Essential Role of Chicken Ovalbumin Upstream Promoter–Transcription Factor II in Insulin Secretion and Insulin Sensitivity Revealed by Conditional Gene Knockout

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Chicken ovalbumin upstream promoter–transcription factor II (COUP-TFII) has been implicated in the control of blood glucose by its potent effect on expression and signaling of various nuclear receptors. To understand the role of COUP-TFII in glucose homeostasis, conditional COUP-TFII–deficient mice were generated and crossed with mice expressing Cre under the control of rat insulin II gene promoter, resulting in deletion of COUP-TFII in pancreatic β-cells. Homozygous mutants died before birth for yet undetermined reasons. Heterozygous mice appeared healthy at birth and showed normal growth and fertility. When challenged intraperitoneally, the animals had glucose intolerance associated with reduced glucose-stimulated insulin secretion. Moreover, these heterozygous mice presented a mild increase in fasting and random-fed circulating insulin levels. In accordance, islets isolated from these animals exhibited higher insulin secretion in low glucose conditions and markedly decreased glucose-stimulated insulin secretion. Their pancreata presented normal microscopic architecture and insulin content up to 16 weeks of study. Altered insulin secretion was associated with peripheral insulin resistance in whole animals. It can be concluded that COUP-TFII is a new, important regulator of glucose homeostasis and insulin sensitivity. *Diabetes* 54:1357–1363, 2005

The function of differentiated β-cells is dependent upon a network of transcription factors that are required for the expression of key genes involved in glucose sensing, insulin biosynthesis, and regulated exocytosis (1–4). Disturbance of this network, even by mutations causing a modest decrease in the protein level, has been shown to lead to important clinical manifestations such as diabetes and/or obesity.

Chicken ovalbumin upstream promoter–transcription factor II (COUP-TFII; also called NR2F and ARP-1) is an orphan member of the steroid/thyroid hormone receptor superfamily that binds DNA by a Zn-finger DNA binding domain (5). COUP-TFII is generally considered to be a functional transcriptional repressor of genes controlled by nuclear hormone receptors such as peroxisome proliferator–activated receptors and hepatocyte nuclear factor (HNF)-4α, which are well known to play important roles in the glucose-responsive phenotype of β-cells (6,7). The possibility that COUP-TFII might play a role in glucose homeostasis is supported by in vitro studies suggesting that this protein is involved in regulating insulin gene expression in pancreatic β-cells (8) but also in the expression of several other genes involved in glucose and lipid metabolism (9,10). It has been shown that COUP-TFII is expressed in the early developing endocrine pancreas and in mature differentiated islet cells (11). However, until now the potential function of COUP-TFII in pancreatic β-cells remains unknown. One way to address this issue is to generate COUP-TFII–deficient mice. Since global COUP-TFII deficiency results in embryonic lethality (12), we generated in this study a conditional knockout of the COUP-TFII gene using the Cre-*loxP* system. Our data show that inactivation of one COUP-TFII allele in β-cells results in disturbed insulin secretion and peripheral insulin resistance.

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COUP-TFII, chicken ovalbumin upstream promoter–transcription factor II; HNF, hepatocyte nuclear factor.

