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Original Article

Expression of 5′-AMP-activated Protein Kinase with Starvation in Murine Thymocytes

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Abstract

The 5′-AMP-activated protein kinase (AMPK) is a key enzyme in the protection of cells during energy crisis. AMPK is a heterotrimer consisting of a catalytic α (α1, 2) subunit and two regulatory subunits, β (β1, 2) and γ (γ1–3). To elucidate the role of AMPK in thymocytes with starvation, we investigated the expression of AMPK in murine thymocytes. The main isoforms expressed were α2, β1, and γ1, of which expression increased time-dependently with starvation, together with an increase in the amount of the active form of AMPK, phospho-AMPKα. In cultured thymocytes, expression of AMPK was induced by dexamethasone, but not by a low glucose concentration in medium. Increased expression was inhibited by glucocorticoid receptor antagonist RU486. Phosphorylation of AMPKα showed an increase with low glucose concentration, but not with dexamethasone. These results suggest that increased expression of AMPK in starved mouse thymocytes is induced by an increase in glucocorticoids and that activation is induced by hypoglycemia.

Key words: AMP-activated protein kinase—Thymocyte—Glucocorticoid—Hypoglycemia—Starvation

Introduction

The 5′-AMP-activated protein kinase (AMPK) was first discovered through its inhibitory effect on a preparation of acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA reductase[4,5]. The role of AMPK in regulating cellular energy balance places the enzyme at a central control point in maintaining energy homeostasis[7,14]. When intracellular concentrations of ATP were reduced by cellular stress, which can be caused by hypoxia or energy source depletion, it was found that AMPK was activated by increased AMP levels or a rise in the AMP/ATP ratio, and that it responded by adjusting the rates of ATP-consuming metabolic pathways (*e.g.*, fatty acid and cholesterol biosynthesis) to active ATP production (*e.g.*, fatty acid and glucose oxidation)[7,14].

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AMPK is a heterotrimeric enzyme composed of a catalytic α subunit and regulatory β and γ subunits[7,14,29]. The α and β subunits each exist in two isoforms (α1, α2, and β1, β2, respectively), and the γ subunit in three isoforms (γ1, γ2 and γ3). AMPK is activated allostelically by AMP and by phosphorylation of the α subunit at T172 by upstream kinases such as LKB1 and/or Ca^{2+}/calmodulin-dependent protein kinase kinase[23,30].

Recent evidence has shown that AMPK activity can also be regulated by physiological or pathological stimuli other than AMP or AMP/ATP ratio, such as vigorous exercise, nutrient starvation, ischemia/hypoxia, or all species of reactive oxygen[7,8,17,31]. In addition, the cellular content of the subunits depends on tissue or cell type[24,28], suggesting a complex regulation of AMPK activity depending on cell type.

Experimental studies in animals have shown that various types of stress induce lymphoid atrophy with immunosuppression[3]. Thymic atrophy is a prominent feature of malnutrition and starvation in both animals and humans[3,6,20]. The mechanism involved in this, however, has yet to be clarified. Robinson *et al.*[21] reported that insulin-induced hypoglycemia increased secretion of glucocorticoids from the adrenal glands by activating the so-called hypothalamic-pituitary-adrenal axis, a major pathway for responding to stress. Glucocorticoids induce apoptosis in thymocytes, particularly in $CD4^+CD8^+$ double-positive cells[1,11]. Stefanelli *et al.*[25] showed that an activator of AMPK, 5-aminoimidazole-4-carboxamide riboside (AICAR), inhibited thymocyte apoptosis induced by glucocorticoids *in vitro*.

To elucidate how AMPK functions in thymocytes under starvation-induced stress, we examined the expression of the isoforms of each of its subunits by quantitative RT-PCR, and of the activated form of AMPK, phospho-AMPKα, by Western blotting using anti-phospho-AMPKα. Expression of AMPK isoforms was also examined in thymocytes cultured in medium containing low or high concentrations of glucose, and in the presence or absence of dexamethasone.

