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Journal of Nutritional Biochemistry 25 (2014) 281–288

Journal of Nutritional Biochemistry

RESEARCH ARTICLES

PKCδ is activated in the liver of obese Zucker rats and mediates diet-induced whole body insulin resistance and hepatocyte cellular insulin resistance☆,☆☆

Michael W. Greene[a,b,c,*], Christine M. Burrington[c], Yuwen Luo[b], Mary S. Ruhoff[c], Darin T. Lynch[c], Niyutchai Chaithongdi[d]

[a]Boshell Metabolic Diseases and Diabetes Program, Auburn University, Auburn, AL 36849, USA
[b]College of Human Sciences, Auburn University, Auburn, AL 36849, USA
[c]Bassett Research Institute, Bassett Healthcare Network, Cooperstown, NY, USA
[d]Department of Internal Medicine, Bassett Medical Center, Bassett Healthcare Network, Cooperstown, NY, USA

Received 5 June 2013; received in revised form 6 September 2013; accepted 8 October 2013

Abstract

Insulin resistance can arise when pathological levels of free fatty acids (FFAs) and proinflammatory cytokines disrupt insulin signaling. Protein kinase C delta (PKCδ) is a FFA- and a proinflammatory cytokine-regulated protein kinase that is associated with inhibition of insulin signaling and action. To gain insight into the role of PKCδ in insulin resistance, PKCδ activation was studied in a genetic model of obesity-linked insulin resistance. PKCδ was found to be activated in the liver of obese insulin-resistant Zucker rats and in isolated cultured hepatocytes. PKCδ was further studied in PKCδ-null mice and their wild-type littermates fed a high-fat or control diet for 10 weeks. PKCδ-null mice on a high-fat diet had improved insulin sensitivity and hepatic insulin signaling compared to wild-type littermates. Additionally, the deleterious effect of a high-fat diet on glucose tolerance in wild-type mice was completely blocked in PKCδ-null mice. To directly test the role of PKCδ in cellular insulin resistance, primary hepatocytes from the high-fat diet mice were isolated and stimulated with insulin. Primary hepatocytes from PKCδ-null mice had improved insulin-stimulated Akt and FOXO phosphorylation compared to hepatocytes from wild-type littermates. Consistent with this result, tumor necrosis factor alpha-mediated inhibition of insulin signaling was blocked in PKCδ knockdown primary hepatocytes. These results indicate that PKCδ plays a role in insulin resistance and is consistent with the hypothesis that PKCδ is a negative regulator of insulin signaling and thus may be a therapeutic target for the treatment of type 2 diabetes.
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Keywords: Protein kinase C; Hepatocyte; Insulin

1. Introduction

Insulin resistance is the inability of cells to respond to normal concentrations of insulin, which functions to stimulate the storage of fuel in muscle, liver and fat. Insulin resistance is often associated with obesity, a major public health problem in the United States. Individuals with insulin resistance have an elevated risk for the development of coronary heart disease [1,2], the number 1 cause of death in the United States. Additionally, insulin resistance is the single best predictor for the development of type 2 diabetes in people with a family history of the disease [3] and is believed to underlie the development of fatty liver disease, which can progress to nonalcoholic steatohepatitis and cirrhosis, an advanced form of liver disease [4].

Because activation of the novel protein kinase C (PKC) isoforms (PKCθ, PKCε and PKCδ), but not classical or atypical PKC isoforms, are consistently observed in the livers of obese diabetic patients compared with lean, nondiabetic control subjects [5–7] and are observed in lipid-induced insulin resistance in human muscle [8], it has been suggested that the novel PKC isoforms constitute a set of potential therapeutic targets that can be exploited to treat insulin resistance. In animal models, hepatic insulin resistance was prevented in mice with reduced expression of PKCε in the liver and white adipose tissue [9]. Glucose tolerance has also been shown to be normalized in the high saturated or unsaturated fat-fed PKCε-null mice [10,11]. Taken together, these results suggest that the PKCε isoform may be a good target for therapeutics to treat type 2 diabetes and an alternative strategy to normalize glucose tolerance. However, the improved glucose tolerance observed in high-fat-fed PKCε-null mice was suggested to be due to enhanced insulin availability rather than an improvement in insulin sensitivity [10,12]. In contrast, PKCδ-null mice fed a high-fat diet (HFD) exhibited increased insulin sensitivity, reduced liver triacylglycerol accumulation and diminished lipogenic enzymes production, which were not observed in high-fat-fed PKCε-null mice [12]. Finally, additional studies focused on PKCδ

☆ Grant support: This work has been supported by the Stephen C. Clark Fund and the E. Donnall Thomas Resident Research Fund.
☆☆ Disclosure statement: The authors have nothing to declare.
* Corresponding author. College of Human Sciences, Auburn University, 101C Poultry Science Building, 260 Lem Morrison Dr., Auburn, AL 36849, USA. Tel.: +1 334 844 8435.
E-mail address: mwgreene@auburn.edu (M.W. Greene).

