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Original Article

Concentrations of total curcuminoids in plasma, but not liver and kidney, are higher in 18- than in 3-months old mice

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ABSTRACT

Background: Curcuminoids (curcumin, demethoxycurcumin, bis-demethoxycurcumin) are lipophilic polyphenols thought to be effective in the prevention and treatment of neurodegenerative disorders, of which mitochondrial dysfunction is a prominent feature. In particular, older people may thus benefit from increasing their curcuminoid intake. However until now, it is not investigated if there exist age differences in the bioavailability of curcuminoids and therefore, it is unclear if curcumin doses have to be adjusted to age. Thus, we explored if the tissue concentrations and biological activities of curcuminoids are affected by age.
Methods: We investigated age-differences in the bioavailability and tissue distribution of curcuminoids and mitochondrial function in 3- and 18-months old mice fed a control diet or identical diets fortified with 500 or 2000 mg curcuminoids/kg for 3 weeks. Therefore, we measured curcuminoid concentrations in plasma, liver, kidney, and brain, basal and stress-induced levels of adenosine triphosphate (ATP) and mitochondrial membrane potential (MMP) in dissociated brain cells and citrate synthase activity of isolated mitochondria.
Results: Plasma but not liver and kidney curcuminoid concentrations were significantly higher in older mice. Age did not affect ATP concentrations and MMP in dissociated brain cells. After damaging cells with nitrosative stress, dissociated brain cells from old mice had a higher MMP than cells from young animals and were therefore more resistant. Furthermore, this effect was enhanced by curcumin.
Conclusion: Our data suggest that age may affect plasma concentrations, but not the tissue distribution of curcuminoids in mice, but has little impact on mitochondrial function in brain cells.
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1. Introduction

Curcuminoids are lipophilic polyphenols derived from the rhizome of the plant *Curcuma longa* and responsible for the yellow color of turmeric. Commercially available curcumin powders contain 75–85% curcumin, 15–20% demethoxycurcumin (DMC) and 2–8% bis-demethoxycurcumin (BDMC) [1]. Curcuminoids are used as a spice and food additive (E100) [2] and have long been used in Ayurvedic medicine for the treatment of respiratory and liver disorders, anorexia, rheumatism, diabetic wounds, runny nose, cough and sinusitis [7]. Curcumin, the major curcuminoid, may be effective in the prevention [4] and treatment [13] of neurodegenerative disorders, of which mitochondrial dysfunction is a prominent feature. Curcuminoids improve mitochondrial function and increase mitochondrial membrane potential (MMP) and adenosine triphosphate (ATP) concentrations in the brain of mice [6].

In particular, older people may thus benefit from increasing their curcuminoid intake. The oral bioavailability of curcuminoids is low [22] and the small fraction of absorbed curcuminoids is quickly metabolized by phase I and II enzymes and rapidly excreted from the organism [19,24]. During phase I metabolism, curcuminoids are reduced to dihydro-, tetrahydro-, hexahydro-, and octahydrocurcminoids and during phase II metabolism the reduced metabolites are conjugated with glucuronic acid and sulphate in the liver and intestine [10,11]. Physiological changes during aging, such as decreases in i) the absorption surface in the gastro-intestinal tract, ii) hepatic blood flow and mass, iii) renal blood flow and glomerular filtration rate, and iv) increases in total body fat, as well as v) changes in the activity of metabolic enzymes and transporters may affect the bioavailability of curcuminoids. It is currently not known, however, if the absorption and tissue concentrations of curcuminoids and consequently their biological activities are affected by age.

We compared young (3-months) and old (18-months) mice fed curcuminoids for three weeks to investigate if age impacts on curcuminoid concentrations in tissues (plasma, liver, kidney, and brain) and alters mitochondrial function in brain cells.

