

« Research Note »

Characterization of Mitochondrial Content and Respiratory Capacities of Broiler Chicken Skeletal Muscles with Different Muscle Fiber Compositions

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Mitochondrial content is regarded a useful feature to distinguish muscle-fiber types in terms of energy metabolism in skeletal muscles. Increasing evidence suggests that specific mitochondrial bioenergetic phenotypes exist in metabolically different muscle fibers. A few studies have examined the energetic properties of skeletal muscle in domestic fowls; however, no information on muscle bioenergetics in broiler chickens selectively bred for faster growth is available. In this study, we aimed to characterize the mitochondrial contents and functions of chicken skeletal muscle consisting entirely of type I (oxidative) (*M. pubo-ischio-femoralis pars medialis*), type IIA (glycolytic/oxidative) (*M. pubo-ischio-femoralis pars lateralis*), and type IIB (glycolytic) (*M. pectoralis*) muscle fibers. Citrate synthase (CS) activity was the highest in type IIA muscle tissues and isolated mitochondria, among the muscle tissues tested. Although no difference was registered in mitochondrial CS activity between type IIB and type I muscles, tissue CS activity was significantly higher in the latter. Histochemical staining for NADH tetrazolium reductase and the ratio of muscle-tissue to mitochondrial CS activity indicated that type I, type IIA, and type IIB muscle-fiber types showed decreasing mitochondrial content. Mitochondria from type I muscle exhibited a higher coupled respiration rate induced by pyruvate/malate, palmitoyl-CoA/malate, and palmitoyl-carnitine, as respiratory substrates, than type IIB-muscle mitochondria, while the response of mitochondria from type IIA muscle to those substrates was comparable to that of mitochondria from type I muscle. Type IIA-muscle mitochondria exhibited the highest carnitine palmitoyltransferase-2 level among all tissues tested, which may contribute to the higher fatty acid oxidation in these mitochondria. The results suggest that mitochondrial abundance is one of the features differentiating metabolic characteristics of different chicken skeletal muscle types. Moreover, the study demonstrated that type IIA-muscle mitochondria may have distinct metabolic capacities.

Key words: carnitine palmitoyltransferase system, citrate synthase, mitochondrial content

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Introduction

Skeletal muscles, which account for approximately 40% of body mass in animals, have been shown to influence whole-body metabolism. Energy production in the tissues and the physiology of the tissues is altered by physiological stimuli,

such as exercise and adaptive thermogenesis (Ju *et al.*, 2016; Kwon *et al.*, 2017; Lowell and Spiegelman, 2000). In most animals, skeletal muscles are composed of several types of muscle fibers, which are classified into slow-twitch (type I) and fast-twitch (type IIA and IIB) types. Type I fibers show slow contractility and exhibit oxidative metabolism based on mitochondrial oxidative phosphorylation, whereas type IIB fibers have fast contractility and exhibit glycolytic metabolism. In chickens, type IIA fibers have fast contractility but exhibit oxidative metabolism, thereby defining these muscle fibers as glycolytic-oxidative (intermediate) type (Baldwin *et al.*, 1972).

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Muscle fibers have acquired metabolic and histochemical characteristics for energy or functional demands. Mitochondrial content, which is expressed as density, number, or volume on a case-by-case basis, is a major feature that differentiates the fiber types in terms of energy metabolism. Mitochondria are centers of aerobic metabolism that generate the bulk of ATP needed to maintain cellular functions based on a highly organized catabolic system consisting of the tricarboxylic acid (TCA) cycle, fatty acid import by the carnitine palmitoyltransferase (CPT) system, and downstream β -oxidation and electron-transferring pathways. Several recent studies have shown that depending on the respiratory capacity of the muscle, specific mitochondrial phenotypes exist in metabolically different muscle fibers in several vertebrates, such as pigs, rats, and rainbow trout (Glancy and Balaban, 2011; Leary *et al.*, 2003; Picard *et al.*, 2012; Ponsot *et al.*, 2005).

