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Original Contribution

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

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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.

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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

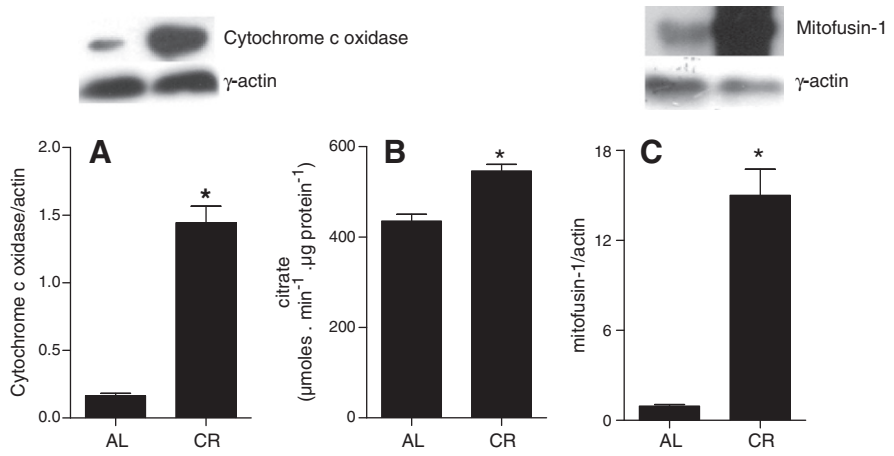

Fig. 1. CR increases brain mitochondrial biogenesis. (A) Cytochrome *c* oxidase expression, (B) citrate synthase activity, and (C) mitofusin-1 expression in AL and CR mouse brains. Averages ± SEM are depicted under representative blots. $^{*}p<0.01$ vs AL.

tricarboxylic acid cycle and is commonly used as a marker for mitochondrial mass [19], was increased by about 20% in CR brain lysates (Fig. 1B). Finally, mitofusin-1, an outer mitochondrial membrane protein involved in mitochondrial fusion [20], controlling morphology and dynamics, was enhanced more than 10 times in CR brains compared to AL (Fig. 1C). Overall, these experiments indicate that there is a large increase in mitochondrial mass in the brains of CR animals.

Mitochondrial biogenesis is a process stimulated by $NO^{\bullet}$ generated by nitric oxide synthases (NOSs). We thus measured the levels of nNOS and eNOS. Fig. 2 shows that both nNOS and eNOS levels are strongly increased in the brains of CR animals and that eNOS also presented a higher rate of phosphorylation. Overall, the higher expression of NOSs in the brains of CR animals is compatible with enhanced mitochondrial biogenesis observed in Fig. 1, although a cause/effect relationship cannot be directly established in this model.

CR serum stimulates $NO^{\bullet}$ signaling in primary cultured neurons

To establish an in vitro system in which the effects of CR could be studied on neurological tissue, we collected sera from CR and AL animals and cultured primary cerebellar granule neurons in the presence of these sera. We found that incubation with CR serum was sufficient to induce many changes in cultured neurons observed in the brains of CR mice. After 24 h incubation with CR serum, nNOS expression was very significantly incremented (Fig. 3A). The functional activation of nNOS was confirmed by measuring NO_2^- accumulated in the culture medium (Fig. 3B), which was significantly increased in CR versus AL serum. We could not detect eNOS in cultured neuronal lysates.

CR serum stimulates mitochondrial biogenesis in primary cultured neurons

To verify if increased $NO^{\bullet}$ signaling induced by CR serum was accompanied by enhanced mitochondrial biogenesis, we measured the levels of cytochrome *c* oxidase and citrate synthase activity. Both were largely enhanced in CR cultures compared to cells incubated in AL serum (Figs. 4A and B).