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2. Materials and methods

2.1. Animals and treatment

Thirty 3-months and thirty 18-months old male C57BL/6 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and divided into six groups of ten mice. Young (Y) and old (O) mice were fed either a pelleted control diet (C1000, Altromin, Lage, Germany; control groups: YC and OC, respectively) or the control diet supplemented with 500 mg curcumin (Y500 and O500, respectively) or 2000 mg curcumin per kg diet (Y2000 and O2000, respectively) for 3 weeks. The curcuminoid extract used in the formulation of the diets was from Jupiter Leys (Cochin, Kerala State, India) and contained 82% curcumin, 16% demethoxycurcumin (DMC), and 2% bis-demethoxycurcumin (BDMC). The mice had free access to feed and water throughout the experiment and were housed in groups of 5 animals per cage in a conditioned room (temperature, 23 °C; relative humidity 55%; 12 h light/dark cycle). All experiments were carried out by individuals with appropriate training and experience according to the requirements of the Federation of European Laboratory Animal Science Associations and the European Communities Council Directive (Directive 2010/63/EU). Body weights were measured weekly and at the end of the trial. The mice were sacrificed by cervical dislocation and decapitation. Blood samples were centrifuged in heparinized vials (1308 ×*g*, 5 min, 4 °C) and plasma samples were stored at −80 °C. Liver and kidney were rinsed with sodium phosphate buffer, snap-frozen in liquid nitrogen and stored at −80 °C. The brain was quickly processed on ice after displacement of the cerebellum and brain stem.

2.2. Quantification of total curcuminoids

Sodium acetate buffer (0.1 M, pH 4–4.5) containing 1.6% EDTA (48.8 μM) and 2.5% ascorbic acid (25 μM) were added to 100 μL plasma. Tissue samples (~300 mg liver or kidney in 400 μL buffer; ~100 mg cerebellum in 200 μL buffer) were homogenized (Miccra D-8 homogenizer, ART Prozess- und Labortechnik GmbH & Co. KG, Müllheim, Germany) in the same buffer. Plasma and tissue samples were incubated with 1000 U β-glucuronidase (from Helix pomatia, Sigma, St. Louis, USA) dissolved in 0.1 M sodium acetate buffer (pH 4–4.5) for 45 min at 37 °C under agitation. After incubation, 1 mL extraction solvent (95% ethyl acetate, 5% methanol, Carl Roth GmbH + Co.KG, Karlsruhe, Germany) was added and vortex -mixed for 30 s. Subsequently, samples were centrifuged (10,500 ×*g*, 5 min, 4 °C) and supernatants collected. This step was repeated twice. The organic layer was evaporated to dryness using an RVC 2–25 CDplus centrifugal evaporator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

Samples were re-suspended in 150 μL methanol, vortex-mixed for 30 s, left in the dark for 10 min, and vortex-mixed again for 20 s, and then transferred to an injection vial. Curcuminoids were quantified on a Jasco HPLC system (Jasco GmbH, Gross-Umstadt, Germany) with a fluorescence detector (excitation wavelength 426 nm, emission wavelength 536 nm) and separated on a Reprosil-Pur C18-AQ column (150 mm × 4 mm, 3 μm particle size; Dr. Maisch GmbH, Ammerbuch, Germany) maintained at 40 °C. The mobile phase consisted of 52% deionized water (adjusted to pH 3 with perchloric acid), 34% acetonitrile and 14% methanol and was delivered at a flow rate of 1.4 mL/min. Curcuminoids were quantified against external standard curves (curcumin, purity ≥97.2%, CAS # 458-37-7; demethoxycurcumin (DMC), purity ≥98.3%, CAS # 22608-11-13; bis-demethoxycurcumin (BDMC), purity ≥99.4%, CAS # 24939-16-0; Chromadex, Irvine, USA).

2.3. Preparation of dissociated brain cells

Dissociated brain cells (DBC) were prepared as previously described [8,9]. After preparation, DBC were diluted in Dulbecco's Modified Eagle Medium (DMEM) without supplements and seeded in 24-well plates (250 μL for measurement of mitochondrial membrane potential) or 96 well-plates (50 μL for measurement of adenosine triphosphate (ATP)) and cultured in a humidified incubator at 37 °C under 5% CO_2. To induce nitrosative damage, DBC were incubated for 3 h with sodium nitroprusside (SNP; 0.1 mmol/L for ATP measurements; 2 mmol/L for mitochondrial membrane potential measurements) in DMEM (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). The degree of damage caused by SNP was calculated by normalization of the MMP and ATP values after SNP incubation to the corresponding basal values.

