Evidence for organic cation transporter-mediated metformin transport and 5′-adenosine monophosphate-activated protein kinase activation in rat skeletal muscles

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ABSTRACT

Objective: 5′-adenosine monophosphate-activated protein kinase (AMPK) is a key molecule of metabolic enhancement in skeletal muscle. We investigated whether metformin (MET) acts directly on skeletal muscle, is transported into skeletal muscle via organic cation transporters (OCTs), and activates AMPK.

Materials/Methods: Isolated rat epitrochlearis and soleus muscles were incubated *in vitro* either in the absence or in the presence of MET. The activation status of AMPK, the intracellular energy status, and glucose and MET transport activity were then evaluated. The effect of cimetidine, which is an OCT inhibitor, on AMPK activation was also examined.

Results: MET (10 mmol/L, ≥ 60 min) increased the phosphorylation of Thr^{172} at the catalytic α subunit of AMPK in both muscles. AMPK activity assays showed that both AMPKα1 and AMPKα2 activity increased significantly. The AMPK activation was associated with energy deprivation, which was estimated from the ATP, phosphocreatine (PCr), and glycogen content, and with increased rates of 3-*O*-methyl-D-glucose (3MG) transport. MET did not change the basal phosphorylation status of insulin receptor signaling molecules. MET was transported into the cytoplasm in a time-dependent manner, and cimetidine suppressed MET-induced AMPK phosphorylation and 3MG transport.

Conclusion: These results suggest that MET is acutely transported into skeletal muscle by OCTs, and stimulates AMPKα1 and α2 activity in both fast- and slow-twitch muscle types, at least in part by reducing the energy state.

Keywords: Metformin, 5′-adenosine monophosphate-activated protein kinase, Organic cation transporter, Glucose transport, Skeletal muscle

Abbreviations: MET, metformin; AMPK, 5′-adenosine monophosphate-activated protein kinase; OCT, organic cation transporter; PCr, phosphocreatine, T2DM, type 2 diabetes mellitus; KRB, Krebs–Ringer bicarbonate buffer; DNP, 2,4-dinitrophenol; AICAR: 5-aminoimidazole-4-carboxamide-1-α-D-ribonucleoside; ACC, acetyl CoA carboxylase; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal protein S6 kinase; 4E-BP1, eukaryotic initiation factor 4-binding protein 1; GSK, glycogen synthase kinase; IRS, insulin receptor substrate; 3MG,3-*O*-methyl-D-glucose; SE, standard error; EDL, extensor digitorum longus.

1. Introduction

Metformin (MET) is the most commonly prescribed medication in the world for type 2 diabetes mellitus (T2DM) patients, and treatment with MET has reduced the incidence of T2DM in the Diabetes Prevention Program [1]. MET is known to lower the blood glucose level mainly through hepatic glucose output as a result of increased hepatic insulin sensitivity [2]. The molecular mechanism of MET action is not fully understood, but Zhou et al. [3] demonstrated in 2001 that MET stimulates the "metabolic regulator" 5′-adenosine monophosphate-activated protein kinase (AMPK) with decreased glucose production in primary cultured rat hepatocytes. Since then, a number of studies have documented MET activation of hepatic AMPK. However, although skeletal muscle has been implicated in the antidiabetic effect of MET [2], only a few studies have examined skeletal muscle AMPK. Zhou et al. [3] found that MET (2 mmol/L, 3 h) stimulated AMPKα1 and α2 activity and glucose transport in isolated rat fast-twitch epitrochlearis muscle, but the effects of MET on slow-twitch muscle and on muscle energy status were not examined. Musi et al. [4] showed that 10 weeks of MET treatment increased AMPKα2 activity and decreased ATP, phosphocreatine (PCr), and glycogen content in vastus lateralis muscle biopsies from T2DM patients, but neither the acute effect of MET administration on AMPK nor the energy status was studied. Suwa et al. [5] and Kristensen et al. [6] showed that a single oral dose of MET promoted AMPK phosphorylation in multiple muscle types in rats [5] and mice [6], but they did not examine AMPK activity or energy status. Furthermore, Kristensen et al. [6] did not detect any increase in glucose transport in skeletal muscles.

