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Article

Branched-Chain Amino Acid Supplementation Promotes Survival and Supports Cardiac and Skeletal Muscle Mitochondrial Biogenesis in Middle-Aged Mice

Giuseppe D'Antona,[1,2] Maurizio Ragni,[3] Annalisa Cardile,[3] Laura Tedesco,[3,4] Marta Dossena,[3,5] Flavia Bruttini,[1,2] Francesca Caliaro,[1,2] Giovanni Corsetti,[6] Roberto Bottinelli,[1,2] Michele O. Carruba,[3,4] Alessandra Valerio,[3,5] and Enzo Nisoli[3,4,*]

[1]Department of Physiology, Human Physiology Unit
[2]Interuniversity Institute of Myology
Pavia University, Pavia 27100, Italy
[3]Center for Study and Research on Obesity, Department of Pharmacology, Chemotherapy and Medical Toxicology, School of Medicine, Milan University, Milan 20129, Italy
[4]Istituto Auxologico Italiano, Milan 20145, Italy
[5]Pharmacology Unit
[6]Human Anatomy Unit
Department of Biomedical Sciences and Biotechnologies, Brescia University, Brescia 25123, Italy
*Correspondence: enzo.nisoli@unimi.it
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SUMMARY

Recent evidence points to a strong relationship between increased mitochondrial biogenesis and increased survival in eukaryotes. Branched-chain amino acids (BCAAs) have been shown to extend chronological life span in yeast. However, the role of these amino acids in mitochondrial biogenesis and longevity in mammals is unknown. Here, we show that a BCAA-enriched mixture (BCAAem) increased the average life span of mice. BCAAem supplementation increased mitochondrial biogenesis and sirtuin 1 expression in primary cardiac and skeletal myocytes and in cardiac and skeletal muscle, but not in adipose tissue and liver of middle-aged mice, and this was accompanied by enhanced physical endurance. Moreover, the reactive oxygen species (ROS) defense system genes were upregulated, and ROS production was reduced by BCAAem supplementation. All of the BCAAem-mediated effects were strongly attenuated in endothelial nitric oxide synthase null mutant mice. These data reveal an important antiaging role of BCAAs mediated by mitochondrial biogenesis in mammals.

INTRODUCTION

Aging is a natural process that affects most biological functions and results in reduced resistance to stress, increased vulnerability to diseases (including cardiovascular disease, cancer, diabetes, sarcopenia, osteoporosis, and kidney disease), and increased probability of death. Among the plethora of biological phenomena affected by aging, the malfunction of mitochondria and the decrease of mitochondrial biogenesis, together with increased oxidative damage, seem to exert some of the most deleterious effects on the organism (Guarente, 2008; López-Lluch et al., 2008). A variety of strategies that alleviate age-related deficits in mitochondrial biogenesis and activity, including calorie restriction (CR) and moderate physical exercise, promote survival in mammals. These interventions increase the expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α, a master regulator of mitochondrial biogenesis and reactive oxygen species [ROS] defense system) and of sirtuin 1 (SIRT1, a member of the sirtuin family linked to life span extension, enhanced mitochondrial biogenesis, and decreased ROS production), thus reducing oxidative damage in metabolically active tissues of mice and humans (Civitarese et al., 2007; Nisoli et al., 2005; Ristow et al., 2009). The CR effects on mitochondrial biogenesis are due, at least in part, to induction of endothelial nitric oxide synthase (eNOS) expression (Nisoli et al., 2005). Indeed, eNOS null mutant (eNOS$^{-/-}$) mice are characterized by a reduced life span (Li et al., 2004), due to age-related diseases (Cook et al., 2003), and by a reduced mitochondrial biogenesis (Nisoli et al., 2003, 2004) and SIRT1 expression (Nisoli et al., 2005).

Although CR has beneficial effects in humans (Heilbronn et al., 2006), such a dietary regimen is unlikely to be widely adopted in the elderly. As such, many researchers have focused on the development of CR mimetic compounds providing some of the benefits of dietary restriction without reduction in caloric intake (Ingram et al., 2004). Such attempts have been only partially successful in experimental models up to now and are not imminently feasible for humans.

Recent intriguing results indicate that amino acids leucine, isoleucine, and valine extend chronological life span in *Saccharomyces cerevisiae* (Alvers et al., 2009), thus identifying branched-chain amino acids (BCAAs) as potential candidates in promoting survival. We investigated whether long-term dietary supplementation with a specific BCAA-enriched mixture (BCAAem) that improves age-related disorders in animals and humans (Pansarasa et al., 2008; Solerte et al., 2008a) also promotes mice survival.

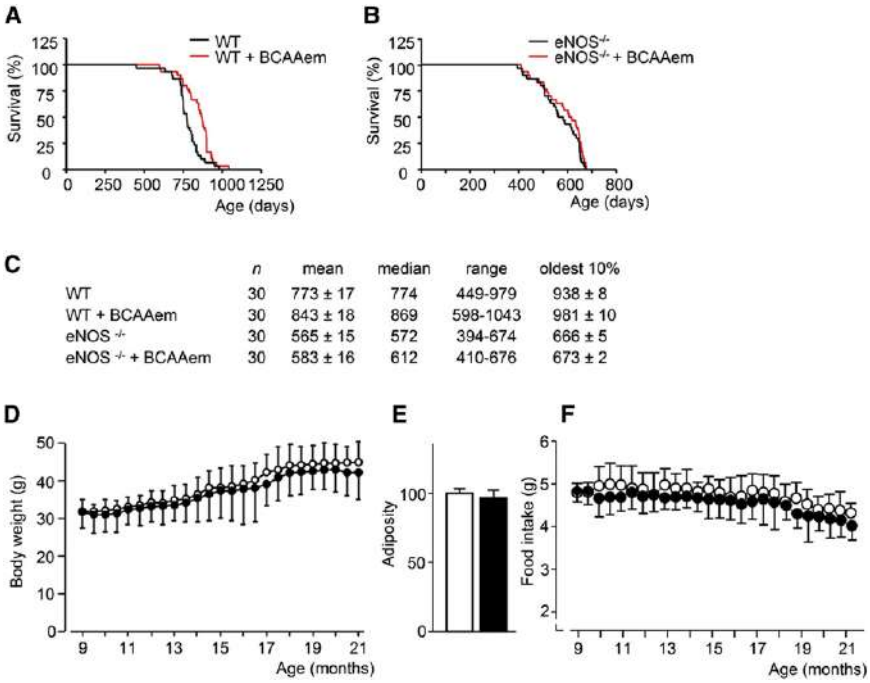

Figure 1. BCAAem Supplementation Increases Average Survival of Male Mice Independently of Adipose Tissue Changes

(A and B) Kaplan-Meier survival curves for WT (A) and eNOS$^{-/-}$ mice (B), with P values calculated using the log rank test. A significant extension in average survival was observed in BCAAem-supplemented WT mice (χ^2 = 8.154; p = 0.0043 versus untreated WT mice; hazard ratio, 0.50; 95% CI of ratio, 0.2499 to 0.7727), while differences between BCAAem-supplemented and untreated eNOS$^{-/-}$ mice did not reach the significance (χ^2 = 1.646; p = 0.1995; hazard ratio, 0.7257; 95% CI of ratio, 0.4179 to 1.200).

