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Creatine supplementation prevents fatty liver in rats fed choline-deficient diet: a burden of one-carbon and fatty acid metabolism

Rafael Deminice[a,c,*], Gabriela Salim Ferreira de Castro[a,1], Lucas Vieira Francisco[a,1], Lilian Eslaine Costa Mendes da Silva[a,1], João Felipe Rito Cardoso[a,1], Fernando Tadeu Trevisan Frajacomo[a,1], Bruno Gonzaga Teodoro[d], Leonardo dos Reis Silveira[b,d,1], Alceu Afonso Jordao[a,1]

[a]Nutrition and Metabolism, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Av. Bandeirantes 3900, Ribeirao Preto, Sao Paulo, Brazil
[b]School of Physical Education of Ribeirao Preto, University of Sao Paulo, Av. Bandeirantes 3900, Ribeirao Preto, Sao Paulo, Brazil
[c]Department of Physical Education, Faculty of Physical Education and Sport, State University of Londrina. Rodovia Celso Garcia Cid | Pr 445 Km 380 | Campus Universitário, Londrina, Paraná, Brazil
[d]Department of Biochesmtry and Imunology, Faculty of Medicine of Ribeirão Preto Av. Bandeirantes 3900, Ribeirao Preto, Sao Paulo, Brazil

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Abstract

Aim: To examine the effects of creatine (Cr) supplementation on liver fat accumulation in rats fed a choline-deficient diet.
Methods: Twenty-four rats were divided into 3 groups of 8 based on 4 weeks of feeding an AIN-93 control diet (C), a choline-deficient diet (CDD) or a CDD supplemented with 2% Cr. The CDD diet was AIN-93 without choline.
Results: The CDD significantly increased plasma homocysteine and TNFα concentration, as well as ALT activity. In liver, the CDD enhanced concentrations of total fat (55%), cholesterol (25%), triglycerides (87%), MDA (30%), TNFα (241%) and decreased SAM concentrations (25%) and the SAM/SAH ratio (33%). Cr supplementation prevented all these metabolic changes, except for hepatic SAM and the SAM/SAH ratio. However, no changes in PEMT gene expression or liver phosphatidylcholine levels were observed among the three experimental groups, and there were no changes in hepatic triglyceride transfer protein (MTP) mRNA level. On the contrary, Cr supplementation normalized expression of the transcription factors PPARα and PPARγ that were altered by the CDD. Further, the downstream targets and fatty acids metabolism genes, UCP2, LCAD and CPT1a, were also normalized in the Cr group as compared to CDD-fed rats.
Conclusion: Cr supplementation prevented fat liver accumulation and hepatic injures in rats fed with a CDD for 4 weeks. Our results demonstrated that one-carbon metabolism may have a small role in mitigating hepatic fat accumulation by Cr supplementation. The modulation of key genes related to fatty acid oxidation pathway suggests a new mechanism by which Cr prevents liver fat accumulation.

Keywords: Creatine supplementation; Choline-deficient diet; Fatty liver; One-carbon metabolism; Fat oxidation

1. Introduction

In the last few years, hepatic fat accumulation and the progression of nonalcoholic steatohepatitis (NASH) have been associated with impairment of hepatic one-carbon metabolism [1]. This has been attributed to the dysfunction of specific enzymes that result in the decreased availability of S-adenosylmethionine (SAM) in the liver and increased homocysteine (Hcy) formation [2, 3]. SAM acts primarily as the universal methyl donor for the methylation of DNA and synthesis of >50-methylated compounds including adrenaline, carnitine, creatine and phosphatidylcholine (PC). A byproduct of these transmethylation reactions is S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to adenosine and Hcy [4]. Once formed, Hcy can be remethylated by two fashions to reform methionine and support SAM synthesis, thus maintaining the methylation capacity [5]. In the first, 5-methyltetrahydrofolate donates a methyl group to the vitamin B-12 containing enzyme methionine synthase, which is subsequently added to Hcy. Alternatively, the remethylation of Hcy is also catalyzed by betaine Hcy methyltransferase (BHMT) that uses betaine as a methyl donor [4]. Because betaine is endogenously synthesized from choline, then it follows that a choline-deficient diet (CDD) impairs remethylation via BHMT and, thus, decreases the availability of SAM [6]. Indeed, it has been demonstrated that a CDD decreased hepatic SAM concentrations and subsequently impaired PC synthesis via phosphatidylethanolamine N-methyltransferase (PEMT). Furthermore, a CDD diminished secretion of liver triglyceride as very low density lipoprotein (VLDL) and led to the accumulation of liver triglycerides and NASH in rats and mice [7–9].