Few studies on differences in respiratory capacities between several types of skeletal muscles in domestic fowls have been conducted. A study in ducklings reported that the basal respiration rate was significantly higher in the *gastrocnemius* muscle (slow-twitch fiber muscle type) than in the *pectoralis* muscle (fast-twitch fiber muscle type) (Rey *et al.*, 2013). A study in chickens showed that substrate utilization differs between mitochondria isolated from *pectoralis* muscle and those isolated from adductor muscle (Thakar, 1977). However, no information on skeletal muscle respiratory capacities in current broiler chickens selectively bred for faster growth is available. Therefore, to improve our understanding of the mitochondrial energetics for energy production, the present study determined the differences in mitochondrial respiratory capacities of chicken skeletal muscles, including type I, type IIA, and type IIB muscle fibers.

Materials and Methods

Ethics Statement

The Animal Care and Use Committee of the Graduate School of Agricultural Science, Tohoku University, approved all procedures, and every effort was made to minimize pain or discomfort to animals.

Animals and Experimental Design

Five newly hatched male broiler chicks (Ross strain, *Gallus gallus domesticus*) were obtained from a commercial hatchery (Matsumoto Poultry Farms & Hatcheries Co., Ltd., Miyagi, Japan). The chicks were housed in electrically heated incubators under continuous light for the first two weeks and were kept under the optimal temperature for chicks according to the Ross breeder's manual. Thereafter, the chicks were reared in individual wire-cages at 24–26°C. The chickens had water available *ad libitum* and they were fed a breeder's recommended diet for meat-type chickens, according to their growth stage. At 5–6 weeks of age, the chicks were euthanized by decapitation, and *M. pectoralis* (Pec), *M. pubo-ischio-femoralis pars lateralis* (PIFL), and *M. pubo-ischio-femoralis pars medialis* (PIFM) were rapidly excised. A portion of each muscle was placed in ice-cold dry

acetone for histochemical staining, and another portion was placed in ice-cold isolation medium (100 mM KCl, 50 mM Tris/HCl [pH 7.4], and 2 mM ethylene glycol-bis [β -aminoethyl ether]-*N,N,N',N'*-tetraacetic acid [EGTA]) for mitochondrial isolation.

Histochemical Staining of Skeletal Muscle

Five frozen blocks of each muscle were cut into 10- μ m-thick sections on a cryostat (CM1850; Leica, Nussloch, Germany) and mounted on glass microslides. The sections were immunostained with antibodies specific for fast myosin heavy chain (MyHC) (M4276; Sigma-Aldrich) and stained for determination of nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) activity, as previously described (Watanabe *et al.*, 2016) with minor modifications for chicken tissues. Images of the enzymatically stained and immunostained sections were obtained using a microscope and were processed using ImageJ. The percentages of the fibers stained in each muscle were calculated as a ratio to the total number of fibers observed in the corresponding field. The colorimetric intensity of NADH-TR activity in the muscle serial sections was quantified relative to that of Pec muscle. Total counted numbers of muscle fibers were 538, 206, 156 in Pec, PIFL, PIFM, respectively. Staining data were obtained from five individual pictures for each muscle. Weak fast MyHC-positive muscle fiber in PIFM was not considered for the determination of type IIA muscle fiber. Size bars in each picture indicate 50 μ m.

Isolation of Skeletal Muscle Mitochondria

Mitochondria were isolated from Pec, PIFL, and PIFM by homogenization, protein digestion, and differential centrifugation at 4°C, as previously described (Kikusato and Toyomizu, 2015). The protein concentration of the isolated mitochondria was determined using bicinchoninic acid (BCA) assays (B9643/C2284; Sigma-Aldrich), with bovine serum albumin (BSA) as the standard. All mitochondria were freshly prepared on the day of the experiment.