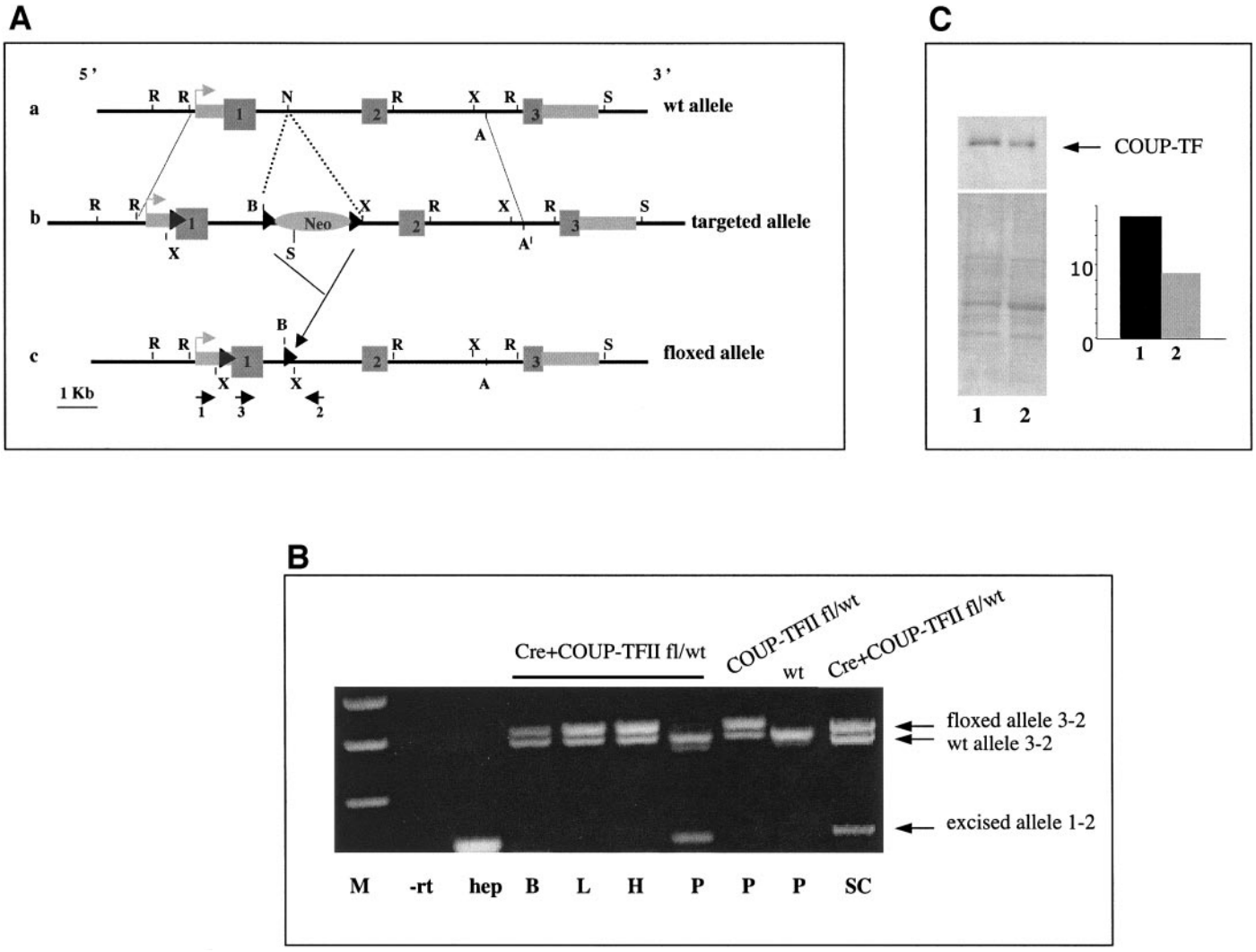

FIG. 1. Assessment of COUP-TFII deletion in pancreatic β-cell and extrapancreatic cells of Rip2-Cre+/−. COUP-TFII fl/wt mice. *A*: Schematic representation of the genomic structure of COUP-TFII: (a) shows the genomic map of the wt COUP-TFII allele. The *lower panels* show the targeted locus before (b) and after (c) excision of the neomycin selection cassette (Neo) that is flanked by one *loxP* upstream and two *loxP* sites downstream of exon 1 and also shows the position of the different primers used in the PCR analysis. Individual letters represent restriction sites. *B*: One representative PCR analysis of DNA samples. B, brain; L, liver; H, hypothalamus; P, pancreatic islet; and SC, spinal cord and hep: HepCre+/−. COUP-TFII fl/fl hepatocytes as control of the profile-excised fragment. *C*: Western blot analysis of islet nuclear extracts of wild-type (*lane 1*) and Cre+/−.COUP-TFII fl/wt (*lane 2*) mice. The *lower panel* represents the blot stained by red Ponceau, and the *upper panel* is the same blot hybridized with COUP-TF antibody.

RESULTS AND DISCUSSION

Conditional inactivation of COUP-TFII in pancreatic β-cells. To achieve conditional knockout of the COUP-TFII gene in pancreatic β-cells using the Cre-*loxP* system, we first generated heterozygous COUP-TFII floxed (fl)/wild-type (wt) mice carrying one fl and one wt COUP-TFII allele (Fig. 1*A*). Genotype analysis of the offspring by intercrossing these mice indicated that both homozygous COUP-TFII fl/fl and heterozygous COUP-TF fl/wt pups were present at normal Mendelian frequency at days E11.5 and E18.5 (Fig. 2). However, half of the COUP-TFII fl/fl pups died after birth (Fig. 2). The surviving homozygous COUP-TFII fl/fl mice were indistinguishable from their wt littermates by visual inspection; they were fertile and had a normal lifespan.

Stage	Genotype COUP-TFII fl/fl x Cre+/-.COUP-TFII fl/wt				
	Cre+/-. fl/fl	fl/fl	fl/wt	Cre+/-. fl/wt	Total
E11.5	5 (13)	17 (13)	14 (13)	16 (13)	52
E18.5	0 (11)	12 (11)	14 (11)	18 (11)	44

	Genotype COUP-TFII fl/wt x Cre+/-.COUP-TFII fl/wt						
	Cre+/-. fl/fl	fl/fl	Cre+/-. fl/wt	fl/wt	Cre+ /-	wt	Total
After birth	0 (12)	7 (12)	26 (25)	19 (25)	36 (12)	14 (12)	102

FIG. 2. Number of viable Cre+/−.COUP-TFII fl/fl embryos produced by breedings between COUP-TFII fl/fl and Cre+/−.COUP-TFII fl/wt mice or COUP-TFII fl/wt and Cre+/−.COUP-TFII fl/wt mice at 11.5 and 18.5 days post coitum and after birth. Values in parentheses indicate the Mendelian frequencies expected from these breedings.

