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Dietary supplementation with acetyl-L-carnitine counteracts age-related alterations of mitochondrial biogenesis, dynamics and antioxidant defenses in brain of old rats

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ABSTRACT

We previously reported the ability of dietary supplementation with acetyl-L-carnitine (ALCAR) to prevent age-related decreases of mitochondrial biogenesis in skeletal muscle and liver of old rats. Here, we investigate the effects of ALCAR supplementation in cerebral hemispheres and cerebellum of old rats by analyzing several parameters linked to mitochondrial biogenesis, mitochondrial dynamics and antioxidant defenses.

We measured the level of the coactivators PGC-1α and PGC-1β and of the factors regulating mitochondrial biogenesis, finding an age-related decrease of PGC-1β, whereas PGC-1α level was unvaried. Twenty eight-month old rats supplemented with ALCAR for one and two months showed increased levels of both factors. Accordingly, the expression of the two transcription factors NRF-1 and TFAM followed the same trend of PGC-1β. The level of mtDNA, ND1 and the activity of citrate synthase, were decreased with aging and increased following ALCAR treatment. Furthermore, ALCAR counteracted the age-related increase of deleted mtDNA. We also analyzed the content of proteins involved in mitochondrial dynamics (Drp1, Fis1, OPA1 and MNF2) and found an age-dependent increase of MFN2 and of the long form of OPA1. ALCAR treatment restored the content of the two proteins to the level of the young rats. No changes with aging and ALCAR were observed for Drp1 and Fis1. ALCAR reduced total cellular levels of oxidized PRXs and counteracted the age-related decrease of PRX3 and SOD2.

Overall, our findings indicate a systemic positive effect of ALCAR dietary treatment and a tissue specific regulation of mitochondrial homeostasis in brain of old rats. Moreover, it appears that ALCAR acts as a nutrient since in most cases its effects were almost completely abolished one month after treatment suspension. Dietary supplementation of old rats with this compound seems a valuable approach to prevent age-related mitochondrial dysfunction and might ultimately represent a strategy to delay age-associated negative consequences in mitochondrial homeostasis.

1. Introduction

Acetyl-L-carnitine (ALCAR) is an acetylated form of L-carnitine, a molecule naturally produced by the body. ALCAR is also administered as a dietary supplement for its ability to maintain and/or restore mitochondrial homeostasis during aging (reviewed in Rosca et al., 2009) and in conditions characterized by neurodegeneration (reviewed in Traina, 2016). Back in 1990, we reported that a single intraperitoneal injection (IJ) of ALCAR reverted the age-related decrease of mitochondrial transcription and translation in different tissues of old (Gadaleta et al., 1990a) as well as hypothyroid rats (Gadaleta et al., 1990b). These effects were already noticeable 1 h after IJ and occurred in a time- and dose-dependent manner (Fernandez-Silva et al., 1991; Gadaleta et al., 1994). Since that, several papers reported that ALCAR

Abbreviations: ALCAR, Acetyl-L-carnitine; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; CS, citrate synthase; PGC-1α, peroxisome proliferator-activated receptor γ [PPARγ] coactivator-1α; PGC-1β, peroxisome proliferator-activated receptor γ [PPARγ] coactivator-1 β; TFAM, mitochondrial transcription factor A; NRF-1, Nuclear respiratory factor-1; ND1, NADH dehydrogenase subunit 1; PRX3, peroxiredoxin 3; SOD2, superoxide dismutase 2; Drp1, dynamin-related protein 1; Fis1, fission protein 1; OPA1, optic atrophy 1; MFN2, mitofusin 2; ANOVA, analysis of variance

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administration to old rats was able to restore mitochondrial bioenergetics efficiency in different tissues (Hagen et al., 1998; Lesnefsky and Hoppel, 2006; Paradies et al., 1992, 1994, 1996; Petruzzella et al., 1992). In line with these findings, we found that dietary supplementation with ALCAR reverted the decrease of mitochondrial biogenesis in skeletal muscle and liver of old rats (Pesce et al., 2004, 2010, 2012), as well as in muscles of unloaded rodents (Cassano et al., 2006). Moreover, we found that ALCAR treatment was able to prevent the age-related accumulation of an overoxidized acidic and irreversibly inactivated form of the mitochondrial antioxidant enzyme peroxyredoxin 3 (PRX3) in the liver of old rats (Musicco et al., 2009).

Since ALCAR can cross the blood-brain barrier through the OCTN2 and $ATB^{(0+)}$ transporters (Kido et al., 2001), we decided to investigate the effects of chronic dietary supplementation with ALCAR in cerebral hemispheres and cerebellum tissues of old rats. To this aim, we analyzed the effect of aging and ALCAR supplementation on mediators of mitochondrial biogenesis, mitochondrial dynamics and oxidative stress. Findings from the present investigation show that ALCAR supplementation is able to revert the age-related decrease of mitochondrial biogenesis in old rat brain, through the activation of peroxisome proliferator-activated receptor-γ coactivator 1 (PGC-1) α and β. Furthermore, ALCAR supplementation counteracted the age-dependent increase of mitofusin 2 (MFN2) and optic atrophy 1 (OPA1), the decrease of PRX3 and superoxide dismutase 2 (SOD2) content, and the increase of mtDNA (mtDNA)^{4834} deletion load. Furthermore, the data reported here confirm that ALCAR behaves as a nutrient (Liu and Ames, 2005).