Materials and Methods

1. Animals

All animal experiments were performed in accordance with the Guidelines on Animal Care and Use established by Tokyo Dental College, number 05-15. Male BALB/c mice (6–7 weeks old, weighing 23–24 g each) were purchased from the Charles River Laboratory Japan (Yokohama, Japan). They were housed in a controlled-light (a 12 hr light/12 hr dark cycle) environment and allowed ad libitum access to standard laboratory chow and water. For starvation, food was withdrawn from the cage at onset of the dark cycle for 12 hr or 24 hr, but ad libitum access to water was allowed.

2. Thymocytes culture

Thymocyte suspensions (6×10^6 cells/ml) were prepared from the thymus glands of the control and starved mice in RPMI 1640 with or without glucose (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 10 mM HEPES, and antibiotics[27]. Under the control conditions, the concentration of glucose in the medium in the presence of fetal calf serum was 208 mg/dl. When glucose-free RPMI 1640 was used, the final glucose concentration of the medium was 8 mg/dl in the presence of fetal calf serum. RU486 (Sigma-Aldrich, St. Louis, MO) was preincubated with cells for 30 min at 10 μM before addition of dexamethasone (Sigma-Aldrich) at 1 μM[2].

3. Analysis of thymocyte subpopulation using flowcytometer

Cell subpopulations were analyzed by 2-color flow cytometry using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) with the appropriate PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 monoclonal antibodies (Becton Dickinson).

4. Western blot analysis of AMPK isoforms

Cells were collected, washed with phosphate-

buffered saline and then sonicated in lysis buffer consisting of 10 mM Tris-HCl (pH7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich) at 20 W for 2 min. Samples were centrifuged at 10,000 × g for 20 min and the supernatants used for SDS-polyacrylamide gel electrophoresis. Proteins were separated on 10% SDS-polyacrylamide gel and transferred to Immobilon P membrane (Millipore, Bedford, MA). Membrane was blocked with 2.5% bovine serum albumin (anti-α and anti-phospho α antibodies) or 5% skimmed milk (other antibodies) and incubated with each antibody to each AMPK subunit isoform (1:1000) and anti-actin (1:5000), and detected with horseradish peroxidase-conjugated anti-rabbit IgG antibody using the ECL plus system (GE Healthcare Bio-Science, Piscataway, NJ). Anti-α1 (63kDa) (#27947) and anti-α2 (63kDa) (AF2850) antibodies were purchased from Upstate (Charlotteseillle, VA) and R & D systems (Mineapolis, MN), respectively. Anti-phosphorylated α (63kDa) (#2535S) antibody was purchased from Cell Signaling Technology (Beverly, MA), and anti-β1 (38kDa) (sc-20163), anti-β2 (30kDa) (sc-20164), anti-γ1 (37kDa) (sc-19138), and anti-γ2 (75kDa) (sc-20165) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-γ3 (54kDa) (AP7050c), and anti-actin (45kDa) (A-2066) antibodies were obtained from Abgent (San Diego, CA) and Sigma-Aldrich, respectively. Immunoblots were scanned and the densitometric value of each band was analyzed using the NIH Image (Scion Corp., Frederick, MD).

5. Quantitative RT-PCR

Quantification of AMPK mRNA expression was performed using real-time RT-PCR with the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and SYBR Green Chemistry. Total RNA isolated from the thymocytes using the RNeasy Kit (Qiagen, Hilden, Germany) was used as the template for cDNA synthesis using ReverScript II (Nippon gene, Tokyo, Japan) and random hexamer primers. All samples were analyzed according to the manufacturer's instructions and the data normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as an internal control. PCR primers specific to the isoforms of the AMPK subunits and GAPDH were designed using Primer Express ver. 1.5 software (Applied Biosystems) and were as follows:

