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PKCδ regulates hepatic triglyceride accumulation and insulin signaling in Lepr$^{db/db}$ mice

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ABSTRACT

PKCδ has been linked to key pathophysiological features of non-alcoholic fatty liver disease (NAFLD). Yet, our knowledge of PKCδ's role in NAFLD development and progression in obese models is limited. PKCδ$^{-/-}$/Lepr$^{db/db}$ mice were generated to evaluate key pathophysiological features of NAFLD in mice. Hepatic histology, oxidative stress, apoptosis, gene expression, insulin signaling, and serum parameters were analyzed in Lepr$^{db/db}$ and PKCδ$^{-/-}$/Lepr$^{db/db}$ mice. The absence of PKCδ did not abrogate the development of obesity in Lepr$^{db/db}$ mice. In contrast, serum triglyceride levels and epididymal white adipose tissue weight normalized to body weight were reduced in PKCδ$^{-/-}$/Lepr$^{db/db}$ mice compared Lepr$^{db/db}$ mice. Analysis of insulin signaling in mice revealed that hepatic Akt and GSK3β phosphorylation were strongly stimulated by insulin in PKCδ$^{-/-}$/Lepr$^{db/db}$ compared Lepr$^{db/db}$ mice. PKCδ may be involved in the development of obesity-associated NAFLD by regulating hepatic lipid metabolism and insulin signaling.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is believed to be initiated by the accumulation of lipids in the liver (steatosis) and is commonly associated with obesity, type 2 diabetes, and the metabolic syndrome [1]. Insulin resistance is believed to underlie the development of NAFLD. Up to 30% of the people who have NAFLD are thought to develop a more advanced form of liver disease termed non-alcoholic steatohepatitis (NASH) which can progress to cirrhosis [2].

The classical (α, β, and γ) and novel (δ, ε, and θ) protein kinase C (PKC) isoforms are intracellular signaling molecules activated acutely and chronically by diacylglycerol (DAG), a free fatty acid metabolite [3,4]. Experimental treatments such as high fat feeding, bolus lipid treatment, and genetic obesity which are known to promote DAG and fat (triglyceride) accumulation in the liver have been shown to activate the novel PKC isoforms, PKCδ and PKCε, and the classical PKC isoform, PKCβ [5–8]. Recent studies in genetically modified mice indicate that PKCβ, PKCδ, and PKCε independently regulate high fat induced triglyceride (TG) accumulation in the liver and hepatic lipogenic gene expression [6,7,9,10]. PKCβ, PKCδ, and PKCε null mice are also protected from high fat diet induced whole body and hepatic insulin resistance [6–10]. Alternatives to the high fat diet model of NAFLD also suggest a role for PKC isoforms in hepatic lipid metabolism. In a non-obese model of severe NAFLD, hepatic PKCδ gene and protein expression are upregulated, and PKCδ null mice were found to have reduced TG accumulation in the liver and altered hepatic lipogenic gene expression [11]. Further, a reduction in oxidative stress and apoptosis, key aspects of the pathophysiology of NAFLD progression, was also observed in PKCδ null mice [11]. PKCδ hepatic gene expression and activation has been detected in Lep$^{ob/ob}$ mice and Zucker Lepr$^{fa/fa}$ rats [6,8], genetic models of obesity in which hyperphagia leads to obesity and is accompanied with insulin resistance and glucose intolerance [12]. Of the three PKC isoforms implicated in NAFLD, only PKCβ has been studied in the background of a genetically obese animal. In Lep$^{ob/ob}$ mice lacking PKCβ, hepatic TG accumulation is reduced and insulin sensitivity is

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PKC, protein kinase C; MCD, methionine and choline-deficient; ALT, alanine aminotransferase; TBARS, thiobarbituric acid reactive substances; 4-HNE, 4-hydroxy-2-nonenal; TG, triglyceride; NEFA, non-esterified fatty acids.

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improved compared to Lep$^{ob/ob}$ mice containing PKCβ [9]. However, a reduction in body and adipose tissue weight in Lep$^{ob/ob}$ mice lacking PKCβ suggest that hepatic changes may be secondary to changes in obesity.

PKCδ appears to modulate hepatic TG accumulation in lean and high fat diet fed mice. Yet, whether PKCδ has a protective role in profound obesity induced NAFLD is unknown. Therefore, in the present study, we investigated the role of PKCδ in the regulation of hepatic lipid metabolism, oxidative stress, apoptosis, and insulin signaling, key pathophysiological features in NAFLD, using PKCδ$^{-/-}$/Lepr$^{db/db}$ mice.

2. Materials and methods

2.1. Antibodies

See the Supplementary Methods for details.

