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Calorie restriction influences key metabolic enzyme activities and markers of oxidative damage in distinct mouse liver mitochondrial sub-populations

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ABSTRACT

Aims: The purpose of the study was to establish if enzyme activities from key metabolic pathways and levels of markers of oxidative damage to proteins and lipids differed between distinct liver mitochondrial sub-populations, and which specific sub-populations contributed to these differences.

Main methods: Male C57BL/6J mice were fed non-purified diet for one month then separated into two groups, control and calorie-restricted (CR). The two groups were fed semi-purified diet (AIN93G), with the CR group receiving 40% less calories than controls. After two months, enzyme activities and markers of oxidative damage in mitochondria were determined.

Key findings: In all mitochondrial sub-populations, enzyme activities and markers of oxidative damage, from control and CR groups, showed a pattern of M1 > M3 > M10. Higher acyl-CoA dehydrogenase (β-oxidation) and β-hydroxybutyrate dehydrogenase (ketogenesis) activities and lower carbonyl and TBARS levels were observed in M1 and M3 fractions from CR mice. ETC enzyme activities did not show a consistent pattern. In the Krebs cycle, citrate synthase and aconitase activities decreased while succinate dehydrogenase and malate dehydrogenase activities increased in the M1 mitochondria from the CR versus control mice.

Significance: CR does not produce uniform changes in enzyme activities or markers of oxidative damage in mitochondrial sub-populations, with changes occurring primarily in the heavy mitochondrial populations. Centrifugation at 10,000 *g* to isolate mitochondria likely dilutes the mitochondrial populations which show the greatest response to CR. Use of lower centrifugal force (3000 *g* or lower) may be beneficial for some studies.

Introduction

Chronic calorie restriction (CR) has been reported to induce shifts in metabolism towards increased capacity for gluconeogenesis (Dhahbi et al., 2001, 1999; Hagopian et al., 2003a) and fatty acid oxidation (Bruss et al., 2010), and decreased capacity for glycolysis (Dhahbi et al., 1999; Hagopian et al., 2003b). Alterations in liver mitochondria have been proposed to play an important role in the metabolic adaptations to CR (Anderson and Weindruch, 2010). In particular, CR-induced mitochondrial changes could influence capacity for β-oxidation, ketogenesis and utilization of proteins for gluconeogenesis. However, it is not known if CR induces uniform changes in all liver mitochondria or if particular sub-populations are primarily responsible for the metabolic changes observed with CR.

Mitochondria are composed of heterogeneous populations that can be separated by differential centrifugation into distinct sub-populations. Previous studies have separated rat (Lanni et al., 1996) and mouse (Hagopian et al., 2011) liver mitochondria into three distinct fractions, with the heaviest showing the highest respiration rates. An association between mitochondrial biogenesis and mitochondrial fractions has been proposed, indicating the presence of a growth cycle where lighter mitochondria are considered to be the precursors of the heavy mitochondria (Gianotti et al., 1998; Justo et al., 2005; Koekemoer and Oelofsen, 2001; Lombardi et al., 2000). Previous results from brown adipose tissue (BAT) studies support this idea, showing that acute cold exposure or fasting influences the lighter mitochondria primarily (Gianotti et al., 1998; Moreno et al., 1994) whereas overfeeding, chronic fasting or cold acclimation also affects the heavy mitochondria (Gianotti et al., 1998; Matamala et al., 1996; Moreno et al., 1994).

Previous studies have indicated that biochemical differences exist between the heavy and light mitochondria. For example, higher oxygen consumption rates, ROS generation and lower antioxidant capacity have been observed in liver heavy mitochondria compared to lighter ones (Venditti et al., 2002, 1996). Additionally, several enzymes from key metabolic pathways have been reported to display increased activities in heavy compared to light mitochondria from both liver and white adipose tissue (Koekemoer and Oelofsen, 2001). Specifically, cytochrome

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c oxidase activity has been reported to be increased in heavy versus light mitochondria from liver (Koekemoer and Oelofsen, 2001; Lanni et al., 1996), white adipose tissue (Koekemoer and Oelofsen, 2001) and brown adipose tissue (Moreno et al., 1994). In addition, we reported recently (Hagopian et al., 2011) that H_2O_2 was generated by M1 and M3 fractions, with M1 showing higher rates than M3.