5′-TCGGTTTCCTGAATCGAAATG-3′ and 5′-TATGTCCGGTCAACTCGTGC-3′ for *α1* (68 bp), 5′-TCAACCGTTCTGTCGCCAC-3′ and 5′-GACATTCGCATCGTAGGAGG-3′ for *α2* (154 bp), 5′-CACACCTGCTGCAGGTC ATC-3′ and 5′-TTGTACCGGTGTGTTGCAC TG-3′ for *β1* (153 bp), 5′-GGTCCTTAGCGC AACCCAT-3′ and 5′-TTCAAAGGCAAGGGA GATGC-3′ for *β2* (127 bp), 5′-TGTACAGCA CCGAGTCTCCG-3′ and 5′-AGGTCTTTTC GGCTGCCAA-3′ for *γ1* (106 bp), 5′-AGAG GCGGTCACTGCGAGT-3′ and 5′-CCACAT CTCCATCCAGGAGC-3′ for *γ2* (74 bp), 5′-AGAGCCTAGGTGAAGTCATTGACA-3′ and 5′-AGATGGCTTGGGTGTGAGGA-3′ for *γ3* (160 bp), 5′-TGCCCAGAACATCATCCCTG-3′ and 5′-TCAGATCCACGACGGACACA-3′ for *GAPDH* (146 bp). TaqMan probe and primers of *SRG3* mRNA were obtained from Applied Biosystems. PCR conditions included an initial incubation at 50°C for 2 min and at 95°C for 10 min followed by 40 cycles comprising 15 sec at 95°C and 1 min at 60°C. The PCR products were electrophoresed on 2% agarose gel in TBE buffer.

Results

1. Expression of AMPK isoforms mRNA in thymocytes

Figure 1A shows relative expression of other subunit isoforms when expression of α2 was 1.0. Expression of α1 subunit mRNA was the lowest, being less than 4% that of α2, with only trace PCR product being detected on the agarose gels (Fig. 1B). Expression of the β1 and β2 isoforms was approximately 55-fold and 6-fold that of α2, respectively. Expression

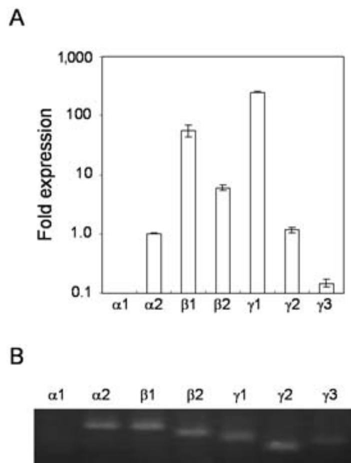

Fig. 1 Expression of AMPK subunit isoforms in mouse thymocytes

A. mRNA expression of major AMPK isoforms in thymocytes was detected by qRT-PCR. Messenger RNA level was normalized using GAPDH expression as an internal control and values expressed are relative to level of $\alpha2$ expression (n-3).

B. qRT-PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

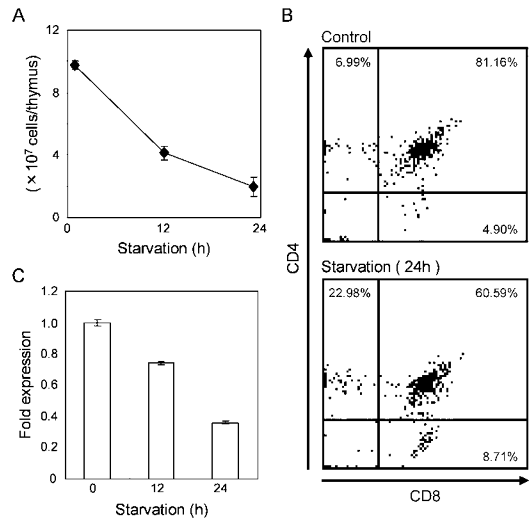

Fig. 2 Effect of starvation on thymocyte number, subpopulation of thymocytes, and expression of *SRG3* mRNA

A. After starvation, thymocyte number in thymus was calculated using Burker-Turk hemocytometer. Data are means ± SD of triplicate experiments.

B. Thymocyte subpopulations were examined at 24 hr starvation by flow cytometry after staining with anti-CD4 and anti-CD8 antibodies.

