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Dietary creatine supplementation lowers hepatic triacylglycerol by increasing lipoprotein secretion in rats fed high-fat diet

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Abstract

Recent studies have shown that dietary creatine supplementation can prevent lipid accumulation in the liver. Creatine is a small molecule that plays a large role in energy metabolism, but since the enzyme creatine kinase is not present in the liver, the classical role in energy metabolism does not hold in this tissue. Fat accumulation in the liver can lead to the development of nonalcoholic fatty liver disease (NAFLD), a progressive disease that is prevalent in humans. We have previously reported that creatine can directly influence lipid metabolism in cell culture to promote lipid secretion and oxidation. Our goal in the current study was to determine whether similar mechanisms that occur in cell culture were present *in vivo*. We also sought to determine whether dietary creatine supplementation could be effective in reversing steatosis. Sprague–Dawley rats were fed a high-fat diet or a high-fat diet supplemented with creatine for 5 weeks. We found that rats supplemented with creatine had significantly improved rates of lipoprotein secretion and alterations in mitochondrial function that were consistent with greater oxidative capacity. We also find that introducing creatine into a high-fat diet halted hepatic lipid accumulation in rats with fatty liver. Our results support our previous report that liver cells in culture with creatine secrete and oxidize more oleic acid, demonstrating that dietary creatine can effectively change hepatic lipid metabolism by increasing lipoprotein secretion and oxidation *in vivo*. Our data suggest that creatine might be an effective therapy for NAFLD.

Keywords: Creatine; Lipid metabolism; Lipoprotein; Mitochondrial oxidation; NAFLD

1. Introduction

Creatine is a small molecule that plays an important role in energy metabolism. Creatine can either be synthesized endogenously using the amino acids arginine, glycine and methionine or can be consumed in the diet. Creatine is not catabolized but spontaneously cyclizes to form creatinine, which is subsequently secreted in the urine. Typical dietary creatine intake is not enough to replace daily losses, and so biosynthesis is required to maintain creatine homeostasis [1]. Although the role of creatine in the maintenance of muscular and neural ATP concentration is well described, we have recently provided evidence that creatine may also exert regulatory effects on energy and nutrient metabolism in other tissues [2–4].

Creatine and creatine phosphate together with the enzyme creatine kinase (CK) serve to buffer ATP in cells that utilize a large amount of ATP over a relatively short period of time [5]. The buffering of ATP with creatine requires CK and thus only occurs in tissues that contain this enzyme activity, primarily the muscle and brain. However, we recently observed that dietary creatine supplementation in rats fed high-fat diet (HFD) resulted in reduced accumulation of triacylglycerol (TG) and improved markers of oxidative stress in the liver [2]. This was a surprising finding that changed the way we thought about creatine. Very recently, Deminice *et al.* [6] have shown that creatine supplementation prevents TG accumulation in the livers of rats fed choline-devoid diet, a different dietary model of nonalcoholic fatty liver. Taken together, the evidence suggests that in addition to expanding the pool of ATP in the muscle and brain, creatine may be regulating the metabolism of lipid in the liver.

The mechanism by which creatine influences hepatic lipid metabolism is unclear. Indeed, there may be more than one mechanism responsible. A study by Kazak *et al.* [7] has provided strong evidence that the CK reaction is involved in substrate oxidation in both the brown and beige adipose tissue of mice. The authors showed that creatine was required for maximal oxygen consumption by mitochondria from brown (human and mouse) and beige fat (mouse). If beige adipose was oxidizing more fatty acids in creatine-supplemented animals, then that may lead to greater clearance of lipid from the plasma and reduced circulating fatty acids. This mechanism could influence hepatic TG content by lowering the total amount of lipid entering the liver, through elevated peripheral lipid clearance or reduced circulating fatty acids arising from adipose TG, or both. This hypothetical mechanism could also work if muscle tissue were oxidizing more substrates for TG synthesis. Indeed, creatine supplementation has been shown to increase oxygen consumption during

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variable exercise [8] and reduce circulating TG [9] in humans. However, we have recently published a study that supports the idea that creatine concentration can directly affect lipid metabolism in liver cells. We used the McArdle RH-7777 cell line and incubated with various concentrations of creatine and oleate showing that added creatine in the cell culture medium did indeed prevent the accumulation of TG in these cells [3]. Further analysis of these cells showed that both fatty acid oxidation and lipid secretion were increased in the cells that were incubated with creatine and that this was brought about by increased PPARα activity. Indeed, there were strong similarities in the mRNA data for PPARα, CPT1α, Acox and the acyl-CoA dehydrogenases between the rat livers from the diet study [2] and the McArdle cells incubated with creatine [3].

The goal of the current study was to determine whether supplemental creatine in an HFD would result in lipoprotein secretion *in vivo*, as it did in our *in vitro* study. We also wanted to investigate whether creatine might be effective in treating a fatty liver. Therefore, we undertook an experiment where we generated a fatty liver by feeding an HFD and then introduced creatine. Previous studies indicate that dietary creatine supplementation has a positive impact on markers of inflammation in the liver; therefore, we decided to measure an array of inflammatory markers. Our current results show that rats on HFD supplemented with creatine have improved lipoprotein secretion and reduced expression of respiratory F_o–F_1 ATPase (complex V) and the voltage-dependent anion channel (VDAC/porin) that suggest greater oxidative capacity in the liver. We also show that creatine treatment was able to prevent further accumulation of fat in the livers of animals that had already developed fatty liver.

2. Materials and methods

All procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. Sprague–Dawley rats were obtained from Charles River Laboratories (Montreal, Canada) and were housed on a 10:14-h light/dark cycle and fed a chow diet (Lab diet 5001; PMI Nutrition International) *ad libitum* for 2 weeks to acclimate to the environment.