Measurement of Mitochondrial Respiratory Capacity

The mitochondrial O₂ consumption rate was measured using a Clark-type O₂-sensitive electrode (Rank Brothers, Cambridge, UK), as previously described (Kikusato *et al.*, 2015). Mitochondria were incubated at 38°C in assay medium (115 mM KCl, 10 mM KH₂PO₄, 3 mM HEPES [pH 7.2], 1 mM EGTA, 2 mM MgCl₂, and 0.3% [*w/v*] defatted BSA), which was assumed to contain atomic oxygen at 402 nmol/mL (Reynafarje *et al.*, 1985). To obtain complex I- or complex II-driven respiration rates, mitochondria were stimulated using 10 mM pyruvate plus 2.5 mM malate or 4 mM succinate plus 5.0 μ M rotenone, which inhibits reverse electron flow from ubiquinol. Mitochondria were incubated with 80 μ M palmitoyl-CoA plus 2.5 mM malate in the presence of 2 mM carnitine or 80 μ M palmitoyl-carnitine plus 2.5 mM malate to measure fatty acid oxidation-supported respiration depending on CPT1 (CPT1-dependent respiration) or independent of CPT1 (CPT1-independent respiration). In the assays, malate was added to promote the oxidation of acetyl-CoA, which was generated by β -oxidation. In each respiration measurement, mitochondria were first

incubated in the absence of ADP to obtain substrate-dependent respiration in which there was no phosphorylation of ADP to ATP (state 2), and subsequently, 73 μ M ADP was added to obtain coupled respiration in which ADP phosphorylation occurred at the maximal rate (state 3). After this phosphorylation was completed, uncoupled respiration without ADP phosphorylation but with ATP retained in the mitochondria (state 4) was measured continuously. The state conditions in the measurement were illustrated in Kikusato *et al.* (2015). The respiratory control ratio (RCR) was calculated as a ratio of the state 3 to state 4 respiration rates to estimate the coupling efficiency of oxidative phosphorylation.

Determination of Citrate Synthase (CS), CPT1, and CPT2 Activities

CS activity was determined as previously described (Azad *et al.*, 2010; Mujahid *et al.*, 2007). Isolated mitochondria and muscle tissue homogenates (10%, v/w) were suspended in assay buffer (250 mM sucrose, 20 mM Tris [pH 7.5], 1 mM EGTA, and 10% Triton X-100) and centrifuged at 1,200 $\times g$ for 10 min at 4°C. The supernatants were mixed with 2 mL of reaction buffer containing 0.1 M Tris/HCl (pH 8.0), 100 μ M 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (freshly prepared), 50 μ M acetyl-CoA, and 250 μ M oxaloacetate, and CS activity was measured using a spectrophotometer (UVmini-124 equipped with CPS-100; Shimadzu Co. Ltd., Japan). Absorbance at 412 nm was recorded over 6 min at 25°C, and the values were expressed as μ mol/min/mg mitochondrial or tissue protein. Protein concentration was determined using the BCA assay. Muscle tissue CS activity was normalized to mitochondrial CS activity to determine mitochondrial content in the skeletal muscles.

Mitochondrial CPT1 and CPT2 activities were measured as previously described (Zhu *et al.*, 2013). Briefly, mitochondria were incubated in assay medium (20 mM HEPES [pH 7.4], 1 mM EGTA, 220 mM sucrose, 40 mM KCl, 100 μ M DTNB, 1.3 mg/mL bovine serum albumin, and 40 μ M palmitoyl-CoA) prewarmed to 25°C, to which 1 mM carnitine was added to measure total CPT activity. The absorbance of the solution at 412 nm was monitored for 8 min with a spectrophotometer. CPT2 activity was measured with the same reaction as for total CPT activity but in the presence of 10 μ M malonyl-CoA, which inhibits CPT1 activity. CPT1 activity was calculated by subtracting CPT2 activity from total CPT activity. The result was normalized to mitochondrial protein content.

Statistical Analysis

All data are presented as the mean \pm standard error (SE) of five individual mitochondrial preparations. Data were analyzed one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test, with *P* values < 0.05 considered significant. Values labeled with different letters are significantly different.

