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SHORT COMMUNICATION

Deletion of protein kinase Cε in mice has limited effects on liver metabolite levels but alters fasting ketogenesis and gluconeogenesis

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Abstract
Aims/hypothesis Protein kinase Cε (PKCε) is emerging as a key mediator of lipid-induced insulin resistance in liver and hepatic lipid metabolism itself. We investigated whether PKCε plays a role in other metabolic processes, to further examine its suitability as a therapeutic target.
Methods We measured amino acid, organic acid and sugar levels by liquid and gas chromatography-mass spectrometry of liver extracts from chow and fat-fed wild-type (WT) and PKCε-deficient (*Prkce*$^{-/-}$) mice. Fed and fasting glucose, ketone and fatty acid levels were measured in blood. Triacylglycerol levels and gluconeogenic and ketogenic enzyme expression were measured in liver. The effect of fasting on epididymal fat pad mass was also determined.
Results Metabolomic analysis indicated that the short-term high-fat diet affected over 20 compounds, including a 50% reduction in the glucogenic amino acid alanine. *Prkce* deletion resulted only in a reduction of 4-hydroxyproline and aspartate and an increase in glutamate. However, upon fasting, *Prkce*$^{-/-}$ mice were better able to maintain blood glucose levels and also exhibited lower levels of the ketone β-hydroxybutyrate compared with WT mice. Upon fasting, *Prkce* deletion also resulted in lower liver and plasma lipids and a smaller reduction in fat pad mass.
Conclusions/interpretation Metabolomic analysis provided new insights into the effects of a high-fat diet on liver metabolite levels. Glucose homeostasis under fasting conditions is improved in *Prkce*$^{-/-}$ mice, which, in turn, may reduce the mobilisation of lipid from adipose tissue, reducing the availability of ketogenic substrate in the liver. Together with the protection against fat-diet-induced glucose intolerance previously observed in the fed state, these findings indicate PKCε as a unique therapeutic target for the improvement of glucose homeostasis.

Keywords Alanine · Gluconeogenesis · β-Hydroxybutyrate · Ketone · Liver · Metabolomics · Protein kinase C

Abbreviations

βHB	β-Hydroxybutyrate
LC-MS	Liquid chromatography-MS
PKCε	Protein kinase Cε
WT	Wild type

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Introduction

Liver insulin resistance is a major characteristic of type 2 diabetes, contributing significantly to the impairment in glucose homeostasis [1]. While the precise mechanisms through which insulin resistance develops are still to be established, a strong association with fat oversupply suggests the involvement of inhibitory lipid intermediates, including diacylglycerol, an activator of protein kinase C (PKC) isoforms such as PKCε [2]. We have demonstrated that the role of PKCε in liver insulin sensitivity extends beyond inhibition of proximal insulin signal transduction [3, 4]. The protection of glucose homeostasis afforded by ablation of the gene encoding PKCε (*Prkce*) is associated with alterations in hepatic lipid partitioning in fat-fed mice [4], suggesting a broader impact on liver metabolism. The effect of a high-fat diet on liver metabolites has been previously addressed mostly on an individual basis, and we have investigated this more comprehensively using an unbiased metabolomic screen. We have also more specifically examined gluconeogenesis and ketogenesis, as well as plasma and tissue lipids. Our findings indicate that while a high-fat diet affects the levels of over 20 liver metabolites, PKCε ablation has relatively minor effects. Yet, in contrast to the high-fat diet, the absence of PKCε has pronounced effects on the metabolism of glucose and ketones under fasting conditions, associated with altered lipid supply to the liver.

Methods

Animals The generation and maintenance of WT and *Prkce* knockout (*Prkce*$^{-/-}$) mice was as described previously [4]. The Garvan/St Vincent's Hospital Animal Ethics Committee granted ethical approval for mouse studies. At 6–8 weeks of age, mice were fed either a standard chow diet (10.88 kJ/g; 8% fat, 21% protein and 71% carbohydrate; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia) or a lard-based high-fat diet prepared in-house (19.67 kJ/g; 45% fat, 20% protein and 35% carbohydrate [16% sucrose]; based on Research Diets D12451, New Brunswick, NJ, USA) for 1 week, as confounding effects of PKCε deletion on insulin secretion are not observed during this time [4]. Detailed ingredients are given in the electronic supplementary material (ESM) Methods. Mice were given free access to food or fasted, as indicated, and blood glucose was determined as described previously [4]. Blood β-hydroxybutyrate (βHB) levels were measured in tail vein blood using an Optium Xceed ketone monitor (Abbott Diabetes Care, Doncaster, VIC, Australia).

