNA That Normally Occurs During Feeding

It has recently been demonstrated that the cAMP signaling cascade induces transcription of peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1), a transcriptional coactivator involved in the initiation of the gluconeogenic program in hepatocytes, via a cAMP-response element binding protein (CREB)-dependent mechanism (Herzig *et al.*, 2001; Yoon *et al.*, 2001). PGC-1, in turn, has been shown to enhance the transcription of gluconeogenic genes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-P) through a coordinated action with CREB and hepatic nuclear factor 4α (HNF-4α) (Yoon *et al.*, 2001). Since PKA is a major regulator of CREB-dependent transcription by phosphorylating serine 133 of the CREB protein, we predicted that the levels of PGC-1, PEPCK, and G-6-P mRNA might be increased in livers from CαR/*alb-cre* mice. Using quantitative real-time RT-PCR methods, we measured mRNA levels for these key gluconeogenic genes from 6-h refed and 24-h fasted mice. The levels of PGC-1, PEPCK, and G-6-P were not significantly different in the fed state among genotypes (Fig. 3c–e). Upon fasting, levels of PGC-1 mRNA were slightly elevated in CαR/*alb-cre* mice compared to controls, although this effect did not reach statistical significance. In contrast, the level of glucokinase mRNA, a critical component in the regulation of glycolysis in the liver, was dramatically reduced in fed CαR/*alb-cre* animals (Fig. 3f). The glucokinase promoter has been shown to be negatively regulated by glucagon (Iynedjian *et al.*, 1989; Magnuson *et al.*, 1989), a hormone that causes increased cAMP in liver, and our results demonstrate that increased PKA activity due to CαR expression also inhibits glucokinase gene transcription. The lack of induction of the gluconeogenic genes PEPCK, PGC-1, and G-6-P may reflect compensatory changes in other known signaling pathways that counterregulate transcription of these genes such as those downstream of the insulin receptor.

CαR/alb-cre Mice Exhibit Fasting Hyperglycemia and Are Glucose-Intolerant

We next performed in vivo analyses to assess overall glucose homeostasis. Fasting blood glucose levels were elevated by ~25% in CαR/*alb-cre* mice (Fig. 4a). Intraperitoneal (IP) glucose tolerance tests performed on 24-h

fasted mice showed that CαR/*alb-cre* animals exhibited an impaired ability to remove glucose from the bloodstream (Fig. 4b). Similar results were observed after oral glucose tolerance testing (data not shown). Mice that were random fed showed a blood glucose elevation of ~20–25% (138.7 ± 4.1, WT; 126.3 ± 4.7, CαR; and 171.8 ± 9.4, CαR/*alb-cre*; n = 18–32 per genotype). Interestingly, mice that were fasted for 24 h and then refed for 6 h had normal blood glucose levels (Fig. 4a).

CαR/alb-cre Mice Exhibit Paradoxical Fasting Hypoinsulinemia and Fed Hyperinsulinemia

Based on the fasting blood glucose data and impaired glucose tolerance tests, we tested whether CαR/*alb-cre* mice were effectively insulin-resistant at the level of the liver due to elevated PKA activity. In other models of liver insulin resistance, most notably the insulin receptor knockout in the liver (Michael *et al.*, 2000), insulin levels rise in an attempt to compensate for this resistance. Surprisingly, measurement of fasting insulin levels indicated that CαR/*alb-cre* mice had lower levels of insulin than either WT or CαR controls. However, after a 6-h refeeding period CαR/*alb-cre* mice now showed a small elevation of insulin levels compared to CαR littermates (Fig. 5a). Measurement of serum triglycerides showed a significant elevation in fasted CαR/*alb-cre* mice (WT 57.7 ± 3.6 mg/dl, CαR 53.8 ± 3.0 mg/dl, CαR/*alb-cre* 71.7 ± 4.2 mg/dl), consistent with decreased insulin levels in this metabolic state. This disparity in triglyceride levels was lost upon refeeding (not shown). Insulin tolerance tests revealed that fasted CαR/*alb-cre* mice showed normal insulin sensitivity at two different doses of insulin (0.75 U/kg (not shown) and 1.5 U/kg, Fig. 5b), indicating that these animals are not grossly insulin-resistant in skeletal muscle. We also performed in vivo insulin secretion assays after an acute 3 mg/gm glucose challenge to assess the first phase of insulin secretion. Control mice showed a significant elevation in insulin level at the 2-min time point after glucose administration; in contrast, a decrease in serum insulin was observed in samples derived from the CαR/*alb-cre* animals (Fig. 5c).