Results

Determination of Muscle Fiber Types

We first characterized muscle fiber types for Pec, PIFL,

and PIFM, each of which is considered a specific muscle consisting entirely of fast-glycolytic (type IIB), fast-oxidative-glycolytic (type IIA), and slow-oxidative (type I) fibers, respectively (Suzuki *et al.*, 1985). Figure 1 shows the results of histochemical staining of the above muscles. A large number of muscle fibers in Pec and PIFL exhibited fast MyHC-positive reactions (Pec, 96.0 \pm 2.4%; PIFL, 87.6 \pm 4.1%), whereas a small percentage of muscle fibers showed a positive reaction in PIFM (4.0 \pm 2.4%). NADH-TR activity in the serial sections was low in Pec fibers, whereas most PIFL fibers showed a positive enzyme reaction. Most PIFM fibers also showed a positive reaction for NADH-TR activity, and the colorimetric intensity (2.3 \pm 0.2 pixels) was significantly higher than that in PIFL (1.7 \pm 0.3). Pec showed a limited NADH-TR activity, and the value (1.0 \pm 0.1) was significantly lower than that in PIFL. Based on these data, we confirmed that Pec, PIFL, and PIFM are composed mostly of type IIB, IIA, and I muscle fibers, respectively.

CS Activities in Skeletal Muscle Tissue and Isolated Mitochondria, and the Ratio of Tissue CS to Mitochondrial CS Activity

CS activity in skeletal muscle tissues and isolated mitochondria was determined. CS is an important enzyme that links the TCA cycle and the β -oxidation pathways in mitochondria. As shown in Table 1, type IIA muscle (PIFL) showed the highest CS activity in both muscle tissue and isolated mitochondria, among the muscle types tested. The tissue CS activity in type I muscle (PIFM) was significantly higher than that registered in type IIB muscle (Pec), while no significant difference in mitochondrial CS activity was observed between these two muscles. CS activity is often used as a mitochondrial marker in tissues (Boushel *et al.*, 2007); however, this would not be possible if CS activity and/or content in mitochondria differ between the muscles. Therefore, the present study calculated the ratio of muscle tissue CS activity to mitochondrial CS activity in each muscle type. The ratio was the highest in type I muscle, and the value in type IIA muscle was significantly higher than that in type IIB muscle.

Mitochondrial Respiratory Capacity

We next determined respiratory capacities in isolated muscle mitochondria exposed to pyruvate plus malate (complex I-driven respiration), succinate (complex II-driven respiration), palmitoyl-CoA (CPT1-dependent respiration), or palmitoyl-carnitine (CPT1-independent respiration). As shown in Fig. 2, for all substrates, state 2 and state 4 respiration rates did not differ considerably between type IIB-, IIA-, and I-rich muscles. Moreover, state 3 respiration rates in mitochondria receiving complex I-linked substrates were significantly higher in type IIA and I muscles than in type IIB muscle, with no significant differences in the rates observed between type IIA- and type I-muscle mitochondria (Fig. 2A). RCR values of complex I-driven respiration did not differ considerably between Pec, PIFL, and PIFM mitochondria. In mitochondria exhibiting complex II-driven respiration, the state 3 respiration rate was significantly higher in type IIA than in type IIB muscles; however, no significant differences

