Free Radical Biology & Medicine 52 (2012) 1236–1241

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Calorie restriction increases cerebral mitochondrial respiratory capacity in a NO•-mediated mechanism: Impact on neuronal survival

Fernanda M. Cerqueira [a], Fernanda M. Cunha [b], Francisco R.M. Laurindo [c], Alicia J. Kowaltowski [a,*]

[a] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil
[b] Escola de Artes, Ciências, e Humanindades, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil
[c] Faculdade de Medicina, Instituto do Coração, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil

ARTICLE INFO

Article history:
Received 23 July 2011
Revised 11 January 2012
Accepted 15 January 2012
Available online 28 January 2012

Keywords:
Caloric restriction
nitric oxide synthase
mitochondrial biogenesis
aging

ABSTRACT

Calorie restriction (CR) enhances animal life span and prevents age-related diseases, including neurological decline. Recent evidence suggests that a mechanism involved in CR-induced life-span extension is NO•-stimulated mitochondrial biogenesis. We examine here the effects of CR on brain mitochondrial content. CR increased eNOS and nNOS and the content of mitochondrial proteins (cytochrome *c* oxidase, citrate synthase, and mitofusin) in the brain. Furthermore, we established an in vitro system to study the neurological effects of CR using serum extracted from animals on this diet. In cultured neurons, CR serum enhanced nNOS expression and increased levels of nitrite (a NO• product). CR serum also enhanced the levels of cytochrome *c* oxidase and increased citrate synthase activity and respiratory rates in neurons. CR serum effects were inhibited by L-NAME and mimicked by the NO• donor SNAP. Furthermore, both CR sera and SNAP were capable of improving neuronal survival. Overall, our results indicate that CR increases mitochondrial biogenesis in a NO•-mediated manner, resulting in enhanced reserve respiratory capacity and improved survival in neurons.

Aging is characterized by progressive loss of function and increased incidence of diseases, often involving the brain. Interestingly, many age-associated neurological diseases have been related to lower mitochondrial respiratory capacity. Indeed, aging involves a limitation of mitochondrial function and decrease in mitochondrial mass in many tissues (reviewed in [1]).

Calorie restriction (CR), or the limitation of ingested calories without malnutrition, increases the life span in a variety of laboratory animals and prevents age-related disease, including neurological deficits, brain atrophy, and cognitive losses [2]. Interestingly, recent data demonstrate that CR increases mitochondrial biogenesis in many tissues, promoting enhanced respiratory capacity [3,4]. Indeed, mitochondrial function is central to life-span extension by CR [5,6], and increased respiratory rates are associated with extended life span [7,8].

Mitochondrial biogenesis is controlled by PGC1-α, a transcriptional coactivator [9–11]. PGC1-α in turn is regulated by nitric oxide (NO•) [9–12]. Prior reports have demonstrated that endothelial nitric oxide synthase (eNOS) is a source of NO• involved in mitochondrial biogenesis promoted by dietary restriction [4,10]. Indeed, Nisoli et al. [10] found that much of the increase in mitochondrial biogenesis induced by diet was absent in eNOS knockout animals, although the effect was not completely abrogated. Because NO• is diffusible, it is reasonable to believe that other sources of this messenger may be involved in the signaling events leading to mitochondrial biogenesis in CR.

In the brain, the effects of CR on mitochondrial mass still remain to be uncovered. Nisoli and co-authors [10] found that mitochondrial markers increase with every-other-day feeding, a dietary intervention that bears some similarity to CR but also has significant differences and yet undetermined effects on the neurological effects of aging [13,14]. This article addresses the effects of CR on brain mitochondrial biogenesis in vitro and in vivo, studies the role of NO• signaling in this process, and measures the impact of CR- and NO•-induced mitochondrial biogenesis on neuronal survival.

Materials and methods

In vivo calorie restriction

All experiments were conducted in strict agreement with the National Institutes of Health guidelines for humane treatment of animals and were reviewed and approved by the local animal care and use committee. Female, 4-week-old Swiss mice were separated into two groups: AL, fed ad libitum with an AIN-93-M diet prepared by Rhoster (Campinas, Brazil), and CR, fed with 60% of the same diet supplemented with micronutrients to reach the vitamin and mineral levels

Abbreviations: AL, ad libitum; CR, calorie restriction; eNOS, endothelial nitric oxide synthase; L-NAME, *N*5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride; nNOS, neuronal nitric oxide synthase; NO•, nitric oxide; NO_2^-, nitrite; SNAP, *S*-nitroso-*N*-acetyl-L,L-penicillamine.

* Corresponding author. Fax: +55 11 38155579.
E-mail address: alicia@iq.usp.br (A.J. Kowaltowski).

0891-5849/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2012.01.011

consumed by AL animals [13]. CR feedings were adjusted weekly by weight based on AL food consumption measured 1 week earlier. Food was offered to CR mice every day at 6:00 PM. The animals were lodged five individuals per cage and given water ad libitum. After 6 months of dietary intervention, the mice were sacrificed after 12 h fasting and the collected forebrains were stored at −80 °C.

In vitro calorie restriction

The sera for studies using cultured cerebella granular neurons were obtained as described by de Cabo et al. [15]. Briefly, male 8-week-old Sprague–Dawley rats were subjected to CR or AL feedings as described above. The animals were lodged three individuals per cage and given free access to water. At 34 weeks, the rats were sacrificed after 12 h fasting. The blood obtained was allowed to clot for 20–30 min at 25 °C and centrifuged for 20 min at 300 *g*. The clear supernatants were then collected and stored at −20 °C. All sera were thawed and heat inactivated at 56 °C for 30 min before use in cell culture experiments.

