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<th>Expression of AMP-activated Protein Kinase Subunit Isoforms in Masseter and Tibialis Anterior Muscles of Mice before and after Weaning</th>
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Expression of AMP-activated Protein Kinase Subunit Isoforms in Masseter and Tibialis Anterior Muscle of Mice Before and After Weaning

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Running title: AMPK in mice masseter and limb muscle during development

Field: Biochemistry
Abstract: The AMP-activated protein kinase (AMPK) acts as a fuel gauge during exercise and stress-induced energy crisis. The function of the masseter muscle changes during postnatal development, along with an alteration in type of muscle fiber. We investigated expression of AMPK subunit isoforms in masseter muscle by quantitative real-time RT-PCR and Western blotting, and compared it with that in tibialis anterior muscle in 2-, 4- and 9-week-old mice. The most abundantly expressed isoform of the catalytic subunit was α2, and those of the regulatory subunits were β1, β2 and γ1 in both types of muscle; mRNA expression of all the main isoforms in masseter muscle was higher than that in tibialis anterior muscle. Expression of α protein increased with development in both types of muscle. Messenger RNA expression of regulatory subunit isoforms β1 and γ1 in masseter muscle increased, together with an increase in the amounts of their corresponding proteins. On the other hand, mRNA expression of these isoforms in tibialis anterior muscle decreased with development, while their corresponding proteins increased at a similar rate to that of the development of the α subunit. This altered expression of AMPK subunit isoforms in each muscle during postnatal development may be related to alteration in function of each type of muscle.
Key Words: AMPK isoform/masseter muscle/limb muscle/development/mouse
Introduction

The AMP-activated protein kinase (AMPK) is a heterotrimeric complex consisting of a catalytic (α) and two regulatory (β, γ) subunits. Two isoforms of the catalytic α subunit (α1, α2), two of the β subunit (β1, β2), and three of the γ subunit (γ1, γ2 and γ3) have been identified in mammalian muscles. AMPK is activated through phosphorylation by upstream kinases such as LKB1 and/or Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase under low intracellular energy levels induced by various stresses. Once activated, AMPK phosphorylates several downstream substrates, resulting in the switching off of ATP-consuming pathways and the switching on of ATP-generating pathways. Therefore, it is believed to serve as a fuel gauge in response to energy demands during muscular exercise, and to protect against energy deprivation.

The cellular content of each subunit depends on type of tissue and/or cell, or type of stress, which suggests that regulation of AMPK activity is complex. In skeletal muscle, AMPK is involved in the regulation of glucose transport in response to hypoxia, electrical stimulation and exercise. Once activated in skeletal muscle, it increases glucose transport.
and regulates lipid and glucose metabolism\textsuperscript{8,9}). Moreover, mutation and genetic modification
of AMPK subunit isoforms, both in humans and animals, have been shown to be related to
metabolic or functional disorders in muscles\textsuperscript{10-12). These findings suggest that AMPK
expression and isoform composition are closely related to the metabolic profiles and functions
of each type of muscle.

Like tibialis anterior muscle, masseter muscle is a kind of skeletal muscle, although the
former is derived from mesenchymal cells in the somites and the latter from unsegmented
mesoderm of the first brachial arch\textsuperscript{13}). In masseter muscle, muscle fiber bundles run in
different directions in the superficial and deep layers\textsuperscript{14). The fiber bundles in the superficial
layer function mainly in chewing, and have been shown to alter the expression of muscle fiber
during postnatal development, presumably in response to the transitional phase from sucking
to mastication\textsuperscript{14-16). Tibialis anterior muscle is a fast contracting limb muscle, and has been
shown to alter its muscle fiber type earlier than masseter muscle during postnatal
development\textsuperscript{15,17).}

In this study, we investigated the expression of AMPK subunit isoforms by quantitative
real-time RT-PCR (qRT-PCR) and Western blotting and compared it between that in tibialis
anterior muscle and that in masseter muscle to determine the relationship between AMPK expression and muscular function during postnatal development.
Material and Methods