C. *SRG3* mRNA expression in thymocytes after 12 and 24 hr starvation was analyzed by qRT-PCR and expressed as control (1.0). Data are means ± SD of triplicate experiments.

of the $\gamma1$ isoform was the highest among all the isoforms, at about 247-fold that of $\alpha2$, and $\gamma2$ expression was at a similar level to that of $\alpha2$, as seen in Fig. 1A. The $\gamma3$ subunit was sparsely expressed. The main subunit isoform mRNAs expressed in the thymocytes were, therefore, catalytic $\alpha2$ and regulatory $\beta1$ and $\gamma1$.

2. Thymocyte number and $CD4^+CD8^+$ double-positive cells reduced with starvation

The number of thymocytes in the thymus of the starved mice was about 45% of that in the freely-fed controls after 12 hr starvation and about 21% after 24 hr (Fig. 2A). Flowcytometric analysis of thymocyte subpopulations showed a significant alteration in the relative proportions of thymocytes in the starved mice. Relative percentages of $CD4^+CD8^+$ double-positive thymocytes fell to about 60% after 24 hr starvation, but those of $CD4^+$ or $CD8^+$ single-positive cells showed an approximately 3-fold or 2-fold increase at 24 hr, respectively (Fig. 2B).

3. *SRG3* mRNA expression with starvation

SRG3, the murine homolog of yeast SW13 and human BAF155, is a gene highly expressed in the thymus[10]. It is critical in determining glucocorticoid sensitivity in thymocytes, and is down-regulated in positively selected single-positive cells[11]. To determine the properties of resting thymocytes after starvation, we analyzed the expression of SRG3 in the thymocytes of the control and starved mice. SRG3 expression in the thymocytes of the starved mice was reduced to approximately 75% and 38% at 12 hr and 24 hr, respectively, with starvation (Fig. 2C), indicating that thymocytes resistant to glucocorticoid were still present in the starved mice.

4. Expression of AMPK mRNAs and proteins in thymocytes with starvation

To determine whether AMPK expression was altered by starvation-induced stress,

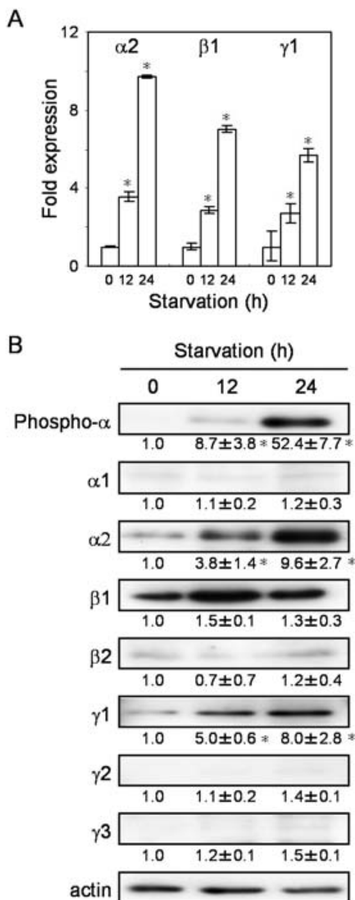

Fig. 3 Effect of starvation on AMPK expression in mouse thymocytes

Thymocytes of control and starved mice were prepared and expression of mRNA (A) and protein (B) of AMPK subunit isoforms was determined by qRT-PCR and Western blotting, respectively. Expression was normalized with GAPDH expression and relative mRNA level is shown from initial level at 0 hr (A). Data are means ± SD of three experiments. Protein expression was normalized to expression of β-actin, and relative protein levels to that at 0 hr are shown. Data are means ± SD of three independent experiments. Data were analyzed with a one-way-ANOVA and differences from level in 0 hr starvation were found to be statistically significant (*$p<0.05$).

mRNA expression of the main subunits, $\alpha 2$, $\beta 1$ and $\gamma 1$, was examined by qRT-PCR at 12 hr and 24 hr starvation. All of these subunits increased approximately 3–3.6-fold and 6–11-fold at 12 hr and 24 hr starvation, respectively (Fig. 3A). Expression of $\alpha 1$ mRNA was extremely low in both the control and starved mice, being approximately 4% that of $\alpha 2$, and showed no change with starvation. Expression of $\gamma 2$ mRNA showed an increase with starvation time (data not shown), although this level was much lower than that of $\gamma 1$ as shown in Fig. 1.