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http://dx.doi.org/10.1016/j.jnutbio.2013.10.008

and metabolic dysfunction have demonstrated that hepatic PKCδ was activated in an acute model of insulin resistance [13,14], that adipocyte PKCδ was activated in HFD-induced insulin resistance [15,16] and that glucose tolerance was normalized in the high-fat-fed PKCδ-null mice [6,12]. However, in chow-fed mice, it was shown that PKCδ regulates the second phase of insulin secretion, resulting in glucose intolerance while maintaining normal insulin sensitivity [17]. Taken together, the *in vivo* evidence suggests that PKCδ is a good target for therapeutics to treat insulin resistance, although it has not been firmly established. In support of the hypothesis that PKCδ is a good target for therapeutics to treat insulin resistance, we have demonstrated in our *in vitro* studies using cells of hepatic origin [18–20] that PKCδ regulates cellular insulin resistance. To further assess the *in vivo* role of PKCδ in insulin resistance, we set out to determine whether hepatic PKCδ is activated in a genetic model of chronic insulin resistance and whether PKCδ modulates HFD-induced whole body insulin resistance.

2. Methods and materials

2.1. Antibodies

Polyclonal antibodies to PKCδ (C-20 and C-17) and monoclonal antibodies to GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies to Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), phosoho-GSK3β, phospho-FOXO1a (Ser256), IκBα (C-21), phospho-PKC(pan) and phospho-PKCδ (Ser643) were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Animals

Male Sprague–Dawley, Zucker obese (*fa/fa*) and Zucker lean (*Fa/fa*) rats were housed two per cage in Thoren units in the Bassett Research Institute AAALAC-accredited animal facility in 12-h light/dark cycle, with a temperature of 22°C, and humidity-controlled rooms. Rats were provided with standard laboratory chow and water *ad libitum* in accordance with an Institutional Animal Care and Use Committee-approved protocol. PKCδ-heterozygous mice and mixed C57BL/6×129/SV background mice [21] were housed and bred in the Bassett Research Institute animal facility. The mice were provided chow and water (*ad libitum*). Heterozygous mice were bred to obtain PKCδ-null mice and their wild-type littermates. Genotype was determined by polymerase chain reaction (PCR) using gene-specific primers [21] to generate a 2.9-kb band for the WT allele and a 1.8-kb band for the null allele. Genotype was verified by immunoblotting for PKCδ in brain protein lysates. PKCδ-null and wild-type littermates were placed on a control or HFD (60 kcal% fat) obtained from Research Diets, Inc. (D12450B and D12451, respectively) for 10 weeks starting at 3 weeks of age. The mean and standard error (S.E.) of the final body, liver and fat weight was determined. All animals had free access to water. No procedures were undertaken that caused more than minimal pain, distress or discomfort.

2.3. Metabolic measurement and insulin stimulation

A blood sample was drawn from the tail vein of unanesthetized rats or mice for measurements of serum glucose using a FreeStyle FLASH glucometer and strips. Animals were fasted overnight and a Glucose Tolerance Test (GTT) was performed using 2 g of glucose per kilogram body weight, administered by intraperitoneal injection. Glucose readings were taken at baseline (time=0) and at 15, 30, 60 and 120 min after injection. One week after the GTT, an ITT was conducted using Novolin R human insulin at 0.75 U/kg body weight in females and 0.85 U/kg body weight in males administered by intraperitoneal injection. Animals were fasted (females, 4 h; males, 5 h), and serum glucose was tested by tail vein at baseline (time=0) and at 30, 45, 60 and 90 min after injection. Mice were fasted overnight and then anesthetized with an intraperitoneal injection of avertin (2,2,2-tribromoethanol) in phosphate-buffered saline (PBS; 0.5 mg/g), and the abdominal cavity was 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.

2.4. Primary hepatocyte isolation and culture

Hepatocytes were isolated by collagenase digestion of isolated, perfused livers. Rats (175–225 g) were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg), and the abdominal cavity was opened. The liver isolation and perfusion techniques are based on a modified version of Seglen [22]. Perfusion began at 40 ml/min (using a 16-gauge catheter) with well-oxygenated, calcium-free Hank's balanced salt solution containing 5 mM glucose, 1.5 mM Na lactate, 0.15 mM Na pyruvate, 0.1 mM EGTA, 10 mM HEPES, 100 IU/ml penicillin (Pen) and 0.1 mg/ml streptomycin (Strep) maintained at 37°C. After 5 min, perfusion was continued with 0.05% collagenase (Sigma Type I) in low-glucose Dulbecco's modified Eagle's medium containing 10 mM HEPES, 40 mM $NaHCO_3$ and Pen/Strep. After 15 min of perfusion, cells were isolated and washed following the protocol of Berry and Friend [23]. Damaged cells were removed by centrifugation in buffered iso-osmotic Percoll [24]. Cells were suspended in Waymouth's MB 752/1 medium containing 5% bovine growth serum and Pen/Strep (Waymouth's Growth Medium). Viability was determined by trypan blue exclusion.