Primary cultures of cerebellar granule neurons

Cerebella from 7-day-old male Sprague–Dawley rats were finely minced and pooled in PBS supplemented with 20 mM glucose and 0.0005% (v/v) trypsin. The tissues were incubated for 40 min at 37 °C. Subsequently, soybean trypsin inhibitor (Sigma; 0.1%) was added and the cells were dispersed manually with a 1-ml pipette (adapted from [16]). The supernatant was centrifuged (300 *g*, 5 min) and cells were suspended in DMEM–F12 (Gibco; 25 mM glucose) with 25 mM Hepes and 2% B27 serum (Gibco). The cells were plated over polylysine in 24-well plates for the viability assays (5×10^4 cells/well); 1×10^7 cells were plated in 75-cm^2 flasks for Western blots and respiratory determinations. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, 1 μM 1-β-D-arabinofuranosylcytosine (Ara-C; Merck) was added to the culture medium to inhibit glia growth. Ara-C was removed after 48 h. On the seventh culture day, B27 serum was substituted for 10% AL or CR rat serum. In some experiments, 10 nM SNAP or 50 μM L-NAME was added at this same time point. As a control, an equal quantity of the solvent dimethyl sulfoxide (DMSO; 0.001%) was used when necessary. Medium was changed every 3 days and SNAP or L-NAME was replaced.

Viability assays

Viable cells were counted from the 7th day on (when rat sera were introduced) for 12 days by photographing with a Snap HQ Roper Scientific camera coupled to a Photometrics Cool microscope using the 20× objective and a bright field. Five regions from each well were chosen randomly and photographed and then analyzed using ImageJ software. There were no detectable differences between the regions in the same well. Data were collected at least in triplicate, and all experiments were repeated at least three times with different preparations.

NO_2^- measurements

NO_2^-, a marker of NO$^\bullet$ level [16], was measured using a NO$^\bullet$ analyzer (Model 208A; Sievers Instruments, Boulder, CO, USA) according to the manufacturer's protocols through the detection of chemiluminescence in the presence of potassium iodide and acetic acid [17]. NO_2^- levels from the AL and CR serum-containing culture media in the absence of cells were subtracted from all measurements.

Respiratory rate measurements

Oxygen consumption was measured in cells (10^6 ml^{-1}) suspended in PBS with 10 mM glucose using a computer-interfaced Oroboros oxygen electrode, at 37 °C, with continuous stirring. The basal oxygen consumption was followed for 3 min, followed by 3 min in the presence of 0.5 μg ml^{-1} oligomycin and 3 min in the presence of 2 μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP).

Citrate synthase activity

Brain and cell samples were homogenized in lysis buffer (50 mM sodium phosphate, pH 7.4, 10% glycerol, 1% octylphenol ethoxylate, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, supplemented with Sigma protease inhibitor mixture). After 30 min over ice, the lysates were centrifuged (13,000 *g*, 20 min, 4 °C), and the resulting supernatants were collected. Total protein (20 μg) was incubated at 37 °C for 5 min in 20 mM Tris–HCl, pH 8.0, 0.42 mM acetyl-coenzyme A, and 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid). The reaction was initiated by the addition of 0.5 mM oxaloacetate, and the reduction of 5′,5′-dithiobis(2-nitrobenzoic acid) by citrate synthase was measured spectrophotometrically for 5 min at 412 nm (extinction coefficient = 13.6 mM^{-1} cm^{-1}). Activities are expressed as nmol of citrate min^{-1} mg^{-1} protein.

Western blots

Total proteins from brain or neuron lysates were diluted in Laemmli sample buffer (100 mM Tris–HCl, 2% w/v SDS, 10% v/v glycerol, 0.1% bromophenol blue) containing 100 mM dithiothreitol, with the exception of eNOS and phospho-eNOS Western blots, which were performed without the reducing agent. After heating at 90 °C for 5 min, proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes. After membranes were blocked with 5% bovine serum albumin, the detection of individual proteins was carried out by blotting with specific primary antibodies against eNOS (Sigma; 1:3000), phospho-eNOS^{Ser1177} (Cell Signaling; C9C3 clone, 1:1000), nNOS (Abcam; 1:2,000), cytochrome *c* oxidase (Sigma; 1:2000), mitofusin-1 (Santa Cruz; H65 clone, 1:2000), and γ-actin (Sigma; 1:2000). Chemiluminescence detection using a secondary peroxidase-linked anti-rabbit (Calbiochem; 1:10,000) or anti-sheep IgG (Calbiochem; 1:13,000) and a detection system from Pierce KLP (Rockford, IL, USA) was performed. Signals were quantified by densitometry using Image J (NIH software), and the detected proteins were normalized either to γ-actin or to the non-phosphorylated titer of the same protein.

Data analysis

Data shown represent means ± SEM or representative blots of at least three equal repetitions. Statistical comparisons were conducted using ANOVA or log-rank Mantel–Cox tests (for survival curves) and GraphPad Prism software.

Results

Brain mitochondrial biogenesis and NO$^\bullet$-generating enzymes are strongly increased by CR

Mice subjected to a CR diet for 6 months are well documented to present more favorable markers of overall health than animals fed AL (reviewed in [18]). In addition, we found that the detection of cytochrome *c* oxidase, an inner mitochondrial membrane component of the electron transport chain, was strikingly increased (approximately seven times) in the brains of CR animals (Fig. 1A). The activity of citrate synthase, a mitochondrial matrix enzyme that is part of the