Cell Metabolism
Short Article

Inhibition of PKCε Improves Glucose-Stimulated Insulin Secretion and Reduces Insulin Clearance

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DOI 10.1016/j.cmet.2007.08.012

SUMMARY

In type 2 diabetes, pancreatic β cells fail to secrete sufficient insulin to overcome peripheral insulin resistance. Intracellular lipid accumulation contributes to β cell failure through poorly defined mechanisms. Here we report a role for the lipid-regulated protein kinase C isoform PKCε in β cell dysfunction. Deletion of PKCε augmented insulin secretion and prevented glucose intolerance in fat-fed mice. Importantly, a PKCε-inhibitory peptide improved insulin availability and glucose tolerance in *db/db* mice with preexisting diabetes. Functional ablation of PKCε selectively enhanced insulin release ex vivo from diabetic or lipid-pretreated islets and optimized the glucose-regulated lipid partitioning that amplifies the secretory response. Independently, PKCε deletion also augmented insulin availability by reducing both whole-body insulin clearance and insulin uptake by hepatocytes. Our findings implicate PKCε in the etiology of β cell dysfunction and highlight that enhancement of insulin availability, through separate effects on liver and β cells, provides a rationale for inhibiting PKCε to treat type 2 diabetes.

INTRODUCTION

Glucose homeostasis is maintained by a complex interplay between liver, skeletal muscle, and adipose tissue and is largely orchestrated by the release of insulin from pancreatic β cells. This relationship is perturbed in obesity, whereby the effectiveness of insulin in regulating glucose fluxes out of liver, and into skeletal muscle, is compromised. In most instances, however, this insulin resistance can be overcome by a compensatory enhancement of insulin secretion. Type 2 diabetes (T2D) arises selectively in that subset of individuals whose β cells fail to compensate for insulin resistance in liver and muscle (Cavaghan et al., 2000; Kahn et al., 2006).

β cell failure is characterized by a reduction in β cell mass compared to that of nondiabetic obese subjects, as well as a number of secretory defects including enhanced basal secretion and a selective loss of responsiveness to glucose (Cavaghan et al., 2000; Grill and Bjorklund, 2000; Kahn et al., 2006; Rhodes, 2005). The molecular basis of β cell failure is poorly understood, although a key role has been proposed for intracellular lipid accumulation resulting from either oversupply or altered cellular metabolism (Grill and Bjorklund, 2000; Muoio and Newgard, 2006; Prentki and Nolan, 2006; Rhodes, 2005; Unger and Zhou, 2001). Dyslipidemia is also implicated in the development of other aspects of the metabolic syndrome, and in some tissues this may involve activation of isozymes of the lipid-regulated protein kinase C (PKC) family (Morino et al., 2006; Schmitz-Peiffer, 2000). Chronic lipid treatment of muscle, liver, and β cells results in preferential activation of the novel subgroup of PKC isozymes (Considine et al., 1995; Schmitz-Peiffer et al., 1997; Wrede et al., 2003). Surprisingly, however, a causative role for PKC in β cell failure has not been addressed directly. Our current goal, therefore, was to evaluate the potential contribution of a member of the novel PKC group, PKCε, in models of glucose intolerance, with particular emphasis on the development of β cell dysfunction.

RESULTS

Fat-Fed *PKCε*$^{-/-}$ Mice Are Protected against Glucose Intolerance

We generated *PKCε*$^{-/-}$ mice, which lack PKCε protein in all tissues examined (see Figure S1 in the Supplemental Data available with this article online). These mice and wild-type littermates were subjected to high-fat feeding, which induced features of the metabolic syndrome in humans, such as obesity and hyperglycemia (Table S1). The diet also provoked gross impairment of glucose disposal during an intraperitoneal glucose tolerance test (i.p. GTT) in wild-type mice (Figure 1A). Remarkably, glucose tolerance was essentially normal in fat-fed *PKCε*$^{-/-}$ mice (Figure 1A). Compared to chow-fed controls,