* Corresponding author at: Department of Physical Education, Faculty of Physical Education and Sport - State University of Londrina. Rodovia Celso Garcia Cid | Pr 445 Km 380 | Campus Universitário, Londrina, Paraná, Brazil. Tel.: +55-16-36024564, +55-43-36024564.
E-mail address: deminice@ig.com.br (R. Deminice).
[1] Tel.: +55-16-36024564.

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Creatine synthesis consumes a considerable amount of hepatic SAM and thus produces an equivalent amount of Hcy [10]. Previous studies have shown that creatine supplementation (CR) down-regulates the endogenous formation of creatine from its substrate guanidinoacetic acid (GAA), via Guanidinoacetate methyltransferase (GAMT), which reduces Hcy synthesis [11–14]. Because both PEMT and GAMT draw from the same pool of hepatic SAM, CR may also increase SAM availability for PC synthesis (via PEMT) and, moreover, increase VLDL secretion and prevent the accumulation of fat in the liver during CDD feeding (Fig. 2). Previous data from our group has demonstrated that CR prevents fatty liver after 3 weeks of feeding a high-fat diet [15]. However, feeding a high-fat diet was unable to explain the specific contribution of one-carbon metabolism to hepatic fat accumulation. Therefore, we proposed that the induction of NASH by feeding a CDD and supplementing with creatine would clarify the relationship between one-carbon metabolism and hepatic fat accumulation. The aim of the present study was to examine the effects of CR on liver fat accumulation and one-carbon metabolism in rats fed a CDD.

2. Methods

Twenty-four male Wistar rats (initial weight ~120 g) were obtained from Central Animal Care at the University of São Paulo, Faculty of Medicine of Ribeirão Preto. All procedures were approved by the Ethics Committee for Animal Use at the same institution and were in accordance with the Guidelines of the COBEA (Brazilian College of experiments with animals). The rats were housed in individual cages on a 12-h-light/-dark cycle at a mean temperature of 22°C and were randomly assigned to three dietary groups of eight rats each: control (C); CDD; CDD plus CR. Group C was fed standard AIN-93 maintenance diet as proposed by Reeves *et al.* [16] which contains 0.25% choline. CDD groups received AIN-93 standard diet without choline. CR was performed by adding 2% (wt:vol) creatine monohydrate to the CDD diet. The rats had free access to food throughout the 4 weeks, and food intake was measured daily to assess consumption of fat, creatine and total energy. Body weight was measured twice a week.

2.1. Tissue preparation

After 4 weeks of experimental feeding, rats were anesthetised with an intraperitoneal injection of sodium pentobarbital (65 mg/kg ip) and killed by decapitation. Animals were sacrificed between 9 and 11 a.m. At necropsy, blood was collected into heparinized tubes and centrifuged. The plasma fraction was separated and stored at −80°C until analysis. Livers were extracted, weighed and freeze-clamped with aluminum tongs. Weighed samples were and stored at −80°C. All procedures were performed under standard RNase-free conditions to avoid exogenous RNase contamination. A portion of fresh liver tissue was weighed and cut in small cubes of approximately 5×5×5 mm and embedded using 10% buffered formalin for histopathologic evaluation. Left kidney was removed and immediately homogenized in ice-cold 50 mmol/L potassium phosphate buffer (pH 7.4) for approximately 30 s. This homogenate was used for the analysis of AGAT activity. Epididymal fat pads were removed and weighed as an indication of visceral fat.