MET has been identified as a substrate of organic cation transporters (OCTs). MET uptake in the liver is mediated by OCT1 [7-9], and deletion of *Oct1* decreased both MET-stimulated AMPK phosphorylation and inhibition of glucose production in mouse hepatocytes [8]. Moreover, reduced-function alleles of *OCT1* have been associated with a reduction in the effect of MET on the oral glucose tolerance test in humans [8]. On the other hand, skeletal muscle expresses OCT1 and OCT3 [10, 11], and the general OCT inhibitor cimetidine was reported to block MET-stimulated AMPK phosphorylation in primary cultured human skeletal muscle cells [11]. However, to our knowledge, no study has tested the ability of mature skeletal muscle to take up MET into the cytoplasm.

We conducted this study to reevaluate the hypothesis that MET acts directly on different muscle types, and that it can activate both AMPK and glucose transport under reduced energy status. We also examined the possibility that MET is acutely transported into skeletal muscle via OCTs.

2. Materials and Methods

2.1. Animals

Male Wistar rats aged 5 weeks (Shimizu Breeding Laboratories, Kyoto, Japan) were fed a standard chow and water *ad libitum*. The animals were randomly divided into the experimental groups after an overnight fast. Experimental protocols were approved by Kyoto University Graduate School of Human and Environmental Studies, and Kyoto University Radioisotope Research Center.

2.2. Muscle treatment *in vivo*

Rats were injected with MET (250 mg/kg body wt) dissolved in saline into the peritoneal cavity without anesthesia. The volume of injection was 10 ml/kg body wt. Control rats were injected with saline. Forty five minutes after MET or saline injection, rats were anesthetized with injection of 40 mg/kg pentobarbital sodium into the peritoneal cavity. Fifteen minutes after anesthesia the blood glucose level was measured from the cut tail tip using Glutest-Ace (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Then the fast-twitch epitrochlearis muscle [12] and slow-twitch soleus muscle [13] were rapidly isolated and frozen in liquid nitrogen. The muscles were then assayed for isoform-specific AMPK activity.

2.3. Muscle preparation *in vitro*

Muscle incubation was performed as we previously described [14]. Epitrochlearis and soleus muscles were isolated immediately after cervical dislocation. The muscles were then attached to an incubation apparatus, and preincubated in Krebs–Ringer bicarbonate buffer (KRB) containing 2 mmol/L pyruvate for 40 min. The muscles were transferred to fresh buffer either in the absence or presence of 2 or 10 mmol/L MET for up to 120 min. The muscles were also incubated in fresh buffer containing 0.5 mmol/L 2,4-dinitrophenol (DNP) for 15 min or 2 mmol/L 5-aminoimidazole-4-carboxamide-1-α-D-ribonucleoside (AICAR) for 30 min for maximal stimulation of AMPK, or 1 μmol/L insulin for 30 min for maximal stimulation of Akt. The OCT inhibitor cimetidine [11], when present, was added in the preincubation and incubation buffer. The concentration of dimethyl sulfoxide was 0.5%, which had no effect in any assay. The muscles were either used for MET transport and glucose transport measurements, or frozen in liquid nitrogen for subsequent analysis. Some muscles, liver, kidney, and heart were frozen after dissection for Western blotting of OCTs.

2.4. Western blotting

Western blotting was conducted as we previously described [14]. Frozen muscles were homogenized in homogenization buffer containing 1% Triton X-100, 20 mmol/L Tris·HCl (pH 7.4), 250 mmol/L sucrose, 50 mmol/L NaCl, 2 mmol/L dithiothreitol, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 50 mg/L trypsin inhibitor, 4 mg/L leupeptin, 0.1 mmol/L benzamidine, and 0.5 mmol/L phenylmethylsulfonyl fluoride at 4°C. The homogenate was centrifuged at 16,000 g for