2.4. Determination of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured in cultivated DBC using the fluorescent dye Rhodamin 123 (R123) [6]. DBC were incubated in the dark for 15 min (37 °C, 5% CO_2) with 0.4 μmol/L R123 and then centrifuged (3000 rpm, 5 min) and washed with Hank's Balanced Salt Solution (HBSS) buffer (supplemented with Mg^{2+}, Ca^{2+}, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); pH 7.4; 37 °C). After supplementing DBC with new HBSS, MMP was assessed by reading the R123 fluorescence at an excitation wave- length of 490 nm and an emission wavelength of 535 nm (Victor X3 2030 multilabel counter, Perkin Elmer, Rodgau-Jügesheim, Germany). Fluorescence values were normalized to cellular protein concentrations.

2.5. Quantification of adenosine triphosphate concentrations

The ViaLight® Plus bioluminescence kit (Lonza, Walkersville, USA) was used for the quantification of ATP in DBC. Briefly, DBC were incubated for 10 min with lysis buffer and at least for 5 min with monitoring reagent. The emitted light (bioluminescence), which is linearly related to ATP concentrations, was recorded using a luminometer (Victor X3). ATP concentrations were quantified with a standard curve and normalized to cellular protein content.

2.6. Isolation of brain mitochondria

Brain (1/4) from the frontal region was homogenized in 2 mL of mitochondrial respiration medium (MiR05, containing EGTA (0.5 mmol/L), magnesium dichloride (3 mmol/L), lactobionic acid (60 mmol/L), taurine (20 mmol/L), potassium dihydrogenphosphate (10 mmol/L), HEPES (20 mmol/L), sucrose (110 mmol/L) and essentially fatty acid-free bovine serum albumin (1 g/L)). Additionally, a protease inhibitor (Roche, Mannheim, Germany) was added to the medium. Samples were homogenized and centrifuged (1400 ×*g*, 7 min, 4 °C). The supernatant was collected and centrifuged again for washing (1400 ×*g*, 3 min, 4 °C). Afterwards, the supernatant was centrifuged to collect mitochondria in the pellet (10,000 ×*g*, 5 min, 4 °C). After resuspension, mitochondria were centrifuged for washing (1400 ×*g*, 3 min, 4 °C) and once again (10,000 ×*g*, 5 min, 4 °C) to collect the mitochondria in the pellet.

2.7. Citrate synthase activity

An aliquot of the isolated mitochondria was immediately frozen in liquid nitrogen for citrate synthase activity measurement. A reaction medium containing 0.1 mmol/L 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 0.5 mmol/L oxaloacetate, 50 μmol/L EDTA, 0.31 mmol/L acetyl coenzyme A, 5 mmol/L triethanolamine hydrochloride, 0.25% Triton X-100 and 0.1 mol/L Tris–HCl was mixed and incubated for 5 min at 30 °C. Then, 10 μL of mitochondria was added to the reaction medium and citrate synthase activity was determined spectrophotometrically at 412 nm.

2.8. Protein quantification

Protein contents were analyzed by the Lowry method using the BioRad DC Protein Assay (Bio-Rad, Munich, Germany). Bovine serum albumin was used as standard.

2.9. Statistics

Two-factorial ANOVA (factors were diet and age) was used to test for effects of the diet and age as well as diet × age interactions. Where an overall effect of the diet or age or a diet × age interaction was observed, Bonferroni post-hoc tests were carried out to test for significant differences between groups. To compare the weight of the mice, two-way repeated-measures ANOVA was used. Data analyses were performed using SPSS (IBM Corp., IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA) and GraphPad Prism version 6.01 (GraphPad Software, San Diego California, USA). Reported values are arithmetic means with their standard error of the mean (SEM).