To evaluate the functional result of increases in mitochondrial enzymes, we measured cellular respiration (Fig. 4C). Baseline respiratory rates in neurons incubated in CR serum were significantly higher than in cells incubated in AL serum. This increase was not due to uncoupling between electron transport and oxidative phosphorylation, because respiration in the presence of the ATP synthase inhibitor oligomycin, dependent only on the proton leak, was similar in both groups. On the other hand, maximal respiratory rates obtained in the presence of the respiratory uncoupler FCCP were increased by CR serum, indicating that CR increases the amount of functional

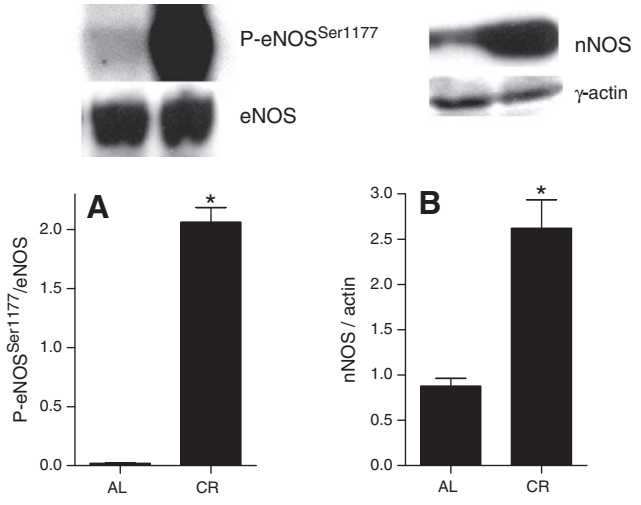

Fig. 2. CR enhances brain eNOS and nNOS. (A) Phospho-eNOS^{Ser1177}/eNOS and (B) nNOS from AL and CR mouse brains. Averages ± SEM of the phosphorylated over total protein ratios are depicted under representative blots. $^{*}p<0.001$ vs AL.

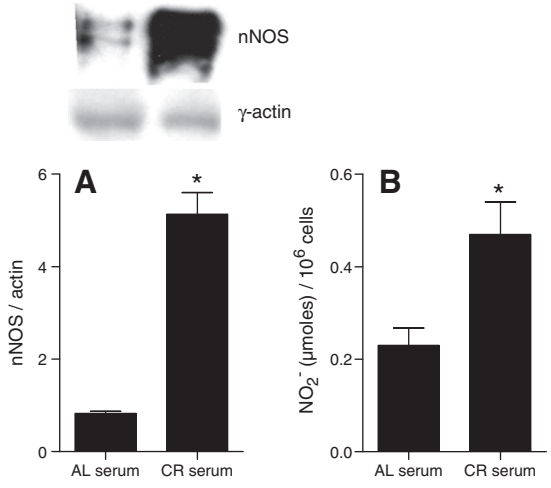

Fig. 3. CR serum increases nNOS in cerebellar neurons, enhancing nitrite release. Neurons were cultured with 10% AL or CR sera for 24 h. (A) Cell lysates were used to measure nNOS expression. Averages ± SEM of the phosphorylated over total protein ratios are depicted under a representative blot. (B) Culture medium NO_2^- levels. $^{*}p<0.05$ vs AL.

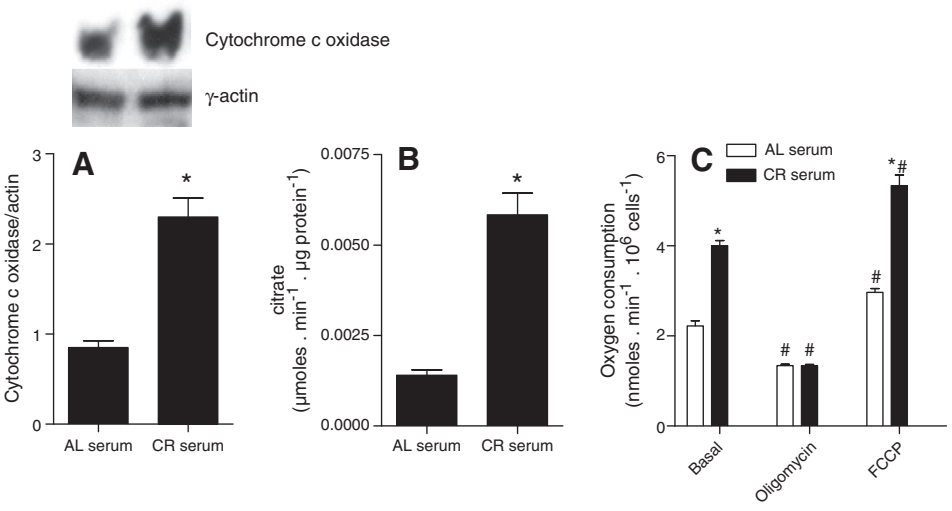

Fig. 4. CR serum increases mitochondrial biogenesis and reserve respiratory capacity in cerebellar neurons. Cells were incubated as described for Fig. 3. Cell lysates were used to measure (A) cytochrome *c* oxidase expression and (B) citrate synthase activity. (C) Respiration was measured in suspended neurons. Oligomycin (0.5 μg ml^{-1}) and FCCP (2 μM) were sequentially added. $^{*}p<0.05$ vs AL. $^{\#}p<0.05$ vs basal respiration.