The proteins of the AMPK subunits and phospho-α subunit were detected by Western blotting (Fig. 3B). Alpha1 protein was scarcely detected using anti-$\alpha 1$ antibody reflecting low expression of its mRNA (Fig. 1), and almost no change with starvation was observed (Fig. 3B). Alfa 1 protein did not changed with starvation, but increased $\alpha 2$ subunit protein was observed with starvation in parallel with its highly increased expression of mRNA (Fig. 3B). Highly increased phosphorylation of the α subunit was observed with starvation. Beta 1 protein was expressed and increased with starvation slightly, while no significant change was seen in $\beta 2$ protein. While $\gamma 1$ protein was also expressed and increased with starvation, no significant change was observed in the other γ isoforms compared to $\gamma 1$.

5. Effect of glucose concentration on expression of AMPK mRNA and protein in cultured thymocytes

With starvation, blood glucose levels decreased to about 44%, and corticosterone levels increased by about 2.6-fold at 24 hr in mice[9]. In this study, to elucidate the underlying mechanism of increased AMPK expression and activation with starvation, we investigated the effects of glucose and dexamethasone in cultured thymocytes. When the thymocytes were incubated in a low glucose medium (8 mg/dl glucose), mRNA expression of the major isoforms did not alter after 2 hr (Fig. 4A). Expression of the $\alpha 2$ subunit isoform mRNA and protein did not alter, even after 24 hr under both low and high glucose conditions, but phospho-α subunit increased time-dependently under low glucose conditions revealing approximately 25-fold increase after 2 hr incubation (Fig. 4B). Neither mRNA nor protein of the $\alpha 1$ subunit isoform altered after 2 hr. This suggests that low glucose does not induce AMPK mRNA or protein expression, but that this enzyme is

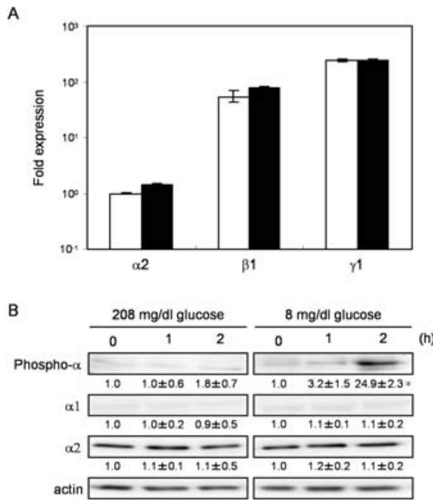

Fig. 4 Effect of glucose concentration in culture medium on expression of AMPK and phosphorylation of AMPKα

Thymocytes were cultured in medium with low glucose concentration (8 mg/dl glucose, closed bar) or in control medium with glucose at 208 mg/dl (open bar).

A. Expression of AMPK mRNA at 2 hr incubation was analyzed by qRT-PCR and values expressed are relative to control at 0 hr. Data are means ± SD of triplicate experiments.

B. AMPKα and phosphorylated AMPKα were analyzed by Western blotting after 1 and 2 hr incubation. Protein expression was normalized to expression of β-actin, and relative protein levels to that at 0 hr are shown. Data are means ± SD of three experiments. Data were analyzed with a one-way-ANOVA and differences from level in control medium were found to be statistically significant (*p<0.05).

activated by phosphorylation under low glucose conditions.

6. Effects of dexamethasone on expression of AMPK mRNA and protein in cultured thymocytes

In the presence of dexamethasone at 1 μM, mRNA expression of the main AMPK isoforms, α2, β1 and γ1, increased time-dependently by about 2–13-fold at 1 hr incubation and 4–11-fold at 2 hr incubation (Fig. 5A). Expression of the main AMPK isoform mRNAs increased dose-dependently (Fig. 5B). Expression of the α1 protein rather decreased slightly, but the α2 protein expression increased by approximately 3.7-fold and 25.6-fold at 1 hr and 2 hr, respectively with incubation time (Fig. 5C). However, dexamethasone did not affect the