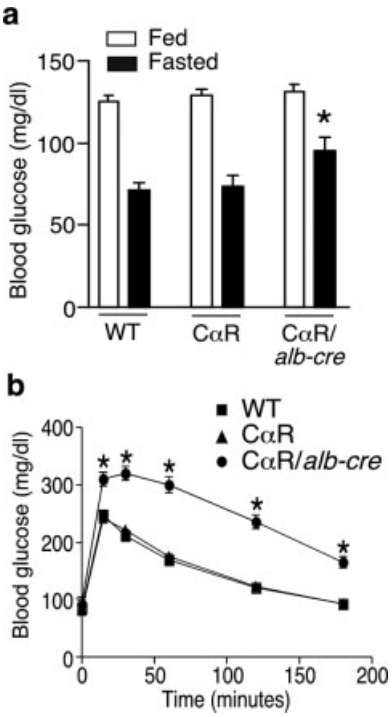

FIG. 4. CαR/*alb-cre* mice are glucose-intolerant and exhibit fasting hyperglycemia. **a:** 6-h fed (white bars) and 24-h fasted (black bars) blood glucose values are shown (*$P < 0.0001$, one-way ANOVA, fasted samples). **b:** Littermates were injected with 1 mg/gm glucose at time 0 and samples were taken at the indicated time points for measurement of blood glucose (*$P < 0.001$ for all time points; n = 10–16 animals per genotype).

There are several possibilities that might explain the altered insulin response characteristic of CαR/*alb-cre* animals. Because the mutation is present in the liver, we tested whether insulin clearance, a process that occurs in hepatocytes (reviewed in Di Guglielmo *et al.*, 1998; Duckworth *et al.*, 1998), might be enhanced. To assess this, we measured the C-peptide-to-insulin ratio in serum from fasted mice. C-peptide and insulin are released from pancreatic β-cells in a 1:1 ratio, but only insulin is cleared in the liver by exocytosis after binding to its receptor (Polonsky and Rubenstein, 1986; Zavaroni *et al.*, 1987). These studies showed that the calculated ratio was not different between CαR/*alb-cre* mice and littermate controls (insulin:C-peptide ratio, mean ± SEM: WT, 6.76 ± 1.4; CαR/*alb-cre,* 5.20 ± 0.98, n = 3–5 per genotype). From these data we conclude that the presence of the CαR enzyme in the liver does not affect insulin clearance.

The second possibility that might explain the observed results is that CαR/*alb-cre* mice have a pancreatic β-cell defect that prevents normal insulin secretion in response to glucose. To address this question, isolated islets from mice fed ad libitum were assessed for their insulin secretory response to glucose and arginine. Islets were incubated in varying concentrations of glucose and insulin secretion was measured. The islets derived from CαR/*alb-cre* mice were defective in their ability to secrete insulin at all glucose concentrations tested (Fig. 6a). The response to the secretagogue arginine, however, which induces calcium influx and membrane depolarization (Weinhaus *et al.*, 1997), was normal (Fig. 6b). Measurement of islet insulin content revealed that CαR/*alb-cre* islets actually contained more insulin than islets derived from WT mice (Fig. 6c), suggesting that defective insulin secretion is not a result of impaired insulin synthesis or storage. Preliminary assessment of islet size, number, area, and proliferation rate has revealed no differences between islets from CαR/*alb-cre* mice and WT or CαR controls (data not shown). To exclude the possibility that the impairment in glucose-stimulated insulin secretion arises from unexpected expression of the CαR protein in islets themselves, we performed DNA and RT-PCR experiments on isolated islets. These studies revealed that no recombination or