(C) Comparative survival characteristics of eNOS$^{-/-}$ and WT mice. Oldest 10% are the mean life span of the longest-living 10% of animals within a genotype (p > 0.05 for BCAAem effect in both mice strain).

(D) Body weight of male WT mice treated (closed circles) or not (open circles) with BCAAem, housed individually (n = 30 animals each group). Time scale shows the treatment periods starting from 9-month-old mice.

(E) Adiposity, expressed as percentage of weight of visceral and subcutaneous fat when untreated mice (open columns) were given a value of 100.

(F) Food intake (g/day) of untreated (open circles) and BCAAem-supplemented (closed circles) mice. Time scale shows the treatment periods starting from 9-month-old mice. Data in (D)–(F) represent mean ±SEM. See also Figure S1.

Here, we demonstrated that the BCAAem supplementation increased average life span of male mice, and this was accompanied by increased mitochondrial biogenesis and SIRT1 expression both in cardiac and skeletal muscles, unlike adipose tissues and liver of middle-aged mice. Further, the muscle ROS defense system genes were upregulated by BCAAem supplementation, resulting in decreased indices of oxidative damage.

RESULTS

Average Life Span Is Extended by BCAAem Supplementation in Mice

Different amino acid mixtures have been used to improve metabolic profile and restore protein content of defective tissues, in particular of skeletal muscles, in aged subjects (Dillon et al., 2009; Volpi et al., 1998). BCAAem supplementation has been reported to improve age-related disorders such as sarcopenia, insulin resistance, type 2 diabetes, and cardiovascular dysfunction (Solerte et al., 2008a, 2008b). Recently, isoleucine, threonine, and valine or extra supplemental leucine were shown to extend chronological life span in *S. cerevisiae* (Alvers et al., 2009). Thus, we investigated the effects of BCAAem on mouse survival and asked whether eNOS expression and activity might be involved in the antiaging effects of BCAAs. Nine-month-old male WT (F2 Hybrid B6.129S2) mice were supplemented with BCAAem (1.5 mg/g body weight/day in drinking water, contributing 6 kcal/kg/day, corresponding to ~1% daily caloric intake) as previously described (Pellegrino et al., 2005). No statistical difference in daily drinking volumes was found between untreated (7.0 ± 1.0 ml, n = 10 animals) and BCAAem-supplemented WT mice (6.5 ± 2.0 ml, n = 10 animals). At ~700 days of age, the survival curves of the supplemented and untreated WT groups began to diverge and remained separated by a mean interval of 70 days (Figure 1A). The median life span was 774 days for all of the untreated controls, a value lower than those recently described in either parental strain (Yuan et al., 2009) but comparable to values reported by others (Conti et al., 2006), as opposed to 869 days for all of the supplemented WT mice (12% increase), without significant change of maximal life span (Figures 1A and 1C). Notably, the body weight, adiposity, and food intake of supplemented and untreated WT mice were not statistically different (Figures 1D–1F). These results suggest that the effect of BCAAem on mice survival is independent of adipose tissue reduction.

Conversely, the survival curves of the supplemented and untreated eNOS$^{-/-}$ mice did not statistically diverge throughout the entire period of treatment (Figures 1B and 1C). No statistical difference in food intake (data not shown) and daily drinking volumes was found between untreated (6.9 ± 1.0 ml, n = 10 animals) and BCAAem-supplemented (6.5 ± 1.5 ml, n = 10 animals) eNOS$^{-/-}$ mice. The acute BCAAem supplementation transiently increased circulating BCAA levels in eNOS$^{-/-}$, similar to what observed in WT mice (Figure S1), suggesting that BCAA intestinal absorption or first-pass metabolism are similar in these mouse strains. These findings suggested that eNOS-mediated phenomena might contribute to the extension of average life span by BCAAem.

Hormonal Profile in BCAAem-Supplemented Mice

Metabolic and endocrine alterations are involved in regulation of aging and antiaging processes (Russell and Kahn, 2007). BCAAem supplementation for 3 months did not change the plasma levels

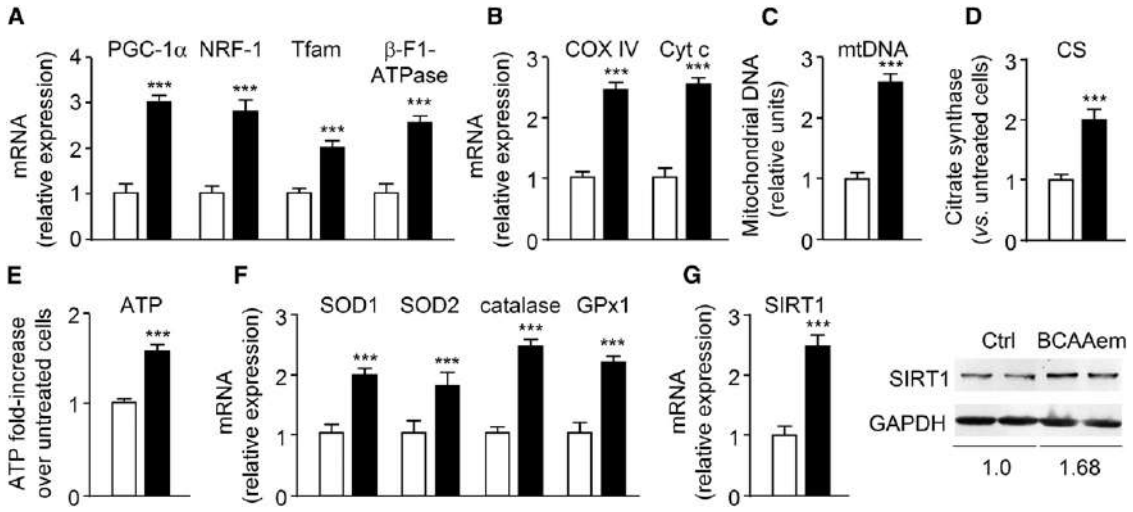

Figure 2. BCAAem Promotes Mitochondrial Biogenesis in HL-1 Cardiomyocytes

(A and B) PGC-1α, NRF-1, Tfam, and β-F1-ATPase (A) and COX IV and CytC (B) mRNA analyzed by means of quantitative RT-PCR. Relative expression values of the untreated cells (open bars) were taken as 1.0 (n = 5 experiments; ***p < 0.001).

(C) Mitochondrial DNA amount analyzed by means of quantitative PCR. Relative units are expressed in comparison to those of untreated cells (open bars) taken as 1.0 (n = 5 experiments; ***p < 0.001).

(D and E) Citrate synthase activity (D) and ATP levels (E). Values are expressed as fold change versus untreated cells (open bars) taken as 1.0 (n = 5 experiments; ***p < 0.001).

(F) Copper/zinc superoxide dismutase (SOD1), manganese SOD (SOD2), catalase, and glutathione peroxidase (GPx1) mRNA analyzed by means of quantitative RT-PCR. Relative expression values of the untreated cells (open bars) were taken as 1.0 (n = 5 experiments; ***p < 0.001).