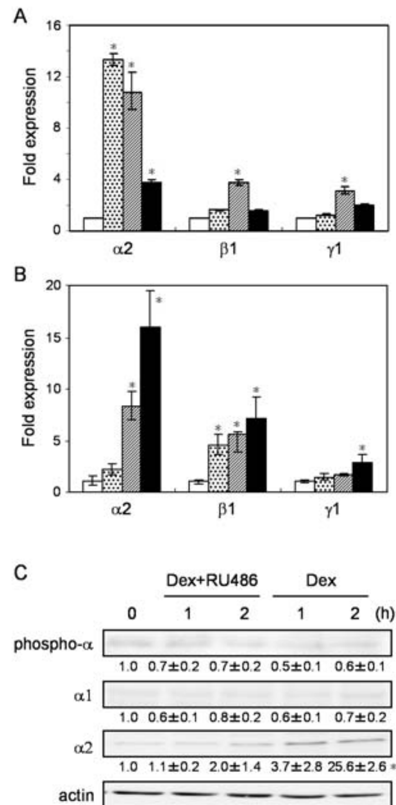

Fig. 5 Effect of dexamethasone on expression of AMPK and phosphorylation of AMPKα

A. Thymocytes were incubated in presence of dexamethasone at 1 μM for 1 (dotted bar) and 2 hr (striated bar), and expression of AMPK mRNA was determined by qRT-PCR. RU486 was preincubated at 10 μM for 30 min before addition of dexamethasone and thymocytes were incubated for additional 2 hr (closed bar). Values of mRNA expression are relative to control at 0 hr (open bar), and data are means ± SD of three experiments. Data were analyzed with a one-way-ANOVA and differences from level in control at 0 hr were found to be statistically significant (*p<0.05).

B. Thymocytes were incubated without (control; open bar) or with 0.01 μM (dotted bar), 0.1 μM (striated bar) or 1 μM (closed bar) of dexamethasone for 2 hr. Values of mRNA expression are relative to the control, and data are means ± SD of three experiments. Data were analyzed with a one-way-ANOVA and differences from level in control at 0 hr were found to be statistically significant (*p<0.05).

C. AMPKα and phospho-AMPKα were analyzed by Western blotting at 1 and 2 hr after addition of dexamethasone in presence or absence of RU486 at 10 μM. Protein expression was normalized to expression of β-actin, and relative protein levels to that at 0 hr are shown. Data are means ± SD of three experiments. Data were analyzed with a one-way-ANOVA and differences from level in control at 0 hr were found to be statistically significant (*p<0.05).

level of the phospho-α subunit. Induction of AMPK mRNA and protein expression was inhibited in the presence of RU 486 (Fig. 5A and C). These results suggest that dexamethasone is partly involved in the induction of AMPK mRNA expression through glucocorticoid receptors.

Discussion

AMPK is a ubiquitously expressed multisubstrate serine/threonine protein kinase which functions as an intracellular fuel sensor activated by depletion of high energy phosphor compounds[7,14]. The present study showed that AMPK was expressed in mouse thymocytes and that the major isoforms were $\alpha 2$, $\beta 1$ and $\gamma 1$. The relative expression of catalytic subunits between $\alpha 1$ and $\alpha 2$ mRNAs is different among tissues, with $\alpha 2$ being predominant in skeletal muscle, although both isoforms were detected in rat liver[24,28]. In this study on mouse thymocytes, the mRNA and protein of the $\alpha 1$ subunit were scarcely detected and showed no change with starvation. Expression of $\alpha 1$ mRNA was detected in mouse skeletal muscle and kidney by using the same primer sets, and low expression of the $\alpha 1$ mRNA in thymocytes was confirmed using other primer sets. Stefanelli *et al.*[25] found $\alpha 1$, but not $\alpha 2$, expression in rat thymocytes. However, we found that expression of $\alpha 2$ mRNA and protein was lowest in mouse thymocytes among mouse tissues examined, at approximately 20–30% of $\alpha 2$ in kidney, heart and skeletal muscle (R. Okoshi, unpublished data). It has been shown that expression of $\alpha 1$ and $\alpha 2$ differs among tissues and species[25,28]. The $\alpha 1$ isoform protein showed no predominant change with starvation (Fig. 3), but the $\alpha 2$ isoform increased with starvation, suggesting that the $\alpha 2$ is the main catalytic subunit responding to starvation stress in mouse thymocytes.