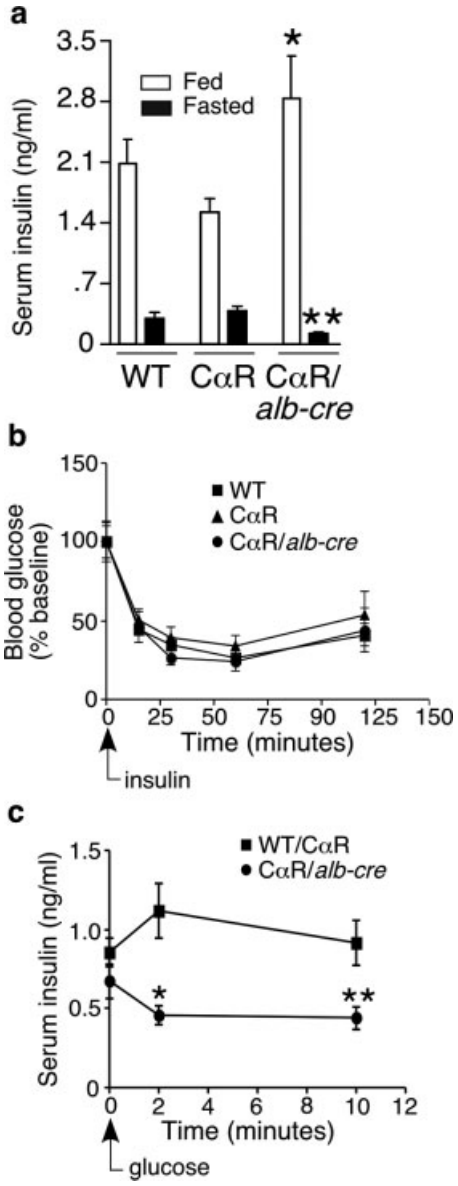

FIG. 5. CαR/*alb-cre* mice exhibit defects in circulating insulin levels dependent on metabolic status and do not show an increase in insulin levels in response to a glucose challenge. **a:** 6-h fed (white bars) and 24-h fasted (black bars) serum insulin levels are shown (*P = 0.0345, one-way ANOVA, fed samples; *t*-tests, $P = 0.093$ for WT versus CαR; $P = 0.183$ for WT versus CαR/*alb-cre*, *$P = 0.0196$ for CαR versus CαR/*alb-cre*. **$P = 0.0087$, one-way ANOVA, fasted samples. n = 14–18 mice per genotype). **b:** Mice were injected with 1.5 U/kg of Humulin insulin at time 0 and samples were taken for measurement of blood glucose. **c:** Mice were injected with 3 mg/gm glucose at time 0 and samples were taken at the indicated time points for measurements of serum insulin. (*$P = 0.0031$ and **P = 0.0130, *t*-tests; WT/CαR controls, n = 15; CαR/*alb-cre*, n = 12.)