		Control	COUP-TFII fl/wt	Cre+/-.COUP-TFII fl/wt
Glucose (mg/dl)	fast	111 ± 4.0	108 ± 4.1	92 ± 3.5*
	fed	122 ± 3.0	121 ± 4.0	122 ± 3.0
Insulin (ng/ml)	fast	0.38 ± 0.01	0.37 ± 0.03	0.7 ± 0.04*
	fed	1.23 ± 0.04	1.21 ± 0.05	2.5 ± 0.1*
Glucagon (pg/ml)	fast	255 ± 34	265 ± 30	235 ± 14
Leptin (ng/ml)	fed	4.05 ± 0.34	4.15 ± 0.54	4.35 ± 0.2
FFA (mM)	fast	0.9 ± 0.14	0.92 ± 0.15	0.77 ± 0.16
	fed	0.43 ± 0.04	0.42 ± 0.04	0.41 ± 0.02
Glycerol (mM)	fast	458 ± 47	460 ± 31	469 ± 23
	fed	211 ± 14	210 ± 15	241 ± 16
Triglycerides (mM)	fast	0.71 ± 0.08	0.72 ± 0.05	0.75 ± 0.03
	fed	0.75 ± 0.1	0.74 ± 0.12	0.95 ± 0.05*
Total cholesterol (mM)	fast	2.1 ± 0.26	2.2 ± 0.05	2.24 ± 0.08
	fed	1.84 ± 0.17	1.79 ± 0.1	1.91 ± 0.08
HDL cholesterol (mM)	fast	1.56 ± 0.1	1.6 ± 0.15	1.31 ± 0.22
	fed	1.22 ± 0.14	1.2 ± 0.1	1.25 ± 0.06
Albumin	fed	26.5 ± 1.44	27 ± 1	26 ± 0.89
Body weight (g)		31.4 ± 0.5	30.5 ± 1.4	30.3 ± 0.9
Fat-free mass (g)[A]		19.8 ± 0.5	nd	20.2 ± 0.3
% fat mass[A]		13.3 ± 1.7	nd	13.8 ± 1.1
Food intake (g/d)		6.5 ± 0.23	6.1 ± 0.31	6.1 ± 0.33

FIG. 3. Plasma hormones and fuels, body composition, and food intake in wt, COUP-TFII fl/wt, and Cre+/−.COUP-TFII fl/wt mice. [A]Body composition was assessed by biphotonic absorptiometry. All data are expressed as means ± SE (n = 12 mice/group). *P < 0.01 Cre+/−.COUP-TFII fl/wt vs. COUP-TFII fl/wt and control mice.

Mice carrying COUP-TFII gene inactivation in the pancreatic β-cells were generated by breeding homozygous COUP-TFII fl/fl mice with transgenic mice expressing Cre driven by the rat insulin II promoter (Rip2-Cre) generated by Magnuson and colleagues (13,14). The resulting Cre+/−.COUP-TFII fl/wt females were mated to COUP-TFII fl/wt males to generate mice with different genotypes. As shown in Fig. 2, homozygous Cre+/−.COUP-TFII fl/fl mice were embryonic lethal. Since there are no precedents for embryonic lethal phenotypes resulting from β-cell–specific gene deletions, this lethality, together with the observations of Magnuson and colleagues (14), led us to examine possible Cre-induced deletion of the floxed COUP-TFII allele in other tissues than endocrine pancreas. Multiplex PCR analysis was performed on DNA prepared from liver, hypothalamus, brain, and spinal cord (Fig. 1*B*). We detected gene deletion in the spinal cord from the Cre+/−.COUP-TFII fl/wt mice, while we previously demonstrated that the COUP-TFII gene is expressed in the ventral neural tube at 9.0 days post coitum (11). Therefore, extrapancreatic COUP-TFII gene knockout could contribute in the observed lethality of homozygous Cre+/−.COUP-TFII fl/fl mice.

Multiplex PCR analysis of DNA prepared from islets isolated from Cre+/−.COUP-TFII fl/wt mice confirmed that one floxed allele was deleted (Fig. 1*B*). As judged by immunoblotting, COUP-TF levels were decreased by ~45% in nuclear extracts prepared from islets from Cre+/−.COUP-TFII fl/wt mice (Fig. 1*C*, *lane 2*) compared with wt mice (Fig. 1*C*, *lane 1*). These results indicate that Cre-induced recombination in heterozygous Cre+/−.COUP-TFII fl/wt mice leads to an efficient inactivation of the COUP-TFII gene in β-cells. The expression of COUP-TFI, a closely related family member, was not altered, suggesting that adaptative upregulation of this isoform does not occur (Fig. 7).

Finally, to understand the lethality of some COUP-TFII fl/fl pups, we examined whether inclusion of the *loxP* sites might interfere with COUP-TFII gene expression. Semiquantitative RT-PCR analysis using the A5HF and R5HF primers showed that liver and kidney mRNA was decreased by ~20% in heterozygous floxed mice with respect to normal animals (data not shown). Accordingly, COUP-TFII protein analyzed by Western blot was also slightly decreased (by 10–20%) in hepatocyte nuclear extracts of heterozygous floxed mice. Therefore, the floxed COUP-TFII allele is hypomorphic, which could explain the death of some homozygous floxed COUP-TFII pups.