Metabolomic analyses of mouse liver Livers from chow- and fat-fed WT and *Prkce*$^{-/-}$ mice (seven per group) were harvested after a 6 h fast. Tissues were extracted, compounds derivatised and metabolomic analysis carried out, using either liquid chromatography-MS (LC-MS) or GC-MS [5], by Metabolomics Australia (University of Melbourne, VIC, Australia). Full details are given in the ESM Methods.

RT-PCR RNA was extracted from *Prkce*$^{-/-}$ and WT liver, reverse-transcribed to cDNA, and gene expression assessed by TaqMan or LightCycler UPL assays, as detailed in the ESM Methods.

Statistical analysis Student's *t* test and factorial ANOVA were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Differences with a p value <0.05 were considered significant.

Results

We performed metabolomic analysis by GC-MS and LC-MS on liver extracts to examine the effects of diet and *Prkce* ablation on levels of sugars, sterols, amino acids and other metabolites (ESM Table 1). The levels of 20 compounds were altered similarly in livers from both WT and *Prkce*$^{-/-}$ high-fat-fed mice, indicating a consistent response to the high-fat diet (Fig. 1). Strikingly, alanine, ethanolamine and γ-aminobutyric acid levels were reduced by approximately 50% by fat feeding, while tocopherol, sucrose and taurine levels were significantly increased. In contrast, few metabolites were affected by PKCε deletion (Fig. 1c). Liver 4-hydroxyproline and aspartate were 25% and 10–15% lower in chow-fed and fat-fed *Prkce*$^{-/-}$ mice, respectively, compared with similarly fed WT mice. Glutamate was 16% and 40% higher in chow-fed and fat-fed *Prkce*$^{-/-}$ mouse livers, respectively.

Alanine is a major gluconeogenic amino acid [6], and large changes in its levels in livers of fat-fed mice might indicate effects on gluconeogenesis. Furthermore, the reciprocal changes in aspartate and glutamate observed in livers from *Prkce*$^{-/-}$ mice are consistent with increased production of oxaloacetate for gluconeogenesis by aspartate transamination, which also produces glutamate [7]. We therefore examined whether diet- and PKCε-dependent changes in amino acids were reflected in alterations in gluconeogenesis. Contrary to our hypothesis, high-fat feeding did not result in altered blood glucose levels in either fed or fasted mice. However, *Prkce*$^{-/-}$ mice did exhibit higher glucose levels compared with WT mice (Fig. 2a) under fasting conditions, independently of diet. This was not associated with transcriptional upregulation of the gluconeogenic enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, which were in fact downregulated in livers from *Prkce*$^{-/-}$ mice (ESM Fig. 1a).