(G) SIRT1 mRNA and protein levels. A representative immunoblot is shown for SIRT1 protein, and numbers below the blots are values of SIRT1 densitometric analysis referred to GAPDH. Values measured in the untreated cells (Ctrl) are taken as 1.0 (n = 5 experiments; ***p < 0.001). BCAAem was used at 1× concentration. All data represent mean ±SEM. See also Figure S2.

of insulin (untreated, 1.85 ± 0.07 ng/ml; treated, 1.90 ± 0.05 ng/ml; n = 14 animals/group), insulin-like growth factor-1 (IGF-1) (untreated, 307 ± 36 ng/ml; treated, 319 ± 25 ng/ml; n = 14 animals/group), and testosterone (untreated, 35 ± 9.7 nmol/l; treated, 35.2 ± 14 nmol/l; n = 8 animals/group) in middle-aged WT mice. Although not statistically changed, growth hormone (GH) levels were increased by BCAAem (untreated, 21.3 ± 8.5 ng/ml; treated, 39.1 ± 11.1 ng/ml; n = 10 animals/group). This and results described below on muscle fiber size do not rule out that GH may play some role in BCAAem effects, even if lack of differences in IGF-1 along with unaltered insulin levels would support the conclusion that GH levels were not affected by supplementation.

Mitochondrial Biogenesis Is Increased by BCAAem in a Tissue-Specific Manner

Boosting mitochondrial activity may provide at least some of the processes that increase life span in yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals (Baur et al., 2006; Bonawitz et al., 2007; Lagouge et al., 2006; Lin et al., 2002; Zid et al., 2009). Moreover, a higher pool of functional mitochondria may lead to reduced ROS production, with amelioration of age-related tissue damages (Barja, 2007). Thus, we aimed to verify the effects of BCAAem in comparison to single amino acids or other amino acid mixtures (Table S1) on mitochondrial biogenesis markers (i.e., mitochondrial DNA [mtDNA] amount and PGC-1α mRNA levels) in HL-1 adult cardiomyocytes. The BCAAem (mixture #3) was more effective than other mixtures in dose-dependently promoting mitochondrial biogenesis (Figure S2). With further investigation, treatment of HL-1 cells with BCAAem increased the mRNAs encoding PGC-1α, nuclear respiratory factor-1 (NRF-1), mtDNA transcription factor A (Tfam), and β subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase) (Figure 2A). Mitochondrial gene targets of PGC-1α involved in oxidative phosphorylation were also upregulated by BCAAem (Figure 2B). These effects on gene expression translated into a 2.6-fold increase in mtDNA content (Figure 2C), a 2-fold increase in citrate synthase activity (Figure 2D), and a 1.5-fold increase in ATP amount (Figure 2E). Moreover, the genes of the ROS defense system, including copper/zinc superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2), catalase, and glutathione peroxidase (GPx1), whose expression is increased by PGC-1α activation (St-Pierre et al., 2006), were upregulated by BCAAem supplementation (Figure 2F). Notably, SIRT1 mRNA and protein levels were increased as well (Figure 2G).

Next, to validate these observations in vivo, we examined the effects of BCAAem supplementation in middle-aged mice. Gene expression of mitochondrial transcriptional regulators and SIRT1 as well as citrate synthase activity were markedly increased by prolonged (3 months) supplementation in heart, diaphragm, soleus, and tibialis muscles, unlike white (WAT) and brown adipose tissue (BAT) and liver (Figures 3A–3C and S3A–S3F). Correspondingly, the expression of genes of the ROS defense system was increased by BCAAem in cardiac and skeletal muscles (Figure 3D), unlike adipose tissues and liver (data not shown). These effects were absent in young animals.

Interestingly, BCAAem supplementation to exercised mice further strengthened the mitochondrial biogenesis (Figure 3A–3C)

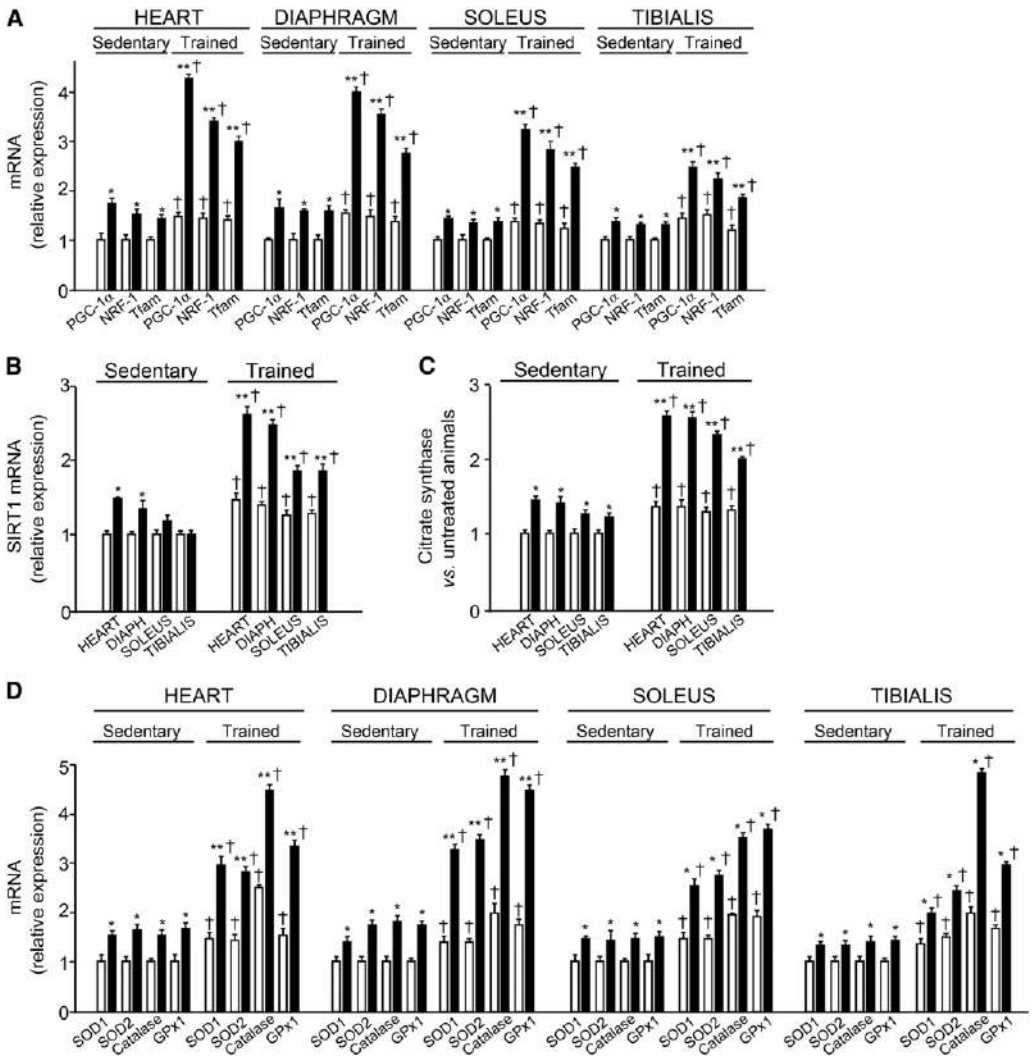

Figure 3. BCAAem Supplementation Promotes Mitochondrial Biogenesis and Increases the ROS Defense System in Cardiac and Skeletal Muscles of Sedentary and Exercise-Trained Middle-Aged Mice

(A and B) PGC-1α, NRF-1, and Tfam (A) and SIRT1 (B) mRNA levels analyzed by means of quantitative RT-PCR. Relative expression values of untreated (open bars) sedentary mice were taken as 1.0 (n = 6 experiments; *p < 0.05, **p < 0.01 versus corresponding untreated animals; †p < 0.05 versus corresponding sedentary animals).