The present study was carried out in the Cre+/−.COUP-TFII fl/wt mice, in which the floxed allele is inactivated in the Cre-expressing cells and shows to be hypomorphic only in other tissues. Therefore, the COUP-TFII protein is expected to be decreased by half in the former cells and by 10–20% in the latter ones. All results obtained from the mutant Cre+/−.COUP-TFII fl/wt mice were compared with both control (Cre+/− or wt) and COUP-TFII fl/wt mice.

Cre+/−.COUP-TFII fl/wt mice exhibit altered insulin secretion after glucose challenge. Body weight, food intake, and body composition, as assessed by biphotonic absorptiometry, in Cre+/−.COUP-TFII fl/wt mice were comparable to the three groups of mice, suggesting that energy metabolism in these animals is not grossly abnormal (Fig. 3). However, a more detailed analysis of metabolic parameters in fasted and fed states revealed differences as shown in Fig. 3. In the fasted state, Cre+/−.COUP-TFII fl/wt mice exhibited slightly lower plasma glucose ($P < 0.01$) and free fatty acids (but do not reach statistical significance) and higher plasma insulin ($P < 0.01$) than control and COUP-TFII fl/wt mice. Despite the observed hypoglycemia, the plasma insulin–to–glucagon ratio was increased twofold in Cre+/−.COUP-TFII fl/wt mice compared with COUP-TFII fl/wt or wt mice (a ratio of 3 in Cre+/−.COUP-TFII fl/wt versus 1.4 in wt and 1.5 in COUP-TFII fl/wt). In the fed state, plasma glucose was normal in Cre+/−.COUP-TFII fl/wt mice, while plasma insulin ($P < 0.01$) and triglyceride ($P < 0.01$) levels remain higher than in COUP-TFII fl/wt and control mice. No difference was found in plasma leptin, total and HDL choles-