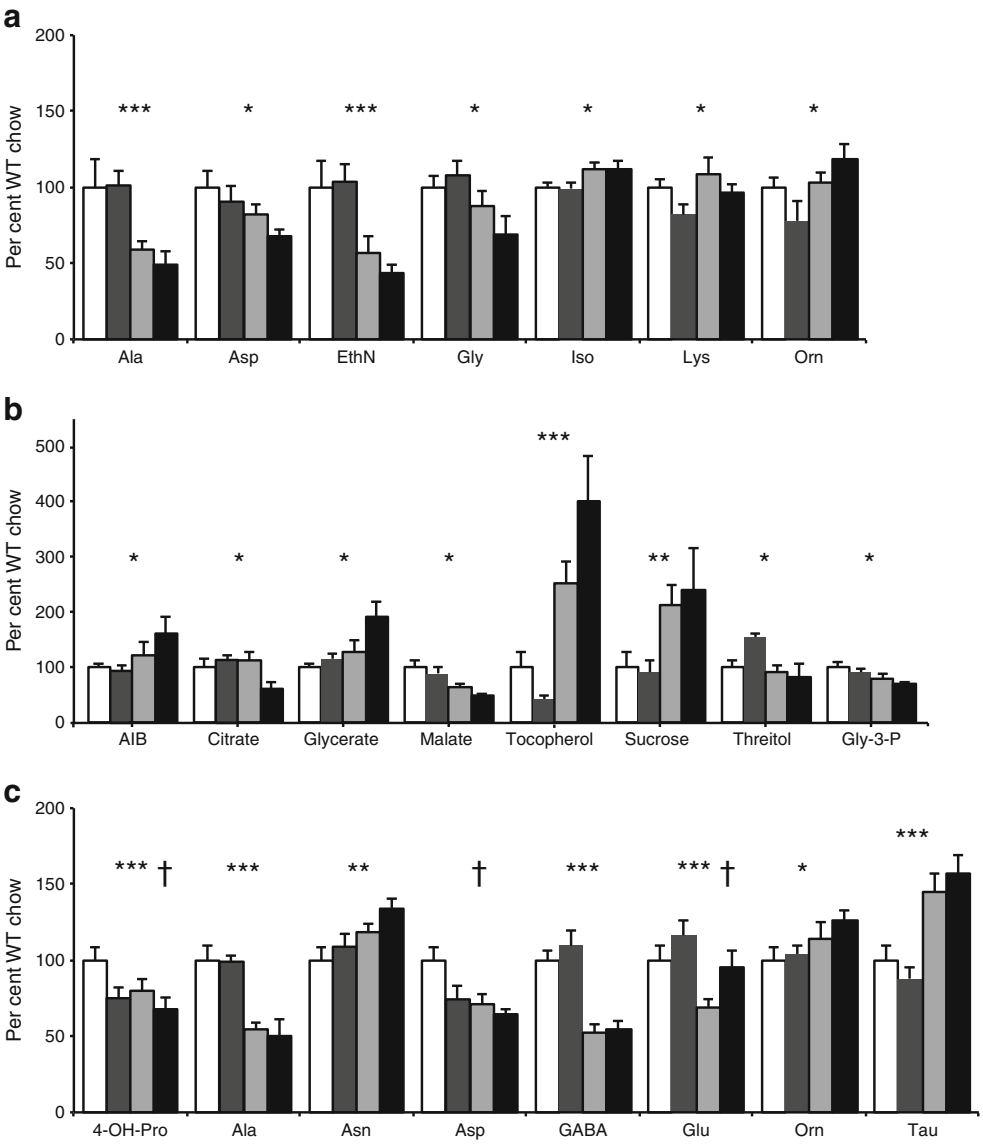

Fig. 1 Liver metabolites altered by either high-fat feeding or PKCε deletion, measured by (**a**) TBS GC-MS, (**b**) TMS GC-MS and (**c**) LC-MS. White bars, chow-fed WT liver; dark grey bars, chow-fed *Prkce*$^{-/-}$ liver; light grey bars, fat-fed WT liver; black bars, fat-fed *Prkce*$^{-/-}$ liver. ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$ for effect of diet; $^{\dagger}p<0.05$ for effect of genotype ($n=7$ per group). AIB, α-aminoisobutyric acid; EthN, ethanolamine; GABA, γ-aminobutyric acid

Fasting also leads to the generation of ketone bodies by the liver, which is highly dependent on the availability of acetyl-CoA produced by β-oxidation of fatty acids. Because the inhibitory effect of PKCε deletion on β-oxidation [4] could limit the production of ketogenic substrate, we also measured blood levels of the ketone body βHB. Low levels of βHB were observed in chow- or fat-fed mice in the fed state (Fig. 2b). In contrast, ketone levels were tenfold higher in WT mice upon fasting for 48 h, while *Prkce* deletion reduced this response by almost 40% (Fig. 2b). This reduction appears to be mediated at multiple levels. The mRNA expression of *Hmgcs2*, which encodes the rate-limiting ketogenic enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 2, was reduced in livers of *Prkce*$^{-/-}$ mice fasted for 48 h, while *Bdh1*, the product of which is also involved in βHB formation, showed a similar tendency (ESM Fig. 1b). In addition, although *Prkce* deletion was associated with increased lipid storage in the fed state, as previously reported by us [4], fasting caused triacylglycerol to increase to a lesser extent in the liver of *Prkce*$^{-/-}$ mice (Fig. 2c), suggesting reduced supply of extrahepatic lipid. This was confirmed by measurement of plasma fatty acid levels, which were reduced in fasted *Prkce*$^{-/-}$ mice (Fig. 2d). In addition, the significant reduction in fat pad mass observed upon fasting in WT mice was far less pronounced in *Prkce*$^{-/-}$ mice (Fig. 2e).

Discussion

We have shown for the first time that a high-fat diet causes alterations in the levels of specific metabolites in the liver, among them the gluconeogenic amino acid alanine. In contrast, the levels of few compounds was found to change in a PKCε-dependent manner, although we have also established

