Effects of Myostatin Propeptide Transgene on Muscle Mitochondrial Quantity

Excessive fat accumulation in obesity leads to insulin resistance, a clinical condition for type 2 diabetes. Previously, transgenic mice with over-expressed myostatin propeptide showed enhanced muscle with enhanced insulin signaling and decreased fat accumulation. A study reported myostatin-knockout mice with heavy muscling had weak force generation due to a mitochondrial depletion. To investigate the mitochondrial function of skeletal muscle of the myostatin propeptide transgenic mice, we initially developed a real-time PCR method to quantify mitochondrial DNA in the transgenic mice and wild type mice. By quantitative real-time PCR amplification of mitochondria-specific MT-CO1 gene copies and a single-copy nuclear gene named Ndufv1 with SYBR Green dye-based reagents, we compared the relative levels of mitochondria of biceps muscle tissues between transgenic and wild-type mice. The results indicated that there was a not significant difference of mtDNA quantification of myostatin propeptide transgenic mice from the wild-type littermate mice although the mean of the relative mitochondrial DNA levels of transgenic mice is 1.6 times more than the wild-type mice. This research may suggest that biceps muscle of the myostatin propeptide transgenic mice have normal or possibly higher level of mitochondria.

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Introduction

Myostatin is a member of the transforming growth factor-β superfamily that regulates muscle growth in mammals. Precursor protein made by the myostatin gene is digested by a protease into two peptides: C-terminal that is the matured form of myostatin and N-terminal called myostatin propeptide. Previously, we generated transgenic mice through muscle-specific expression of the cDNA sequence (5′-region 886 nucleotides) encoding for the propeptide of myostatin.[1] The myostatin propeptide transgenic mice were heavier than the wild-type animals on the normal diet. In contrast, high-fat diet induced wild-type mice had 170–214% more fat mass than transgenic mice and developed impaired glucose tolerance and insulin resistance. Interestingly, high-fat diet significantly increased adiponectin secretion while blood insulin, resistin and leptin levels remained normal in the transgenic mice.[2] A study reported myostatin-knockout mice with heavy muscling had weak force generation due to a mitochondrial depletion.[3] It was reported that despite a larger muscle mass relative to age-matched wild types, there was no increase in maximum tetanic force generation. These results suggest a mitochondrial depletion. Mitochondria play a central role in energy production. Mitochondrial oxidization produces electrons converted to energy stored in high phosphate bonds of adenosine triphosphate (ATP). Various reactions in the cell determine the adding or subtracting of a phosphate to ADP in the metabolic process. The ratio of mitochondrial DNA to nuclear DNA and mitochondria number were decreased in myostatin-deficient muscle. Our principal investigation is to clarify the paradoxical situation in myostatin propeptide transgenic mice, which had similar muscling phenotype as the knockout mice.

Materials and Methods

DNA extraction

Approximately 50 mg of biceps muscle of 6 transgenic and 6 littermate wild-type mice were used for DNA isolation. Muscle samples were obtained from mice at the age of 18 weeks. Animal experimental work was approved by The University of Hawaii IACUC. Tissue samples were digested by proteinase K at 50°C overnight with rotation. 500 µl of phenol: chloroform was added to each tube, vortexed (5–10 s), and then centrifuged at full speed for 5 min. 500 µl of top phase were carefully transferred to newly labeled tubes. 500 µl of chloroform was added to each tube, vortexed (5–10 s), and then centrifuged for 2 min at full speed. 450 µl of top phase transferred to newly labeled spin columns and centrifuged for 1 min at 8,000 rpm. Collection tubes were emptied and 500 µl of aqueous buffer was added to spin columns then centrifuged for 1 min at 8,000 rpm. Collection tubes were emptied and 500 µl of wash buffer was added to spin columns then centrifuged for 3 min at 14,000 rpm. Collection tube was discarded and spin columns were placed into fresh, labeled collection tube (1.5 ml). 50 µl of dH_2O was added to spin columns, and then centrifuged for 1 min at 8,000 rpm. Final product of dissolved DNA was

collected. DNA product was analyzed in agarose gel electrophoresis.

Polymerase Chain Reaction (PCR)

1 µl of each primer added to 9 µl of dH_2O in separate tubes to make primer stock. Master Mix of each primer (MtCO1 and Ndufvl) was made for two practice samples.

Master mix MtCO1 consists of 10 µl Standard Taq Reaction Buffer Mg Free 10×, 6 µl 25 mM MgCl2, 2 µl 10 mM dNTP Mx, .8 µl Taq DNA Polymerase U/ml, 71.2 µl of distilled H_2O, 1 µl forward MtCO1 primer and 1 µl reverse MyCO1 primer stock. The same was done to make Master mix Ndufvl, using reverse and forward primer Ndufvl stock. Negative block was made in 0.25 ml tube with 5 µl Standard Taq Reaction Buffer Mg Free 10×, 3 µl 25 mM MgCl2, 1 µl 10 mM dNTP Mx, 0.4 µl Taq DNA Polymerase U/ml, 38.6 µl of distilled H_2O, 1 µl reverse and forward primer Ndufvl stock. PCR application was set as 95°C for 10 min followed by 35 cycles 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 5 min. PCR product was analyzed in 1% agarose gel electrophoresis.

Relative Quantitative Real Time PCR

The relative quantification 96-well plate was labeled with sample numbers on each column and detector on each row. New primer stock was made (0.1 µl of 1 nmol/µl primer: 99.9 µl DEPC H20). Master Mix MtCO1 of 75 µl of SYBR Green dye, 57 µl of distilled H_2O and primer stock was made. Master Mix Ndufvl was also made using Ndufvl primer. Master Mixes were distributed evenly in correct wells. 3 µl of each diluted DNA sample (1 µl DNA: 399 µl H20) were added to correct well. The plate was sealed and placed in Real Time PCR machine. The machine was set to run. Results were then calculated for relative mtDNA quantification. Acquired threshold cycle (Ct) of real-time PCR was obtained for both MtCO1 and Ndufvl. The relative mitochondrial gene copy number in ΔCt value was calculated by subtraction of Ct value of MtCO1 from the Ct value of Ndufvl. The relative mitochondrial gene copy number in fold change was calculated by using the mean of wild-type group as base reference value. The effect of genotype on the mitochondrial DNA levels was analyzed by two-tailed Student's t-test. Least square means and their standard errors were reported. Significance was determined at $P<.05$.

Results and Discussion

The data obtained from real-time PCR reactions were expressed as average Ct value in transgenic mice compared to wild type mice. It is known that a lower Ct value is equated to more DNA templates and mitochondrial DNA. Therefore, with the apparent difference found, it can be assumed that transgenic mice retain more relative mitochondrial DNA copy number. Calculations of fold change also showed that transgenic mice have more mitochondrial copy number. However, due to varying Ct values in the transgenic group, these conclusions cannot be statistically supported as the *P* value of t-test between these two groups was more than 0.05. The results indicated that there was a not significant difference of mtDNA quantification of myostatin propeptide transgenic mice from the wild-type littermate mice although the mean of the relative mitochondrial DNA levels of transgenic mice is 1.6 times more than the wild-type mice.

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