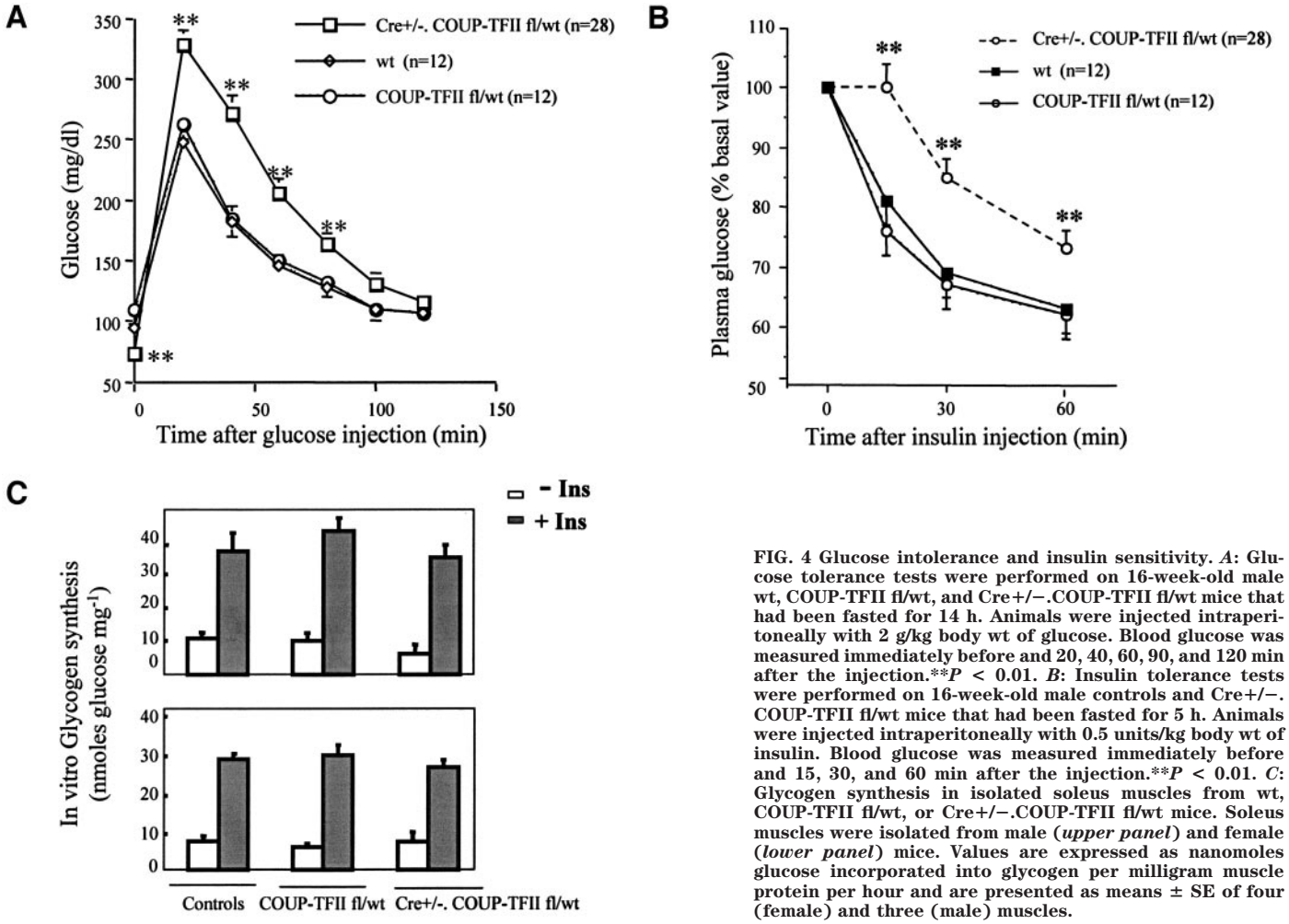

FIG. 4 Glucose intolerance and insulin sensitivity. *A*: Glucose tolerance tests were performed on 16-week-old male wt, COUP-TFII fl/wt, and Cre+/−.COUP-TFII fl/wt mice that had been fasted for 14 h. Animals were injected intraperitoneally with 2 *g*/kg body wt of glucose. Blood glucose was measured immediately before and 20, 40, 60, 90, and 120 min after the injection.$P < 0.01$. *B*: Insulin tolerance tests were performed on 16-week-old male controls and Cre+/−.COUP-TFII fl/wt mice that had been fasted for 5 h. Animals were injected intraperitoneally with 0.5 units/kg body wt of insulin. Blood glucose was measured immediately before and 15, 30, and 60 min after the injection.**$P < 0.01$. *C*: Glycogen synthesis in isolated soleus muscles from wt, COUP-TFII fl/wt, or Cre+/−.COUP-TFII fl/wt mice. Soleus muscles were isolated from male (*upper panel*) and female (*lower panel*) mice. Values are expressed as nanomoles glucose incorporated into glycogen per milligram muscle protein per hour and are presented as means ± SE of four (female) and three (male) muscles.**

terol, glycerol, and albumin levels among the three groups of mice (Fig. 3).

Intraperitoneal glucose tolerance tests in overnight-fasted Cre+/−.COUP-TFII fl/wt mice showed pronounced glucose intolerance with markedly increased blood glucose levels during the first minutes following the glucose challenge (Fig. 4*A*). Moreover, the plasma insulin levels at 20 min after glucose administration were found to be lower in the mutant mice (44 ± 10 μU/ml) than in control mice (132 ± 27 μU/ml) ($P < 0.05$). In summary, the Cre+/−.COUP-TFII fl/wt mice showed elevated basal plasma insulin levels during fasted and random-fed periods and reduced acute glucose-stimulated insulin secretion during intraperitoneal glucose tolerance tests. To determine the role played by this intrinsic defect of glucose-dependent insulin secretion, the insulin secretion in isolated islets of Langerhans from Cre+/−.COUP-TFII fl/wt mice was investigated.

COUP-TFII haploinsufficiency leads to impaired insulin secretion in isolated islets. To identify the consequence of COUP-TFII haploinsufficiency at the level of the β-cell secretory function, we studied glucose- and L-arginine–induced insulin release in an islet perifusion protocol (15). In the basal state, measured in 3 mmol/l glucose, Cre+/−.COUP-TFII fl/wt islets showed a trend (not significant) to release more insulin than the COUP-TFII fl/wt islets, especially at early time points in the perifusion protocol (Fig. 5*A*). Upon glucose challenge, a profound defect in insulin release was observed. In COUP-TFII fl/wt islets, glucose induced insulin release in a concentration-dependent manner between 5 and 10 mmol/l glucose; whereas, Cre+/−.COUP-TFII fl/wt islets showed virtually no response to 7.5 mmol/l glucose, while their response to 10 and 20 mmol/l glucose was only one-third of that seen in the control islets (Fig. 5*A* and *B*). Finally, the insulin secretion in response to the combined stimulation of 20 mmol/l arginine with 10 mmol/l glucose was also significantly lower in the Cre+/−.COUP-TFII fl/wt islets. As the stimulation by arginine was similar in COUP-TFII fl/wt and Cre+/−.COUP-TFII fl/wt islets (3.3- and 4.1-fold, respectively, Fig. 5*B*), we conclude that in Cre+/−.COUP-TFII fl/wt islets, the glucose-induced insulin secretion is specifically affected.

These abnormalities of the insulin secretion by COUP-TFII–deficient islets were not associated with altered islet morphology (data not shown). Intensity of insulin and glucagon immunostaining appeared normal, as was the estimated size of the insulin storage pool (Fig. 6). In addition, the insulin and glucagon mRNA concentrations, assessed by semiquantitative RT-PCR (Fig. 7), were not changed in mutant mice. This suggests that the reduced insulin release from Cre+/−.COUP-TFII fl/wt islets is unlikely to be caused by abnormalities in insulin or glucagon gene expression. The β-cell mass, estimated by quantitative mor-