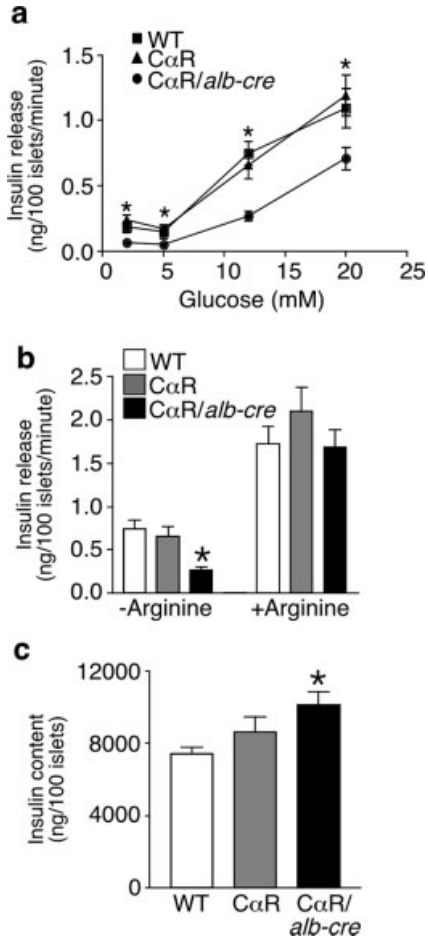

FIG. 6. Islets from CαR/*alb-cre* mice exhibit defective glucose-stimulated insulin secretion in vitro despite an intact response to arginine and elevated insulin levels. **a:** Islets were isolated from WT, CαR, and CαR/*alb-cre* mice and cultured overnight in RPMI as described in Materials and Methods. Islets were incubated with increasing concentrations of glucose and insulin release within a 1-h time period was measured. Squares, WT, triangles, CαR, and circles, CαR/*alb-cre* (*$P < 0.0431$, one-way ANOVA). **b:** Effect of 15 mM arginine on insulin release in the presence of 12 mM glucose. WT (white), CαR (gray), and CαR/*alb-cre* (black) (*$P = 0.0004$, one-way ANOVA). **c:** Insulin content determined at the end of the experimental period is indicated (*$P = 0.022$, one-way ANOVA; *t*-tests, $P = 0.213$ for WT versus CαR; *$P = 0.0125$ for WT versus CαR/*alb-cre*, $P = 0.185$ for CαR versus CαR/*alb-cre*. n = 24–28 samples per genotype for **a,c,** n = 12 for **b**).

expression of mutant RNA was observed in islets from CαR/*alb-cre* mice (data not shown). Therefore, the phenotype does not derive from aberrant expression of the mutant PKA subunit in islets, but rather stems from physiological cross-talk between CαR-expressing liver and islets.

DISCUSSION

We created a line of mice in which PKA activity can be increased by tissue-specific expression of cre recombinase. A tryptophan-to-arginine mutation at position 196 was introduced into one allele of the mouse Cα gene by homologous recombination. Based on the crystal structure of Cα, tryptophan 196 resides on the surface of the catalytic protein and is predicted to provide a hydrophobic interaction site for the R subunit but is not directly involved in catalytic activity. Expression of this mutant C protein (CαR) would result in the formation of a holoen-

zyme that has such high sensitivity to cAMP-mediated dissociation that under normal physiological cAMP concentrations it would be in a constitutively active state (Gibson and Taylor, 1997; Orellana *et al.*, 1993; Orellana and McKnight, 1992).

Expression of the CαR protein in our mutant mouse line is regulated by cre-*loxP* recombination (reviewed in Sauer, 1993, 1998). In both ES cells and in mice, we have shown that the lox-flanked neomycin cassette used for these studies effectively silences the mutant CαR gene and that expression occurs only when the neomycin cassette is removed by the action of cre recombinase. The exception to this finding appears to be in testis, where we observe a small amount of mutant protein expression in the absence of cre recombinase. Males carrying the CαR allele in the absence of Cre recombinase are fertile and we detected no phenotypes associated with the small amount of expression of CαR in testis. We used the same lox-flanked neomycin resistance cassette to silence a dominant negative mutation within the mouse RIα gene and this also proved successful (Willis et al., in prep.).

In both ES cells and in vivo, recombination of the mutant CαR allele produced high levels of mRNA encoding the CαR subunit. C subunit is unstable when not tightly complexed with R proteins in a holoenzyme (Amieux *et al.*, 1997; Hemmings, 1986; Richardson *et al.*, 1990); by extension, we predict that the half-life of CαR is very short, since it does not bind R subunits effectively at basal concentrations of cAMP. The stability of CαR in different tissues would be expected to be influenced by both R subunit expression levels, the activity of the ubiquitin/proteasome machinery, and cAMP levels (Boundy *et al.*, 1998; Hemmings, 1986; Richardson *et al.*, 1990). Despite potential differences in the ability to measure increases in basal PKA activity, we have shown that expression of CαR results in biochemical and phenotypic consequences in hepatocytes. We have also recently activated the CαR mutation using the fat cell-specific aP2 promoter (aP2-Cre), the β-cell-specific rat insulin promoter (RIP-Cre), and a dopamine receptor promoter, (D1R-Cre). In all of these cases the mice displayed either perinatal lethality (aP2-Cre and RIP-Cre) or growth retardation and seizures (D1R-Cre) (Niswender, J.P. Jones, and McKnight, unpubl. data). These results suggest that the utility of the CαR line is limited only by the efficiency and specificity of existing and future lines of cre transgenics.