40 min at 4°C. Aliquots of the supernatant (15 μg protein) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes. The membranes were blocked with skim milk, and then incubated overnight at 4°C with primary antibodies [AMPKα Thr^{172}, AMPKα, acetyl CoA carboxylase (ACC), mammalian target of rapamycin (mTOR) Ser^{2448}, mTOR, p70 ribosomal protein S6 kinase (p70S6K) Thr^{389}, p70S6K, eukaryotic initiation factor 4-binding protein 1 (4E-BP1) $Thr^{37/46}$, 4E-BP1, Akt Ser^{473}, Akt, glycogen synthase kinase (GSK)-3β Ser^{9}, GSK-3β, actin (Cell Signaling Technology, Danvers, MA), insulin receptor substrate (IRS)-1 Tyr^{612} (Life Technologies, Carlsbad, CA), ACC Ser^{79}, IRS-1 (Millipore, Billerica, MA), GLUT4 glucose transporter (Biogenesis, Poole, UK), OCT1, OCT2, OCT3 (Santa Cruz Biotechnology, CA)]. The membranes were then incubated with anti-rabbit IgG or anti-goat IgG. Protein signals were developed using enhanced chemiluminescence (Millipore) and detected with ImageCapture G3 (Liponics, Tokyo, Japan).

2.5. Isoform-specific AMPK activity assay

Skeletal muscle expresses two catalytic subunits of AMPK, α1 and α2 [15]. The α-isoform-specific kinase activity was determined as we previously described [14]. Frozen muscles were homogenized as described in "Western blotting." The supernatant (100 μg protein) was incubated with either anti-AMPKα1 or α2 antibody and Protein A Sepharose beads (Amersham Biosciences, Uppsala, Sweden) at 4 °C overnight. The beads were then subjected to kinase reaction using the SAMS peptide as substrate. The kinase activity was expressed as incorporated ATP per min per immunoprecipitated protein.

2.6. MET transport and glucose transport assay

The MET transport was measured using the double-isotope ([^{14}C]MET and D-[1-^{3}H(N)]mannitol) method. Muscles were preincubated, and incubated in KRB containing 10 mmol/L [^{14}C]MET (0.3 μCi/ml, American Radiolabeled Chemicals, St. Louis, MO) and 1 mmol/L D-[1-^{3}H(N)]mannitol (1.5 μCi/ml, American Radiolabeled Chemicals) at 37°C for up to 60 min. Muscles were then digested in 1 mol/L NaOH at 80°C for 10 min. Digestates were neutralized with 1 mol/L HCl and centrifuged at 20,000 g for 3 min, and radioactivity in aliquots of supernatant was measured by a scintillation counter. The intracellular space was determined as described [16], and the intracellular MET concentration was calculated. The 3-*O*-methyl-D-glucose (3MG) transport was also measured using the double-isotope ([^{3}H]3MG and D-[1-^{14}C]mannitol) method, as we previously described [14]. The transport activity was expressed as 3MG taken up per intracellular space per hour.

2.7. ATP, PCr, and glycogen assay

ATP and PCr contents were measured enzymatically as we previously described [17]. Glycogen content was measured using a glucose assay reagent (Glucose CII Test , Wako, Osaka, Japan) as described previously [18].

2.8. Statistical analysis

Data are expressed as mean ± standard error (SE). Differences between two groups were compared with Student's t-test. Multiple means were analyzed using one-way ANOVA followed by post hoc comparison with Tukey's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Intraperitoneal MET administration increases AMPKα1 and α2 activity in rat skeletal muscles

To confirm that MET acutely increases AMPK activity *in vivo*, α-isoform-specific AMPK activity was measured 60 min after intraperitoneal injection of either MET (250 mg/kg) or saline. MET increased AMPKα1 activity by 1.2- and 1.6-fold and AMPKα2 activity by 1.4- and 1.3-fold in epitrochlearis (Fig. 1A) and soleus (Fig. 1B) muscles, respectively. Blood glucose levels 60 min after MET injection (2.8 ± 0.1 mmol/L, n=6) were significantly lower than those after saline injection (4.7 ± 0.2 mmol/L, n=7, $P < 0.001$ vs. MET group).