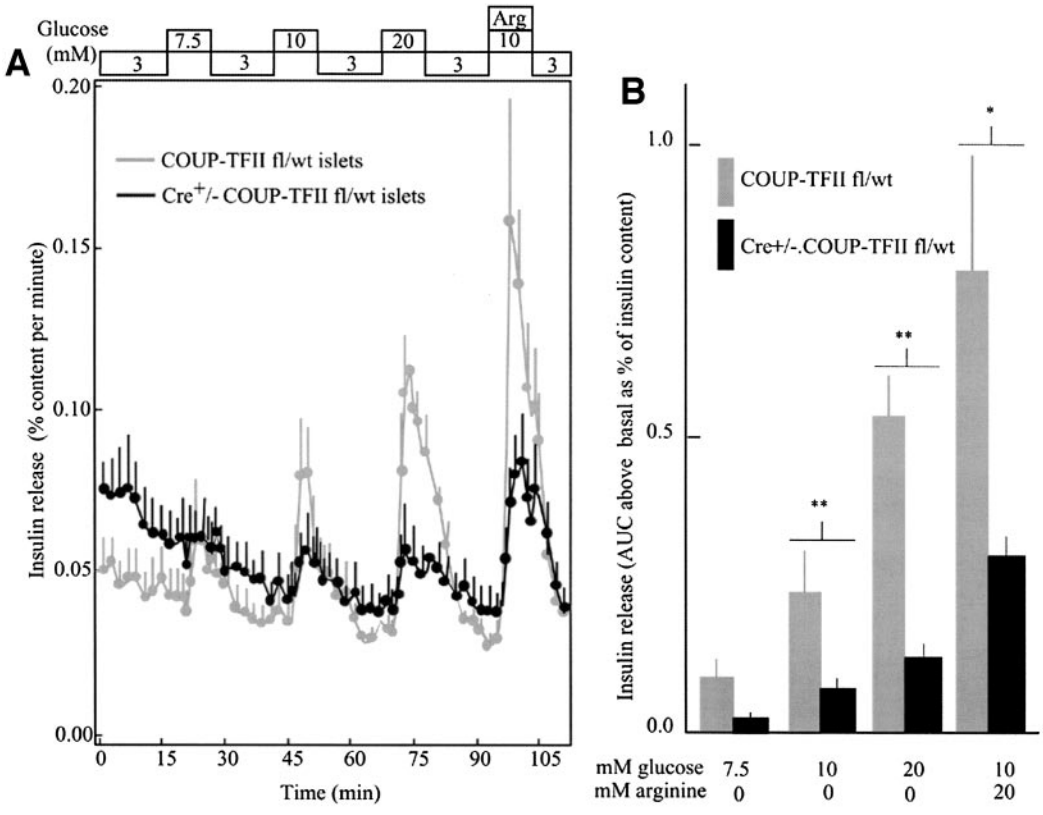

FIG. 5 Blunted glucose- and L-arginine–stimulated (Arg) insulin secretion in isolated Cre+/−. COUP-TFII fl/wt pancreatic islets. *A*: Dynamic insulin secretory response to four successive nutrient stimulations (10 min each) alternating with basal (3 mmol/l) glucose. *B*: Integrated insulin release above basal following each stimulation. Results are expressed as mean insulin release (percent of islet insulin content) ± SE (n = 4). *P < 0.05; **P < 0.01.

phometric analysis, showed no differences between wt, COUP-TFII fl/wt, and Cre+/−.COUP-TFII fl/wt mice (Fig. 6).

The effects of COUP-TFII haploinsufficiency in β-cells on the expression of genes critical for glucose sensing and insulin secretion were examined by semiquantitative RT-PCR experiments. All the transcripts studied were similarly abundant in controls (wt and COUP-TFII fl/wt mice) and heterozygous mice, except the glucose-responsive gene encoding GLUT2, whose mRNA abundance was significantly increased (by 60%) in the islet cells of knockout animals (Fig. 7). The GLUT2 transporter has been shown to play a key role in murine β-cells for glucose signaling to insulin secretion (16) but is not rate limiting for glucose entry to β-cells (17). Nevertheless, increased abundance of the GLUT2 mRNA in the islets of the Cre+/−.COUP-TFII fl/wt mice could result from a decreased repression by COUP-TFII, maybe acting as a hepatocyte nuclear factor (HNF)-4α competitor (7). More biology of the β-cell and genomewide expression analysis in purified pancreatic β-cells will be necessary to enable us to further define more systematically potential mediators of glucose recognition in our model.

Cre+/−.COUP-TFII fl/wt mice exhibit altered insulin sensitivity in vivo. Since altered insulin secretion can modify insulin sensitivity in vivo, an insulin tolerance test was performed showing that Cre+/−.COUP-TFII fl/wt animals were resistant to the glucose-lowering effect of exogenous insulin (Fig. 4*B*). The normal insulin sensitivity of the COUP-TFII fl/wt mice allowed us to rule out a role of the hypomorphic floxed allele by itself. Interestingly, in isolated soleus muscle, basal and insulin-stimulated glycogen synthesis is similar when wt is compared with COUP-TFII fl/wt or Cre+/−.COUP-TFII fl/wt mice, indicating that insulin resistance is unlikely a primary defect in skeletal muscle (Fig. 4*C*). In addition, primary abnormalities in hepatocytes can be ruled out, since mice with the hepatocyte-specific conditional COUP-TFII gene inactivation exhibit normal insulin sensitivity (M.V-C., unpublished data).