In order to study the multiple roles of PKA activity in hepatic glucose metabolism and overall glucose homeostasis, we crossed the CαR mice to a transgenic line expressing cre recombinase from the albumin promoter (*alb-cre*). This *alb-cre* transgenic line of mice has been used in several previous studies to produce effective recombination (Michael *et al.*, 2000; Postic and Magnuson, 2000) and we found nearly complete recombination and activation of the CαR allele in hepatocytes. CαR/*alb-cre* mice display a complex and interesting phenotype and provide a good demonstration of how the CαR mice can be utilized to study the role of specific cell types in physiological pathways. CαR/*alb-cre* mice exhibit fasting hyperglycemia, a 10% reduction in body weight, and a 5% decrease in body length. In the fed state the content of liver glycogen was reduced by 20% and the level of the key regulator of PFK-1, F-2,6-P_2, was reduced dramatically. These changes in glycogen and F-2,6-P_2, as well as the fasting hyperglycemia, were predictable based on the known targets of PKA, although the biochemical mechanisms appear to be more complex than anticipated.

The changes observed in liver gene expression were somewhat unexpected. The genes typically induced by fasting and thought to be PKA-responsive, including PGC-1, PEPCK, and G-6-P, were essentially unchanged and continued to be regulated normally by fasting and refeeding. This might reflect the homeostatic counterregulation of these genes by the insulin signaling pathway; these results provide evidence that the insulin signal is dominant in shutting down transcription of these genes in the fed state even in the presence of elevated PKA activity. In contrast, the 4–5-fold induction of glucokinase mRNA caused by feeding was almost completely blocked in the CαR-expressing hepatocytes. Glucokinase (GK) expression is also inhibited by glucagon, an effect presumably mediated by cAMP/PKA signaling (Iynedjian *et al.*, 1989; Magnuson *et al.*, 1989). GK gene expression in the liver is stimulated by insulin, a phenomenon shown to involve sterol response-element binding protein-1c in cultured hepatocytes (Foretz *et al.*, 1999). Interestingly, F-2,6-P_2 is also able to regulate GK gene transcription in the absence of insulin (Wu *et al.*, 2004). Wu et al. have shown that increasing F-2,6-P_2 levels in mice treated with streptozotocin results in a corresponding increase in GK mRNA levels. These observations suggest that the low levels of F-2,6-P_2 that we observe in fed CαR/*alb-cre* mice could contribute to the lack of GK gene induction observed during feeding. Our results suggest that the inhibition of GK gene transcription by PKA acts in a dominant fashion even in the presence of elevated insulin. Future studies will use these mice as a model system to determine the dominance of the cAMP versus insulin axes in regulating the expression of liver genes such as PEPCK, G-6-P, and GK.

The regulation of insulin secretion in these animals was clearly altered, although the mutant CαR allele was not activated in pancreatic islet cells. When fasted, CαR/*alb-cre* mice demonstrated impaired glucose tolerance, and we observed that this defect in glucose disposal becomes further impaired if the mice are placed on a diabetogenic diet (Niswender, Willis, and McKnight, unpubl. data). Mice with impaired glucose disposal typically have elevated insulin and peripheral insulin resistance, hallmarks of the diabetic state. In contrast, fasted CαR/*alb-cre* mice are actually hypoinsulinemic compared to controls. Glucose administration did not elicit an increase in plasma insulin, as it did in controls. Islets isolated from CαR mice continued to show a blunted ability to release insulin in response to glucose when cultured in vitro. Nevertheless, the insulin content of

mutant islets was high and arginine-elicited release in vivo (Niswender, Willis, and McKnight, unpubl. data) or in vitro was normal. After refeeding, the CαR mice become hyperinsulinemic, indicating that the islet cells respond robustly to nutrient signals other than glucose.

It is interesting to note that the phenotype of CαR/*alb-cre* mice is quite similar to that of the GK liver-specific knockout (Postic *et al.*, 2001). In both cases there is an unexpected insulin secretion defect induced by a liver-specific mutation; similar changes in insulin levels are also observed during feeding and fasting in both mouse models. Since one of the most dramatic changes in CαR/*alb-cre* mice is the loss of GK mRNA expression in the fed state, we speculate that the loss of glucokinase expression may be a common signal that links the insulin secretion phenotype of these two quite different mouse mutants.