3.2. MET promotes AMPKα Thr^{172} phosphorylation and stimulates both AMPK α1 and α2 activity in isolated rat skeletal muscles

To verify that MET acts directly on skeletal muscle, isolated muscles were incubated *in vitro* to avoid confounding effects of humoral, circulatory, gastrointestinal, and neuronal factors. The time-course data revealed that phosphorylation of AMPKα Thr^{172} increased 60 and 120 min after exposure to 10 mmol/L MET in epitrochlearis (Fig. 2A) and soleus (Fig. 2B) muscles, respectively. The total amount of AMPK did not change during the study. The dose–response data showed that 2 mmol/L MET did not significantly promote phosphorylation of AMPKα Thr^{172} (data not shown). The α-isoform-specific AMPK activity assay showed that MET (10 mmol/L, 120 min)

increased AMPKα1 activity by 4.3- and 3.4-fold, and AMPKα2 activity by 1.6- and 2.9-fold in epitrochlearis (Fig. 2C) and soleus (Fig. 2D) muscles, respectively. The increase of AMPKα1 activity was significantly greater than that of AMPKα2 in epitrochlearis muscle ($P < 0.001$) (Fig. 2C). MET (10 mmol/L, 120 min) also increased the extent of phosphorylation of ACC Ser^{79} in epitrochlearis (Fig. 2E) and soleus (Fig. 2F) muscles. The phosphorylation of ACC Ser^{79} reflects the extent of intracellular activation of AMPK in skeletal muscle [19, 20]. Known AMPK activators DNP and AICAR elicited moderate to robust phosphorylation of AMPK and ACC (Figs. 2E and 2F).

3.3. MET is transported into isolated rat skeletal muscles via OCTs

To our knowledge, no study has shown that MET is transported into skeletal muscle. We examined the OCT protein expression levels by Western blot analysis, and clearly detected OCT1 and OCT3 protein in epitrochlearis, soleus, and fast-twitch extensor digitorum longus (EDL) muscles (Fig. 3A). In contrast, OCT2 protein was not detectable in any of the muscle types (data not shown), which is consistent with previous studies showing no detectable expression of OCT2 in skeletal muscle [10, 11]. We found that MET was taken up into both epitrochlearis (Fig. 3B) and soleus (Fig. 3C) muscles in a time-dependent manner. Furthermore, simultaneous incubation with cimetidine suppressed the MET-induced phosphorylation of AMPKα Thr^{172} in epitrochlearis (Fig. 3D) and soleus (Fig. 3E) muscles. The total amount of AMPK did not change during the study.

3.4. MET decreases energy status and stimulates glucose transport in isolated rat skeletal muscles

AMPK is implicated in the mechanism of insulin-independent glucose transport, which is elicited by energy-depriving stimuli including hypoxia, inhibition of oxidative phosphorylation, and contraction [21, 22]. MET inhibits complex 1 of the mitochondrial respiratory chain and impairs oxidative phosphorylation [23, 24]. To determine the energy status of skeletal muscle, we measured ATP, PCr, and glycogen content. MET (10 mmol/L, 120 min) significantly decreased these parameters in both epitrochlearis and soleus muscles (Table 1). Correspondingly, MET (10 mmol/L, 120 min) increased 3MG transport activity 2.4- and 1.7-fold above the basal level in epitrochlearis (Fig. 4A) and soleus (Fig. 4B) muscles, respectively. In parallel with the MET-induced AMPK phosphorylation (Figs. 3D and 3E), MET-induced 3MG transport activity was suppressed by cimetidine in both epitrochlearis (Fig. 4A) and soleus (Fig. 4B) muscles.

3.5. MET does not affect insulin receptor signaling molecules in isolated rat skeletal muscles

We examined whether MET affects the activation status of insulin receptor signaling molecules in skeletal muscle. Similar to the known AMPK activators (DNP, AICAR), MET (2 mmol/L, 120 min) did not change the phosphorylation of IRS-1, Akt,

GSK-3β, mTOR, p70S6K, or 4E-BP1 in either epitrochlearis (Fig. 5A) or soleus (Fig. 5B) muscles. GLUT4 content was not affected by MET in either muscle type.