AMPK functions as a heterotrimer, and the contribution made by the relative amount of each catalytic and regulatory subunit isoform may be related to the tissue-specific function of AMPK. It has been found to function as a regulator of appetite in the hypothalamus, increase glucose transport in skeletal muscle, and inhibit lipogenesis in adipose tissue and liver[7,14]. However, the reason for higher levels of regulatory $\beta 1$ and $\gamma 1$ subunits than catalytic subunit $\alpha 2$ in thymocytes has yet to be determined.

The thymus is an important immune center in T lymphocyte production, maturation, and selection and AMPK is known to be dispensable for immune cell development and function[18]. Thymocyte apoptosis plays a key role in the ontogeny of T lymphocytes[2]. In this study, thymocyte number in the thymus was about 45% that of the initial level after 12 hr starvation, decreasing to about 20% after 24 hr (Fig. 2A). The relative percentage of $CD4^{+}CD8^{+}$ double-positive cells was about 60% at 24 hr (Fig. 2B), revealing an approximately 75% reduction in double-positive cells.

Starvation is accompanied by significant metabolic and endocrine changes that could potentially contribute to lymphoid atrophy. Starvation induced thymic atrophy with increased expression of AMPK mRNA and protein, together with activation of AMPK, namely increased phospho-AMPKα. Starvation is accompanied by hypoglycemia and hypercorticosteronemia in mice[9]. In this study, incubation of thymocytes in low glucose medium did not induce AMPK mRNA expression, but did increase profoundly the amount of phospho-AMPKα. Dexamethasone did not increase the level of the phospho-α subunit, but did induce significantly higher expression of $\alpha 2$ subunit isoform mRNA. Ru486, however, inhibited expression induced by dexamethasone. Transcriptional regulation of AMPK expression remains to be clarified, although the present results suggest that it is regulated, in part, by glucocorticoids. Indeed, DNA data base analysis of the $\alpha 2$, $\beta 1$ and $\gamma 1$ genes showed the presence of 2–3 glucocorticoid-responsive elements in 1 kb upstream regions of the genes. Therefore, the high expression of AMPK in the thymocytes of the starved mice in this study was probably caused by hypercorticosteronemia, and the increased

amount of phospho-AMPKα by hypoglycemia. The markedly reduced number of double-positive cells (Fig. 2B) that were glucocorticoid-sensitive and the reduced expression of *SRG3* mRNA, which is a critical determinant of glucocorticoid sensitivity, also suggest hypercorticosteronemia (Fig. 2C)[12]. Other endocrine alterations have been reported in starved mice such as suppression of gonadal, growth and thyroid hormones[22], leptin[16], and also other cytokines[26]. However, in this study, we found that leptin had no effect on expression of AMPK or the phospho-α subunit (data not shown). In addition to glucocorticoids, endocannabinoides and ghrelin, which are involved in appetite control during fasting, have been shown to modulate AMPK activity[13]. At present, the effects of these compounds on the immune system during starvation are not known.

Although transcriptional regulation of AMPK expression remains to be clarified, we found that glucocorticoids induced expression. The results of this study suggest that AMPK in thymocytes acts as a glucocorticoid-responsive stress enzyme. Stefanelli *et al.*[25] reported that AMPK activation by AICAR inhibited glucocorticoid-induced thymocyte apoptosis in rat. However, we found that AICAR inhibited thymocyte apoptosis, even in the presence of inhibitors of nucleoside transporter and adenosine kinase (K. Ohta, unpublished data). Lopez *et al.*[15] showed that AICAR inhibited apoptosis in Jurkat cells by a mechanism other than AMPK activation. In addition, it has been reported that sustained activation of AMPK induced apoptosis in liver cells[19]. Therefore, whether expression and activation of AMPK in thymocytes helps the immune system to avoid dysfunction under starvation conditions or exerts a toxic influence on the immune cells by responding to the action of glucocorticoid, remains to be clarified.

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