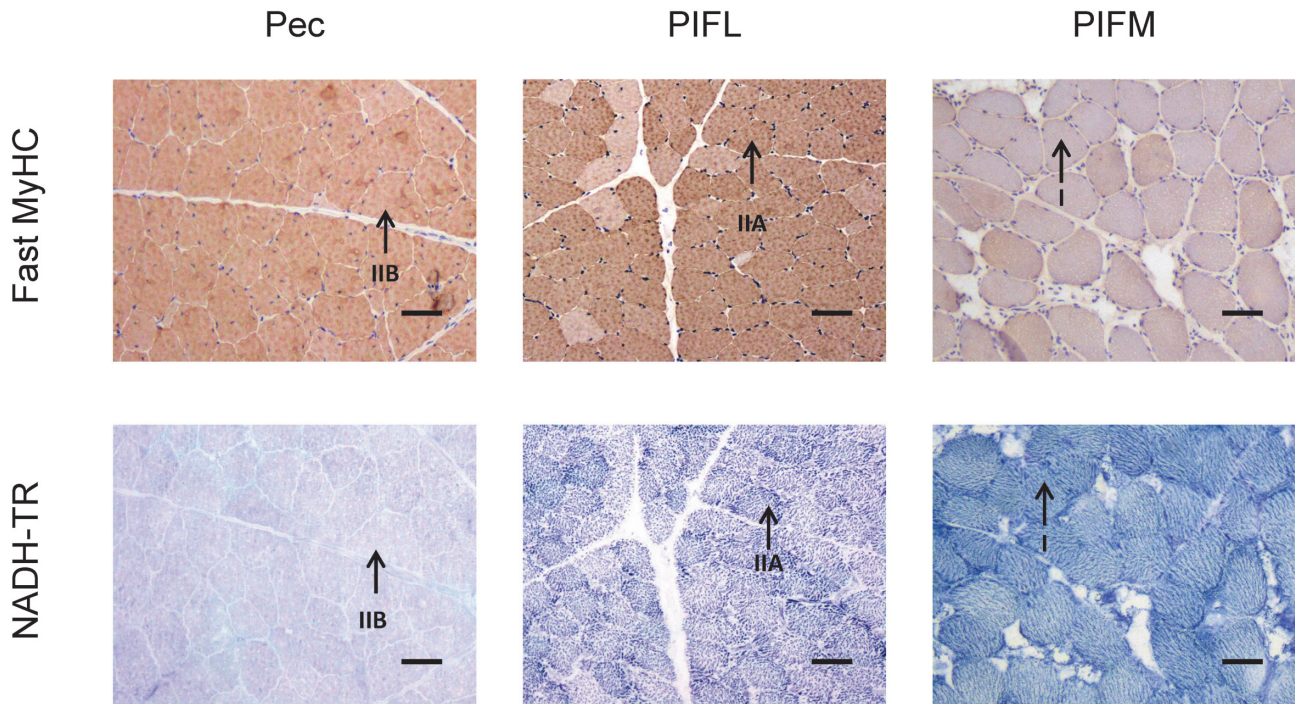


Fig. 1. Histochemical MyHC (top) and NADH-TR (bottom) staining of Pec, PIFL, and PIFM in chickens. Size bars in each picture indicate 50 μm .

Table 1. CS activities in skeletal muscle tissue and isolated mitochondria, and the ratio of tissue CS activity to mitochondrial CS activity

CS activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	Type IIB (Pec)	Type IIA (PIFL)	Type I (PIFM)
Skeletal muscle tissue	4.5 ± 0.4^c	20.1 ± 3.3^a	14.7 ± 1.1^b
Isolated mitochondria	845 ± 118.1^b	1610 ± 126.5^a	826 ± 79.3^b
Ratio of tissue CS to mitochondrial CS activity	0.006 ± 0.001^c	0.012 ± 0.001^b	0.018 ± 0.002^a

Values are means \pm SEs, $n=4-5$. ^{a, b, c} $P < 0.05$ for each muscle; values with different letters are significantly different.

were observed between type IIA and I muscle mitochondria (Fig. 2B). RCR values of complex II-driven respiration did not differ between the muscle mitochondria. In mitochondria exhibiting CPT1-dependent (Fig. 2C) or CPT1-independent (Fig. 2D) respiration, the state 3 respiration rates were markedly higher in type IIA and I muscles than in type IIB muscle ($P < 0.05$), with no significant differences observed between type IIA and type I muscles. Under both substrate conditions, RCR values were significantly higher in type IIA and I muscle mitochondria than in type I muscle mitochondria, with no significant differences observed between type IIA and I muscle mitochondria.