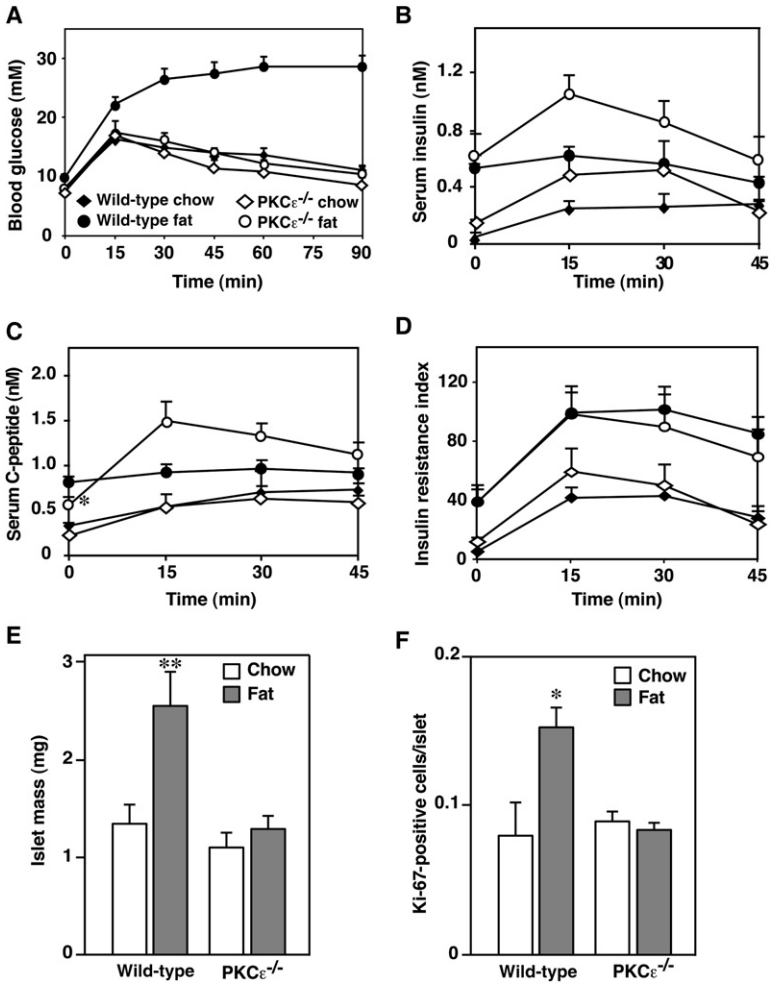

Figure 1. Fat Diet Effect on Glucose Tolerance and Islet Histology in *PKCε*$^{-/-}$ Mice

(A) Blood glucose levels during an intraperitoneal glucose tolerance test (i.p. GTT) of wild-type (n = 9) and *PKCε*$^{-/-}$ mice (n = 8) fed a saturated fat diet for 16 weeks and wild-type (n = 10) and *PKCε*$^{-/-}$ mice (n = 5) fed a standard chow diet as controls. ANOVA: $p < 0.001$ for effect of fat diet in wild-type mice; $p < 0.001$ for effect of PKCε deletion in fat-fed mice. In this and all other figures, error bars represent SEM.

(B) Serum insulin levels during i.p. GTT. ANOVA: $p < 0.001$ for effect of fat diet; $p < 0.002$ for effect of PKCε deletion.

(C) Serum C-peptide levels during i.p. GTT. ANOVA: $p < 0.005$ for effect of fat diet; ANOVA: $p < 0.001$ for effect of PKCε deletion in fat-fed mice. Student's t test: *p < 0.025, fat-fed *PKCε*$^{-/-}$ versus fat-fed wild-type mice at 0 min.

(D) Insulin resistance index (glucose concentration (mM) × insulin concentration (mU/l) ÷ 22.5) calculated from glucose and insulin levels during i.p. GTT. ANOVA: $p < 0.001$ for effect of fat diet.

(E) Comparison of islet mass in wild-type and *PKCε*$^{-/-}$ mice fed either a chow diet or a saturated fat diet. Student's t test: **p < 0.01, fat-fed wild-type versus chow-fed wild-type mice. Results shown in (E) and (F) are from pancreatic sections of at least five animals per group.

(F) Cell proliferation in islets as indicated by the number of Ki-67-positive cells per islet. Student's t test: *p < 0.05, fat-fed wild-type versus chow-fed wild-type mice.

fat-fed wild-type mice exhibited higher fasting insulin levels, but these did not increase further during the i.p. GTTs despite the accompanying hyperglycemia (Figure 1B). Insulin excursions were, however, significantly increased in the *PKCε*$^{-/-}$ mice, especially those fed the fat diet (Figure 1B). PKCε might therefore inhibit insulin secretion under conditions of lipid oversupply.

This conclusion was supported by the analysis of C-peptide levels during the i.p. GTT. Although cosecreted with insulin, C-peptide is cleared much more slowly by the liver (Polonsky and Rubenstein, 1986) and is thus a more direct indicator of β cell secretory responsiveness than circulating insulin levels (Bergman, 2000). The serum C-peptide profiles observed in fat-fed mice (Figure 1C) were, like those of insulin, greatly enhanced in *PKCε* null mice compared to wild-type animals. These results suggest that deletion of PKCε facilitates a compensatory enhancement of insulin secretion specifically in fat-fed mice and that this contributes to the accompanying improvements in glucose tolerance (Figure 1A). This was more clearly demonstrated using an insulin resistance index (Cai et al., 2005) calculated from the product of glucose and insulin levels during the i.p. GTT. This index was increased by high-fat feeding irrespective of genotype, suggestive of fat-induced insulin resistance in both groups of mice (Figure 1D). It therefore seems that the restoration of glucose homeostasis following deletion of PKCε in fat-fed mice is explained better by an improvement in β cell function than by changes in insulin sensitivity.

PKCε Deletion Reconstitutes Glucose-Stimulated Insulin Secretion

We consequently examined the pancreatic islet phenotype in more detail. Total pancreas weight was unaffected by diet or genotype (Table S1), and there was no difference in islet-specific mass between chow-fed wild-type and *PKCε*$^{-/-}$ mice as determined in pancreatic sections (Figure 1E). As documented previously (Rhodes, 2005), islet mass was increased in fat-fed wild-type mice but, surprisingly, not in the *PKCε*$^{-/-}$ animals (Figure 1E). This was due not to differences in apoptosis between the different experimental groups (data not shown) but to an absence of increased islet cell proliferation in fat-fed *PKCε*$^{-/-}$ mice (Figure 1F). This probably reflects the restoration of glucose tolerance and the reduced need for compensatory increases in β cell mass under these conditions. In any event, these results clearly demonstrate that the