In the current study, our aim was to establish if enzyme activities from several intermediary metabolism pathways, levels of markers of oxidative damage to proteins and lipids, and the activities of the ETC enzymes differed between distinct liver mitochondrial sub-populations. Furthermore, a goal of the study was also to determine which specific mitochondrial sub-populations contributed to changes in enzyme activities and oxidative stress with CR.

Materials and methods

Animals and diets

Male mice of the C57BL/6J strain were purchased from Jackson Laboratories (West Sacramento, CA) at three months of age and housed singly. Animal maintenance and care protocols were approved by UC Davis Institutional Animal Care and Use Committee and were in accordance with all local and federal guidelines. Mice were kept at 22 °C on a 12 h dark 12 h light cycle with lights on from 0600 to 1800 h, with free access to water. All mice were fed a 7012 Teklad non-purified diet (Harlan Laboratories, Madison, WI), ad libitum for one month until they were four months old. At four months of age, the mice were assigned to either a control or CR group and fed a semi-purified diet (AIN93G) with the CR group being fed 40% less calories than the control group. All mice were on their respective diets for two months, which made them six months old when sacrificed for the experiments.

Chemicals

All chemicals were purchased from Sigma Chemical Company (St Louis, MO). Bio-Rad protein assay kit was from Bio-Rad laboratories (Hercules, CA) and Pierce BCA protein assay kit was from Thermo Scientific (Waltham, MA).

Liver mitochondrial isolation

Mice were sacrificed by cervical dislocation, body weights recorded and livers were removed immediately, weighed and processed for mitochondrial isolation. Livers were placed in ice-cold isolation medium (220 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, 1 mM EDTA and 0.1% (w/v) fatty acid-free BSA, pH 7.4 at 4 °C) and minced with scissors into small pieces. The buffer was aspirated and fresh buffer added to rinse the liver pieces and help in the removal of blood. This step was repeated three times and the final liver suspension was homogenized, using a cold glass/Teflon homogenizer using isolation medium (at 1:10 w/v ratio), with 4–6 strokes of the Teflon pestle at 500 rpm. The homogenizer was kept in an ice-packed beaker during the homogenization process. The homogenate was centrifuged at 500 g for 10 min at 4 °C and the resulting supernatant was kept for further analysis while the pellet was discarded. The crude supernatant from above was centrifuged at 1000 g for 10 min at 4 °C and the resulting supernatant (S1) was retained while the pellet was washed twice in suspension medium (same as the isolation medium but without BSA) and re-suspended in a minimal volume of suspension medium, labeled M1 and kept on ice. The supernatant S1 was centrifuged at 3000 g for 10 min at 4 °C and the resulting supernatant S3 was retained while the pellet was washed twice in suspension medium and re-suspended in a minimal volume of suspension medium, labeled M3 and kept on ice. The supernatant S3 was centrifuged at 10,000 g for 10 min at 4 °C and the resulting pellet was washed twice in suspension medium and re-suspended in a minimal volume of suspension medium, labeled M10 and kept on ice. The final supernatant from this step was used for assays of lactate dehydrogenase and citrate synthase as marker enzymes.