In conclusion, we developed a novel mouse line that permits the manipulation of cAMP-mediated signal transduction. This mutant enzyme can be controlled in a temporal and spatial fashion using cre recombinase. Using lines of cre recombinase that express in adipose tissue or in hepatocytes, we have shown that expression of this mutant PKA subunit can lead to biologically interesting phenotypes. We used the *alb-cre* line to uncover a novel role for hepatic PKA in the regulation of glucose-stimulated insulin secretion. It is anticipated, due to the widespread expression of the Cα subunit and the growing number of lines of cre recombinase transgenics, that this general strategy will prove useful for the study of PKA activity in many physiological systems.

MATERIALS AND METHODS

Targeting Vector, Gene Targeting, and Blastocyst Injections

The tryptophan 196 to arginine mutation was introduced into exon 7 of a mouse Cα genomic clone previously described (Chrivia *et al.*, 1988) (Fig. 1a). This mutation plus one additional point mutation at the wobble position of Thr 195 results in the creation of an *Mlu I* restriction site specific to the mutated allele. A *loxP*-flanked neomycin selection cassette containing the SV40 promoter, the neomycin phosphotransferase gene, and the SV40 polyadenylation sequences was inserted into a unique SnaBI restriction site within the intron between exons 5 and 6. The targeting vector also contains a 1.6 kb diphtheria toxin A chain cassette as a negative selection marker (Howell *et al.*, 1997). As previously described (Howe *et al.*, 2002), 120 μg of the targeting vector was linearized with *Not I* and electroporated into 1.2×10^7 embryonic stem cells; clones were selected in the presence of G418 (280 μg/ml) and 1,000 U/ml leukemia inhibitory factor (LIF) for ~7 days. DNA was isolated from ES cell clones, digested with *Sac I,* and Southern analyses were performed with a fragment amplified from mouse genomic DNA using primers within exons 9 and 10 of the Cα gene; (forward) 5′ ACTTCAGCTCTGACTTGAACC and (reverse) 5′ GGTATGAAGGGAGCTTCC. The presence of the CαR mutation was confirmed by amplification of both WT and mutant CαR alleles using primers within intron 5 and exon 8 (forward 5′ GGCTTGGAACTCATGATGTAG and reverse 5′ GATGAGGACTCCGAGAGC primers). Fragments were digested with *Mlu I* and resolved on a 2% agarose gel (WT fragment, 835 bp; CαR allele-specific fragments, 609 and 226 bp). Two independent clones were microinjected into C57Bl/6J blastocysts and implanted into pseudopregnant 129Sv/J female recipients. Male chimeras were mated with C57Bl/6J females and progeny were screened using PCR and Southern analyses. Mice were housed in a specific pathogen-free facility on a 12-h light/dark cycle and were fed standard rodent chow. All protocols used in these studies were in accordance with the University of Washington Institutional Animal Care and Use Committee.

In Vitro Recombination of the CαR Allele and Analysis of Wildtype, CαR, and CαR/Cre Embryonic Stem Cells

Fifty μg of pOG-231, an expression vector encoding cre recombinase driven by the cytomegalovirus promoter (a generous gift from S. O'Gorman), was electroporated into 1.2×10^7 CαR ES cells as described above. Cells were plated at low density to isolate single clones and screened by PCR analysis across the *loxP* site predicted to remain after recombination (forward 5′ CTAGAACTCATAGGCTTCTG and reverse 5′ GGGAGTGCAGATACTCAAAGGTCAGG primers). RT-PCR analysis using primers within exons 3 and 10 of the Cα cDNA (forward 5′ GGTGGTGAAGCTAAAGCA and reverse 5′ GGTATGAAGGGAGCTTCC primers), followed by *Mlu I* digestion, resulted in a 713-bp fragment corresponding to WT mRNA versus fragments of 350 and 363 bp if the PCR product contained the CαR mutation. PCR fragments were separated on a 2% agarose gel and the ratio of the 713 and 350 bp bands was determined using Southern analysis with an oligonucleotide within exon 6 (5′ CCCCATGCCCGTTTCTAC). PKA activity assays were performed as previously described (Clegg *et al.*, 1987) in the presence and absence of 5 μM cAMP. Activity in the presence of 5 μM protein kinase inhibitor was subtracted from both basal and cAMP-dependent kinase activity to reflect activity specific for PKA. Western analyses for Cα and RIα were performed as previously described (Amieux *et al.*, 1997).