respiratory chain, leading to enhanced reserve respiratory capacity [21–23]. Respiratory rates in the presence of FCCP normalized to rates in the presence of oligomycin (a cellular measurement similar to respiratory control ratio, which quantifies mitochondrial coupling) were 4.00 ± 0.41 (CR) versus 2.20 ± 0.06 (AL). This indicates that CR serum increases both mitochondrial activity and coupling in neurons.

To verify if the increase in mitochondrial biogenesis was related to enhanced $NO^{\bullet}$ signaling, we measured cytochrome *c* oxidase expression in the presence of the NOS inhibitor L-NAME (Fig. 5). We found that, although L-NAME had little effect on cytochrome *c* oxidase contents in cells cultured in AL sera, it completely reversed the enhanced detection of this mitochondrial respiratory complex promoted by CR serum.

$NO^{\bullet}$ promotes mitochondrial biogenesis and increased respiratory capacity

To further test the hypothesis that enhanced mitochondrial biogenesis and stimulated $NO^{\bullet}$ production observed in CR serum were mechanistically linked, we verified if increasing $NO^{\bullet}$ levels in neuronal cultures using a $NO^{\bullet}$ donor, SNAP, was sufficient to enhance mitochondrial biogenesis (Fig. 6).

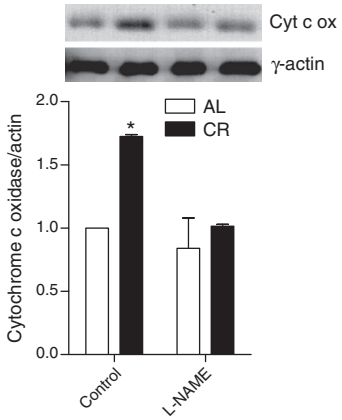

Fig. 5. Mitochondrial biogenesis promoted by CR serum is dependent on NOS. Cells were incubated as described for Fig. 3 in the presence or absence of L-NAME, as described under Materials and methods. Cell lysates were used to measure cytochrome *c* oxidase expression. $^{*}p<0.05$ vs AL.

Various titrations were tested (results not shown), and a low dose of SNAP (10 nM) was chosen, leading to an estimated $NO^{\bullet}$ release rate of 30 pmol min^{-1} [24]. At these concentrations, SNAP promoted, in 24 h, a significant increment in cytochrome *c* oxidase expression and citrate synthase activity (Figs. 6A and B), similar to that promoted by CR serum. In addition, SNAP induced an increase in basal and FCCP-stimulated cellular respiratory rates, as well as oligomycin-inhibited respiration (Fig. 6C). FCCP/oligomycin ratios were 2.20 ± 0.06 (DMSO) versus 1.90 ± 0.19 (SNAP), indicating that mitochondrial coupling was unaffected by SNAP.

CR serum and $NO^{\bullet}$ enhance neuronal survival

Neurodegeneration is an important consequence of aging, and CR is well established as preventing this process in vivo [2,25]. We tested if the protective effects of CR on neurons could be reproduced in our in vitro model by following neuronal survival over time in CR and AL sera (Fig. 7). We found that CR serum alone was capable of significantly extending the in vitro survival of cultured neurons ($p = 0.02$ versus AL).

Next, we questioned if the enhanced survival of these neurons was related to enhanced $NO^{\bullet}$ signaling promoted by CR serum. Previous studies have indicated that CR increases $NO^{\bullet}$ [4], but a direct correlation with survival has not been established in vivo or in vitro. We found that treating neuronal cultures with the same low concentrations of SNAP that increased mitochondrial biogenesis promotes enhanced survival in vitro (Fig. 7, $p<0.0001$ for both AL + SNAP and CR + SNAP versus AL) and similar survival times in CR and AL serum-incubated cells ($p = 0.519$).