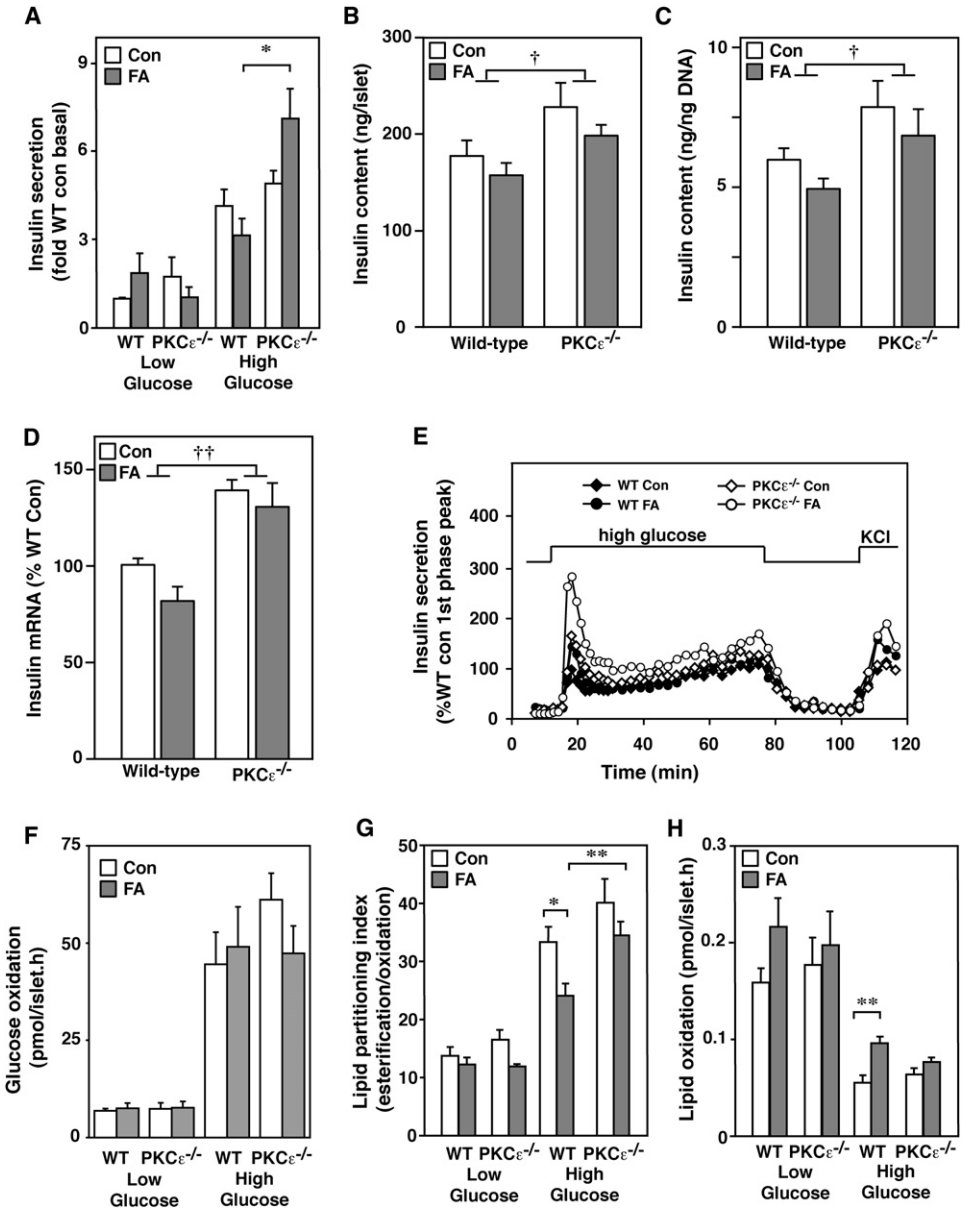

Figure 2. Insulin Secretion and Metabolism in Wild-Type and *PKCε*$^{-/-}$ Islets

Islets were precultured for 48 hr with or without 0.4 mM palmitate (FA).

(A) Insulin secretion at low (2.8 mM) or high glucose (20 mM) in 1 hr batch incubations (n = 4–5). Student's t test: *p < 0.03, FA-treated *PKCε*$^{-/-}$ versus FA-treated wild-type islets.

(B and C) Insulin content expressed per islet (B) and per ng DNA (C). ANOVA: †p < 0.02 for effect of genotype.

(D) Insulin mRNA expression. ANOVA: ††p < 0.005 for effect of genotype.

(E) Insulin secretion by islets perifused at high glucose (16.7 mM) or low glucose (2.8 mM) with or without 24 mM KCl as indicated (n = 5).

(F) $^{14}CO_2$ generation over 2 hr by islets incubated in KRB containing low (2.8 mM) or high glucose (20 mM) (n = 4).

(G) Ratio of [^{14}C]palmitate esterification versus oxidation over 2 hr by islets incubated in KRB containing low (2.8 mM) or high glucose (20 mM) (n = 3). Student's t test: *p < 0.05; **p < 0.025.

(H) $^{14}CO_2$ generation from palmitate over 2 hr by islets incubated in KRB containing low (2.8 mM) or high glucose (20 mM) (n = 4–5). Student's t test: **p < 0.02.

enhanced insulin secretion of fat-fed *PKCε*$^{-/-}$ mice is not explained by increased islet mass.

We next examined glucose-stimulated insulin secretion (GSIS) from isolated pancreatic islets that had been maintained in the presence or absence of elevated fatty acid (FA) for 48 hr ex vivo. Neither basal nor glucose-stimulated responses were altered in *PKCε* null versus wild-type islets when cultured under control conditions (Figure 2A). Following FA pretreatment, however, glucose-stimulated (but not basal) insulin secretion was increased over

2-fold in *PKCε*$^{-/-}$ compared to wild-type islets (Figure 2A). This is broadly consistent with the in vivo data (Figure 1C) and confirms that chronic lipid exposure is required for the enhanced responsiveness of *PKCε*$^{-/-}$ islets to become manifest. Insulin content was also increased in *PKCε*$^{-/-}$ islets (Figure 2B), even after controlling for potential alterations in islet size by normalizing the data to DNA content (Figure 2C). However, because these effects were both small and independent of pretreatment conditions, they are unlikely to explain either the magnitude or the specificity of the observed increases in GSIS (Figure 2A). The increased insulin content of *PKCε*$^{-/-}$ islets was paralleled by changes in insulin (I and II) mRNA (Figure 2D), but not in the expression of another secretory product, *Iapp* (Figure S2). Investigating the secretory phenotype in more detail, we observed significant increases in both the first phase (2.1-fold, $p < 0.01$) and the second phase of GSIS (1.5-fold, $p < 0.05$), but not in response to a depolarizing concentration of KCl (Figure 2E). This argues against a distal site of action and suggests an involvement of PKCε in the generation of proximal metabolic signals.