3. Results

3.1. Body weight development

Body weights were measured weekly (Fig. 1). Old mice were significantly heavier than young mice ($P < 0.000$) at all times. No significant differences between control and curcumin-fed animals were observed within each of the two age groups, with the exception of groups OC and O500 at week 0 ($P < 0.002$). Diet (curcuminoids) did not influence the weight of the mice.

3.2. Curcuminoid tissue concentrations

Total curcuminoid concentrations (the sum of free and conjugated curcuminoids) were measured in the cerebellum, plasma, liver and kidney of mice. Curcuminoids were neither detected in the brain (cerebellum) of any of the groups, nor in any of the tissues in control animals. Significant effects for age and diet were detected for all three curcuminoids in plasma (Fig. 2A). Total plasma curcumin and DMC concentrations were significantly higher in old compared to young animals on the same diets (Fig. 2A). BDMC concentrations were also higher in plasma of old mice after the administration of 2000 mg curcuminoids per kg diet compared to the corresponding young mice ($P = 0.000$, Fig 2A) and concentrations were significantly higher in the O2000 group compared to the O500 group ($P = 0.001$). For plasma BDMC concentrations, an age × diet interaction ($P = 0.008$) was observed. Such an interaction was not seen for curcumin or DMC. In comparison to the corresponding control group (OC), curcumin (P(O500 vs. OC) = 0.038, P(O2000 vs. OC) = 0.003) and DMC (P(O500 vs. OC) = 0.039, P(O2000 vs. OC) = 0.001) concentrations were significantly higher in the curcumin-fed old mice but not in young mice and BDMC differed between O2000 and OC ($P = 0.000$).

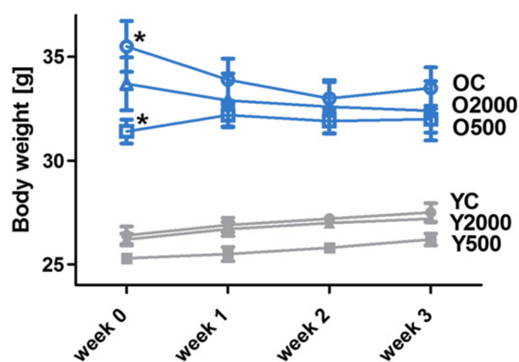

Fig. 1. Mean (±SEM) body weights of young (3 months) and old (18 months) male mice fed a control diet (YC and OC, respectively) or identical diets supplemented with curcuminoids at doses of 500 mg (Y500 and O500, respectively) or 2000 mg (Y2000 and O2000, respectively) per kg diet for three weeks. Old mice were significantly heavier than young mice at each time point ($P < 0.000$; two-way repeated-measures ANOVA with Bonferroni post-hoc tests). *Groups OC and O500 differed significantly in body weight at week 0 ($P < 0.002$), but no other differences in body weight were observed between curcumin-fed animals and their respective age-matched control groups.

In the liver, only diet influenced curcuminoid concentrations and interactions between age and diet were not observed. Liver curcumin and DMC concentrations in the Y2000 and O2000 groups were significantly higher compared to the Y500 (curcumin: $P = 0.007$, DMC: $P = 0.000$) and O500 groups (curcumin: $P = 0.006$, DMC: $P = 0.004$), respectively. DMC was undetectable after the consumption of 500 mg curcumin per kg diet. BDMC contents were significantly higher in O2000 compared to O500 ($P = 0.001$, Fig. 2B). Young and old mice consuming 2000 mg curcuminoids per kg diet had significantly higher curcuminoid concentrations compared to the corresponding control groups (YC and OC, respectively, $P < 0.05$).

In the kidney, diet did, but age did not influence curcuminoid concentrations. No age × diet interactions were detected. Kidney curcumin and BDMC contents were elevated in Y2000 and O2000 in comparison to Y500 and O500, respectively (Fig. 2C). DMC concentrations could only be measured after the administration of the higher curcumin dose and O2000 had significantly higher concentrations than O500 ($P = 0.017$, Fig 2C). Curcumin and BDMC were higher in the Y2000 and O2000 groups, and DMC only in O2000 compared to the young and old control groups ($P < 0.05$).

