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Creatine reduces hepatic TG accumulation in hepatocytes by stimulating fatty acid oxidation

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ABSTRACT

Non-alcoholic fatty liver disease encompasses a wide spectrum of liver damage including steatosis, non-alcoholic steatohepatitis, fibrosis and cirrhosis. We have previously reported that creatine supplementation prevents hepatic steatosis and lipid peroxidation in rats fed a high-fat diet. In this study, we employed oleate-treated McArdle RH-7777 rat hepatoma cells to investigate the role of creatine in regulating hepatic lipid metabolism. Creatine, but not structural analogs, reduced cellular TG accumulation in a dose-dependent manner. Incubating cells with the pan-lipase inhibitor diethyl p-nitrophenylphosphate (E600) did not diminish the effect of creatine, demonstrating that the TG reduction brought about by creatine does not depend on lipolysis. Radiolabeled tracer experiments indicate that creatine increases fatty acid oxidation and TG secretion. In line with increased fatty acid oxidation, mRNA analysis revealed that creatine-treated cells had increased expression of PPARα and several of its transcriptional targets. Taken together, this study provides direct evidence that creatine reduces lipid accumulation in hepatocytes by the stimulation of fatty acid oxidation and TG secretion.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has been associated with obesity and decreased insulin sensitivity, and a fatty liver is considered the hepatic manifestation of the metabolic syndrome [1]. The hallmark of NAFLD is the accumulation of triglycerides (TG) in lipid droplets within hepatocytes. Current data suggests that 20–30% of North Americans have NAFLD, which could progress to more severe liver damage if left untreated [2]. Current clinical treatments for fatty liver are limited and so the search for safe and effective therapy is important.

In vivo, phosphatidylcholine (PC) synthesis is a major consumer of hepatic methyl groups accounting for approximately 40% of all transmethylation reactions, and is an important determinant of hepatic TG metabolism [3]. Hepatocytes have the highest activity of phosphatidylethanolamine *N*-methyltransferase (PEMT) and they synthesize a significant portion of PC via the sequential methylation of phosphatidylethanolamine (PE) [4]. This means that synthesizing a relatively small amount of PC via the PEMT enzyme could have a significant impact on methylation reactions. Fat accumulation and NASH progression have been associated with decreased availability of *S*-adenosylmethionine (AdoMet) as well as an increase in homocysteine levels and oxidative stress [5]. Dietary betaine, an important methyl donor for the remethylation of homocysteine to methionine, protects the liver from fat accumulation and lipid peroxidation in rodent models of both alcoholic and non-alcoholic fatty liver [6,7]. Betaine has been shown to elevate AdoMet, reduce homocysteine, and beneficially alter glutathione redox reactions in alcoholic liver injury [8]. It is possible that the provision of excess methyl groups leads to increased synthesis of PC thereby increasing lipid efflux from this tissue; PC plays a functional role in the packaging of lipoproteins and is the most abundant phospholipid found in these structures. In fact, mice that have a deletion of PEMT develop a fatty liver and have reduced hepatic VLDL secretion when fed a high-fat diet [9].

De novo creatine biosynthesis occurs in the liver via the AdoMet-dependent methylation of guanidinoacetate (GAA) and is a major consumer of hepatic methyl groups, estimated to account for 40% of total methylation reactions in the body [10]. Dietary creatine supplementation can reduce plasma GAA levels by 90% and therefore reduces demand on hepatic methylation [11]. Previously, we hypothesized that dietary creatine supplementation may spare AdoMet for PC synthesis, thus protecting the liver from TG accumulation. Dietary creatine supplementation prevented TG accumulation and the lowering of AdoMet in the liver of rats fed a high-fat diet (HFD) [12]. Interestingly, dietary creatine did not alter hepatic PC levels or PEMT activity; therefore, the

Abbreviations: ABCA1, ATP binding cassette protein; AMPK, adenosine monophosphate-activated protein kinase; CT, CTP:phosphocholine cytidylyltransferase; DGAT, acyl CoA:diacylglycerol acyltransferase; HDL, high density lipoproteins; HFD, high-fat diet; HCD, high-carbohydrate diet; HMGCS2, hydroxymethylglutaryl CoA synthase 2; GPAT, glycerol-3-phosphate acyltransferase; MHFD, moderate-high fat diet; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OA, oleate; PEMT, phosphatidylethanolamine *N*-methyltransferease; McA, McArdle RH-7777 cells

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mechanism(s) through which creatine reduces fatty liver does not appear to be related to AdoMet availability. In the current study, we utilized the McArdle RH-7777 (McA) immortalized hepatoma cell line, an established model for the study of hepatic lipid metabolism that does not express PEMT [13–16], to assess whether creatine might have a direct action on TG synthesis in liver cells. We have now demonstrated that creatine prevents TG accumulation in McA cells incubated with exogenous oleic acid. Our data suggest that creatine, potentially through increased PPARα activity, stimulates fatty acid oxidation and TG secretion.

2. Materials and methods

2.1. Cell culture conditions for McA cells

McArdle RH-7777 cells were obtained from American Type Culture Collection (ATCC) and cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies) containing 10% fetal bovine serum and 10% horse serum within a humidified incubator maintained at 37 °C and 5% CO_2. Concentrated stock solutions of compounds were made in PBS and a volume of 200 μL or less was added to 10 mL of DMEM containing the 20% serum noted above. Where appropriate, oleic acid (Sigma) was delivered to cells in DMEM complexed with BSA (BSA-OA).