PKCε Deletion Alters FA Partitioning in β Cells

Glucose metabolism in pancreatic β cells couples to insulin secretion via separate initiation and amplification pathways. GSIS is initiated by the coupling of oxidative phosphorylation to changes in plasma membrane ion fluxes. However, in control or FA-pretreated islets, PKCε deletion did not significantly alter glucose oxidation at either basal or stimulatory glucose concentrations (Figure 2F). We therefore examined the amplification pathway, thought to involve the switching of endogenous FA metabolism from β-oxidation toward esterification, as a result of glucose-stimulated flux through the tricarboxylic acid cycle (Prentki and Nolan, 2006). In Figure 2G, we document this lipid partitioning as the ratio of [^{14}C]palmitate incorporation into total esterification products (triglyceride, diacylglycerol, and phospholipids) versus oxidative metabolism (CO_2 generation plus water-soluble oxidation products). Although acute glucose stimulation enhanced the esterification/oxidation ratio in all treatment groups, this enhancement was diminished following FA pretreatment of wild-type, but not *PKCε*$^{-/-}$, islets. Conversely, preexposure to FA augmented [^{14}C]palmitate oxidation in wild-type, but not *PKCε*$^{-/-}$, islets in the presence of high glucose (Figure 2H). Tracer incorporation into other metabolites under these conditions is shown in Figure S3. Overall, these results suggest that PKCε inhibition reconstitutes GSIS by restoring the glucose-regulated balance between lipid esterification and oxidation that is otherwise compromised by FA pretreatment. This is unlikely to reflect a role in transcription, as the expression of key genes of lipid oxidation and/or partitioning was largely unaltered by PKCε deletion (Figure S2).

PKCε Modulates Hepatic Insulin Clearance

An effect of PKCε deletion on insulin availability, independent of secretion, was also suggested by the observation that, in chow-fed *PKCε*$^{-/-}$ mice, insulin levels were elevated during i.p. GTT in the absence of any change in C-peptide (cf. Figures 1B and 1C). The fasting C-peptide:insulin molar ratio was also reduced in *PKCε*$^{-/-}$ mice (Figure 3A). Enhanced insulin levels, without changes in secretion, are consistent with a reduction in insulin clearance. To investigate this more directly, we injected chow-fed wild-type and *PKCε*$^{-/-}$ mice with a large bolus of insulin, resulting in a 30-fold increase in plasma insulin levels that far outweighed any contribution of endogenous insulin secretion (Wang et al., 2005b). Under these conditions, plasma insulin reached a higher peak in *PKCε*$^{-/-}$ mice and remained higher throughout the experiment, also consistent with a decrease in insulin clearance (Figure 3B).

As measured in liver extracts and primary cultured hepatocytes, deletion of PKCε was not associated with reductions in the number of total insulin receptors (Figure 3C) or surface-associated insulin receptors (Figure 3D), or their affinity for insulin (Figure 3E). We did, however, observe that PKCε deletion diminished [^{125}I]insulin internalization by hepatocytes (Figure 3F), corresponding to a 30% reduction in the area under the insulin uptake curve (Figure 3G). These data suggest that *PKCε*$^{-/-}$ mice have a defect in receptor-mediated insulin endocytosis. This is likely to augment circulating insulin levels because secreted insulin is partially cleared by the liver prior to reaching peripheral tissues such as muscle and fat (Bergman, 2000).

There was no difference in insulin signaling between wild-type and *PKCε*$^{-/-}$ cells, either using several insulin doses and time points with isolated hepatocytes (Figure S4) or as measured in vivo using livers from chow-fed animals (Figure S5). Likewise, the impaired insulin signaling caused by chronic high-fat feeding was not altered by PKCε deletion (Figure S5). It therefore appears that insulin uptake can be modestly reduced without significantly affecting insulin signaling in the liver, in agreement with other animal models exhibiting diminished clearance (Poy et al., 2002).

Improvement of Preexisting Diabetes by PKCε Inhibition

Our data point to a strategy for regulating insulin availability as a treatment for T2D. Therapeutic utility, however, would be substantiated by the treatment of established disease, as opposed to preventing its onset in fat-fed *PKCε*$^{-/-}$ mice. We therefore made use of the *db/db* mouse, a genetic model with similarities to human T2D (Coleman, 1978). We employed a well-documented PKCε-inhibitory peptide (εV1-2), derived from the PKCε sequence itself, that specifically blocks downstream signaling by preventing interaction with a PKCε binding partner (Hundle et al., 1997; Johnson et al., 1996). We coupled this peptide and a scrambled control to an HIV-TAT sequence that facilitates cellular uptake (Begley et al., 2004; Chen et al., 2001). Mice were injected daily for 5 days with saline, the inhibitor, or control peptide. Although body weights were unaltered between the 3 groups