3.3. Mitochondrial membrane potential in dissociated brain cells

The MMP serves as an indicator of the bioenergetic state of mitochondria [18]. For basal MMP we could not detect any age or diet effects or an interaction between age and diet (Fig. 3A). After damaging dissociated brain cells with SNP, age ($P = 0.000$) and diet ($P = 0.003$) effects, but no age × diet interactions were observed. The MMP was significantly elevated in the old mice compared to the corresponding young group (Fig. 3B). Within the old group, the higher dose of curcumin lead to significantly higher MMP compared to the control ($P = 0.016$).

3.4. ATP concentrations in dissociated brain cells

Age, but not diet, significantly affected ($P = 0.026$) basal ATP concentrations in dissociated brain cells. No age × diet interactions were found. Basal ATP concentrations were significantly higher in Y2000 compared to O2000 ($P = 0.04$, Fig. 3C), but did not differ between any of the remaining groups. ATP concentrations in SNP-challenged cells were similar in all groups (Fig. 3D) and no effects of age, diet or age × diet interactions were observed.

3.5. Citrate synthase activity

Citrate synthase activity is a common quantitative marker for the content of intact mitochondria [12]. Age affected citrate synthase activity ($P = 0.045$), whereas neither diet effects nor age × diet interactions were evident (Fig. 3E). Old mice fed 500 mg curcuminoids per kg diet (O500) had a significantly higher citrate synthase activity compared to the corresponding young animals (Y500; $P = 0.045$, Fig. 3E).

4. Discussion

We measured curcuminoid concentrations in cerebellum, plasma, liver and kidney of 3-months young and 18 months-old mice fed 500 or 2000 mg curcumin per kg diet for three weeks to investigate if the tissue concentrations of these compounds are affected by age. Plasma but not liver and kidney concentrations of curcumin were higher in the old than in the young mice (Fig. 2). This is