1. Animals and treatment

All animal experiments were performed in accordance with the Guidelines on Animal Care and Use established by Tokyo Dental College. ICR mice (male 2-, 4-, and 9-week-old mice) were purchased from Sankyo Labo Service (Tokyo, Japan). Since the mean weaning age of ICR mice is approximately 3 weeks, mice at 2 weeks old were at immediately before the weaning and sucking phase. After 3 weeks of age, the mice were weaned from their dams and kept on a chow diet. Mice at 4 weeks old were at immediately after weaning, and mice at 9 weeks old were of adult age and at mastication phase\(^{(14)}\). At weeks 2 and 4, five mice were used; at week 9, three mice were used. The superficial layers of the masseter and tibialis anterior muscles were dissected and used for protein and mRNA extraction.

2. qRT-PCR

Total RNA was isolated using BioRobot EZ1 (Qiagen, Hilden, Germany) with the EZ1 RNA Tissue Mini Kit (Qiagen) according to the manufacturer’s instructions. Five µg of
extracted RNA was used as a template for cDNA synthesis using ReverScript II (Nippon Gene, Tokyo, Japan) and oligo dT primer. Quantification of mRNA of AMPK subunit isoforms was performed using real-time RT-PCR with the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and qPCR Mastermix Plus for SYBR Green I (Eurogentec, Seraing, Belgium) according to the manufacturer’s instructions. The relative quantities of mRNA were calculated after normalization of the data against a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the competitive $C_T$ method. The PCR primers specific to the AMPK subunit isoforms and GAPDH were designed using Primer Express software (Applied Biosystems) and their sequences are presented in Table 1. Gamma3 primers were prepared according to the report of Mahlapuu et al.\textsuperscript{18}) PCR conditions included an initial incubation at 95°C for 10 min, followed by 40 cycles comprising 15 s at 95°C and 60 s at 60°C. qRT-PCR products were electrophoresed on 2% agarose gels.

3. Western blot analysis

Tissues were homogenized in lysis buffer consisting of 10 mM Tris-HCl(pH7.4), 10
mM NaCl, 3 mM MgCl$_2$, complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich, St. Louis, MO, USA) at 20 W for 2 min using the TissueLyser (Qiagen) and then kept for 20 min on ice. Homogenates were centrifuged at 10,000 × g for 20 min and the supernatants used for Western blot analysis.

Proteins were separated on 10% SDS-polyacrylamide gel and transferred to Immobilon P membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skimmed milk, incubated with each antibody, and then detected with horseradish peroxidase-conjugated anti-rabbit IgG antibody or anti-goat IgG antibody with the ECL plus system (GE Healthcare, Buckinghamshire, UK). Anti-AMPK$\alpha$ and anti-phospho-AMPK$\alpha$ antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA); anti-β1, anti-β2, anti-γ1 and anti-γ2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-γ3 antibody was from Abgent (San Diego, CA, USA); anti-acetyl-CoA carboxylase (ACC) and anti-phospho-ACC were from UP State (Charlottesville, VA, USA); and anti-actin antibody was from Sigma-Aldrich.
Results

1. Expression of AMPK subunit isoform mRNAs in masseter and tibialis anterior muscle of adult mice

Messenger RNA expression of each isoform was calculated relative to α2 in masseter muscle, which was provisionally set at 1.0 (Fig. 1A). Expression of α1 mRNA was scarcely detected in either muscle by qRT-PCR (Fig. 1A) or agarose gel electrophoresis (Fig. 1B).

Expression of β1 and β2 in masseter muscle was slightly lower than that of α2, at approximately 80% and 67% of that of α2, respectively. While expression of γ1 in masseter muscle was about 7.7-fold higher than that of α2, γ3 was about half that of α2 and about 6.5% of that of γ1 (Fig. 1). In tibialis anterior muscle, the most highly expressed components of the AMPK subunit isoforms were α2, β2 and γ1, which was a similar pattern to that seen in masseter muscle, except that β1 expression was approximately 20% of that of β2 in the former.