Electron transport chain (ETC) enzyme assays

The activities of ETC enzymes (Complexes I–IV) were determined in all three mitochondrial fractions. All assays were performed at 30 °C using 25 mM potassium phosphate assay buffer, pH 7.2, in a final volume of 1 ml, using a PerkinElmer Lambda 25 UV/VIS spectrophotometer equipped with a Peltier heating system, as described before (Hagopian et al., 2010). Briefly, Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) activity was measured at 340 nm ($\varepsilon = 6.22\ \text{mM}^{-1}.\text{cm}^{-1}$) in the assay buffer containing (final concentrations) 5 mM $MgCl_2$, 0.13 mM NADH, 65 μM ubiquinone-1, 2.5 mg/ml BSA and 5 μg/ml antimycin A. Activity was measured for 1–2 min after which rotenone (5 μg/ml) was added and activity measured for a further 2 min, allowing the determination of rotenone-sensitive activity. Complex II (succinate: ubiquinone oxidoreductase, EC 1.3.5.1) activity was measured at 600 nm ($\varepsilon = 19.1\ \text{mM}^{-1}.\text{cm}^{-1}$) by initially incubating the sample in the assay buffer containing 20 mM succinate for 10 min at assay temperature, followed by the addition of (final concentrations) 5 mM $MgCl_2$, 2 mM KCN, 5 μg/ml antimycin A, 5 μg/ml rotenone, 50 μM dichlorophenolindophenol and 65 μM ubiquinone-1 to start the assay. Complex III (ubiquinol:ferricytochrome c oxidoreductase, EC 1.10.2.2) activity was measured at 550 nm ($\varepsilon = 19.1\ \text{mM}^{-1}.\text{cm}^{-1}$) in the assay buffer containing (final concentrations) 5 mM $MgCl_2$, 2 mM KCN, 5 μg/ml rotenone, 2.5 mg/ml BSA, 50 μM oxidized cytochrome c and mitochondrial sample. Assays were started by adding 60 μM decylubiquinol, in the presence (5 μg/ml) or absence of antimycin A to distinguish the reduction of ferricytochrome c by decylubiquinol from the non-enzymic reduction. Decylubiquinol was prepared as previously described (Trounce et al., 1996). Complex IV (cytochrome c oxidase, EC 1.9.3.1) activity was determined at 550 nm ($\varepsilon = 19.1\ \text{mM}^{-1}.\text{cm}^{-1}$) in the assay buffer containing 15 μM reduced cytochrome c and mitochondrial sample, for 2 min. Reduced cytochrome c was prepared as previously described (Trounce et al., 1996). The activities of the ETC enzymes were expressed as μmol/min/mg protein and presented as mean ± SEM ($n = 6$).

Metabolic enzyme activities

Mitochondrial enzymes from several metabolic pathways were also measured in the three fractions. From the Krebs cycle, the activities of citrate synthase (CS) (EC 2.3.3.1), aconitase (ACO) (EC 4.2.1.3) and malate dehydrogenase (MDH) (EC 1.1.1.37) were measured at 412 nm ($\varepsilon = 13.6\ \text{mM}^{-1}.\text{cm}^{-1}$), 240 nm ($\varepsilon = 3.6\ \text{mM}^{-1}.\text{cm}^{-1}$) and 340 nm ($\varepsilon = 6.22\ \text{mM}^{-1}.\text{cm}^{-1}$), respectively, as previously described (Hagopian et al., 2004). Succinate dehydrogenase (SDH; Complex II of the ETC) was also measured, as described in the previous section. Acyl-CoA dehydrogenase (ACDH) (β-oxidation pathway, EC 1.3.99.13) was assayed at 600 nm ($\varepsilon = 21\ \text{mM}^{-1}.\text{cm}^{-1}$), using palmitoyl-Co-A as substrate and β-hydroxybutyrate dehydrogenase (HBDH) (ketogenesis pathway, EC 1.1.1.3) was measured at 340 nm ($\varepsilon = 6.22\ \text{mM}^{-1}.\text{cm}^{-1}$), as previously described (Hagopian et al., 2012). Also measured was lactate dehydrogenase (LDH) (EC 1.1.1.27), at 340 nm ($\varepsilon = 6.22\ \text{mM}^{-1}.\text{cm}^{-1}$), as described before (Hagopian et al., 2011). CS and LDH were used as marker enzymes to confirm mitochondrial purity.

Mitochondrial protein carbonyl measurements

Mitochondrial protein carbonyls were measured in all three fractions. Protein carbonyls were determined by using 2,4-dinitropheylhydrazine (DNPH) (Levine et al., 1990). Briefly, mitochondrial fractions were treated with streptomycin sulfate (1% final concentration), incubated at room