Breeding of CαR Animals

CαR animals on a mixed 50% C57Bl6/J / 50% 129 Sv/J mixed F1 background were mated together to produce CαR homozygotes. All subsequent matings were between animals heterozygous for the silent CαR gene and mice expressing cre recombinase in selected tissues. Albumin-cre (*alb-cre*) mice were obtained from Jackson Laboratories (Bar Harbor, ME; strain number 003574) on a >99% C57Bl/6J background. Prior to matings with *alb-*

cre animals, CαR mice were bred at least six generations onto the C57Bl/6J background.

In Vivo Studies and Analytical Procedures

For glucose tolerance tests, mice were fasted for 24 h (9 AM to 9 AM). An initial bleed was performed to assess basal glucose levels. A second bleed was performed after 15 min (time 0) and an IP injection of 1 mg/gm of glucose was administered. Glucose levels were assessed using a One Touch Blood Glucose Monitor (LifeScan, Milpitas, CA). For insulin tolerance tests, 0.75 U/kg or 1.5 U/kg Humulin insulin was injected IP at time 0. For insulin secretion assays, a 3 mg/gm IP glucose bolus was administered at time 0 and 50 μl of blood was taken immediately before and 2 and 10 min after injection. Serum insulin levels were determined using ELISA (Mercodia, Metuchen, NJ). Serum C-peptide was measured by radioimmunoassay (Linco Research, St. Charles, MO), serum glycerol and triglycerides by colorimetric assay (GPO-Trinder; Sigma Diagnostics, St. Louis, MO), and free fatty acids by colorimetric assay (Wako Chemical USA, Richmond, VA). Glycogen levels were assessed by dissolving 20 mg of tissue in 100 μl 0.3 M KOH at 70°C for 20 min. Samples were precipitated three times with ethanol and resuspended in 300 μl 0.2 M sodium acetate, pH 4.8, containing 0.15 mg/ml amyloglucosidase (Sigma-Aldrich, St. Louis, MO) and incubated at 40°C for 3 h. Glucosyl units were determined using a Glucose-Trinder kit (Sigma-Aldrich). Hepatic fructose-2,6-bisphosphate was extracted from frozen liver tissue in 10 volumes of 50 mM NaOH and incubated at 80°C for 5 min. The extract was cooled to 0°C and neutralized by addition of ice-cold 1 M acetic acid in the presence of 20 mM Hepes. After centrifugation at 8,000*g* for 10 min, the supernatant was collected and assayed for F-2,6-P_2 by the 6-phosphofructo-1-kinase activation method as previously described (Wu *et al.*, 2001).

Real-Time Quantitative RT-PCR

One-step real-time PCR was performed using the Mx3000P and Brilliant QRT-PCR reagents (Stratagene, Cedar Creek, TX.) Total RNA was extracted and purified from frozen mouse livers using RNAeasy columns (Qiagen, Valencia, CA). A common wildtype liver RNA sample was used to perform a standard curve consisting of five RNA concentrations assayed in duplicate for each primer set. Relative amounts of unknown mRNA were extracted from the standard curve using Mx3000P software. Mouse primers and probes (PGC-1, PEPCK, G-6-P, GK ordered from Integrated DNA Technologies, Coralville, IA; ARBP ordered from Applied Biosystems, Foster City, CA) were chosen using Primer Express (Applied Biosystems) and are available on request.

β-Cell Morphometry

Beta cell mass was measured as previously described (Liu *et al.*, 2002). Measurements of β-cell proliferation were performed by immunohistochemical costaining for the Ki-67 proliferation marker (1:500; Transduction Laboratories, Lexington, KY), and insulin (Linco Research, St. Charles, MO).

Islet Isolation and Insulin Secretion Assays

Islets were harvested from 12–16-week-old male mice as previously described (Sweet *et al.*, 2004). Islets were handpicked twice into dishes containing 2 mM glucose in RPMI1640 culture media and incubated for 60 min. Batches of 10 islets (in quadruplicate) were transferred into 96-well plates containing 200 μl of RPMI1640 with varying concentrations of glucose, with or without 15 mM arginine, for 60 min. Insulin in the supernatant was then assessed using ELISA (ALPCO, Windham, NH). To extract insulin from the islets, 1 ml of a mixture of ethanol and hydrochloric acid (2% v/v HCl to 95% ethanol) was added and the supernatant was collected after 60 min.

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