4. Discussion

Growing evidence suggests that AMPK is involved in acute and chronic metabolic activation processes in skeletal muscle, such as insulin-independent glucose transport, fatty acid oxidation, glycogen regulation, expression of glucose transporter GLUT4, activation of peroxisome proliferator-activated receptor γ coactivator 1α, mitochondrial biogenesis, and enhanced insulin sensitivity [25, 26]. Exercise (muscle contraction) is a strong activator of AMPK in skeletal muscle, but recent reports suggest that there are a number of physiologically relevant stimuli of AMPK in skeletal muscle, such as adipokines including leptin and adiponectin, antidiabetic drugs such as MET and thiazolidinediones, and functional foods and their natural components [25, 27, 28]. In our earlier studies, we demonstrated that *Morus alba* leaf extract [29], caffeine [17], berberine [30], *Coptidis rhizoma* extract [31], and caffeic acid [32], all of which have been associated with antidiabetic properties, acutely (<30 min) stimulate AMPK activity in isolated rat skeletal muscles.

We examined the effects of MET in different types of skeletal muscle. Bikman et al. [33] reported that chronic oral administration of MET for 4 weeks increased AMPK and ACC phosphorylation in glycolytic muscle (white gastrocnemius), but not in oxidative muscles (soleus and red gastrocnemius) in lean and obese Zucker rats. On the other hand, Kristensen et al. [6] showed that oral treatment with MET for 2 weeks induced significant metabolic activation (increased insulin-stimulated glucose transport) in soleus but not in EDL muscle in mice. In the present study, we found stimulation of AMPK that was not specific to muscle type, indicating that most skeletal

muscles respond to MET *in vivo*. However, we found that MET elicits greater activation of AMPKα1 (by 4.3-fold) than AMPKα2 (by 1.6-fold) in isolated rat epitrochlearis muscle (Fig. 2C). Zhou et al. [3] also reported that MET treatment (2 mmol/L, 3 h) stimulated AMPKα1 activity (by approximately 2.5-fold) to a greater extent than AMPKα2 (by approximately 1.5-fold) in isolated rat epitrochlearis muscle. In contrast, MET increased AMPKα1 activity to a similar extent to that of AMPKα2 in soleus muscle (Fig. 2D). Thus, the magnitude of the effect of MET may vary depending on the muscle type.

We have found that the isolated skeletal muscle preparation is a useful tool with which to elucidate the actual effect of MET on AMPK. AMPKα2 activity accounts for the major part (70%–80%) of basal AMPK activity in rat skeletal muscle, with AMPKα1 making up the remaining 20%–30% [34]. The α2-isoform is also the major α-isoform in biopsied human vastus lateralis muscle [35]. Importantly, AMPK, with the α1 isoform in particular, is easily activated during dissection as a postmortem artifact [36]. Even when actual AMPK activity is increased *in vivo*, it may be exceeded by additional activation of AMPK during dissection. Therefore, sufficient preincubation is required to stabilize AMPK to the basal level [36]. It is also noteworthy that, in contrast to mature muscle, α1 is the predominant α-isoform, and α2 activity is minimal in cultured muscle cells including L6 [37].

The physiological significance of AMPKα1, the minor isoform in skeletal muscle, has been documented in contraction-stimulated glucose transport. Jensen et al. [38] investigated the impact of low-intensity contractions, and demonstrated that glucose transport was activated in soleus muscle of AMPKα2-knockout mice but not in soleus muscle of AMPKα1-knockout and AMPK kinase-dead (KD) mice. Jørgensen et al.

[39] investigated high-intensity contractions, and showed that glucose transport activity was significantly reduced in the soleus muscle of AMPKα1-knockout mouse. These observations indicate that AMPKα1 is relevant for the activation of glucose transport by low-intensity contractions, and is essential for additional activation of glucose transport by high-intensity contractions. The importance of AMPKα1 has also been suggested by our earlier studies, which showed that oxidative stress (H_2O_2, hypoxanthine/xanthine oxidase) [14], low-intensity contraction [36], and the presence of caffeine in low concentration (1 mmol/L) [40] increased AMPKα1 activity and insulin-independent glucose transport without AMPKα2 activation in isolated epitrochlearis muscle.