Messenger RNA expression of the major components in tibialis anterior muscle was lower than that in masseter muscle, at 15-30% of that of the latter, apart from β2 and γ3.

Expression of the β2 and γ3 isoforms in tibialis anterior muscle was about 80% of that in
masseter muscle.

2. Expression of AMPK subunit isoform mRNA during postnatal development

We analyzed mRNA expression of the major AMPK subunit isoforms, α2, β1, β2 and γ1 in masseter muscle and tibialis anterior muscle of 2-, 4- and 9-week-old mice. Relative mRNA levels in both types of muscle were calculated from each expression level in 2-week-old-mice. In masseter muscle, expression of α2 isoform mRNA showed no alteration during postnatal development. Expression of β1 mRNA showed an increase during postnatal development, rising 2.7-fold in 9-week-old-mice (Fig. 2A). Expression of β2 mRNA showed almost no alteration during postnatal development, although the level in 4-week-old mice showed a transient decrease (Fig. 2A). In tibialis anterior muscle, mRNA expression of the α2, β1, γ1 and γ3 isoforms decreased in adult mice, while β2 isoform expression showed a transient increase in 4-week-old-mice, returning to its original level at 9 weeks after birth (Fig. 2B).

3. Expression of AMPK subunit isoform proteins during postnatal development
In general, expression of AMPK subunit isoform mRNA in tibialis anterior muscle was lower than that in masseter muscle (Fig. 1). However, the amounts of the proteins of each isoform in tibialis anterior muscle were similar to, or slightly higher than that in masseter muscle (Fig. 3). Catalytic α subunit protein in both muscles showed a slight increase with postnatal development. Regulatory subunit isoform β1 protein in masseter muscle showed an increase with increased expression of its mRNA, although no marked increase was seen in β2 protein. In tibialis anterior muscle, amount of β1 isoform protein showed an increase, in spite of decreased mRNA expression, and that of β2 isoform protein showed a decrease to about 50% in 9-week-old-mice (Fig. 3). Expression of γ1 isoform protein in masseter muscle showed no significant change during postnatal development, but increased in tibialis anterior muscle with development, in spite of a decrease in mRNA expression (Fig. 2B).

Expression of γ2 and γ3 proteins showed no significant change during development in either type of muscle.

4. Activation of AMPK

To evaluate activation status of AMPK, we analyzed phosphorylation of AMPKα and ACC in
both types of muscle by Western blotting using anti-phospho-AMPKα, anti-ACC and anti-phospho-ACC. ACC is a downstream target of AMPK, which phosphorylates and inactivates ACC, resulting in a reduction in fatty acid synthesis\(^6,7\). Inhibition of ACC also results in a decrease in malonyl-CoA, relieving inhibition of carnitine palmitoyl-transferase and allowing fatty acid oxidation to proceed\(^{19}\). In masseter muscle, phospho-AMPKα in 2-week-old-mice was at a high level, which was maintained during postnatal development, while in tibialis anterior muscle, it decreased with development (Fig. 4), although the level of total AMPKα protein showed a slight increase with development (Fig. 3). ACC level showed no change in either type of muscle (Fig. 4). Phospho-ACC was detected in both types of muscle in 2-week-old-mice, showing a decrease during postnatal development in both types of muscle (Fig. 4).
Discussion

The results of present study showed that AMPK subunit isoform mRNAs and proteins were expressed in both masseter and tibialis anterior muscle. Catalytic subunit isoform α2 mRNA was expressed in both types of muscle, although α1 subunit mRNA was scarcely detected. This low expression of α1 mRNA was confirmed using other primer sets (data not shown). However, α2 protein was not directly detected in mouse muscle, kidney, heart or skeletal muscle using a commercially available anti-α2 antibody (Santa Cruz Biotechnology, sc-19131) using Western blot analysis. After immunoprecipitation using anti-α2, α protein was detected by anti-α antibody (data not shown), suggesting the presence of α2 protein.