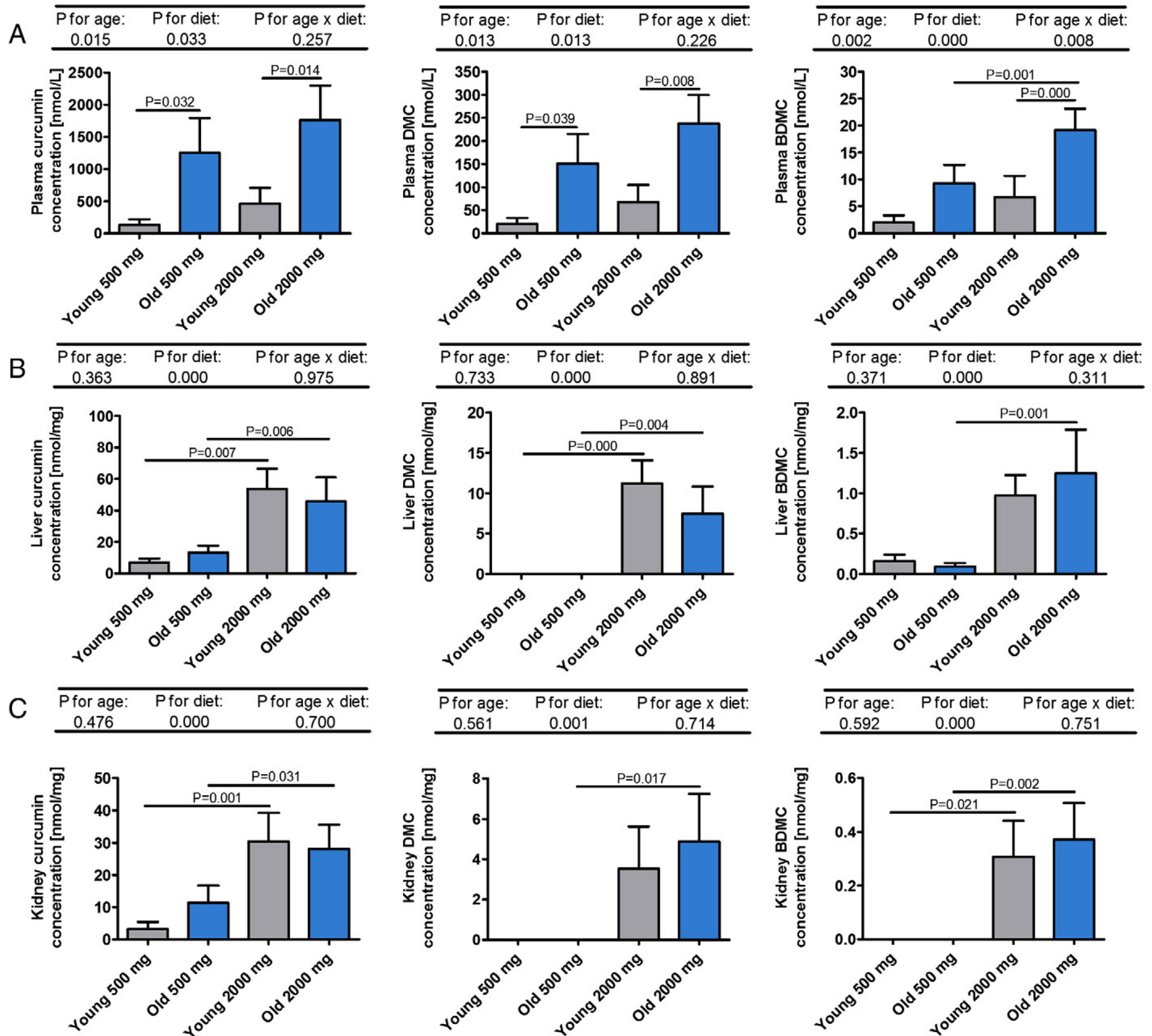

Fig. 2. Mean (± SEM) concentrations of total curcumin, demethoxycurcumin (DMC) and bis-demethoxycurcumin (BDMC) in plasma [A], liver [B] and kidney [C] of 3-months young and 18-months old male mice fed a control diet (YC and OC, respectively) or identical diets supplemented with curcuminoids at doses of 500 mg (Y500 and O500, respectively) or 2000 mg (Y2000 and O2000, respectively) per kg diet for three weeks. Curcuminoids were not detected in the control groups (YC and OC). Two-way ANOVA with Bonferroni post-hoc tests were calculated to detect significant differences. P-values for the effects of age, diet and age × diet interactions are reported above each graph. The P-values of Bonferroni post-hoc tests indicate significant differences between groups.

particularly remarkable since the old animals were heavier than the young mice (Fig. 1) and thus ingested smaller doses per kg bodyweight than their younger counterparts. One possible explanation for these age-differences in curcumin plasma concentrations could be that old mice may have decreased UDP-glucuronosyl transferase activities and thus a reduced metabolism and excretion [25]. The higher bodyweight of the older animals is also associated with changes in body composition, such as increased total body fat and reduced total body water, which may alter the apparent volume of distribution [14] and thus pharmacokinetics of curcuminoids. Our observation that liver and kidney tissue concentrations of the curcuminoids did not differ significantly between young and old mice (Fig. 2) is in line with this notion and suggests that the curcuminoids may have been distributed differently to peripheral tissues in young and old animals.

We did not detect any curcuminoids in the cerebellum of our animals, although curcuminoids, due to their low molecular weight and lipophilicity, are generally able to cross the blood–brain-barrier [3]. These findings are in agreement with our earlier observations that orally ingested curcumin, probably because of limited absorption and rapid conjugation by phase II enzymes, does not lead to measurable concentrations of curcumin in the brain of mice [21]. Circumventing the gastro-intestinal tract and thereby first-pass metabolism in the small intestine and liver by intraperitoneal injection of curcumin (100 mg/kg bodyweight) results in quantifiable concentrations of curcumin in the brain of mice [21]. However, even though curcuminoids were not detectable in the cerebellum of our mice, their intake resulted in measurable differences in the mitochondrial function in dissociated brain cells (Fig. 3).