2.2. Hcy and related metabolites

Plasma Hcy and other sulfur-containg amino acids were derivatized with commercially available kit EZ:Faast Amino Acid Analysis (Phenomenex®) and assayed by gas-chomatography (GC-FID, GC-17A Shimadzu®, Kyoto, Japan). For SAM and SAH determinations, freeze-clamped liver samples were homogenized in ice-cold 8% (wt/vol) trichloroacetic acid. Homogenates were centrifuged at 13,000 g for 5 min at 4°C. The supernatants were removed and analyzed by HPLC using a Phenomenex® C_{18} column equilibrated with 96% of *buffer A* (50-mmol/L NaH_2PO_4 containing 10-mmol/L heptanesulfonic acid at pH 3.2) and 4% acetonitrile. SAM and SAH were separated by means of a gradient of 96–80% of *buffer A* and 4–20% of acetonitrile for 15 min. SAM and SAH peaks were detected at 258 nm and quantified using Millennium^{32} (Version 2) software (Waters, Milford, MA, USA). PC concentrations in liver and plasma were assayed using a commercially available kit from Cayman Chemical Company catalogue #10009926 (Ann Harbor, MI, USA).

Creatine concentrations in plasma and liver were determined using previously described methods [13]. Kidney AGAT activity was determined as described by Van Pilsum *et al.* [17], and protein was assayed using the Biuret method.

2.3. Hepatic histology and lipid analysis

To examine liver morphology, the tissue was preserved in 10% phosphate-buffered formalin, pH 7.0. Formalin-preserved liver samples were exposed to hematoxylin and eosin (H&E) staining. Slides were scored for fat droplets using a conventional light microscope (200×). Total liver fat was determined by homogenizing 0.5 g of liver in 1.0 ml of distilled water. Subsequently, 5 ml of chloroform-methanol (2:1) was added, and the tubes were thoroughly mixed and centrifuged to separate fats. After centrifugation, the organic phase was transferred to a preweighed tube. This chloroform:methanol extraction was repeated twice and the organic phases combined. The extracts were evaporated to dryness under a stream of nitrogen and re-weighed. The hepatic fat was re-suspended in 1 ml of 1-propanol for the quantification of triglycerides and cholesterol, using commercially available kits from Labtest (Lagoa Santa, Minas Gerais, Brazil).

2.4. Inflammatory and oxidative stress markers

Plasma alanine aminotransferase (ALT) activity was performed using a commercially available kit (Labtest, Lagoa Santa, Minas Gerais, Brazil). Liver malondialdehyde (MDA) was determined on an HPLC (LC-20A Shimadzu®, Kyoto, Japan) according to Spirlandeli *et al.* [18]. Hepatic reduced (GSH) and oxidized glutathione (GSSG) were performed according to described by Rahman *et al.* [19]. Plasma and liver TNF-α were determined using a commercially available kit (eBioscience®, USA).

2.5. Gene expression and western blotting

RNA was isolated from 50 mg of frozen liver using a RiboPure Kit (Ambion, part number AM 1924, USA) according to the manufacturer's instructions. Total RNA was quantified by spectrophotometry at OD 260/280 (NanoDrop2000c, ThermoScientific, USA). The quality and integrity of the isolated RNA were assessed using a 1.2% agarose gel and electrophoresis. An additional DNase I treatment (DNA-free Kit, Ambion, part number AM1906, USA) was performed to remove contaminating DNA from the isolated RNA. cDNA was synthesized from 1000 ηg of RNA using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, part number 4374966, USA). Quantitative PCR was performed using the 7500 Fast Real-time PCR System (Applied Biosystems, USA). The following Taqman® Gene Expression probes (Applied Biosystems, USA) were used in this study: Rn01644299_m1 (ChDh), Rn00567492_m1 (Chka), Rn00755199_g1 (Chkb), Rn00589584_m1 (Pcyt 1a), Rn00564517_m1 (PEMT), Rn00578255_m1 (BHMT), Rn00567215_m1 (GNMT), Rn00560948_m1 (CBS), Rn00566193_m1 (Ppara), Rn01754856_m1 (Ucp2), Rn00580241_m1 (Ppargc1a) Rn00690933_m1 (cyclophilin A).SYBR green master mix was used for qPCR of the following primers: