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Dietary L-methionine restriction decreases oxidative stress in porcine liver mitochondria

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ABSTRACT

Dietary methionine restriction (MetR) has been reported to improve hepatocyte function in mammals. However, the underlying mechanisms remain largely unknown. This study was conducted with a swine model to test the hypothesis that MetR decreases generation of reactive oxygen species (ROS) and attenuates oxidative damage in hepatic mitochondria. Twenty-four 35-day old pigs were fed a control diet or a Met-restricted diet for two weeks. Liver mitochondria were isolated to determine: 8-oxodG in mitochondrial DNA, oxidative-derived proteins markers, including glutamic semialdehyde (GSA), aminoadipic semialdehydes (AASA), carboxyethyl-lysine (CEL), carboxymethyl-lysine (CML), and malondialdehyde lysine (MDAL), mitochondrial H_2O_2 generation rate; rates of oxygen consumption; free radical leak (FRL); anti-oxidative capacity, electron transport complex activity; and protein abundances of respiratory chain complex subunits (NDUFA9, SDHA, Core 2, and Cox 1), manganese superoxide dismutase (MnSOD), and apoptosis-inducing factor (AIF). Compared with the control, MetR decreased mitochondrial 8-oxodG content, H_2O_2 generation, FRL ($P < 0.05$), and increased rates of oxygen consumption. Abundances of markers for protein oxidative damage, including GSA, AASA, CEL, and CML, were decreased ($P < 0.05$) by 40%, 30%, 32%, and 28%, respectively, compared with the control. Western blot analysis revealed that MetR decreased ($P < 0.05$) the protein abundances of complex subunits, NDUFA9 and AIF without affecting expression of SDHA, Core 2, Cox 1 or MnSOD. The complex I activity ($P < 0.05$) were lowered in MetR group as compared with that of control. Collectively, our findings indicate that dietary MetR decreases mitochondrial ROS generation primarily via inhibiting complex I activity and ROS generation rather than augmenting anti-oxidative capacity, thereby ameliorating oxidative damage to hepatic mitochondrial DNA and proteins.

1. Introduction

Methionine restriction (MetR) extends life span in diverse organisms, including rodents (Miller et al., 2005; Orentreich et al., 1993; Pamplona and Barja, 2006; Perrone et al., 2013; Sanchez-Roman and Barja, 2013), *Drosophila melanogaster* (Grandison et al., 2009; Kabil et al., 2011; Troen et al., 2007), and yeast (Petti et al., 2011). Besides the longevity-extension effect, lower methionine ingestion has been reported to reduce the incidence, time of onset, and progression of many neurodegenerative disorders (Miller et al., 2005); decrease mitochondrial oxidative damage (Naudi et al., 2007); remodel metabolic program (Perrone et al., 2013); and increase mitochondrial biogenesis (Naudi et al., 2007; Perrone et al., 2010). Biochemically, MetR leads to changes in gene expression, alterations in immunity response, and modifications of endocrine function (Sanchez-Roman and Barja, 2013), which are associated with the development of various metabolic disorders.

Despite a large number of scientific studies during the last decade, the underlying mechanisms through which these beneficial effects act upon MetR remain largely unknown. Accumulating evidence suggests that improved mitochondrial function and decrease in mitochondrial reactive oxygen species (ROS) generation might be critical factors that contribute to the effects observed (Perrone et al., 2012, 2013). Mitochondria are the major intracellular organelles responsible for biological oxidation in mammalian cells. The primary function of mitochondria is to produce cellular adenosine triphosphate (ATP) through electron transport and oxidative phosphorylation in conjunction with the oxidation of metabolites by tricarboxylic acid (TCA) cycle and catabolism of fatty acids by oxidation (Sena and Chandel, 2012). In addition to supplying cellular energy, mitochondria are also the main intracellular site that producing ROS, especially at level of the respiratory chain complexes I and III, during the electron transport process in mammalians (Bratic and Larsson, 2013). Under physiological conditions, ROS produced

Abbreviations: AASA, aminoadipic semialdehyde; AdoMet, S-adenosylmethionine; ADP, adenosine diphosphate; AIF, apoptosis inducing factor; ATP, adenosine triphosphate; CEL, carboxyethyl-lysine; CML, carboxymethyl-lysine; dG, deoxyguanosine; FRL, free radical leak; GSA, glutamic semialdehyde; MDAL, malondialdehyde-lysine; MetR, methionine restriction; mtDNA, mitochondrial DNA; 8-oxodG, 8-oxo-2′-deoxyguanosine; GSH, Glutathione; MnSOD, manganese superoxide dismutase; NADH, nicotinamide adenine dinucleotide; HRP, horseradish peroxidase; ROS, reactive oxygen species; SAA, sulfur-containing amino acids; TCA, tricarboxylic acid.

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during natural metabolic reactions is scavenged by the endogenous antioxidants, and, therefore, maintains intracellular homeostasis. Under pathological conditions, the accumulating ROS reacts with lipids, protein, and DNA, generating oxidative damage and cause harmful effects on health (Sena and Chandel, 2012). Dietary MetR has been reported to decrease the concentrations of mitochondrial complexes, and reduce mitochondrial ROS generation in rodents, suggesting a regulatory effect of methionine on mitochondrial electron transport system (Gomez et al., 2011; Sanchez-Roman and Barja, 2013; Sanchez-Roman et al., 2011; Sanz et al., 2006). Consistently, several genes, including uncoupling proteins, peroxisome proliferator activated receptor γ coactivator 1α and its target genes, have been reported to be regulated by MetR in liver and skeletal muscle of rats (Perrone et al., 2010). It should be noted that most of the experimental studies on MetR were conducted in rodents with a relative long-term period of intervention (more than seven weeks) (Lopez-Torres and Barja, 2008; Perrone et al., 2013; Sanchez-Roman and Barja, 2013). However, little is known about the short-term effect of MetR in animals.

The pig is very similar to the human in anatomy, nutrition, physiology and the immune system, and is an excellent animal model to study various human diseases (Meurens et al., 2012; Wolf et al., 2014). The use of pig as animal model has numerous advantages and contributes to the acquisition of new knowledge to improve both animal and human health (Meurens et al., 2012). We are not aware of studies regarding effects of MetR on liver mitochondrial ROS generation or oxidative damage in pigs. In a recent study, Kalhan et al. demonstrated that dietary isocaloric protein restriction for one week resulted in significant gene expression alteration in the rat (Kalhan et al., 2011). This result suggests that MetR could potentially induce gene expression and result in functional alterations in a couple of days as reported for the protein-restricted rat (Kalhan et al., 2011).

In this study, 35-day old piglets were provided a basal diet (0.24% methionine, serving as methionine restriction diet) or were provided a basal diet supplemented with an extra methionine (0.12%) to meet the nutrient requirement according to the National Research Council (1998), serving as the control diet for 14 days. Liver mitochondria were isolated and the mitochondrial H_2O_2 generation rate; rates of oxygen consumption in both resting and phosphorylating state; free radical leak (FRL); oxidative damage to mitochondrial DNA (mtDNA) and proteins; the protein abundance of mitochondrial complexes subunits, mitochondrial enzyme MnSOD, and AIF; the mitochondrial complex activity were evaluated to test our hypothesis that MetR would affect mitochondrial ROS generation and related oxidative damage in liver mitochondria of piglets.

2. Material and methods

2.1. Animals

The animal handling procedures were approved by the Institutional Animal Care and Use Committee of the China Agricultural University. A total of 24 crossbred health barrows (Duroc × Landrace × Yorkshire), with similar body weight at 28 days of age were weaned to a commercial diet between day 28 and 35 of age. After an adaptation period of 7 days, the piglets were randomly allotted to two groups. Piglets in the control group were provided a corn and soybean meal basal diet containing: 52.91% corn, 20% soybean meal, 10% extruded soybean meal, 12.00% dried whey, 1% soybean oil, 1.56% dicalcium phosphate, 0.50% Limestone, 0.34% NaCl, 0.36% L-lysine, 0.2% L-Threonine, 0.05% L-Tryptophan, 1.00% vitamin–mineral mix. The control and the MetR diet were isocaloric and isonitrogenous (adjusted with L-alanine). The methionine content in the basal diet is 0.24% without providing extra methionine and served as methionine-restricted (MetR) diet. Piglets in the control group were fed the basal diet supplemented with an extra methionine (0.12%, to get a total amount of methionine 0.35%) to meet the nutrient requirement according to the National Research Council (1998), serving as control diet. All Piglets had free access to drinking water and diets. After two weeks of dietary treatment, the animals were sacrificed and liver mitochondria were isolated to measure H_2O_2 generation, rates of oxygen consumption and oxidative damage to mitochondrial DNA and proteins. Tissues were stored at −80 °C for the analysis of protein expression by Western blot analysis.

2.2. Analysis of Met-related metabolites by HPLC

Met and Met-related metabolites in liver tissue were determined by reversed-phase HPLC after derivatization with o-phthaldialdehyde using the method as we described previously (Wu et al., 1997).

2.3. Isolation of mitochondria

Liver mitochondria were isolated as previously described (Duee et al., 1994; Mersmann et al., 1972) with minor modifications. Briefly, fresh livers were rinsed and homogenized in cold isolation buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, and 0.1 mM EDTA (pH 7.4). The nuclei and cell debris were removed by centrifugation at 800 *g* for 15 min at 4 °C. The supernatants were centrifuged at 8000 *g* for 15 min at 4 °C and the resulting pellets were re-suspended in cold isolation buffer without EDTA and centrifuged at 1000 *g* for 10 min at 4 °C. Mitochondria were obtained after centrifugation of the supernatants at 10,000 *g* for 10 min at 4 °C and resuspended in l ml of isolation buffer without EDTA. The mitochondrial protein was measured by the Lowry method according to the protocol provided by the manufacturer. The mitochondrial suspensions were maintained over ice and were immediately used for the measurements of oxygen consumption and H_2O_2 production.

2.4. Measurement of 8-oxodG in mitochondrial DNA

Mitochondrial DNA (mtDNA) was isolated by the methods as previously described (Caro et al., 2008; Sanz et al., 2006). The isolated mtDNA was digested to deoxynucleoside level for assessment of the concentration of 8-oxodG (8-oxo-2′-deoxyguanosine), referred to that of the non-oxidized base (deoxyguanosine, dG). 8-oxodG and dG were analyzed by HPLC with online electrochemical and ultraviolet detection, respectively. 8-oxodG was detected with an ESA CoulochemII electrochemical coulometric detector (ESA, Inc., Bedford, MA), and dG was detected with an ultraviolet detector at 254 nM (Bio-Rad). For quantification, peak areas of dG standards and of three concentration calibration pure 8-oxodG standards were analyzed during each HPLC run.

2.5. Oxidative-derived protein damage markers determination

Protein oxidation markers, including glutamic semialdehyde (GSA) and aminoadipic semialdehydes (AASA); glycoxidation markers, carboxyethyl-lysine (CEL), and carboxymethyl-lysine (CML); lipoxidation markers, malondialdehyde lysine (MDAL), and CML were determined as trifluoroacetic acid methyl esters derivatives in acid hydrolyzed delipidated and reduced mitochondrial protein samples by gas chromatography/mass spectrometry (GC/MS) as previously described (Caro et al., 2008).

2.6. Mitochondrial H_2O_2 production

The rate of mitochondrial H_2O_2 production was determined by the increase in fluorescence intensity (excitation at 312 nm, emission at 420 nm) due to the oxidation of homovanillic acid by H_2O_2 in the presence of horseradish peroxidase (Barja, 2002; Caro et al., 2008). The reaction was stopped by transferring the samples to a cold bath and adding 0.5 ml of stop buffer (2.0 M glycine, 2.2 M NaOH, 50 mM EDTA, pH 12.0) and the fluorescence was read in a fluorometer. Known amounts of H_2O_2 generated in parallel by glucose oxidase with glucose as substrate