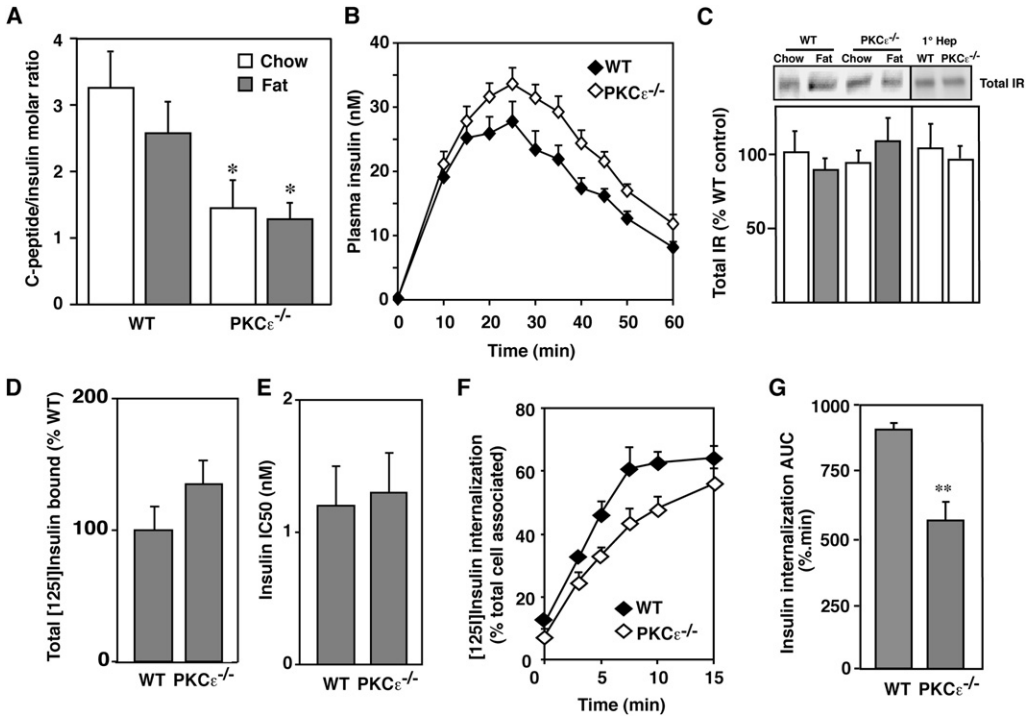

Figure 3. Insulin Clearance In Vivo and Insulin Uptake by Isolated Hepatocytes

(A) The fasting C-peptide:insulin molar ratio in chow-fed wild-type (n = 14) and *PKC*ε$^{-/-}$ mice (n = 12) and fat-fed wild-type (n = 14) and *PKC*ε$^{-/-}$ (n = 12) mice. Student's t test: *p < 0.05 versus wild-type mice fed the same diet.

(B) Plasma insulin levels after i.p. injection of 10 U/kg insulin in chow-fed wild-type and *PKC*ε$^{-/-}$ mice (n = 18 per group). ANOVA: p < 0.001 for effect of PKCε deletion on insulin levels.

(C) Representative immunoblots and results from densitometry of insulin receptor expression in either liver extracts from mice maintained as described in Figure 1A or hepatocyte lysates from chow-fed mice.

(D) Cell-surface-associated insulin receptor estimated as maximal [^{125}I]insulin binding to primary wild-type and *PKC*ε$^{-/-}$ hepatocytes at 4°C.

(E) Affinity of insulin binding to hepatocytes at 4°C calculated from [^{125}I]insulin binding curves.

(F) [^{125}I]insulin internalization by hepatocytes from wild-type and *PKC*ε$^{-/-}$ mice (n = 4–7 independent experiments per time point). ANOVA: p < 0.005 for effect of PKCε deletion.

(G) Area under the curve (AUC) for insulin uptake (n = 4). Student's t test: **p < 0.02.

(data not shown), glucose tolerance during subsequent i.p. GTTs was significantly improved by prior treatment with the inhibitor peptide. Doses of 10 mg/kg (Figure 4A) and 3 mg/kg (data not shown) were similarly effective, whereas the control peptide had no effect (Figure 4A). The improvement in glucose tolerance was accompanied by an enhanced availability of insulin as witnessed by the insulin and C-peptide profiles during the i.p. GTT. Thus, in the PKCε inhibitor-treated mice, plasma insulin levels at 30 min were almost double those of control mice, which did not respond to an observable extent (Figure 4B). A similar distinction was observed in C-peptide levels (Figure 4C). Consistent with these data, we observed that the impaired GSIS of isolated *db*/*db* islets was overcome in a dose-dependent manner by ex vivo exposure to the inhibitory peptide, but not the control peptide (Figure 4D). In contrast, PKCε inhibition had no effect on the robust response of control *db*/+ islets. This is consistent with the in vivo and ex vivo data obtained using *PKC*ε$^{-/-}$ mice (Figure 1C and Figure 2A) and indicates that inhibition of PKCε does not stimulate insulin secretion per se but rather acts selectively under conditions of secretory compromise.

DISCUSSION

We have shown that functional ablation of PKCε improves whole-body glucose disposal by augmenting insulin availability to compensate for insulin resistance. This is explained by two phenotypic characteristics: a restoration of GSIS from defective β cells and a reduction in hepatic insulin clearance. These findings suggest that PKCε plays an unexpectedly diverse role in the development of glucose intolerance.