We have recently reported that caffeine (3 mmol/L, 15 min) stimulated AMPK and also dephosphorylated insulin receptor signaling including IRS-1 (Tyr^{612}), Akt (Ser^{473}), and GSK3β (Ser^{9}) in isolated rat epitrochlearis muscle [41]. In contrast, *Morus alba* leaf extract [29], berberine [30], and caffeic acid [32] acutely stimulated AMPK, but they did not affect Akt Ser^{473} phosphorylation in isolated rat epitrochlearis muscle. In the present study, MET did not change the phosphorylation status of insulin signaling molecules in either muscle type (Figs. 5A and B). MET may not have a modulatory effect on these molecules, at least in the absence of insulin and during the acute time period.

We showed that MET is transported into skeletal muscle and that within 60 min the concentration of MET was comparable with that of incubation buffer (10 mmol/L) (Figs. 3B and C). It is notable that a typical clinical dose of MET is 1000–2000 mg/day, and peak plasma concentrations of MET are about 3 mg/L (2.5 μmol/L) after an oral dose of 1000–1500 mg in humans [42]. Thus, it is unlikely that a single dose of MET

is sufficient to provoke clinically relevant activation of AMPK in human skeletal muscle. As demonstrated by Musi et al. [4], who found significantly increased AMPKα2 activity and decreased ACC activity as well as reduced energy status in biopsied muscle samples from T2DM after 10-week treatment, long-term MET administration might be needed.

Our data provide fundamental evidence that confirms the stimulatory actions of MET on AMPK signaling. MET acutely increased AMPKα Thr^{172} phosphorylation and the activities of both AMPKα1 and α2, in association with decreased ATP, PCr, and glycogen levels. The enhanced levels of phosphorylation of cellular substrate ACC and increased levels of glucose transport indicated a substantial activation of AMPK *in vivo*. MET was transported into the cytoplasm, and the stimulatory effects of AMPK phosphorylation and glucose transport were inhibited by cimetidine. All these effects occurred in both fast-twitch epitrochlearis and slow-twitch soleus muscles. In conclusion, we propose that MET is transported into skeletal muscle by OCTs, and that it stimulates AMPKα1 and α2 activity under reduced energy state in multiple muscle types.

Author contributions

RO and TH designed the experiments; RO, MY, EK, YO, YS, ST, XM and TE performed experiments; RO, MY and TH analyzed data; RO and TH wrote the manuscript.

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Conflict of Interest

The authors state no conflict of interest.

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Figure and table legends

Fig. 1　A single intraperitoneal injection of MET acutely increases AMPK activity in skeletal muscles.

Epitrochlearis (A) and soleus (B) muscles were removed 60 min after MET (250 mg/kg) or saline administration. AMPK activity was measured in anti-α1 and anti-α2 immunoprecipitates. Values are means ± SE; n=5–8. * $P < 0.05$ and *** $P < 0.001$ vs. control group.

Fig. 2　MET activates AMPK in isolated skeletal muscles.

Epitrochlearis (A) and soleus (B) muscles were incubated with MET (10 mmol/L) for 0 (control), 15, 30, 60, or 120 min. The lysate was analyzed by Western blotting. The values are means ± SE; n=5–7. *$P < 0.05$ and **$P < 0.01$ vs. control. Epitrochlearis (C) and soleus (D) muscles were incubated in the absence (control) or presence of 10 mmol/L MET for 120 min. The lysate was subjected to α-isoform-specific AMPK activity assay. Values are means ± SE; n=13–21. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. Epitrochlearis (E) and soleus (F) muscles were incubated in the absence (control) or presence of MET (10 mmol/L) for 120 min, AICAR (2 mmol/L) or insulin (1 μmol/L) for 30 min, or DNP (0.5 mmol/L) for 15 min. The lysate was analyzed by Western blotting. Representative immunoblots are shown.