Discussion

Aging promotes significant impairments in neurological function associated with neuronal loss. Interestingly, cognitive loss associated with aging is prevented by CR, as demonstrated in many animal models (reviewed in [2,25]).

Some authors have proposed that a critical predictor of neuronal survival in aging is reserve respiratory capacity, or the ability to enhance mitochondrial oxidative phosphorylation in response to a heightened energy demand [21,22]. Indeed, larger reserve respiratory capacity enhances survival under damaging conditions in many cellular models (reviewed in [21,23]). Interestingly, CR enhances mitochondrial biogenesis [3,4], leading to higher reserve respiratory

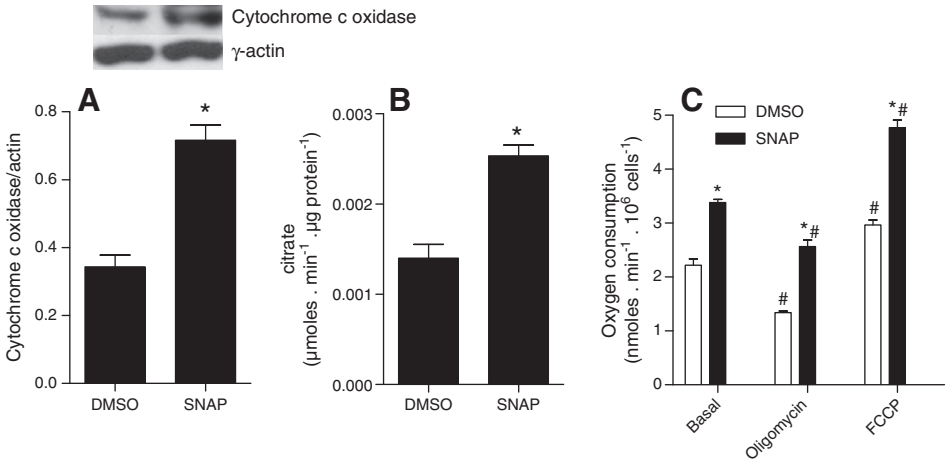

Fig. 6. NO• increases mitochondrial biogenesis and reserve respiratory capacity in cerebellar neurons to levels similar to those of CR. Cells were incubated as described for Fig. 3 in AL serum with 0.001% DMSO or 10 nM SNAP dissolved in the same quantity of DMSO. Cell lysates were used to measure (A) cytochrome *c* oxidase expression and (B) citrate synthase activity. (C) Respiration was measured in suspended neurons. Oligomycin (0.5 μg ml^{-1}) and FCCP (2 μM) were sequentially added. $^{*}p<0.05$ vs DMSO. $^{\#}p<0.05$ vs basal respiration.

capacity in insulin-sensitive tissues. Furthermore, mitochondrial biogenesis has been shown to be enhanced in the brains of animals on a restrictive diet in which food was offered every other day [10].

Mitochondrial biogenesis is induced by the activation of a NO•-sensitive pathway [9] downstream of the activation of eNOS. Indeed, eNOS phosphorylation is increased in CR [4] and the effects of dietary restriction on mitochondrial biogenesis are largely abrogated in eNOS knockout animals [10].

Based on the importance of reserve respiratory capacity in aging brains [26] and the ability of CR to enhance NO•-mediated mitochondrial biogenesis in many tissues [4], we investigated if CR promoted NO•-mediated mitochondrial biogenesis in the brain, and if this enhanced survival. This investigation is important because NO• can be deleterious in the brain even at low, physiological, levels [27].

We found that mice on a CR diet had strikingly higher levels of mitochondrial markers in their brains (Fig. 1), in addition to highly increased phosphorylation of eNOS (Fig. 2). Interestingly, we also found that nNOS was more highly expressed in CR animals (Fig. 2). nNOS is the main source of cerebral NO• [28] and has not, to our knowledge, been previously shown to be activated by CR. Because NO• is diffusible, it is expected that it can lead to the activation of mitochondrial biogenesis regardless of its source. However, in whole forebrains of animals kept on a CR diet, it is not possible to establish if nNOS is also a source of mitochondrial biogenesis-inducing NO•, nor to determine a relationship between CR, NO•, mitochondrial biogenesis, and neuronal survival.