2. Materials and methods

2.1. Animals

The study was approved by the Institutional Animal Care and Use Committee at the University of Bari and all procedures were in accordance with the Italian Ministry of Health Guidelines (no. 86/609/EEC). Cerebral hemispheres and the cerebellum of five groups of male Fisher 344 (Charles River) rats, including young (6-month-old), old (28-month-old) and old rats supplemented daily with ALCAR in drinking water at a dose of 100 mg/kg body weight for 1 or 2 months, were collected. A fifth group of animals consisting of old rats treated for 2 months with ALCAR and sacrificed 1 month after treatment suspension was also included. Rats were obtained from Sigma-Tau Industrie Farmaceutiche Riunite S.p.A. (Rome, Italy) and were housed in individual cages on a 12:12 light–dark cycle with free access to standard rodent chow. No difference in food and water consumption was noticed among experimental groups.

Animals were anesthetized before being sacrificed, weighed, and the cerebral hemispheres and cerebellum were immediately removed, weighed, snap-frozen in isopentane cooled by liquid nitrogen and stored in liquid nitrogen until analysis.

2.2. MtDNA content

Genomic DNA was isolated as reported by Lezza et al. (2008). Briefly, 30–40 mg of cerebral hemispheres and cerebellum were used. DNA integrity was verified by gel electrophoresis in 0.8% agarose gel in 1 × TBE (90 mM Tris-borate pH 7.4, 90 mM boric acid, 2.5 mM EDTA) and quantified using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

MtDNA content was measured using quantitative Real Time-PCR (qRT-PCR), as described by Picca et al. (2013). Briefly, qRT-PCR amplification reactions were performed using SYBR Green chemistry on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers were designed with the Primer Express software (Applied Biosystems) for the rat mitochondrial D-loop region (D-loopFor: 5′-GGTTCTTACTTCAGGGCCATC-3′; D-loopRev: 5′-GATTAGACCCGTTACCATCGAGAT-3′, GenBank™ accession number AY172581) and for the rat nuclear β-actin gene (β-actinFor: 5′-CCCAGCCATGTACGTAGCCA-3′; β-actinRev: 5′-CGTGTCCGGAGTCCATCAC-3′, GenBank™ accession number VO1217.1). The method was validated by primer-limiting experiments (200 nM for each primer pair concentrations) and by evaluating the equal reaction efficiency of the two amplicons. Melting curve analysis and gel electrophoresis were used to control the amplification specificity. Each sample was analyzed three times in triplicate in 25 μL final volume. The reaction mixture consisted of 21 μL iTaq SYBR Green Supermix PCR 1 × Master Mix (Bio-Rad Laboratories Inc., Hercules, CA), 0.2 μM forward and reverse primers, and DNA template (25 ng). Amplification proceeded for 40 cycles. The quantification of the relative mtDNA content was performed according to the Pfaffl (2001). In particular, the difference in threshold cycle values (ΔCt, namely Ct D-loop – Ct β-actin) was used as a measure of the relative abundance of the mitochondrial genome. To compare the mtDNA quantity among experimental groups, the relative amount of mtDNA to nuclear DNA was calculated using the following equation: $R = 2^{\Delta\Delta Ct}$, where R is the calculated ratio and ΔΔCt is the ΔCt analyzed class-ΔCt reference class value, with the younger age class taken as the reference (set to 1).

2.3. $MtDNA^{4834}$ deletion content

The content of the 4.8-kb deletion was measured by qRT-PCR as reported by Picca et al. (2013). Briefly, qRT-PCR amplification reactions were performed via SYBR Green chemistry on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The primers were designed with the Primer Express software (Applied Biosystems), respectively, for the rat mtDNA 4.8-kb deleted region (4.8-kb Del For 5′-AAGGACGAACCTGAGCCCTAATA-3′ and 4.8-kb Del Rev 5′-CGAAGTAGATGATGCGTATACTGTA-3′) and for the rat mtDNA D-loop region, generally undeleted (mtDNA For 5′-GGTTCTTACTTCAGGGCCATCA-3′ and mtDNA Rev 5′-TGATTAGACCCGTTACCATCGA-3′).

The method was validated by primer-limiting experiments and by evaluating the equal reaction efficiency of the two amplicons. Amplification specificity was controlled by melting curve analysis and gel electrophoresis. Each sample was analyzed in triplicate in 25 μL of final volume and fluorescence spectra were monitored through the ABI PRISM7000 Sequence Detection System (Applied Biosystems). The reaction mixture consisted of iTaq SYBR Green Supermix PCR 1 × Master Mix (Bio-Rad Laboratories Inc.), 0.2 μM forward and reverse primers, and DNA template (2.5 μL of diluted 1:10). After 10 min of denaturation at 95 °C, amplification proceeded for 40 cycles, each consisting of denaturation at 95 °C for 15 s, annealing, and extension at 60 °C for 1 min. The relative abundance of the 4.8-kb deleted mtDNA in young, old and ALCAR-treated old rats, all normalized to the corresponding total mtDNA, was performed according to the Pfaffl (2001) using the equation: $R = 2^{\Delta\Delta Ct}$ as described above.

2.4. Citrate synthase activity

Total proteins were extracted from 15 mg of frozen cerebral hemispheres and cerebellum samples by homogenization in a buffer containing 100 mM mannitol, 1 mM ATP, 0.2% BSA, 100 mM KCl, 3 mM $MgCl_2$, 5 mM Tris-buffer, pH 7.4, 1 mM EDTA. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories) according to the supplier's instructions. Citrate synthase (CS) (EC 2.3.3.1) activity ($\mu mol \times min^{-1} \times g\ tissue^{-1}$) was determined in tissue homogenates according to the method developed by Srere (1969). Briefly, 100 μg of total proteins were incubated in 1 mL of assay buffer containing 0.31 mM acetyl-CoA, 100 mM Tris buffer (pH 8.1), 0.25% Triton X-100, 0.1 mM DTNB and 0.5 mM oxaloacetate at 30 °C, and CS activity was determined spectrophotometrically by measuring the rate of production of thionitrobenzoic acid (TNB) at 412 nm.