Viollet et al.\textsuperscript{20,21}, using a knockout mouse model, reported that catalytic isoform α2, but not α1, played a major role as a fuel sensor, not only in muscle, but also in whole-body insulin sensitivity. This suggested that the main isoform of catalytic subunit in masseter and tibialis anterior muscle would be α2. Therefore we used anti-α antibody to investigate catalytic subunit in this study. The main regulatory subunit isoforms were β1, β2 and γ1 in both types of muscle (Fig. 1 and 3). Although the specific roles of β isoforms remain to be clarified,
both $\beta_1$ and $\beta_2$ have been identified in human skeletal muscle and found to respond to
endurance training differently\(^2\). Here, the major $\gamma$ subunit isoform in both masseter and
tibialis anterior muscle was $\gamma_1$. Although regulatory subunit $\gamma_3$ has been shown to play a
major role in certain murine white skeletal muscles such as the gastrocnemius and quadriceps
muscles, $\gamma_1$ has also been revealed to be highly expressed\(^{18}\). It was also reported that, in
soleus muscle, $\gamma_1$ was the major $\gamma$ subunit isoform, while $\gamma_3$ was not detected\(^{18}\). The results
of this study suggest that there is a close relationship between AMPK subunit isoform
organization and function of each muscle type, including masseter and tibialis anterior
muscle.

During the transitional period from suckling to mastication, mRNA expression of $\beta_1$
and $\gamma_1$ in masseter muscle showed an increase, with only minor changes in $\alpha_2$ (Fig. 2). In
parallel with this increase in mRNA expression in masseter muscle, the amount of AMPK
subunit proteins also increased. In tibialis anterior muscle, protein content of each isoform
increased, in spite of decreased mRNA expression. This suggests that regulation at the
mRNA level may not always be manifested at the protein level, indicating that expression of
mRNAs and proteins may be regulated by different mechanisms in each type of muscle.
In masseter muscle, AMPK was highly activated, as evidenced by the presence of phospho-AMPKα and phospho-ACC in 2-week-old-mice, although phospho-ACC level decreased during postnatal development (Fig. 4). In tibialis anterior muscle, phospho-AMPKα was at a high level in 2-week-old-mice, then decreasing to about half level of that in 9-week-old-mice (Fig. 4). This difference in behavior of phospho-AMPK between masseter and tibialis anterior muscle may reflect different metabolic demands and/or other roles in transcriptional control. The duration of muscle activity in juvenile rabbit masseter muscle has been shown to be significantly longer than that of other muscles\(^{23}\), and contraction-induced glucose transport is elicited by metabolic demand rather than by events occurring early during the excitation-contraction coupling\(^{24}\). This suggests that murine masseter muscle is probably continuously active making weaning and masticatory movements, thereby creating metabolic demands resulting in maintenance of high phospho-AMPKα levels during postnatal development. Tibialis anterior muscle may adapt to changing energy demands during postnatal development, resulting in a later decrease in phospho-AMPKα. The fact that phospho-ACC decreased with development in both muscles suggests a role for activated AMPK other than that of the processing of fatty acid oxidation through \(^{16}\).
phospho-ACC.

In masseter muscle, the composition of MHC isoforms has been found to alter from Type I to Type II during postnatal development\(^{15-17}\), and some types of human masticatory muscle are very different from the skeletal muscle of the limbs or abdomen\(^{13,25}\). Fiber type expression in the skeletal muscle is regulated by multiple signaling pathways and transcription factors related to ontogeny, physiological function or aging\(^{26}\). Recently, AMPK has been shown to be involved in the differentiation of cultured skeletal muscle cells\(^{27,28}\) and in the regulation of the cell cycle\(^{29,30}\). The alteration of AMPK subunit isoforms observed in this study in developing mice suggests a relationship with differentiation of muscle fiber type. However, the precise role of AMPK in the differentiation and development of muscle fibers in masseter muscle remains to be elucidated.
Acknowledgement