CPT1 and CPT2 Activities in Isolated Chickens Muscle Mitochondria

CPT1 and CPT2 activities in isolated mitochondria were measured to elucidate the possible mechanisms underlying the higher respiratory capacities due to fatty acid oxidation in

type IIA and I muscles. The CPT system controls fatty acid import into mitochondria, wherein CPT1 functions to transfer acyl groups from acyl-CoA to carnitine to form acyl-carnitine. Type IIB- and IIA-muscle mitochondria showed significantly higher CPT1 activity than type I-muscle mitochondria, with no significant difference observed between type IIB- and IIA-muscle mitochondria (Fig. 3A). CPT2 reconverts acyl-carnitine to acyl-CoA to provide acyl-CoA for β -oxidation. Among the mitochondria tested, the transferase activity was highest in type IIA-muscle mitochondria, and the activity of type I-muscle mitochondria was significantly higher than that of type IIB-muscle mitochondria. Total CPT activities showed a trend similar to CPT2 activities for the different muscle mitochondria samples.

Discussion

A major objective of the present study was to characterize

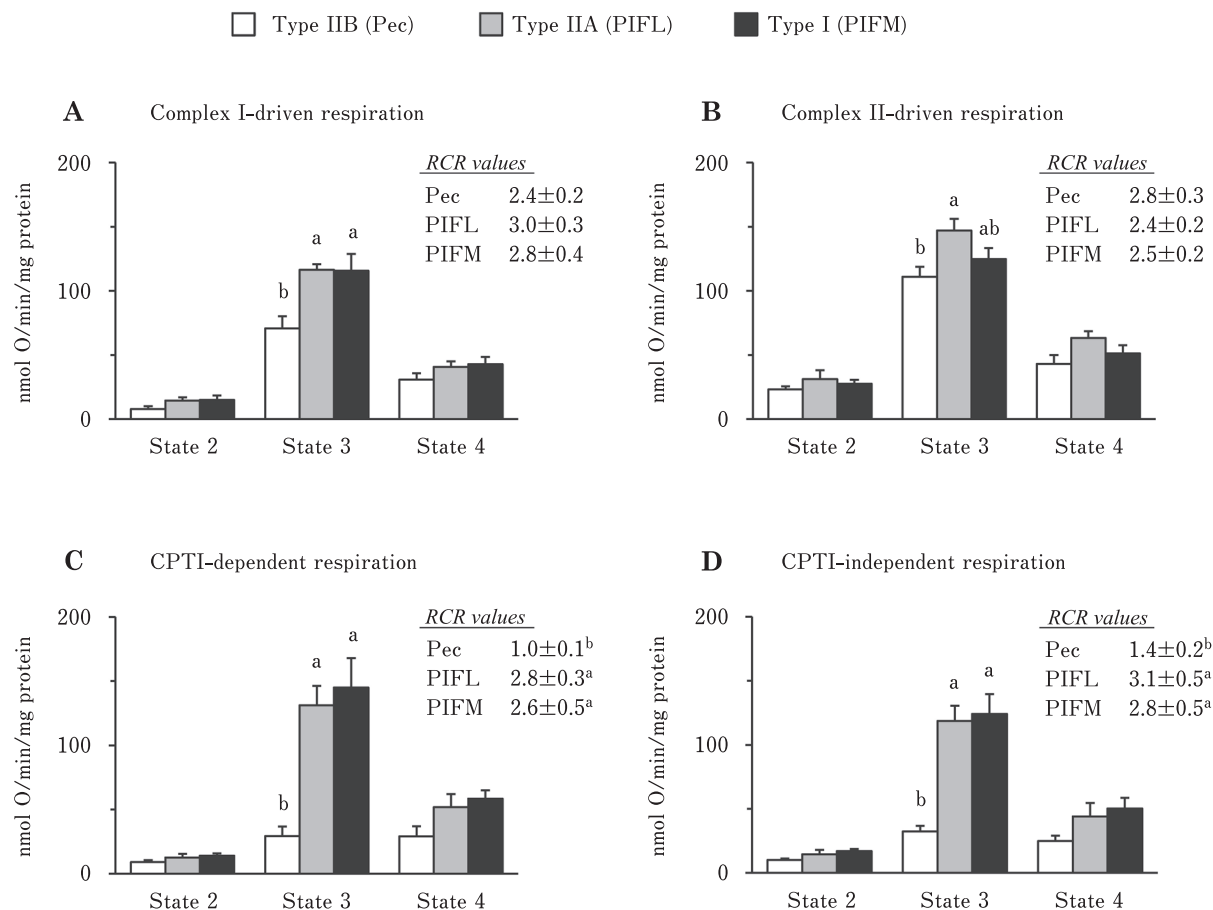


Fig. 2. O_2 consumption rates and RCR values of chicken Pec, PIFL, and PIFM mitochondria exhibiting complex I-driven (pyruvate/malate) (A), complex II-driven (succinate plus rotenone) (B), CPTI-dependent (palmitoyl-CoA/malate plus carnitine) (C), and CPTI-independent (palmitoyl-carnitine/malate) (D) respiration. The respiration rates were determined by a polarographic method. Values are means \pm SEs ($n=5$). ^{a, b, c} $P < 0.05$ for each muscle; values with different letters are significantly different.

mitochondrial respiratory functions of chicken skeletal muscles having different muscle fiber compositions. Histochemical analyses demonstrated that Pec, PIFL, and PIFM consisted entirely of type IIB, IIA, or I fibers, and the muscles could be defined as glycolytic, oxidative-glycolytic (intermediate), and oxidative muscles, respectively (Fig. 1). Moreover, based on NADH-TR staining results and the CS activity ratio (Table 1), we found that in chicken, the PIFM, PIFL, and Pec may show decreasing mitochondrial content.