2.2. Animals

Heterozygous PKCδ$^{-/+}$ mice in a mixed 129SX1 × C57BL/6 background were backcrossed up to six times with C57BL/6 N mice from Harlan Laboratories (Somerville, NJ). PKCδ genotyping was performed as previously described [13]. Heterozygous PKCδ$^{-/+}$ mice in a C57BL/6N background were backcrossed two times with Lepr$^{db/+}$ C57BL/6J mice from Jackson Laboratories (Bar Harbor, MA) and then interbred to generate PKCδ$^{-/-}$/Lepr$^{db/db}$ mice and Lepr$^{db/db}$ littermates. Mice were placed on a low fat diet (MP Biomedical, Cat #96044) for 4 weeks. Mice were housed 2–4 per cage in Thoren units in the Bassett Research Institute, an AAALAC accredited animal facility, in light/dark (12L:12D), temperature 22 °C, and humidity controlled rooms. Mice were provided with standard laboratory chow and water ad libitum in accordance with an Institutional Animal Care and Use Committee approved protocol. The mean and standard error (SE) of the final body, liver, and fat weight was determined. No procedures were undertaken that caused more than minimal pain, distress, or discomfort.

2.3. Histological analysis and special staining of liver tissue

Paraffin embedded sections were stained with hematoxylin and eosin and examined in a blinded fashion by a board certified pathologist, grading for steatosis as previously described [14]. TG was extracted using the Bligh and Dyer method [15] and assayed using a kit from Thermo Scientific (Rockford, IL) and normalized to the protein content measured using the BCA protein assay reagent (Thermo Scientific, Rockford, IL).

2.4. Serum metabolic parameters

Alanine aminotransferase (ALT) and triglycerides were assayed as previously described [14]. Insulin was assayed using the Ultra Sensitive Mouse Insulin ELISA Kit from Crystal Chem Inc (Downers Grove, IL). NEFA was assayed using the kit from Zen-Bio, Inc. (Research Triangle Park, NC).

2.5. Liver oxidative stress analysis

Liver samples were flash frozen and ground in liquid nitrogen. Ground tissue (50–100 mg) was homogenized on ice in PBS pH 7.4 buffer. The homogenate was tested for thiobarbituric acid reactive substances (TBARS) (ZeptoMetrix, Buffalo, NY) following manufacturer's instructions. Protein content was determined using the Pierce BCA Protein assay (Thermo Scientific/Pierce, Rockford, IL). TBARS units (nmol/ml) were normalized to protein concentration. 4-Hydroxy-2-nonenal (4-HNE) staining was performed using a 4-HNE (HNE11-S) antibody (Alpha Diagnostics, San Antonio, TX). Five random fields per slide were scored and the results were determined from an average of those scores.

2.6. Apoptosis analysis

TUNEL positive cells were detected using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) and the manufacturer's recommendations for controls. Propidium iodide (0.25 mg/ml) was used as the counterstain. An average score was generated based on a ratio of positive nuclei to total nuclei in 3 random fields.

2.7. Immunoblotting

See the Supplementary Methods for details.

2.8. RNA extraction and qRT-PCR

See the Supplementary Methods for details.

2.9. Insulin stimulation

Mice were fasted overnight, then anesthetized with an intraperitoneal injection of avertin (2,2,2-tribromoethanol) in PBS (0.5 mg/g), and the abdominal cavity opened. Insulin (12 mU/g) or sterile PBS was injected into the inferior vena cava and then the liver was harvested after 2 min and flash frozen in liquid N_2.

2.10. Statistical analysis

All data are presented as the mean ± 1 standard error (SE). Statistical significance was determined by Student's *t*-test ($\alpha = 0.05$) using the XLSTAT 2009 program (Addinsoft, New York, NY). Pairwise comparisons were made using Tukey's test ($\alpha = 0.05$).

3. Results

3.1. Body and organ weights and serum parameters

As expected, PKCδ protein expression was not detected in the liver of PKCδ$^{-/-}$/Lepr$^{db/db}$ mice (Supplemental Fig. 1). No differences in body weight, glucose, liver weight, NEFA, ALT, and insulin were observed in PKCδ$^{-/-}$/Lepr$^{db/db}$ compared to Lepr$^{db/db}$ mice. In contrast, a reduction in fat pad weight normalized to body weight and serum TG was observed in PKCδ$^{-/-}$/Lepr$^{db/db}$ compared to Lepr$^{db/db}$ mice (Table 1).

Table 1
Body, liver, and fat pad weights and serum metabolic parameters from male Lepr$^{db/db}$ and PKCδ$^{-/-}$/Lepr$^{db/db}$ mice.

	Lepr$^{db/db}$	PKCδ$^{-/-}$/Lepr$^{db/db}$
Body weight (g)	48.9 ± 1.2[b]	53.1 ± 1.4
Glucose (mg/dL)	305 ± 43	251 ± 28
Liver weight (g)	3.58 ± 0.30	4.15 ± 0.17
Fat pad weight (g)	3.83 ± 0.28	3.43 ± 0.10
Liver-body weight (%)	7.30 ± 0.54	7.79 ± 0.18
Fat pad-body weight (%)	7.80 ± 0.43	6.47 ± 0.17**[c]
Triglyceride (mg/dL)	115.3 ± 23.7	75.9 ± 3.8*
NEFA[a] (mM)	1.83 ± 0.18	1.63 ± 0.14
ALT[a] (U/L)	10.34 ± 1.99	17.62 ± 2.49
Insulin (ng/ml)	12.54 ± 3.00	10.77 ± 1.95

[a] NEFA, non-esterified fatty acids; ALT, alanine aminotransferase.
[b] Values represent the means ± SEM for $n = 4–5$.
[c] Compared to Lepr$^{db/db}$ *$P < 0.05$, **$P < 0.01$.