Mice (20–45 g) were anesthetized with an intraperitoneal injection of pentobarbital (80 mg/kg), and the abdominal cavity was opened. Perfusion began at 13 ml/min using a 22-gauge catheter. Solutions used for rat liver perfusion were also used for the mouse, except for the collagenase. Collagenase H (75 ml; Roche Applied Science) at 0.02% was pumped *in situ* through the liver. After 8 min of perfusion, hepatocytes were isolated, washed and suspended as described above. Viability was determined by trypan blue exclusion.

2.5. Infection of primary rat hepatocytes and treatment

Primary hepatocytes were cultured for 1 h in Waymouth's growth medium, then washed and infected with ~100 ng/ml of lentivirus in the presence of 50 μM vitamin E and 3 μg/ml polybrene for 6 h. Following 16 h in Waymouth's growth media, the cells were infected a second time as described above. Seventy-two hours after the infection, cells were subjected to experimental treatments as described in the figures. Cells were serum deprived 2 h in Waymouth's MB 752/1 medium containing 0.2% bovine serum albumin, then treated with 10 μg/ml recombinant rat tumor necrosis factor alpha (TNFα) for time as indicated.

2.6. Subcellular fractionation

Frozen liver tissue was ground with mortar and pestle in liquid N_2 and then 100–150 mg ground-frozen tissue was placed in a 1.5-ml homogenization buffer containing 10 mM Tris (pH 7.4), 20 mM sucrose, 0.1 mM Na_3VO_4, 100 nM okadaic acid and 1× protease inhibitor cocktail Set I (Calbiochem/EMD Biosciences, La Jolla, CA, USA) and then precleared by centrifugation at 250×*g* for 5 min at 4°C. Precleared tissue was homogenized by passage through a 25-gauge needle 7–8× on ice in buffer. Homogenates were centrifuged at 100,000×*g* for 30 min at 4°C. The supernatant was removed and designated as the cytosolic fraction. The pellet was resuspended in a buffer containing 10 mM Tris (pH 7.4), 2 mM NaCl, 1% Triton X-100, 0.1 mM Na_3VO_4, 100 nM okadaic acid and 1× protease inhibitor cocktail (Pierce), incubated on ice for 30 min, and then centrifuged at 100,000×*g* for 30 min at 4°C. The resultant supernatant was transferred to a fresh tube and designated as the Triton-soluble (membrane) fraction.

2.7. PKCδ kinase assay

Cells were lysed in 0.3 ml Lysis Buffer [50 mM HEPES (pH 7.4), 30 mM NaCl, 10 mM protease inhibitor cocktail, 0.1 mM Na_3VO_4, 0.01 mM okadaic acid, 1% Tween-20, 50 mM sodium β-glycerolphosphate and 10% glycerol]. Cellular debris was removed by centrifugation at 16,000×*g* for 15 min at 4°C. Three micrograms of PKCδ (C-17) and 30 μl of Protein A-agarose (50% slurry) were added to the cleared lysate and mixed for 3 h at 4°C. The agarose was washed once with 100 nM Tris (pH 7.4)/500 mM NaCl, twice with Lysis buffer without inhibitors, and finally with 10 mM HEPES (pH 7.4)/50 mM NaCl/50 mM β-glycerophosphate/10% glycerol. The agarose was resuspended in 20 μl kinase buffer [130 mM HEPES (pH 7.4), 32.5 mM β-glycerophosphate, 15% glycerol, 0.65 mM okadaic acid, 6.5 mM Na_3VO_4, 6.5 mM dithiothreitol] and 2.8 μg GST-MARKS substrate. Ten microliters of Mg-ATP [20 mM HEPES (pH 7.4), 5 mM β-glycerophosphate, 0.1 mM Okadaic acid, 1 mM Na Vanadate, 1 mM DTT, 30 mM $MgCl_2$, 6 mM ATP and 5 μCi γ-^{32}P ATP] was added and incubated at 30°C for 30 min. The mixture was placed on ice, and the reaction was stopped with 10 μl of 4× sodium dodecyl sulfate (SDS) sample buffer containing DTT.

2.8. Immunoblotting

Routine methods for SDS polyacrylamide gel electrophoresis and immunoblotting were performed. Protein determination of samples was done using bicinchoninic acid (BCA) reagents from Thermo Scientific (Rockford, IL, USA). Enhanced chemiluminescence substrate was from GE Healthcare (Piscataway, NJ, USA). Goat antirabbit and antimouse Alexa Fluor 635 (Molecular Probes, Eugene, OR, USA) or horseradish peroxidase-conjugated (Sigma, St. Louis, MO, USA) secondary antibodies were used for detection and quantitation of immunoblots.

2.9. Statistical analysis

Chemiluminescent and fluorescent signals were directly quantitated using Storm 860 Imager and ImageQuant v5.1 software. The absolute integration value of the immunoreactive bands minus background was determined. Statistical significance was determined by Student's *t*-test (α=.05) or a one-way repeated-measures analysis of variance (α=.05) using the XLSTAT 2009 program (Addinsoft, New York, NY, USA). Pairwise comparisons were made using Tukey's test (α=.05).

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