500,000 McA cells were plated in 10 cm culture plates and made to a final volume of 10 mL with DMEM containing serum. Concentrated stock solutions of creatinine, guanidinoacetate (GAA), guanidinopropionic acid (GPA) or methylguanidine (MGU) were added to medium and cells were grown for 48 h. After 48 h cells were washed three times in PBS and fresh DMEM (with no serum) containing the respective compounds, with or without oleic acid, and were incubated for a further 4 h at 37 °C. After 4 h the medium was removed and the cells were washed 3 times with PBS and collected in 1 mL of ice-cold lysis buffer containing 20 mM Tris–HCl, 50 mM NaCl, 50 mM NaF, 5 mM NaPyrophosphate and 250 mM sucrose. For RNA isolation, cells were treated in a similar fashion but instead of lysis buffer, 2 mL of ice-cold Trizol® (Invitrogen) was added directly to the petri dish. Cell viability was greater than 95% for all treatments as assessed by trypan blue exclusion.

2.2. Lipid analysis

Total lipids were extracted from cells using a Folch-type extraction using 2:1 chloroform:methanol [17]. The lipid was re-suspended in 2-propanol and assayed for total triglycerides using a commercially available kit from Sekisui Diagnostics (Charlottetown, PEI, Canada, catalogue # 236-60). Hepatic phosphatidylcholine and phosphatidylethanolamine were measured by a phosphorous assay after separation of lipid species by thin-layer chromatography, as described by Jacobs et al. [18]. TG mass in the medium was determined using the GC-FID method of Kuksis [19] using tridecanoylglycerol as an internal standard.

2.3. Lipid synthesis

For experiments measuring the incorporation of oleic acid into lipid species, 9,10-^{3}H-oleic acid (Perkin Elmer) was added to unlabeled oleic acid and complexed to BSA. Cells were incubated with the BSA–^{3}H-oleic acid and lipids were extracted from the cell lysates and separated on TLC plates, as described previously [20]. Bands were detected using iodine vapor, scraped into scintillation vials containing 10 mL of Ultima Gold scintillation fluid (Perkin Elmer). For de novo fatty acid synthesis, ^{14}C-acetate (Perkin Elmer) was added directly into DMEM and incubated for 4 h at 37 °C. Cells were rinsed 3 times with PBS and collected in 1 mL of ice cold lysis buffer (above).

2.4. Fatty acid oxidation

Total fatty acid oxidation was measured by incubating McA cells with BSA–oleic acid labeled with 9,10-^{3}H-oleic acid for 4 h after which the cell culture medium was collected, acidified with 1 M ascorbic acid and extracted using 2:1-chloroform:methanol. The aqueous phase (acid soluble oxidation products) was collected and radioactivity was determined by scintillation counting using Ultima Gold (Perkin Elmer) liquid scintillation fluid. This measurement represents the total catabolism of oleate to acid soluble aqueous metabolites. In a separate method, we determined complete oxidation of fatty acids (oxidation to CO_2) by incubating McA cells in air-tight flasks with 1-^{14}C-oleic acid (Perkin Elmer) labeled BSA–oleic acid complex for 4 h at 37 °C. After this time, 400 μL of concentrated perchloric acid was injected into the culture medium through a septum. CO_2 was collected for 2 h after injecting 300 μL of 1 M KOH into the center-well containing a piece of filter paper. The center-well and contents were added to Ultima Gold (Perkin Elmer) liquid scintillation fluid and radioactivity was determined.

2.5. Western blots

Cell lysates were added to Laemmli sample buffer and resolved using SDS-PAGE. Proteins were subsequently transferred to PVDF membranes, incubated with antibodies using an HRP-conjugated secondary antibody and detected using a Chemidoc (Bio-rad). All antibodies were obtained from Cell Signaling Technology; we used rabbit anti-AMPKα (#2532), rabbit anti-(phospho-Thr172) AMPKα (#2531), rabbit anti-ACC (#3662) and rabbit anti-(phospho-Ser79) ACC (#3661).

2.6. Gene expression analysis

Total RNA was isolated from McA cells using the Trizol® (Invitrogen) method. RNA quality was assessed with an Agilent 2100 bioanalyser, using an RNA 6000 Nano kit (Agilent Technologies). RNA was then reverse transcribed using Superscript II (Invitrogen). Primer sets and a corresponding probe for each gene of interest were designed using the Universal Probe Library (Roche Diagnostics) based on the NCBI reference nucleotide sequences for *Rattus norvegicus*. Each primer pair and probe combination was tested by qPCR (StepOnePlus, Applied Biosystems) to confirm that amplification conditions were suitable for use in the Biomark™ gene chip (Fluidigm). A mix containing primers for all genes combined in a single assay was used to pre-amplify the cDNA in each sample. Pre-amplification was performed in order to enrich the template cDNA for use in the Biomark™ gene chip. All pre-amplified samples were tested on the StepOnePlus qPCR machine using the probe for cyclophilin (Ppia) before loading of the Biomark™ gene chip. Ninety-six primer pairs and pre-amplified samples were loaded into separate wells on a 96-by-96 gene chip (Fluidigm). qPCR was run on the Biomark™ system (Fluidigm) for 40 cycles. Relative RNA expression for each gene in a sample was calculated using the comparative threshold (ΔΔCT) method. Values were normalized to the endogenous housekeeping gene cyclophilin. All samples were assayed in triplicate for each primer pair.

2.7. Statistical analysis

Data are expressed as the means ± standard deviation ($N = 4$–6 for each measurement). Data were analyzed using one-way ANOVA or Student's *t*-test where appropriate. The time course experiments were fitted using non-linear regression. All analyses were done using GraphPad Prism software. A p-value <0.05 was taken as statistically significant.

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