Metabolic differences between oxidative and glycolytic muscle tissues and the mitochondria have been extensively investigated. With regard to the respiratory capacities of mammalian muscles, a previous study investigating mitochondrial functions of rabbit skeletal muscles showed that oxidative muscle (*soleus*, 98% type I) exhibited higher complex I- and palmitoyl-carnitine-driven respiration rates than glycolytic muscle (*gracilis*, 99% type IIB) (Jackman and

Willis, 1996), and similar results were obtained in rat skeletal muscle (*soleus* versus *superficial gastrocnemius*) (Ponsot *et al.*, 2005). Additionally, in a study comparing porcine skeletal muscle characteristics, no differences in complex I-driven mitochondrial respiration rates were observed between *vastus intermedius* (70%, type I) and *gracilis* (70%, type IIB) muscles, and palmitoyl-carnitine-driven respiration was higher in the oxidative muscle (Glancy and Balaban, 2011). Consistent herewith, our current findings in chicken muscle mitochondria demonstrated that type I-muscle (PIFM) mitochondria exhibited higher coupled respiration capacities in response to two different forms of fatty acids or complex I-linked substrates than type IIB-muscle (Pec) mitochondria (Fig. 2). From previous findings and our current experimental results, it is likely that chicken type I muscle is characterized by higher fatty acid import and oxidation in the mitochondria.

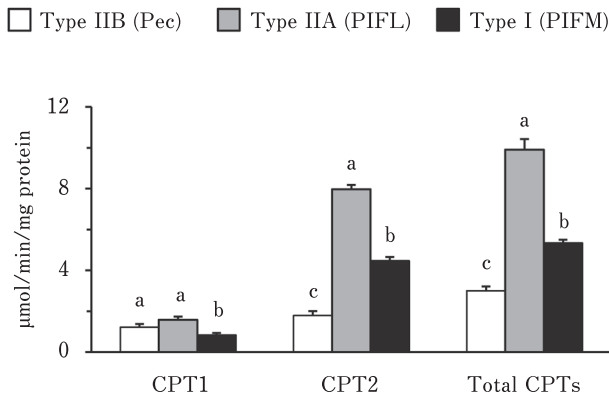


Fig. 3. Activities of CPT1, CPT2, and total CPT in isolated mitochondria from chicken Pec, PIFL, and PIFM. Values are given as the fold change relative to Pec values. Values are means \pm SE ($n=5$). ^{a,b,c} $P < 0.05$ for each muscle; values with different letters are significantly different.