(C) Citrate synthase activity. Values in untreated (open bars) sedentary mice taken as 1.0 (n = 3 experiments; *p < 0.05, **p < 0.01 versus corresponding untreated animals; †p < 0.05 versus corresponding sedentary animals).

(D) SOD1, SOD2, catalase, and GPx1 mRNA levels analyzed by means of quantitative RT-PCR. Relative expression values of untreated (open bars) sedentary mice were taken as 1.0 (n = 6 experiments; *p < 0.05, **p < 0.01 versus corresponding untreated animals; †p < 0.05 versus corresponding sedentary animals). All data represent mean ±SEM. See also Figure S3.

and ROS defense system in cardiac and skeletal muscles (Figure 3D). In addition, electron microscopy analysis confirmed increased mitochondrial mass in cardiac and skeletal muscle of sedentary and trained mice (Figure 4) following BCAAem supplementation.

BCAAem Preserves Muscle Morphology and Improves Functional Capacity

It is well known that aging causes a gradual loss of skeletal muscle efficiency, and several lines of evidence suggest mitochondrial involvement in this condition (Marzetti et al., 2009). Here, we observed that although weight and muscle mass relative to the body weight of vastus, gastrocnemius, and tibialis muscle were unchanged or minimally decreased in the middle-aged compared to adult WT animals (data not shown), the fiber cross-sectional areas of these muscles were significantly decreased in middle-aged mice (Figure 5A) and were restored to the levels of adult mice by BCAAem supplementation (Figure 5A). Consistently, BCAAem supplementation improved whole endurance capacity in treadmill tests of sedentary and trained mice (Figure 5B). Moreover, in motor coordination tests on a rotating rod, BCAAem-supplemented mice performed better than untreated animals (Figure 5C), particularly after exercise training.

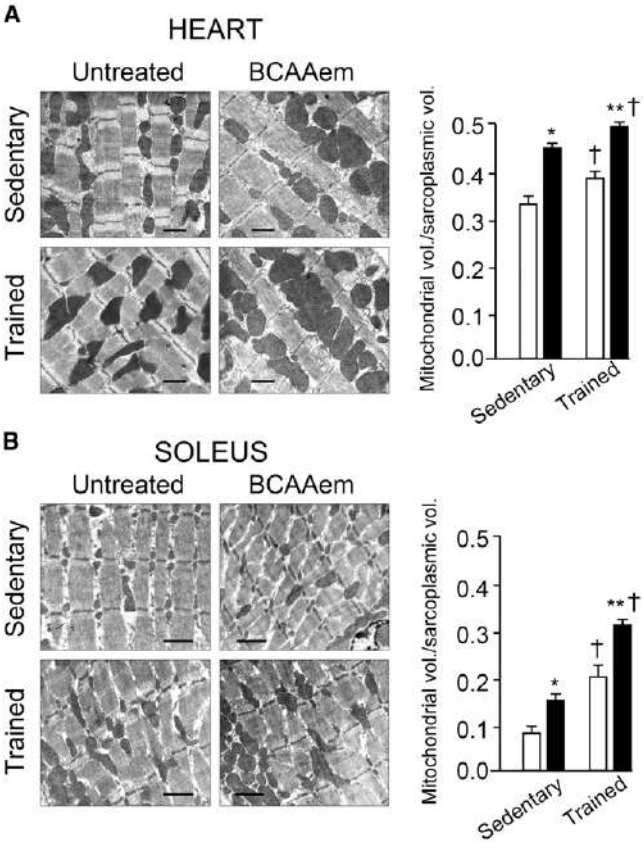

Figure 4. BCAAem Supplementation Increases Mitochondrial Mass in Cardiac and Skeletal Muscles of Middle-Aged Mice

(A and B) Electron microscopy analysis of heart (A) and soleus muscle (B) from sedentary and trained animals, with (closed bars) or without (open bars) BCAAem supplementation. Mitochondrial mass was analyzed as mitochondrial volume/sarcoplasmic volume ratio (*$p < 0.05$, **$p < 0.01$ versus corresponding untreated animals; †$p < 0.05$ versus corresponding sedentary animals; n = 3 per group). Scale bar, 1 μm. All data represent mean ±SEM.

BCAAem Decreases Mitochondrial Oxidative Stress in Cardiac and Skeletal Muscles

Increased oxidative damage due to ROS accumulation is a major cause of aging in lower organisms and mammals (Finkel and Holbrook, 2000). When compared to untreated mice, BCAAem-supplemented animals showed a reduced mitochondrial H_2O_2 release (an index of mitochondrial superoxide anion production), decreased mitochondrial ROS production (as assessed by the basal/total aconitase activity ratio, a sensitive marker of oxidative stress), and increased capacity to eliminate superoxides at the mitochondrial level (since SOD activity was significantly stimulated) in heart (Figure 6A) and soleus muscle (Figure 6B). Furthermore, unlike adipose tissues, lipid peroxidation (an indirect index of ROS levels) was lower in skeletal muscles of BCAAem-treated than untreated mice (Figure 6C).

eNOS Is Involved in BCAAem Effects on Mitochondrial Biogenesis and ROS Defense System

Since eNOS-derived nitric oxide (NO) was demonstrated to increase mitochondrial biogenesis (Nisoli et al., 2003, 2004; Nagy et al., 2004; Xu et al., 2007) and suggested to play a role in life span of mammals (Nisoli et al., 2005), we sought whether eNOS mediated the effects of BCAAem on mitochondrial biogenesis and function. BCAAem addition increased eNOS expression and activation (eNOS-Ser1177 phosphorylation) in HL-1 cardiomyocytes (Figure 7A). Moreover, knocking down eNOS (Figure 7B) resulted in decreased expression of mitochondrial biogenesis markers, and the cells became insensitive to BCAAem (Figure 7C). mtDNA content and citrate synthase activity followed the same pattern (Figures 7D and 7E).

To confirm that BCAAem supplementation induced mitochondrial biogenesis and ROS defense system through eNOS expression, primary adult cardiac and gastrocnemius myocytes were isolated from $eNOS^{-/-}$ and WT littermates and treated or nor with BCAAem for 48 hr. BCAAem markedly increased mitochondrial biogenesis and function as well as SOD1 expression in the primary cardiac myocytes (Figures S4A, S4C, and S4D) and gastrocnemius myocytes (Figures S4B, S4E, and S4F) of WT mice. On the contrary, eNOS gene deletion completely precluded the BCAAem-mediated effects (Figures S4A–S4F).