To do so, we established an in vitro model testing the effects of sera isolated from animals on AL and CR diets on primary cultured neurons. This model is based on previous studies pioneered by de Cabo's group studying the effects of serum from CR animals on cultured cells [15,29]. This in vitro model is interesting, because it demonstrates that hormonal changes in sera can induce effects of CR independent of glucose levels, which are high in cell culture media. We found that CR serum has profound effects on cultured neurons. Survival of the cells in culture, a measure that correlates with neuronal resistance to stress and neurodegenerative stimuli [30,31], was enhanced in CR serum (Fig. 7).

Furthermore, compared to neurons cultured in AL serum, CR serum-cultured neurons exhibited largely enhanced release of nitrite, indicative of enhanced NO• signaling. Indeed, nNOS expression was very largely enhanced by CR serum. Because the eNOS isoform is absent in cerebellar granule neurons [32], our finding indicates that nNOS also has an important role in mitochondrial biogenesis [33,34]. Indeed, nNOS is the most important source of NO• in the brain [28], and it is the only NOS detected in cerebellar neurons, where it is highly expressed [32].

To verify if enhanced NO• production induced by CR serum was related to improved neuronal survival, we inhibited NO• synthesis using L-NAME and found that it completely reversed the enhanced levels of cytochrome *c* oxidase found in CR serum (Fig. 5). We also treated the cells with a NO• donor, SNAP (Fig. 6). Various concentrations were tested in preliminary experiments (2–100 nM) and we found that 10 nM SNAP added every 48 h significantly enhanced neuronal life span and equaled survival levels in CR and AL sera.

The nervous system is particularly sensitive to NO• [35]. Increased levels in biomarkers of nitrosative stress are found in many conditions

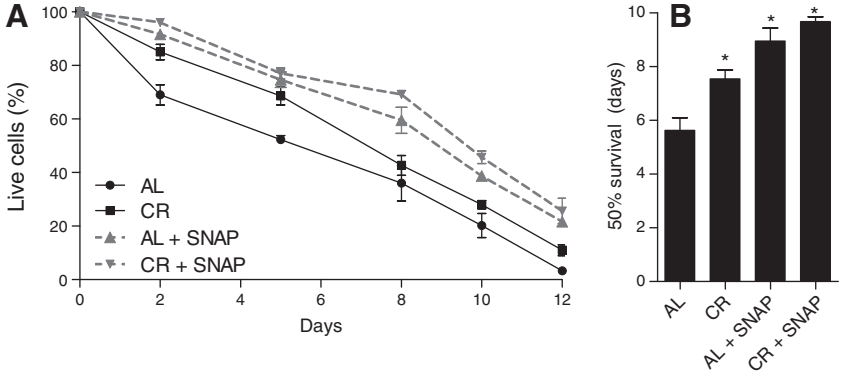

Fig. 7. CR and NO• increase neuronal survival. Cerebellar neurons were cultured in medium containing CR or AL sera and 0.001% DMSO or 10 nM SNAP, as indicated. Medium was replaced and cells were counted every 48 h. $^{*}p<0.05$ vs AL.

involving neurodegeneration (reviewed in [36]). In many of these circumstances, NO• was associated with mitochondrial fission [37]. However, NO• concentrations 4–5 orders of magnitude higher than those used in this study were necessary to promote mitochondrial fragmentation in cortical neurons [38] or damage cerebellar granule neurons [39]. Thus, it seems reasonable to suggest that low doses of NO• are neuroprotective, whereas high concentrations, leading to oxidative and nitrosative stress, are damaging. In neurons, NO• is also implicated as a signaling molecule in neuromodulation, synaptic plasticity, and other fundamental neurological processes (reviewed in [40]).

SNAP and other NO• donors have been previously shown to induce mitochondrial biogenesis in other tissues [41]. Indeed, we found that low SNAP concentrations capable of increasing neuronal survival also promoted enhanced mitochondrial biogenesis in a manner similar to incubation with CR serum (Fig. 7). Respiration was faster under basal conditions and also in the presence of the uncoupler FCCP, indicating higher maximal respiratory rates. Recently, the importance of this reserve respiratory capacity has been recognized as a predictor of survival under conditions of cellular stress [21,22], allowing enhanced oxidative phosphorylation under conditions that require superior energy demand. In this sense, our demonstration that CR increases neuronal NO• signaling and mitochondrial biogenesis, resulting in longer survival, provides insight into mechanisms involved in the prevention of neurodegeneration by CR.

Acknowledgments

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