Dissociated brain cells of old mice had significantly higher MMP than those isolated from young mice upon stimulation with the stressor SNP,

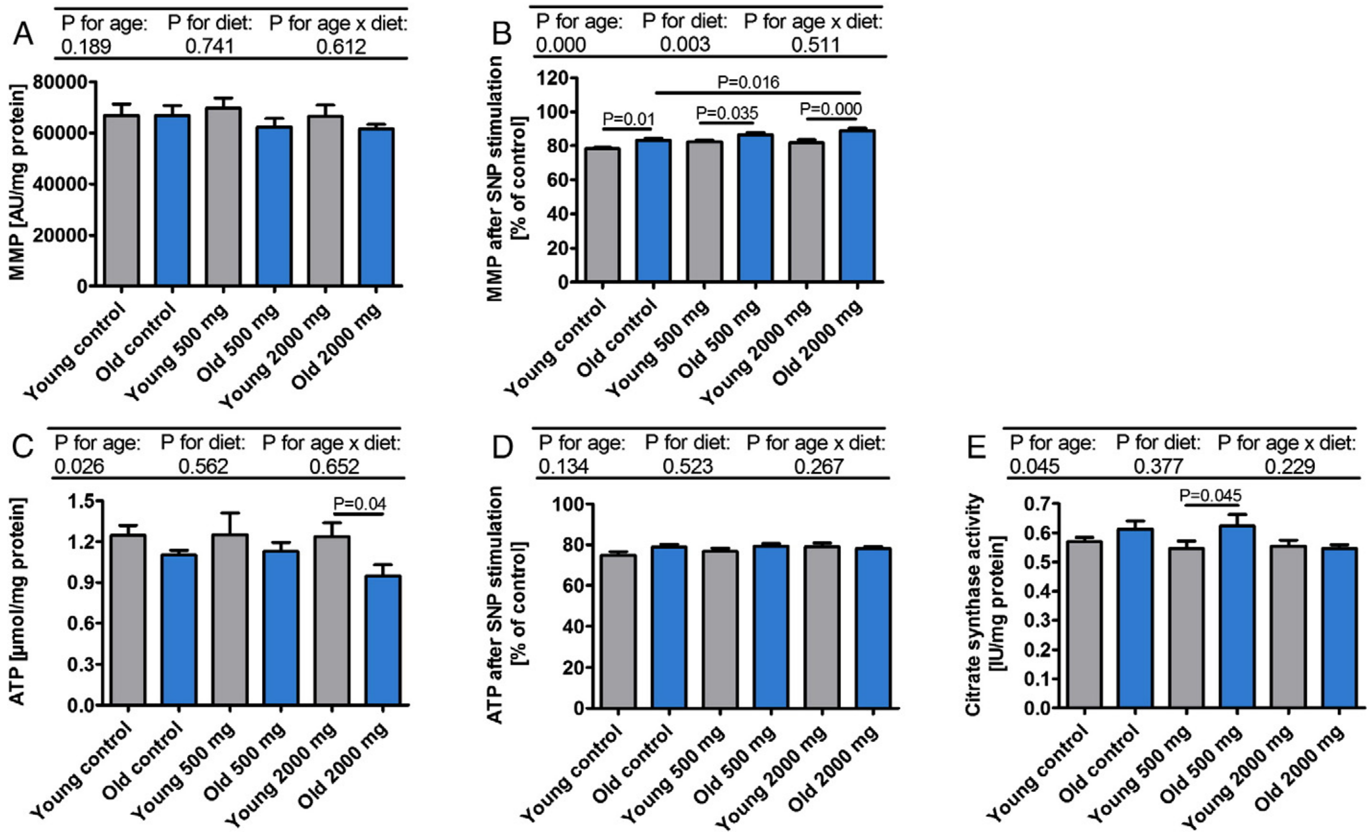

Fig. 3. Basal MMP [A] and ATP [C], and MMP [B] and ATP [D] after treatment with SNP measured in dissociated brain cells and mean (±SEM) citrate synthase activity [E] measured in isolated brain mitochondria of male young (3 months) and old (18 months) mice fed a control diet (YC and OC, respectively) or identical diets supplemented with curcuminoids at doses of 500 mg (Y500 and O500, respectively) or 2000 mg (Y2000 and O2000, respectively) per kg diet for three weeks. Values are means with their standard errors. Two-way ANOVA with Bonferroni post-hoc tests were calculated to detect significant differences. P-values for the effects of age, diet and age × diet interactions are reported above each graph. The P-values of Bonferroni post-hoc tests indicate significant differences between groups.

which inhibits cellular respiration and causes a decrease in ATP production [26], and curcuminoid intake reinforced this effect (Fig. 3B). The MMP of un-stimulated cells were similar between young and old as well as between control-fed and curcuminoid-fed mice (Fig. 3A). These observations suggest that old mitochondria are less susceptible to stress-induced damage than young ones. In agreement, mitochondria from senescent human umbilical vein endothelial cells were better protected against oxidative stress than young cells; photodamage-induced formation of reactive oxygen species led to loss of MMP in young cells, whereas senescent cells were resistant [17]. Furthermore, activities of antioxidant enzymes in brains of adult and aged mice are higher than in young mice, which might result in a better protection of mitochondria from reactive species [16].

The mitochondrial–lysosomal axis theory of aging suggests that the number of defective mitochondria within long-lived postmitotic cells increases throughout the aging process and is accompanied by mitochondrial enlargement and decreased ATP production [23]. In accordance with this, we observed a trend toward higher basal ATP concentrations in the young animals fed control diets or 500 mg curcuminoids per kg diet compared to their respective old counterparts, and significantly higher ATP in the Y2000 compared to the O2000 group ($P < 0.05$, Fig. 3C). Curcuminoid-feeding, however, did not affect ATP