The expression of mitochondrial biogenesis markers, SIRT1, and citrate synthase activity were unchanged in different muscles of middle-aged $eNOS^{-/-}$ mice chronically (3 months) supplemented or not with BCAAem (Figures S3G–S3I). Correspondingly, the expression of genes of the ROS defense system was unchanged by BCAAem supplementation in cardiac and skeletal muscles of $eNOS^{-/-}$ mice (Figure S3J). Not even a long-term exercise training improved BCAAem effects in

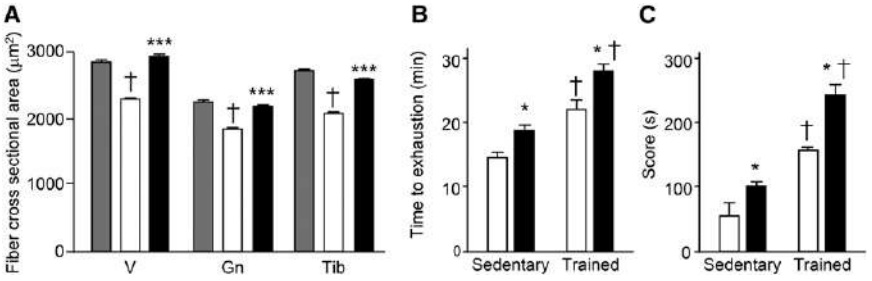

Figure 5. BCAAem Supplementation Restores Fiber Size of Skeletal Muscles and Improves Endurance Capacity and Coordination Function in Middle-Aged Mice

(A) Fiber cross-sectional area of skeletal muscles. V, vastus muscle; Gn, gastrocnemius muscle; Tib, tibialis muscle from WT mice, untreated or supplemented with BCAAem (n = 5 mice per group). Gray bars, untreated adult mice; open bars, untreated middle-aged mice; black bars, BCAAem-treated middle-aged mice. †$p < 0.001$ versus adult mice; ***$p < 0.001$ versus untreated middle-aged mice.

(B) Time to reach exhaustion following treadmill tests in untreated or BCAAem-supplemented (closed bars) mice (*$p < 0.05$ versus corresponding untreated mice; †$p < 0.05$ versus corresponding sedentary mice; n = 20 per group).

(C) Rotarod score in untreated or BCAAem-supplemented (closed bars) mice (*$p < 0.05$ versus corresponding untreated mice; †$p < 0.05$ versus corresponding sedentary mice; n = 10 experiments). All data represent mean ±SEM. See also Figure S6.

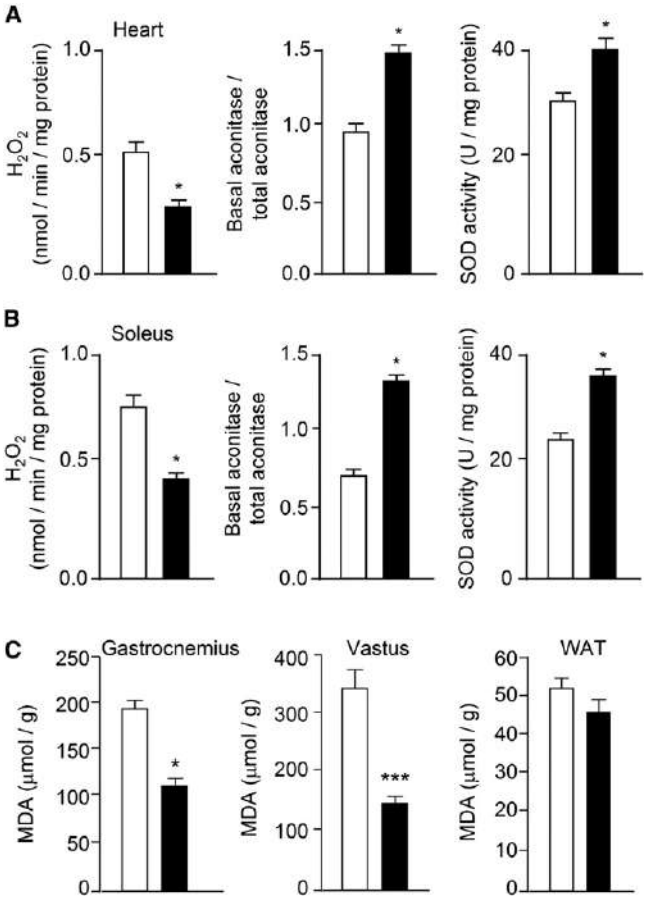

Figure 6. BCAAem Supplementation Reduces Oxidative Damage in Middle-Aged Mice

(A and B) Mitochondrial H_2O_2 release, basal aconitase/total aconitase ratio, and superoxide dismutase activity (SOD) in heart (A) and soleus muscle (B) from mice supplemented (closed bars) or not (open bars) with BCAAem (*$p < 0.05$ versus untreated mice; n = 10 experiments).

(C) Lipid peroxidation measured as malondialdehyde (MDA) production in skeletal muscles and white adipose tissue (WAT) of BCAAem-treated (closed bars) or not (open bars) animals (n = 5 experiments). All data represent mean ±SEM. See also Figure S5.

muscles of eNOS$^{-/-}$ mice (Figures S3G–S3J). Moreover, the ROS production was unchanged in muscles and adipose tissues of BCAAem-supplemented eNOS$^{-/-}$ mice with respect to untreated knockout mice (Figures S5). Muscle fiber cross-sectional area was slightly increased by BCAAem in gastrocnemius and tibialis but not in vastus muscle of knockout animals (Figure S6A). Finally, the impaired whole-endurance capacity and motor coordination of knockout mice were not improved by BCAAem supplementation in sedentary nor in trained conditions (Figures S6B and S6C).

mTOR Signaling Is Involved in BCAAem Effects on Mitochondrial Biogenesis

Since BCAAs are known to activate the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) (Avruch et al., 2009), while the mTOR inhibitor rapamycin was shown to downregulate PGC-1α and its target genes (Cunningham et al., 2007), we investigated the role of mTOR signaling in BCAAem-induced mitochondrial biogenesis. BCAAem supplementation increased activation of mTOR and of the best-characterized mTOR substrates, ribosomal S6 protein kinase (S6K) and eukaryotic translation initiation factor (eIF4E)-binding protein (4E-BP1), in HL-1 cardiomyocytes (Figure 7F). Unlike what was observed in C2C12 cells (Cunningham et al., 2007), rapamycin did not downregulate per se mtDNA content in HL-1 cells (Figure 7G). However, it strongly antagonized the effects of BCAAem on mtDNA content in HL-1 cells (Figure 7G). Mitochondrial gene expression and citrate synthase activity followed the same pattern (Figures S4G and S4H).

Notably, NO is able to upregulate mTOR activity and its downstream targets (Pervin et al., 2007), while rapamycin inhibits eNOS expression and activity (Barilli et al., 2008). Consistently, knockdown of eNOS reduced BCAAem-mediated mTOR activation in HL-1 cells (Figure 7H). Activity of mTOR was unchanged by BCAAem supplementation of exercise-trained eNOS$^{-/-}$ unlike WT mice (Figure S4I). Moreover, rapamycin blocked eNOS activation by BCAAem in HL-1 cells (Figure 7I).