Our present findings showed that there were no differences between the CS activities of type I- and IIB-muscle mitochondria (Table 1). Type I-muscle mitochondria exhibited higher CPT2 and total CPT activities than type IIB-muscle mitochondria (Fig. 3). These results suggest that the activities may contribute to the higher respiration rate due to fatty acid oxidation in type I-muscle mitochondria of chickens. Meanwhile, the potential mechanism underlying the higher complex I-driven respiration rate in the oxidative muscle has to be considered (Fig. 2A). A proteomic study reported that protein expression levels of malate dehydrogenase and aconitase are higher in oxidative than in glycolytic muscle tissues in rats (Okumura *et al.*, 2005). From these findings, it is conceivable that TCA cycle enzymes other than CS may be associated with differences in complex I-driven respiration between type I- and IIB-muscle mitochondria in chickens. It is possible that mitochondrial respiratory complex abundance and/or supercomplex formation might contribute to the higher complex I-driven respiration in type I versus type IIB muscle, given recent findings that the above factors can affect the respiration capacity (Amo *et al.*, 2014; Greggio *et al.*, 2017; Lapunte-Brun *et al.*, 2013; Rosca *et al.*, 2008). Since no difference in complex II-driven respiration was observed between type I- and type IIB-muscle mitochondria, further proteomic investigations are required to identify the factors inducing the higher respiration capacity in type I-muscle mitochondria.

Chicken type IIA muscle (PIFL), which was characterized as intermediate type (glycolytic/oxidative) muscle, had the highest mitochondrial CPT and CS activities among the muscles tested, but exhibited a respiratory capacity comparable to that of oxidative (type I) muscle. One can assume that this discrepancy might be due to a lower activity of the β -oxidation pathway, which generates acetyl-CoA from acyl-CoA provided via the CPT system, in type IIA than in type I muscle fibers.

Metabolic characteristics of type IIA muscle have not been studied in other animals. Regarding the molecular properties of type IIA muscle, a recent study in a rodent model and humans reported that type IIA muscle exhibited the highest peroxisome proliferator-activated receptor γ coactivator 1- α (PGC-1 α) content among several muscle types tested (Gouspillou *et al.*, 2014). PGC-1 α is a cotranscription factor governing mitochondrial biogenesis, in which the cofactor is associated with mitochondrial metabolic genes, including those encoding CPT2 and CS (Arany *et al.*, 2005; Choi *et al.*, 2008). In relation to this idea, we found that type IIA, type I, and type IIB showed decreasing CS activity per skeletal muscle tissue. Based on the findings, it is conceivable that the metabolic characteristics of chicken type IIA-muscle mitochondria could be attributed to PGC-1 α content and/or its cotranscriptional activity. Further comparative investigation is required to determine if the characteristics are specific for broiler chickens.

PIFL and PIFM in chickens are located next to each other inside of each leg. In the current study, we found that both muscles contained larger amounts of fats than Pec (data not shown); this, along with the observation that the muscles had greater fatty acid oxidation capacities, suggested that both leg muscles can utilize fats as energy sources to generate the mechanical forces for standing. Moreover, our study suggested that the energy production abilities of PIFL and PIFM could be attributed to mitochondrial contents and respiration capacities, respectively.

In conclusion, we found that the mitochondrial content differs among chicken skeletal muscles with different fiber compositions. The study also revealed that fast-oxidative-glycolytic type IIA muscle showed increased respiratory capacity, comparable to oxidative type I muscle, although it is still unclear how type IIA muscle acquired distinct bioenergetic properties in chickens. Our findings provide important insights into the muscle fiber type-specific bioenergetic characteristics of skeletal muscle mitochondria of current broiler chickens.

Acknowledgments

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