We would like thank Shinichi Abe, Department of Anatomy, Tokyo Dental College, for their stimulating discussions. This work was in part supported by a research grant from the Ministry of Education, Culture, Sports, Science and Technology in Japan (#15591978 for H. K.). We also thank Jeremy Williams, Laboratory of International Dental Information, Tokyo Dental College, for his assistance with the language.
References


Figure Legends

Fig. 1  Expression of AMPK subunit isoform mRNA in masseter and anterior tibialis muscle of mice

(A) RNA was extracted from masseter muscle and tibialis anterior muscle of 9-week-old-mice; expression of each mRNA was determined by qRT-PCR and normalized to GAPDH as an endogenous control.  Expression was calculated as relative to that of α2 in masseter muscle which was set at 1.0.  Data are means ± SD of three different experiments.

(B) PCR products were analyzed by electrophoresis on 2% agarose gel.

Fig. 2  Expression of major AMPK isoform mRNA during postnatal development in masseter muscle (A) and anterior tibialis muscle (B) of mice

Expression of major AMPK subunit isoforms, α2, β1, β2 and γ1, was analyzed by qRT-PCR in 2 (open-bar)-, 4 (cross-bar)- and 9 (filled-bar)-week-old mice.  Values were normalized to GAPDH as an endogenous control.  Expression was calculated as relative to that of α2 in 2-week-old-mice which was set at 1.0.  Data are means ± SD of three different
experiments. Data were statistically analyzed by one-way ANOVA and the differences from
the level in 2-week-old-mice were found to be statistically significant (*p<0.05).

Fig. 3  Protein expression of AMPK subunit isoforms in masseter muscle and tibialis anterior muscle during postnatal development

Protein was extracted from each muscle of 2-, 4- and 9-week-old mice and separated on
10% SDS-polyacrylamide gel. Protein expression was determined by Western blotting using
each corresponding antibody as described in the text. Experiments shown are representative
of three experiments.

Fig. 4  Protein expression of phospho-AMPK, phospho-ACC and ACC in masseter muscle and tibialis anterior muscle during postnatal development

Protein was extracted from each muscle of 2-, 4- and 9-week-old-mice and separated on
10% SDS-polyacrylamide gel. Protein expression was determined by Western blotting using
each corresponding antibody as described in the text. Experiments shown are representative
of three experiments.
### Table 1  Sequences of forward and reverse primers used for qRT-PCR

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<th>Reverse primer</th>
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<td>α1</td>
<td>5'-TCGGTTTCTGAAATCGAATG-3'</td>
<td>5'-TATGTCCCGTGCACTCGTG-3'</td>
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<tr>
<td>α2</td>
<td>5'-TCAACCCTTTAGTGCCAC-3'</td>
<td>5'-GACATTTCGATCGTAGGAGG-3'</td>
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<tr>
<td>β1</td>
<td>5'-CACACCTGCTGCAGGTGTCATC-3'</td>
<td>5'-TTGTACCCTGTTGTGCTGACTG-3'</td>
<td>153</td>
</tr>
<tr>
<td>β2</td>
<td>5'-GGTCTTTAGCGAAACCAGAT-3'</td>
<td>5'-TTCAAGGGCAAGGGAGATG-3'</td>
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<td>γ1</td>
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<td>5'-AGGTCTTTTCCGGCTGCC-3'</td>
<td>106</td>
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<td>5'-CCACATCTCCATCCAGGAG-3'</td>
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<td>γ31)</td>
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<td>5'-AGATGGCTTGGGTGAGG-3'</td>
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<td>GAPDH</td>
<td>5'-TGCCCAGAACATCATCCCTG-3'</td>
<td>5'-TCAGATCCAGACCGACACA-3'</td>
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1) Sequences of γ3 primers described by Mahlapuu et al. (18).
Fig. 1 Okoshi et al.
Fig. 2 Okoshi et al.
Fig. 3 Okoshi et al.
<table>
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<td>p-AMPKα</td>
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Fig. 4 Okoshi et al.