DISCUSSION

In the present study, we have demonstrated that oral supplementation of a BCAAem increased the average life span of male mice. This is in line with recent studies showing the role of isoleucine, threonine, and valine or extra supplemental leucine in extension of chronological life span in *S. cerevisiae* (Alvers et al., 2009). The increased survival of BCAAem-supplemented mice was accompanied by increased mitochondrial biogenesis and function in cardiac and skeletal muscles, although not in adipose tissue or liver of middle-aged mice. The BCAAem preserved muscle fiber size and improved physical endurance and motor coordination.

Notably, recent evidence supports the notion that increased mitochondrial activity plays a causative and not only a correlative role in extension of yeast and *Drosophila* life span (Bonawitz et al., 2007; Zid et al., 2009). Consistently, in a mouse model of defective mitochondrial biogenesis such as eNOS null mutant mice, BCAAem was unable to affect muscle mitochondrial density and function. More important, average life span of eNOS$^{-/-}$ mice, which was lower than that of WT animals, as previously found (Li et al., 2004), was insensitive to BCAAem supplementation. Our findings do not rule out that an earlier BCAAem supplementation could extend also eNOS$^{-/-}$ mice survival. However, this hypothesis seems unlikely, since prolonged (1–3 months) BCAAem supplementation neither improves insulin resistance nor induces mitochondrial biogenesis and ROS defense system in young (2- to 4-month-old) knockout mice (G.D. and E.N., unpublished data).

BCAAem supplementation also upregulated SIRT1, the mammalian equivalent of the yeast longevity gene *SIR2*, in cardiac and skeletal muscles. In line with our results, the production of SIRT1 is known to be induced in several mouse tissues, except in liver, by CR (Chen et al., 2008; Civitarese et al., 2007; Nisoli et al., 2005), a dietary regimen that extends life span, increases mitochondrial biogenesis, and decreases ROS production in lower and higher organisms (López-Lluch et al., 2008). A number of nutrient restriction effects require eNOS,

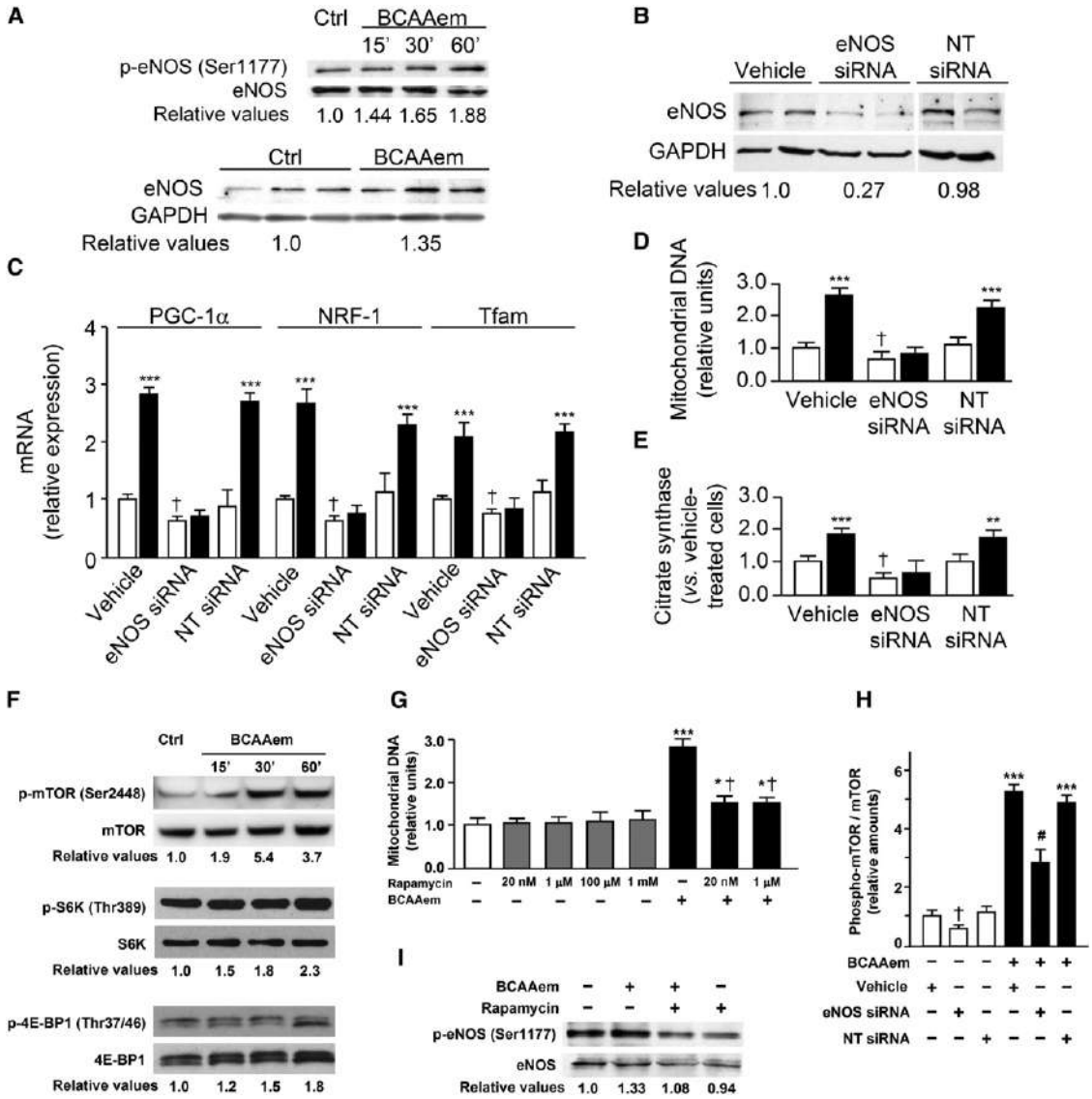

Figure 7. BCAAem Exposure Increases Mitochondrial Biogenesis in HL-1 Cells through eNOS and mTOR Activation

(A) Upper panel: eNOS phosphorylation in untreated (Ctrl) and BCAAem-treated cells. Lower panel: eNOS expression measured after 48 hr treatment.

(B) eNOS expression in HL-1 cells transfected with either eNOS siRNA or nontargeting (NT) siRNA. Vehicle, transfection reagent.

(C) PGC-1α, NRF-1, and Tfam mRNA levels in untreated (open bars) or BCAAem-treated (closed bars) cells (***$p < 0.001$ versus corresponding BCAAem-untreated cells, †$p < 0.05$ versus corresponding untransfected cells; n = 3).

(D and E) Mitochondrial DNA content (D) and citrate synthase activity (E) in untreated (open bars) or BCAAem-treated (closed bars) cells (***$p < 0.001$ versus corresponding BCAAem-untreated cells, †$p < 0.05$ versus corresponding untransfected cells; n = 3).

(F) mTOR, S6K, and 4E-BP1 phosphorylation in untreated (Ctrl) and BCAAem-treated cells.

(G) The increase of mtDNA, induced by 48 hr BCAAem (closed bars), was antagonized by rapamycin (n = 3 experiments; *$p < 0.05$ and ***$p < 0.001$ versus corresponding BCAAem-untreated cells; †$p < 0.05$ versus BCAAem-treated cells).