Deletion of PKCε in β cells results in multiple effects, including augmentation of both insulin content and GSIS, alterations in fuel utilization, and a relative reduction in β cell mass. The regulation of insulin content is probably explained by accompanying changes in insulin mRNA, which suggests that PKCε should be added to the list of potential regulators of insulin gene expression (Poitout et al., 2006). Although repletion of insulin content would therefore represent an additional benefit of PKCε inhibition, this feature is unlikely to explain the reconstitution of GSIS since the latter, in contrast to the regulation of insulin content, was absolutely dependent on prior lipid exposure. This was

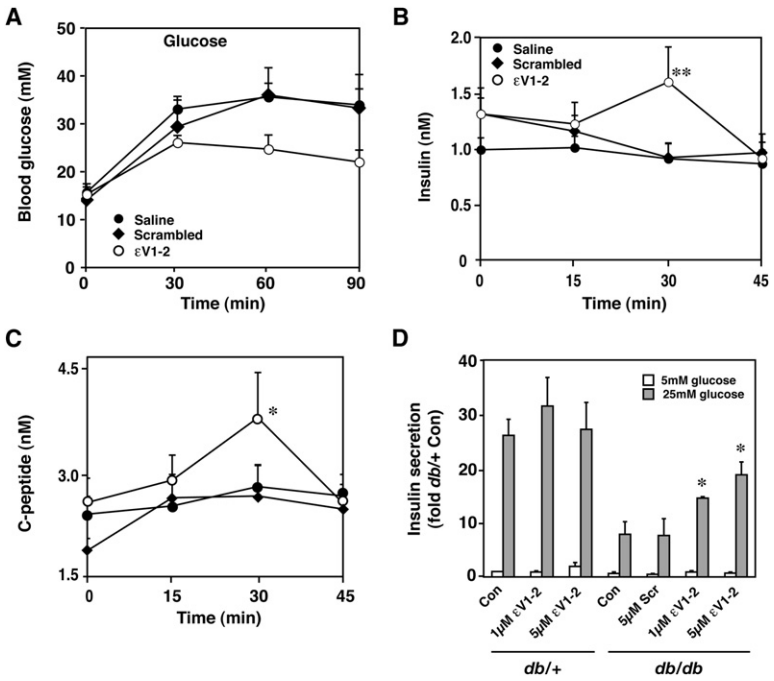

Figure 4. Effects of PKCε Inhibition on Glucose Tolerance in *db/db* Mice

(A) Blood glucose levels during i.p. GTTs performed on fasted *db/db* mice treated with 10 mg/kg εV1-2•HIV-TAT (n = 5), 10 mg/kg scrambled control peptide•HIV-TAT (n = 4), or saline (n = 7). ANOVA: $p < 0.001$, εV1-2•HIV-TAT versus saline; $p < 0.02$, εV1-2•HIV-TAT versus scrambled.

(B) Insulin levels in εV1-2•HIV-TAT-treated, scrambled peptide-treated, and saline-treated mice during i.p. GTT. ANOVA: $p < 0.01$ for effect of PKCε inhibition 0–45 min. **$p < 0.03$ for εV1-2 versus saline at 30 min by t test.

(C) C-peptide levels during i.p. GTT. ANOVA: $p < 0.05$ for effect of PKCε inhibition 0–45 min. *$p < 0.05$ for εV1-2 versus saline at 30 min by t test.

(D) Glucose-stimulated insulin secretion by islets from *db/+* and *db/db* mice pretreated for 24 hr in the absence or presence of 1 μM or 5 μM εV1-2•HIV-TAT or 5 μM scrambled control peptide•HIV-TAT (n = 3 independent experiments per group). Student's t test: *$p < 0.05$, εV1-2•HIV-TAT-treated versus control *db/db* islets.

most apparent in high-fat-fed mice, which show defects in insulin and C-peptide secretion similar to those of T2D subjects: an enhanced fasting insulin secretion and a relative loss of responsiveness to glucose (Kahn et al., 2006). Under these conditions, deletion of PKCε both lowered fasting C-peptide levels and facilitated a secretory response to glucose that was much greater than that seen in fat-fed wild-type mice. This protection was mediated directly at the level of the β cell, since the secretory defects observed in islets isolated from *db/db* mice or in wild-type islets following chronic FA exposure in vitro were reversed in the absence of functional PKCε.

PKC activation has generally been considered stimulatory for insulin secretion, but the evidence for this is much stronger for receptor-binding agonists than for nutrient stimuli (Carpenter et al., 2004; Jones and Persaud, 1998). In our hands, deletion or inhibition of PKCε did not alter GSIS in the absence of FA pretreatment, as demonstrated by both the i.p. GTTs and studies with isolated islets. This clearly demonstrates that PKCε is not normally needed for secretion in response to glucose, although earlier work suggested that PKCε is activated under these conditions (Mendez et al., 2003; Yedovitzky et al., 1997) and might contribute to glucose-dependent alterations in membrane capacitance (Mendez et al., 2003). This is similar to the situation with classical PKC isoforms, which are also activated but not required during GSIS (Carpenter et al., 2004). Our results therefore implicate PKCε activation as a necessary and proximal step in the sequence by which FAs selectively induce secretory dysfunction. While our direct focus has been lipotoxicity, we do not exclude the possibility that activation of PKCε might also contribute to secretory defects induced by chronic glucose exposure. This is consistent with the restoration of GSIS by inhibition of PKCε in *db/db* islets ex vivo, a model in which defective secretion is dependent on prior hyperglycemia (Kjorholt et al., 2005).

Although additional mechanisms may contribute, the best explanation for the improvement in secretory response appears to be a restoration of the efficiency of glucose to regulate lipid partitioning, whereby FA esterification is increased at the expense of β-oxidation. This is a key event since GSIS is reciprocally modulated by manipulations of β-oxidation (Herrero et al., 2005; Roduit et al., 2004; Rubi et al., 2002). Importantly, the secretory impairments evoked by chronic lipid exposure are also accompanied by an enhancement of β-oxidation, especially during acute glucose stimulation (Brun et al., 1997; Liang et al., 1997). Our results show that PKCε deletion appears to normalize β-oxidation under these conditions. This allows reconstitution of an amplification signal, which according to two hypotheses might comprise long-chain acyl-CoAs or lipid esterification products themselves (Prentki and Nolan, 2006) or, alternatively, the metabolic cycling of glucose-derived pyruvate (Muoio and Newgard, 2006). Because the exact identity of this signal is unknown, the site of action of PKCε is difficult to pinpoint. Alterations in the expression of key genes controlling lipid oxidation and/or partitioning were not implicated, but this key interface between lipid and carbohydrate metabolism is likely to be regulated by many other genes, as well as by posttranslational mechanisms. Interestingly, PKCε deletion did reverse the lipid-dependent downregulation of glycolytic genes such as *Glut2* (glucose transporter 2), *Gck* (glucokinase), and *Gpdh* (glycerol phosphate dehydrogenase 2) (Figure S2), but the effects were subtle and were not associated with any alteration in glucose oxidation (Figure 2E). Therefore, these changes are potentially

consequences, rather than causes, of the overall improvement in β cell function.