In conclusion, the observed insulin resistance in heterozygous mice could be, at least in part, the consequence of persistent higher insulin levels. In some mice models and in humans, chronic hyperinsulinemia was shown to be a self-perpetuating cause of the defects in insulin action and not only a compensatory response to insulin resistance (18–21).

Summary. Our results demonstrate that a heterozygous COUP-TFII deletion in islet β-cells leads to an impaired glucose sensitivity and then to an abnormal insulin secretion and secondary insulin resistance. The results obtained

(n=5)	Insulin content	β– cell mass	β– cell density
	ng/islet	mg	μm²/nuclei
Control	66.1 ± 8.3	0.83 ± 0.25	171 ± 14
COUP-TFII fl/wt	52.4 ± 7.7	0.78 ±0.22	149 ± 12
Cre+/-. COUP-TFII fl/wt	66.9 ± 11.3	0.62 ± 0.15	162 ± 9

FIG. 6. Pancreatic insulin content, β-cell mass, and β-cell density in 14-week-old mice. Results are the mean values ± SE for three to five animals per genotype.

	Cre+/-.COUP-TFII fl/wt/Cre+/-	COUP-TFII fl/wt/Cre+/-
β actine	1.3	1.17
-RT	-	-
GLUT2	1.6*	1.0
Glucokinase	1.1	0.9
Hexokinase 1	1.0	1.0
Sur1	1.1	1.1
Kir6.2	1.1	1.3
Insulin R	1.1	1.3
Igf1R	1.3	1.1
Insulin	1.1	1.0
Glucagon	1.1	1.1
COUP-TFI	1.1	1.1

FIG. 7. Steady-state mRNA levels of target genes in Cre+/−.COUP-TFII fl/wt and COUP-TFII fl/wt pancreatic islets of 3- to 4-month-old mice. RT-PCR analysis for islet glucose-sensing proteins and COUP-TFI transcription factor in isolated islets. Eight different animals for each genotype we used for RT-PCR using [α-^{32}P] dCTP. Mutant/Cre+/− and COUP-TFII fl/wt/Cre+/− indicate the ratio of expression levels of the mean values of Cre+/−.COUP-TFII fl/wt (mutant) and COUP-TFII fl/wt to controls (Cre+/− or wt). *P < 0.01 Cre+/−.COUP-TFII fl/wt vs. Cre+/−.

in vivo and in isolated islets allow us to exclude any participation of the autonomic nervous system in the alteration of insulin secretion. In addition, pleiotropic effects of reduced intracellular COUP-TFII activity on β-cell metabolism, which may be detrimental to the generation of ATP, seem to be excluded since genes analyzed by RT-PCR show no major change in expression level. Moreover, as COUP-TFII deficiency is genetically imposed upon β-cells, we interpret the lack of α-cell response to hypoglycemia as secondary e.g., caused by a high rate of insulin release. It is well known that β-cells can suppress α-cell function (22). Tissue-specific deletion of HNF-3β in pancreatic β-cells in mice suggested in vivo coupling of α-cell function to β-cells (23). It is worthy of note that the abnormalities due to a partial COUP-TFII deficiency in pancreatic β-cells mimic some of the disorders observed in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

Gene targeting and conditional allele generation. A mouse 129SV PAC genomic clone containing the entire COUP-TFII gene was isolated by screening the RPCI21 library (from the resource center of the German Human Genoma Project). A conditional allele was generated by flanking the first exon with *loxP* sites for the Cre recombinase. A *loxP* site was inserted into a *Bgl*II site located 445 bp upstream of the Met-ATG codon, and the pgk-neo selection gene flanked by two additional *loxP* sites was introduced into a *Nde*I site located in the first intron. The targeting construct was obtained after digestion by *Hind*III-*Avr*II, and the insertion was electroporated into CK35 embryonic stem cells (24). After selection, the genotype of G418-resistant clones were identified by Southern blot analysis after *Xba*I digestion using an external flanking 5′ *Eco*RI-*Eco*RI DNA fragment as a hybridization probe, after *Spe*I digestion using an external 3′ noncoding DNA fragment and after PCR analysis. Ten percent of correctly recombined clones were identified. Cre-mediated recombination was obtained after electroporation of 10^7 recombinant embryonic stem cells using 4 μg pIC-Cre vector (25). Of 288 clones isolated after Cre expression, 4 had lost the selection gene, as identified by Southern blot analysis of *Xba*I-digested DNA with E1 probe. Two clones containing an fl first exon were isolated. Mice strains carrying a COUP-TFII fl allele were established on a mixed C57BL/6N × 129SVJ background. Genotyping was performed by PCR analysis using DNA isolated from the tail tip of newborn mice using the primers flanking the 5′ *loxP* site: A5HF, GCA AGT CGA TTG TCT GGC TTC; R5HF, AAC TCC TCC GCT GCA CAC TA.