(H) mTOR activation in untreated or 30 min BCAAem-treated (closed bars) cells was decreased by eNOS silencing (n = 3 experiments; ***$p < 0.001$ versus corresponding BCAAem-untreated cells; †$p < 0.05$ versus untransfected cells; #$p < 0.05$ versus untransfected, BCAAem-treated cells).

(I) Exposure to rapamycin (20 nM) prevented eNOS phosphorylation induced by 30 min BCAAem treatment. All data represent mean ±SEM. See also Figure S4.

and NO is known to increase SIRT1 expression in cultured cells (Nisoli et al., 2005). Further investigation is needed to assess the role of SIRT1 induction in BCAAem effects on mice survival.

Of interest, the prolonged survival due to BCAAem supplementation was associated with increased expression of genes involved in antioxidant defense and marked reduction of ROS production in cardiac and skeletal muscles of WT but not eNOS$^{-/-}$ mice. Failure of eNOS null mutant mice to reduce ROS in response to BCAAem supplementation may partly explain the inability of this amino acid mixture to extend their average life span. Notably, it has recently been reported that NO-dependent mechanisms activate AMP-activated protein

kinase (AMPKα1), which increases the expression of genes involved in antioxidant defense in endothelial cells (Colombo and Moncada, 2009). These and the present results indicate that NO plays a role in maintaining ''antioxidant homeostasis.'' Diverse reports point out that aging renders cells and tissues increasingly vulnerable to oxidative stress (see for review Finkel and Holbrook, 2000). Oxidative damage to mitochondria could account for some of the age-related changes in their function. Tissues from aged animals show mitochondrial dysfunctions with increased superoxide and H_2O_2 generation and decreased energy production (Sohal et al., 1994). Thus, the increased expression of PGC-1α, a powerful transcription regulator of ROS defense system (St-Pierre et al., 2006), could play a relevant role in the extension of average life span evoked by BCAAem in middle-aged mice. Otherwise, hormonal changes, including insulin/IGF-1, testosterone, or GH, whose role is not conclusively defined both in aging processes and survival effects of nutrient restriction (Masoro, 2009), are unlikely to take part in the BCAAem effects.

Exercise promotes survival and, in particular, extends average although not maximal life span (Holloszy, 1993), specifically improving insulin sensitivity (James et al., 1984) that is defective in aging. This survival-promoting effect may be independent of changes in body weight (Holloszy, 1993). Similarly, the survival-extending property of BCAAem was not accompanied by modification of food intake, body weight, or adiposity and was independent of changes in mitochondrial biogenesis, SIRT1, or antioxidant genes in adipose tissue and liver. A drawback of exercise is that it also increases mitochondrial ROS production. Unexpectedly, Ristow et al. (2009) have recently demonstrated that antioxidants such as vitamin C and vitamin E abolished the health-promoting effects (i.e., increased parameters of insulin sensitivity, including plasma adiponectin) of exercise in humans, since these compounds prevented the induction of the ROS sensors PGC-1α/β and consequent activation of ROS defense. Conversely, our present results demonstrate that BCAAem supplementation increased expression of PGC-1α and of antioxidant defense enzymes also in long-term exercise-trained middle-aged mice. Moreover, the BCAAem has been described to increase plasma adiponectin in slightly trained elderly subjects (B. Solerte, personal communication). Thus, the BCAAem could be a valid substitute for dietary supplementation with antioxidants.

A relevant point is the molecular mechanism(s) involved in the BCAAem-increased mitochondrial biogenesis and survival. Interestingly, BCAAs have been reported to increase mTORC1 activity (Avruch et al., 2009), which correlates positively with cell oxidative capacity (Schieke et al., 2006) and regulates PGC-1α coactivation of its own promoter and mitochondrial gene expression in muscle cells (Cunningham et al., 2007). Moreover, rapamycin was shown to reduce eNOS expression and NO production (Barilli et al., 2008). Based on our results, one can hypothesize that BCAAem-activated mTOR signaling could enhance mitochondrial biogenesis partly through increasing the NO-generating system. In turn, our findings are consistent with recent reports showing that NO upregulates mTOR activity and its downstream targets (Pervin et al., 2007), suggesting that a positive feedback mechanism between eNOS and mTOR exists. Interestingly, selective knockout of either mTOR or the mTORC1 component raptor in skeletal muscle decreased oxidative capacity, mitochondrial gene expression, and survival (Bentzinger et al., 2008; Risson et al., 2009). In contrast, the absence of raptor in adipose tissue results in lean mice with enhanced mitochondrial respiration (Polak et al., 2008). Accordingly, BCAAem supplementation induces mitochondrial biogenesis in muscle but not in fat or liver.

Several studies indicate that reduced TOR signaling underlies life span extension by CR (see Stanfel et al., 2009 for review). Mice fed rapamycin live longer than control mice fed unsupplemented chow, even when treatment begins in late life (Harrison et al., 2009), and mice with deletion of S6K have increased life span and resistance to age-related pathologies (Selman et al., 2009). However, the reported prolongevity effects of chronic rapamycin treatment in mice do not conclusively prove that mTOR inhibition is the mechanism involved. Notably, rapamycin has been recently shown to be ineffective to increase life span in *Drosophila* (Harrison et al., 2010). Moreover, unlike CR, rapamycin is more efficacious in female than in male mice (Harrison et al., 2009), while S6K deletion increases life span in female but not in male mice (Selman et al., 2009). Again, the role of mTOR in CR is probably tissue specific, in that CR reduces mTOR signaling in liver (Jiang et al., 2008) but increases it in WAT and heart (Linford et al., 2007) and increases mitochondrial function in different tissues (Nisoli et al., 2005), a finding that is not consistent with reduced mTOR signaling. Although we did not specifically investigate the contribution of mTOR in BCAAem-mediated increase of survival, our findings support the notion that the role of mTOR in CR mechanisms is complex and not conclusively clarified at the moment (Anderson and Weindruch, 2010).

Among drug strategies to extend life span, SIRT1 activators (e.g., 3,5,4-trihydroxystilbene or resveratrol) are currently under investigation in humans. Although able to improve health under all dietary conditions, resveratrol promotes survival only in high-fat diet (Baur et al., 2006) but not in standard-diet-fed mice (Pearson et al., 2008). The BCAAem, besides increasing average life span in normally fed mice, was found to promote several healthy effects in humans, since it reduced sarcopenia (Solerte et al., 2008b) and decreased inflammatory markers in chronic heart failure patients (Kalantar-Zadeh et al., 2008).

In summary, we have provided evidence that an original BCAA mixture increases average life span in male mice. This was likely the consequence of increased mitochondrial biogenesis and reduced oxidative stress in cardiac and skeletal muscles via eNOS-mediated mechanisms. Our study offers a rationale for deeply exploring the role of amino acids in prevention and control of age-related disorders in humans.