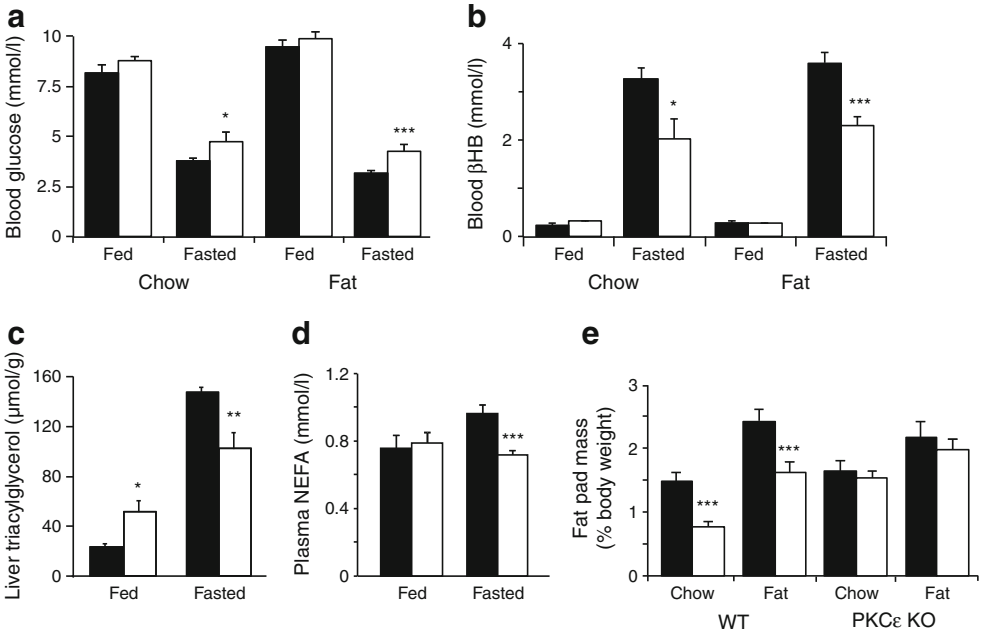

Fig. 2 Effect of PKCε deletion on metabolic variables in fed and fasted mice. (**a**) Blood glucose levels in WT (filled bars, $n=19$) and *Prkce*$^{-/-}$ mice (white bars, $n=19$), fed either a chow or fat diet for 1 week, and subjected to fasting for 48 h as indicated. (**b**) βHB levels were measured in blood from mice treated as in (**a**). (**c**) Hepatic triacylglycerol content and (**d**) plasma NEFA levels in WT ($n=10$–11) and *Prkce*$^{-/-}$ mice ($n=6$–11) fed or fasted for 48 h after 1 week of fat feeding. (**e**) Epididymal fat pad mass in mice treated as in (**a**). Student's *t* test: *$p<0.05$, **$p<0.01$, ***$p<0.005$, *Prkce*$^{-/-}$ vs WT (**a**–**d**) or fed vs fasted (**e**)

that *Prkce*$^{-/-}$ mice exhibit higher glucose levels but lower ketone body levels upon fasting. Thus, in addition to enhanced glucose tolerance [4], these animals are better able to maintain glucose homeostasis in the absence of energy supply.

NMR and MS-based analyses of changes in liver metabolites in response to a high-fat diet have been reported previously [8–10], but with a major focus on lipids. We now report the effect of a commonly used high-fat diet composition on over 20 compounds, including several amino acids and organic acids. While the elevated hepatic sucrose content we observed is likely to be due to its abundance in the diet itself, which is modelled on a Western diet, major alterations in metabolites such as alanine and taurine may indicate specific changes in hepatic metabolism.

Despite the contribution of alanine to gluconeogenesis [6], we did not observe a diet-dependent effect on blood glucose in fed or fasted mice. On the other hand, *Prkce* deletion resulted in a reduction in aspartate and an increase in glutamate. The enhanced preservation of blood glucose in fasted *Prkce*$^{-/-}$ mice may be related to the repricocal effects on these amino acids, which are consistent with the production of the gluconeogenic substrate oxaloacetate. This effect appeared to be independent of the expression of gluconeogenic enzymes, which were in fact decreased, and the precise explanation for the higher glucose levels remains to be determined.

However, the partly improved glucose availability in fasted *Prkce*$^{-/-}$ mice may, in turn, have reduced the demand for ketogenesis. This could explain the reduced mobilisation of lipid from adipose tissue, as evidenced by the smaller reduction in fat pad mass, the reduced plasma fatty acid levels and the lower hepatic triacylglycerol content in these mice. Together with the decreased expression of *Hmgcs2* in liver, these alterations most likely account for the reduced βHB levels measured in fasted *Prkce*$^{-/-}$ mice, in contrast to our initial hypothesis concerning the reduction in β-oxidation we have previously reported [4]. Interestingly, the similarity in the glucose and ketone responses of chow and fat-fed *Prkce*$^{-/-}$ mice suggest that dietary lipid oversupply is not a key factor in these phenotypes.

In summary, we have further defined the metabolic changes induced in the liver by the deletion of PKCε, which is already known to improve glucose homeostasis as well as alter lipid partitioning in the fed state. We have uncovered effects on ketogenesis and glucose homeostasis under fasting conditions, providing strong support for a wider role of PKCε in the regulation of metabolism, beyond its function as an effector of lipid second messengers to inhibit proximal insulin signalling. The improved ability to maintain fasting blood glucose levels complements the enhanced insulin secretion and action upon glucose challenge [3, 4], increasing the value of PKCε as a target for the therapy of insulin resistance.

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Contribution statement KR, GF, BD and BML, ML and CSP all designed and performed studies and analysed data. CSP directed the study, interpreted the data and wrote the paper. All authors critically revised the manuscript and approved the final version.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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