Insulin resistance is generally accompanied by a compensatory expansion of β cell mass. This occurs in response to circulating trophic factors that remain poorly characterized (Del Prato et al., 2004). No compensatory expansion was observed in fat-fed *PKCε*$^{-/-}$ mice, suggesting that β cell plasticity is perhaps influenced more by alterations in overall glucose tolerance than by insulin resistance per se. Thus, the improvement in glucose tolerance is presumably accompanied by a reduction in trophic factors, potentially including glucose itself, but probably not including insulin, which was increased under these conditions. Alternatively, PKCε might form part of the signaling cascade whereby an unknown trophic factor stimulates β cell expansion. Much further work is required to resolve these possibilities.

The second major phenotype that we have observed in *PKCε*$^{-/-}$ mice is the reduction in hepatic insulin clearance. Supporting evidence includes the observations that, relative to controls, these mice less readily cleared an exogenous insulin load and displayed considerably higher levels of circulating insulin during an i.p. GTT despite exhibiting a similar rate of C-peptide secretion. In addition, assessment of insulin uptake by isolated hepatocytes confirmed that PKCε deletion in these cells reduces insulin internalization. Although the inhibition we observed in insulin uptake by *PKCε*$^{-/-}$ hepatocytes was relatively subtle, it is likely to have a significant influence on whole-body insulin clearance because of the direct circulatory link between pancreas and liver. Perhaps surprisingly, insulin signaling in liver was unaffected by PKCε deletion, but it is becoming increasingly apparent that insulin receptor internalization is not always directly coupled to insulin receptor signaling (Ceresa et al., 1998; Poy et al., 2002). Moreover, insulin signaling was still severely compromised in *PKCε*$^{-/-}$ animals maintained on a high-fat diet (Figure S5). This contrasts with recent findings using mice pretreated with antisense oligonucleotides for *PKCε* and then fat-fed for 3 days (Samuel et al., 2007). Glucose tolerance curves were not presented in that study, although a slight reduction in whole-body insulin resistance was observed. In our model, a profound reversal of glucose intolerance was accompanied by only minor alterations in the insulin resistance index. These differences are probably explained by our protocol of longer-term fat feeding (16 weeks versus 3 days) with a correspondingly greater impairment in glucose tolerance. Thus, while we do not exclude the possibility that deletion of PKCε may have resulted in slight decreases in insulin resistance in our dietary model, we do not believe that this makes a major contribution to the improved glucose tolerance that we observe under these conditions.

The findings presented here provide a strong impetus for the development of PKCε inhibitors to treat T2D. Not only was glucose intolerance prevented in dietary models by long-term deletion of this PKC isoform, it was also improved in a model of preexisting diabetes by as few as five once-daily injections of a PKCε inhibitor. In the main, T2D therapeutics target separate tissues and act as muscle insulin sensitizers (thiazolidinediones), suppressors of hepatic glucose output (biguanides), or relatively nonselective stimulators of insulin secretion (sulfonylureas). Our results raise the possibility of an alternative strategy for improving insulin availability with several beneficial features. First, loss of PKCε did not alter GSIS from noncompromised islets, which is of therapeutic interest because it suggests that PKCε inhibition specifically reprograms glucose responsiveness in dysfunctional β cells rather than simply reversing a nondiscriminatory brake on secretion. Second, secretion was augmented in the first phase, which is the key regulator of glucose tolerance (Bruttomesso et al., 1999) and is selectively diminished in T2D (Cavaghan et al., 2000; Grill and Bjorklund, 2000). Third, PKCε inhibitors do not initiate secretion and so should be inactive in the absence of hyperglycemia. Finally, the effects of PKCε deletion on β cell function were complemented by diminished hepatic insulin clearance, with both aspects contributing to an enhanced availability of insulin. These features convey advantages in terms of specificity, selectivity, and potentially synergy compared to existing therapies for enhancing insulin secretion.

EXPERIMENTAL PROCEDURES

Mice

A LacZ/neo cassette was inserted into the first transcribed exon of the *PKCε* gene (Figure S1A) using standard gene targeting techniques. As a consequence of the insertion, transcription was abolished, which led to a null allele, with no evidence for smaller transcripts. Ethical approval for mouse studies was granted by the St. Vincent's Hospital Animal Experimentation Ethics Committee. Mice were maintained on a hybrid 129/Sv × C57BL/6 background using *PKCε* heterozygous breeding pairs; age-matched wild-type and *PKCε*$^{-/-}$ littermates were used for experiments. To induce insulin resistance, 7-week-old mice were fed a coconut fat/sucrose-based diet adapted from Research Diets, Inc. diet D12451 (Hennige et al., 2003) for 16 weeks. *db/db* mice were on a C57BL/KsJ background. For i.p. GTTs, mice were fasted overnight and injected intraperitoneally with glucose (2 g/kg total body weight for diet-fed C57BL/6 mice; 1 g/kg lean body weight [i.e., body weight of nonobese littermates] for *db/db* mice). Blood samples were obtained from tail tips, and glucose concentrations were measured using an Accu-Chek Advantage II glucometer (Roche). Insulin was measured by ELISA (Mercodia). C-peptide was measured by RIA (Linco).