concentrations. We previously observed a similar trend toward increased MMP and ATP in mice of the fast-aging strain senescence-accelerated mouse-prone 8 (SAMP8) fed with 500 mg curcuminoids per kg diet for five months [6]. The supplementation of transgenic APOE3 and APOE4 mice with 2000 mg curcuminoids/kg diet for three months increased ATP concentrations numerically in APOE3 and significantly in APOE4 mice in comparison with their respective control-fed animals [5]. In both studies, the expression of the master regulator of mitochondrial biogenesis peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) was higher in the curcumin-fed than in the respective control animals [5,6], suggesting that curcumin may improve mitochondrial function by increasing mitochondrial content. In our present study, citrate synthase activity, a quantitative marker for the amount of intact mitochondria, differed in young and old animals fed 500 mg curcuminoids per kg diet, but not in those fed 2000 mg/kg diet (Fig. 3E) [15]. This is in contrast to a previously reported study feeding Thy1-APPSL transgenic mice, a mouse model for Alzheimer's disease, for three weeks with 500 mg curcumin/kg diet of a highly bioavailable micellar formulation [22] resulting in significantly increased MMP and numerically increased ATP levels, but unchanged citrate synthase activity compared to the control [20]. This discrepancy in the activity of curcuminoids on mitogenesis might be due to the use of different curcuminoid formulations (highly bioavailable curcuminoid micelles vs. native curcumin powder) and animal models (genetically modified mice (Alzheimer's disease model) vs. young and old wildtype mice (aging model)).

5. Conclusions

To the best of our knowledge, our findings of higher curcuminoid plasma concentrations in 18- compared to 3-months-old mice fed identical doses of curcuminoids are the first evidence that age may be an important determinant of curcumin plasma concentrations. Further studies are warranted to better understand potential age-differences in the absorption, metabolism, distribution, and elimination of curcuminoids. Dissociated brain cells from curcumin-fed animals were somewhat more resistant to stress-induced decreases in mitochondrial function, but overall, only minor differences in mitochondrial function were observed between young and old mice.

Conflicts of interest

None.

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