EXPERIMENTAL PROCEDURES

Animals, Diets, and Treatments

Young (8-week-old; n = 20) and middle-aged (16-month-old) male WT (F2 Hybrid B6.129S2 obtained from crossing C57BL/6J and 129S1/SvImJ mice) as well as middle-aged B6.129P2-Nos3^{tm1Unc}/J (eNOS$^{-/-}$) mice (Shesely et al., 1996) (Jackson Laboratory), housed one per cage, were treated according to the EU guidelines and with the approval of the Institutional Ethical Committee. Animals were given unrestricted access to a standard diet (4.3 kcal % fat, 18.8 kcal % protein, 76.9 kcal % carbohydrate, Laboratorio Dottori Piccioni) and tap water. Both middle-aged WT and eNOS$^{-/-}$ mice were divided into two groups, sedentary and exercise-trained groups, each

further subdivided into untreated and BCAAem-supplemented groups (20 mice/group). BCAAem supplementation was performed for 3 months, while the exercise training was performed during the third month of supplementation. BCAAem (1.5 mg/g body weight/day in drinking water with percent composition detailed as mixture #3 in Table S1) (Pellegrino et al., 2005) was dissolved in tap water by calculating average daily drinking 2 weeks before starting treatment and stored at 4°C before daily administration. Drinking volume was checked weekly. Mice were sacrificed by cervical dislocation. Heart, diaphragm, posterior limb muscles (soleus, tibialis, and gastrocnemius), epididymal white fat, interscapular brown fat, and liver were dissected under a stereomicroscope, frozen in liquid nitrogen, and stored at −80°C.

Study Design for Survival Analysis

For survival analysis, 9-month-old WT (n = 60) and eNOS$^{-/-}$ mice (n = 60) were randomly assigned to control and BCAAem-supplemented groups (BCAAem, 1.5 mg/g body weight/day in drinking water) (Pellegrino et al., 2005). Most mice in the survival study died natural deaths; there were not accidental or external causes. In particular, lymphomas, lung and liver tumors, kidney diseases, and infection/septicemia were the main causes of death for WT mice (Haines et al., 2001), while cardiovascular and metabolic disorders were mainly involved in eNOS$^{-/-}$ mice death (Li et al., 2004). Moribund mice or those showing clear signs of distress were euthanized to mitigate suffering. Survival curves were plotted using the Kaplan-Meier method. Adiposity was measured as wet weight of visceral and subcutaneous fat. Body weight and food intake were monitored every other week for the time of the experiment.

Analytical Procedures

Glucose concentrations were determined using the Glucocard Gmeter (Menarini; Florence, Italy). ELISA kits were used to measure insulin (LINCO Research Inc.; St. Charles, MO), IGF-1 and GH (Diagnostic Systems Laboratories; Webster, TX), and testosterone (Diagnostic Products Corporation; Los Angeles, CA). To study the BCAA absorption rate, plasma BCAA analysis was performed. Details can be found in the Supplemental Experimental Procedures.

HL-1 Cell Cultures and Treatments

HL-1 cardiomyocytes (a gift from W.C. Claycomb) were cultured as described in the Supplemental Experimental Procedures. Cells were treated with single amino acids or different amino acid mixtures, including BCAAem, with percent composition and final concentrations indicated in Table S1 for 48 hr, except for time- or dose-response experiments. For eNOS knockdown experiments, HL-1 cells were transfected with 30 nM eNOS siRNA SMARTpool (Dharmacon; Lafayette, CO) or siGENOME nontargeting siRNA using Dharmafect 3 transfection reagent. Details can be found in the Supplemental Experimental Procedures.

Cardiomyocyte and Myocyte Preparation and Treatment

Cardiac myocytes were isolated from left ventricle free wall of WT or eNOS$^{-/-}$ mice, while satellite skeletal muscle cells were prepared from limbs of WT and eNOS$^{-/-}$ postnatal mice. Mature cardiac and skeletal myocytes were treated with BCAAem for 48 hr prior to cell harvesting. Details are in Supplemental Experimental Procedures.

Gene Expression and Mitochondrial Biogenesis Methods

Quantitative RT-PCR reactions were performed as described (Tedesco et al., 2008) and run with the iQ SybrGreenI SuperMix (Bio-Rad; Segrate, Italy) on an iCycler iQ Real-Time PCR detection system (Bio-Rad). Calculations were performed by a comparative method ($2^{-\Delta\Delta Ct}$) using 18S rRNA as an internal control. For mtDNA analysis, total DNA was extracted with QIAamp DNA extraction kit (QIAGEN). mtDNA was amplified using primers specific for the mitochondrial cytochrome *b* (CytB) gene and normalized to genomic DNA by amplification of the large ribosomal protein p0 (36B4) nuclear gene. Primers were designed using Beacon Designer 2.6 software (Premier Biosoft International; Palo Alto, CA). Immunoblot analysis was performed as described (Tedesco et al., 2008). The whole amount of ATP in cells was measured by using the ATP determination kit from Molecular Probes (Eugene, OR) as described (Tedesco et al., 2008). Citrate synthase activity was measured spectrophotometrically in either tissue or whole-cell extracts and expressed as nmol citrate produced/min/mg protein as described (Tedesco et al., 2008). Electron microscopy studies were conducted on heart and soleus muscle as previously described (Nisoli et al., 2004; Weibel, 1979). Details are in Supplemental Experimental Procedures.

Mitochondrial Oxidative Stress

Mitochondria were isolated using the Qproteome Mitochondria Isolation Kit (QIAGEN). Aconitase-specific activity was measured as previously described (Lionetti et al., 2007). SOD activity was measured with the Superoxide Dismutase Assay Kit (Calbiochem; San Diego, CA). Mitochondrial H_2O_2 release was assayed by the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes). To assay lipid peroxidation (LPO), malondialdehyde (MDA) derived from polyunsaturated fatty acid peroxides was evaluated by means of the LPO-586 colorimetric assay kit (OxisResearch; Portland, OR). Details are in Supplemental Experimental Procedures.

Training, Exercise Tolerance, and Motor Coordination Tests

Exercise training (5 days/week for 4 weeks as detailed in Supplemental Experimental Procedures) and exercise exhaustion tests were performed on the belt of a 6-lane motorized treadmill (Exer 3/6 Treadmill, Columbus Instruments; Columbus, OH), supplied with shocker plates (electrical stimulus: 200 ms, 0.34 mA, 1 Hz) as previously described (Benchaouir et al., 2007). Constant speed rotarod (47600 Model, Ugo Basile; Comerio, Italy) was used to measure fore- and hindlimb motor coordination and balance in 12-month-old mice, as detailed in Supplemental Experimental Procedures (Serradj and Jamon, 2007).

Muscle Fiber Size

Vastus, tibialis, and gastrocnemius muscles, obtained from adult (6-month-old) or middle-aged male WT and eNOS$^{-/-}$ mice, supplemented or not with BCAAem, were frozen in liquid nitrogen. Serial transverse sections (10 μm thick) were cut with a LeicaCM 1850 cryostat and stained with hematoxylin and eosin. To evaluate fiber cross-sectional area, morphometric analyses were performed on ~150 fibers per muscle (n = 5 mice per group) by using an Image 1.63 software (Scion Corporation; Frederick, MD).

Statistical Analysis

Data were analyzed by two-tailed unpaired Student's t test or by one-way ANOVA with Tukey's post hoc test in instances of multiple comparisons. Two-way ANOVA was used to evaluate the supplementation and exercise effects and their interaction. P values for survival analyses were calculated using the log rank test. All data are reported as mean ±SEM unless otherwise stated. Statistical analyses were performed using GraphPad Prism version 4.0 software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, six figures, and two tables and can be found with this article online at doi:10.1016/j.cmet.2010.08.016.

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