Determination of Islet Mass, β Cell Apoptosis, and Cell Proliferation

Islet mass was quantified from pancreas sections stained with hematoxylin and eosin using a digitizing tablet and BIOQUANT software (R&M Biometrics). Islet mass was calculated from relative cross-sectional islet area and total pancreas mass. Apoptosis was assessed in pancreas sections using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling technique (In Situ Cell Death Detection Kit, POD, Roche). Cell proliferation was assessed using a Ki-67 antibody (1:100; gift from Dako) counterstained with hematoxylin.

Measurement of GSIS in Pancreatic Islets

Islets were isolated (Carpenter et al., 2004) from wild-type and *PKCε*$^{-/-}$ mice and cultured for 48 hr in RPMI 1640 supplemented with either 0.4 mM palmitate coupled to 0.9% (w/v) BSA, giving a molar ratio of 3:1, or BSA alone (Busch et al., 2002). Islets from *db/+* and *db/db* mice were cultured for 24 hr in RPMI 1640 in the absence or

presence of 5 μM either εV1-2•HIV-TAT heterodimer or scrambled control peptide. For measurement of insulin secretion in batch incubations, groups of 4–5 islets were incubated in 0.5 ml Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) containing low and high concentrations of glucose as indicated for 1 hr (Busch et al., 2002). For perifusion studies, groups of 25 islets in Swinnex chambers (Millipore) were perifused at 0.5 ml/min with 2.8 mmol/l glucose in KRB at 37°C for 10 min before experimental additions as shown (Carpenter et al., 2004). Insulin content in fractions collected over 1 min (0–20 min) or 2 min intervals (20–120 min) was analyzed by RIA (Linco).

Islet Metabolic Studies

Islets cultured as above were distributed into batches of 25 in 0.1 ml KRB containing 2.8 or 20 mM glucose with 13.6 or 1.9 Ci/mol [U-^{14}C]glucose, respectively. Alternatively, 0.4 mM palmitate coupled to 0.9% (w/v) BSA was included with [U-^{14}C]palmitate (47.6 Ci/mol). After 2 hr incubation at 37°C, an aliquot of the media was acidified for oxidation studies, and $^{14}CO_2$ was trapped onto a glass fiber filter and quantified by liquid scintillation spectrometry (Busch et al., 2005). For FA esterification studies, cell pellets were extracted overnight in 1 ml chloroform:methanol (2:1, vol/vol). After two washes in H_2O, a sample of the aqueous phase was removed for determining water-soluble oxidation products. The dried organic phase was redissolved and spotted onto silica plates (K6, Whatman), and lipids were separated by thin-layer chromatography in petroleum ether:diethyl ether:methanol:acetic acid (180:14:4:1 by volume). Spots comigrating with triglyceride, diacylglycerol, and phospholipid standards were individually scraped and counted by liquid scintillation spectrometry (Busch et al., 2005).

In Vivo Insulin Clearance

In vivo clearance of exogenous insulin was assessed as previously described (Wang et al., 2005a, 2005b). Briefly, we injected nonfasted wild-type and *PKCε*$^{-/-}$ mice intraperitoneally with 10 U/kg insulin (Novo Nordisk Actrapid) and took 10 μl blood samples from the tail vein at 10 min intervals for the determination of serum insulin levels by RIA.

Insulin Internalization by Primary Hepatocytes

Hepatocytes were isolated from livers of chow-fed wild-type and *PKCε*$^{-/-}$ mice (Scott et al., 1985). Cells were incubated with 30 pM [^{125}I]insulin on ice for 4 hr in serum-free RPMI 1640 containing 0.2% BSA, washed in PBS/0.2% BSA, and then incubated at 37°C for 0–15 min in RPMI/0.2% BSA before washing in 0.2% BSA-PBS (pH 3.0) and PBS (pH 7.4) and lysing with 1 M KOH. ^{125}I in the acid wash was counted as surface-bound, noninternalized insulin, and ^{125}I in the KOH-solubilized cells was counted as internalized cell-associated ligand. Internalized insulin was calculated as a percentage of total cell-associated insulin (the sum of surface-bound plus cell-associated ligand) (Formisano et al., 1994; Poy et al., 2002).

Treatment of *db/db* Mice with PKCε-Inhibitory Peptide

The PKCε-specific peptide εV1-2 (C-EAVSLKPT) (Johnson et al., 1996) was conjugated to a cell-permeating carrier peptide derived from the HIV-TAT sequence (C-YGRKKRRQRRR) (Vives et al., 1997) via an N-terminal cysteine bridge (Mimotopes), which enables intracellular release of the εV1-2 peptide and avoids problems with TAT-induced subcellular localization (Begley et al., 2004). Ten-week old *db/db* mice were injected intraperitoneally daily for 5 days with saline, a scrambled control peptide (10 mg/kg), or the εV1-2 heterodimer (3 or 10 mg/kg). Intraperitoneal glucose tolerance tests were carried out on day 5 after overnight fasting of the mice and final injection.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one table, and five figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/6/4/320/DC1/.

ACKNOWLEDGMENTS

This research was supported by the National Health and Medical Research Council of Australia and the Diabetes Australia Research Trust. The authors wish to acknowledge T. Jensen and C. Scott for specialist advice, S. Maleki and L. Young for Ki-67 staining, and the expert technical assistance of the Garvan Institute Biological Testing Facility, particularly E. Schmied. We also thank R. Daly, L. Campbell, D. Chisholm, B. Hegarty, D. James, and L. Holt for critical evaluation of the manuscript.

Received: December 19, 2006
Revised: July 13, 2007
Accepted: August 22, 2007
Published: October 2, 2007

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