2.1. Diet experiments

Rats were fed control diet (CON; BioServ #F4031) and HFD (BioServ #F3282) obtained from Cedar Lane. For the creatine-supplemented diets, 2% creatine monohydrate (20 g/kg) was added to the HFD from BioServ (CHFD). Two percent creatine in the diet is sufficient to down-regulate endogenous creatine synthesis and prevent hepatic lipid accumulation as previously reported [2]. The CON profile was 0.82 kcal/g protein, 0.64 kcal/g fat and 2.46 kcal/g carbohydrate for a total of 3.93 kcal/g, and the HFD profile was 0.82 kcal/g protein, 3.24 kcal/g fat and 1.43 kcal/g carbohydrate for a total of 5.49 kcal/g. The sources of protein, fat and carbohydrates for the diets were casein, lard, maltodextrin and sucrose. The amino acid profiles were identical in the experimental and control diets. Specifically, methionine content was 7.1 g/kg in all three experimental diets. Experimental diets were provided *ad libitum* for either 4 or 8 weeks where indicated. For the 8-week feeding trial, all rats were fed the HFD for 4 weeks and then split into randomly assigned groups. One group was fed the HFD and the other group was fed the CHFD for an additional 4 weeks.

2.2. Tissue collection

A portion of liver tissue was snap-frozen in liquid N_2 and stored at −80°C until further analysis. A portion of tissue to be used for histology was added to 10% phosphate-buffered formalin (0.4% formaldehyde). Rat blood was collected in EDTA treated tubes and centrifuged at 1000*g* for 10 min; plasma was separated and stored at −80°C until further analysis. Tissues were homogenized in either ice-cold lysis buffer containing 20 mM Tris–HCl, 50 mM sodium chloride, 50 mM sodium fluoride, 5 mM sodium pyrophosphate and 250 mM sucrose, except for cytokine analysis where phosphate-buffered saline (PBS) was used.

2.3. Western blotting

Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Immobilon). Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST). Membranes were probed with primary antibodies diluted in 1% skim milk solution overnight at 4°C and then probed with secondary antibody in 1% skim milk in TBST on a rocker at room temperature for 1 h. Proteins were visualized using ECL chemiluminescence (GE Healthcare) solution and a Chemidoc Imager (Bio-Rad). Antibodies were obtained for mouse anti-mitochondrial respiration complexes (MitoSciences #MS604) and the antibody against rabbit anti-VDAC (Affinity Bioreagents PA1-954A), goat anti-ApoB (Chemicon/Millipore AB742), anti-MTTP (BD Biosciences #612022) and anti-PDI (Cell Signalling #2446).

2.4. Lipid analysis

Tissues were extracted using a modified Folch extraction [10], lipids were dissolved in either 100 µl hexane and mass was determined using the GC-FID method of Myher and Kuksis [11]; tridecanoylglycerol was added before extraction and used as an internal standard.

2.5. Histology

Gross morphology and lipid droplets in the liver were visually assessed after hematoxylin–eosin (H/E) staining. Briefly, liver tissues that were fixed in 10% phosphate-buffered formalin were embedded in paraffin blocks. Slices that were 10 µm thick were placed on slides and stained with H/E using a standard procedure. Slides were imaged using an Axio Observer A.1 light microscope (Zeiss) equipped with an AxioCam MRc at 20× magnification.

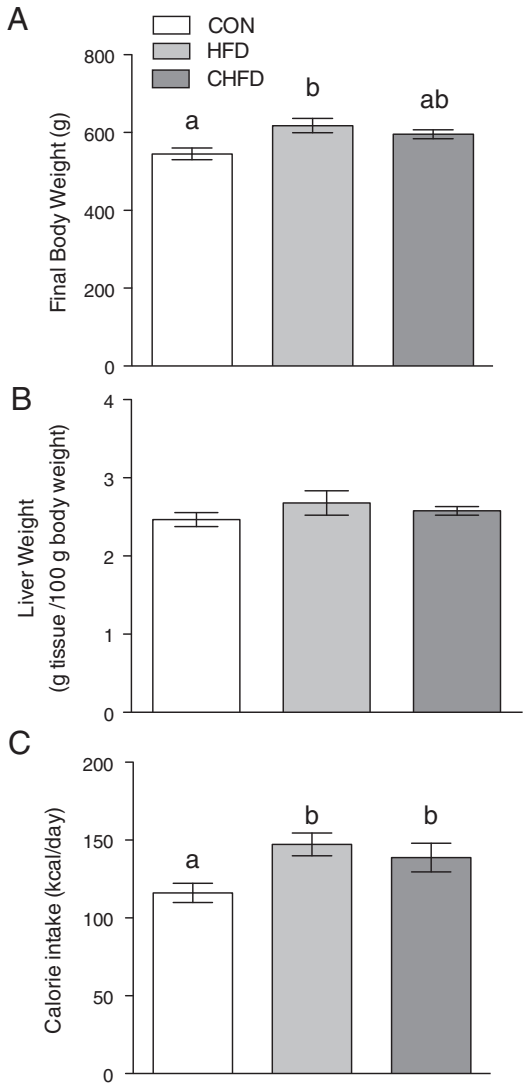

Fig. 1. Body and tissue weights for rat fed for experimental diets 5 weeks. Final body weights in grams (A), liver weight per 100 g body weight (B), average daily caloric intake (C); white bars and black circles represent CON, light gray bars and black squares represent HFD, and dark gray bars and open circles represent CHFD. Different letters denote a significant difference between values or curves, and the same letter denotes statistically similar values ($P<.05$, $n=6$).