Rip-Cre–mediated recombination of the COUP-TFII gene. Transgenic mice expressing Cre recombinase under the control of rat insulin II promoter (Rip-Cre transgenic line [13,14]) were used to produce COUP-TFII β-cell–specific inactivation. The intercrossing COUP-TFII fl mice and Rip2-Cre mice were genotyped by PCR using the primers described above and primers against the Cre transgene and an internal upstream stimulatory factor 1 control as described previously (26). Cre recombination efficiency was assessed on liver, brain, spinal cord, hypothalamus, and islet DNA prepared from isolated islets, as described below, and by PCR. The first one, 5′ to the *loxP* site (1), was located in the 5′ noncoding region with the sequence TGA TTT CGA TGG CTT TCC TG, while the second primer (3) was located in exon 1 with the sequence CGG AGG AAC CTG AGC TAC AC. The third primer in intron 1 (2) was located at the 3′ of the *loxP* site with the sequence TGC CCA CAC TTT CCT ACT CC (Fig. 1*A*). An 850 bp indicated an intact exon 1 from the fl allele, an 810 bp an intact exon 1 from the wt allele, while a 500 bp indicated Cre-mediated recombination.

Analysis of COUP-TFII expression by Western blotting. A total of 1,000 islets were cultured for 16 h in Ham's F10 medium containing 10 mmol/l glucose and 10% FCS. Nuclear proteins were prepared as described (27), and 20 μg were subjected to a standard Western blot protocol using our COUP-TF–specific antibodies at a 1:1,000 dilution (11).

Animals. Control animals used in this study were either wt or Cre+/−. wt mice. Adult male mice were studied at 14–16 weeks of age. Animals had free access to water and standard mouse diet. All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

Analytical procedures. For glucagon, blood samples obtained by retroorbital phlebotomy were collected in tubes containing aprotinin, and radioimmunoassay was performed (Biochem ImmunoSystems-Pharmacia and Upjohn-France). In a fed state, blood was collected at 9:30 P.M. For fasting experiments, food was removed at 9:00 A.M. and the mice were kept for 4 h before blood sampling. Plasma insulin and leptin concentrations were assessed using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem, Chicago, IL) and a mouse leptin enzyme-linked immunosorbent assay kit (Crystal Chem), respectively. Plasma concentrations of triglycerides, free fatty acids, glycerol, total and HDL cholesterol, and albumin were determined using an automated Monarch device (Instrumentation Laboratory, Lexington, MA).

Measurement of insulin release from perifused islets. Isolation of islets of Langerhans, overnight culture, and measurement of insulin release were carried out as described previously (15). Approximately 200 islets were loaded onto a Biogel P2 column and preperifused for 20 min in Ham's F10 medium, supplemented with 0.5% BSA, 2 mmol/l glutamine, 2 mmol/l CaCl2, and 3 mmol/l glucose equilibrated with 95% air/5% CO_2. Then, at a flow rate of 0.5 ml/min, pulses of 10 min were given with 7.5, 10, and 20 mmol/l glucose and 10 mmol/l glucose with 20 mmol/l L-arginine. Samples were collected every minute and assayed for immunoreactive insulin with guinea pig anti-insulin serum (Linco Research, St. Louis, MO). As islet insulin contents between models were similar, results were expressed as percentage of islet insulin content. Insulin was extracted by sonicating the Biogel P2 containing the islets in 5 ml of 2 mmol/l acetic acid/0.25% BSA.

Immunohistochemistry, quantification of β-cell mass, and β-cell density. β-Cell mass was determined as described previously (28) by insulin immunostaining (Dako) on 10 subsequent sections of pancreas tissue separated by >50 μm followed by measurement of the surface area of the sections using Biocom VisioL@b 1000 software. β-Cell mass was calculated by multiplying the pancreatic weight by the relative β-cell volume. β-Cell density was assessed by counting the number of nuclei in a 30,000-μm^2 islet total area that contained only insulin-positive cells. Values are the means ± SE in five mice of each genotype.

Gene expression analysis. Total pancreatic islet RNA isolated using Trizol (Gibco-BRL) were treated with RNase-free DNase-I before use for semiquantitative RT-PCR. The PCR cycle numbers were estimated for each primer pair to assure linear range amplification. All results were verified in seven control COUP-TFII fl/wt and mutant mice. We quantified bands using a STORM850 PhosphoImager and Image-Quant 5.0.

Determination of glycogen synthesis in isolated muscle. The two soleus muscles were isolated as described previously (29) and incubated with or without 10 nmol/l insulin for 60 min at 37°C in 1 ml of Krebs-Ringer bicarbonate buffer (pH 7.3) supplemented with 1% BSA and with 3-[3H] glucose

(5 mmol/l, 1 μCi/ml). Muscles were dissolved in 1 N NaOH, aliquots were spotted onto Whatmann paper, and the filters were washed three times in ice-cold 60% ethanol before counting.

Statistical analysis. Data are expressed as means ± SE. Statistical analyses were carried out using an unpaired two-tailed Student's *t* test for dual samples and Mann-Whitney for groups, and null hypothesis was rejected at 0.05.

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