Fig. 3　MET is transported into isolated skeletal muscles, and the OCT inhibitor cimetidine suppresses MET-stimulated AMPK phosphorylation.

(A) Epitrochlearis (EPI), soleus (SOL), and extensor digitorum longus (EDL) muscles were dissected and analyzed by Western blotting. Representative immunoblots are shown. Epitrochlearis (B) and soleus (C) muscles were incubated with 10 mmol/L MET for 30 or 60 min. Intracellular MET concentration was determined at each time point. Values are means ± SE; n=6–7. *** $P < 0.001$ vs. 0-min group. ### $P < 0.001$ vs. 30-min incubation group. Epitrochlearis (D) and soleus (E) muscles were incubated in the absence (control) or presence of 10 mmol/L MET for 120 min either with or without cimetidine (C: 0.1, 1.0, or 10 mmol/L). The lysate was analyzed by Western blotting. Representative immunoblots are shown.

Fig. 4　The OCT inhibitor cimetidine suppresses MET-stimulated glucose transport activity in isolated skeletal muscles.

Epitrochlearis (A) and soleus (B) muscles were incubated in the absence (control) or presence of 10 mmol/L MET for 120 min with or without cimetidine (C: 10 mmol/L), and the rate of 3MG transport was measured. Values are means ± SE; n=7–10. *** $P < 0.001$ vs. control group. # $P < 0.05$ and ### $P < 0.001$ vs. MET group.

Fig. 5　MET does not affect insulin signaling in isolated skeletal muscles.

Epitrochlearis (A) and soleus (B) muscles were incubated in the absence (control) or presence of MET (10 mmol/L) for 120 min, DNP (0.5 mmol/L) for 15 min, or AICAR (2 mmol/L) or insulin (1 μmol/L) for 30 min. The lysate was analyzed by Western blotting. Representative immunoblots are shown.

Table 1

ATP, PCr, and glycogen content in isolated skeletal muscles. Epitrochlearis (EPI) and soleus (SOL) muscles were incubated in the absence (control) or presence of 10 mmol/L MET for 120 min. ATP, PCr, and glycogen content were determined. Values are expressed as nanomoles per milligram wet weight. Values are means ± SE; n=6–13. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control group.

References

[1] Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med. 2002;346:393-403.

[2] DeFronzo RA. Pharmacologic therapy for type 2 diabetes mellitus. Ann Intern Med. 1999;131:281-303.

[3] Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001;108:1167-74.

[4] Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes. 2002;51:2074-81.

[5] Suwa M, Egashira T, Nakano H, Sasaki H, Kumagai S. Metformin increases the PGC-1alpha protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. J Appl Physiol. 2006;101:1685-92.

[6] Kristensen JM, Treebak JT, Schjerling P, Goodyear L, Wojtaszewski JF. Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin-stimulated glucose uptake in mouse soleus muscle. Am J Physiol Endocrinol Metab. 2014;306:E1099-109.

[7] Umehara KI, Iwatsubo T, Noguchi K, Kamimura H. Functional involvement of organic cation transporter1 (OCT1/Oct1) in the hepatic uptake of organic cations in humans and rats. Xenobiotica. 2007;37:818-31.

[8] Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. J Clin Invest. 2007;117:1422-31.

[9] Sogame Y, Kitamura A, Yabuki M, Komuro S. A comparison of uptake of metformin and phenformin mediated by hOCT1 in human hepatocytes. Biopharm Drug Dispos. 2009;30:476-84.

[10] Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm Res. 2007;24:1227-51.

[11] Chen L, Pawlikowski B, Schlessinger A, More SS, Stryke D, Johns SJ, et al. Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. Pharmacogenet Genomics. 2010;20:687-99.

[12] Nesher R, Karl IE, Kaiser KE, Kipnis DM. Epitrochlearis muscle. I. Mechanical performance, energetics, and fiber composition. Am J Physiol. 1980;239:E454-60.

[13] Armstrong RB, Phelps RO. Muscle fiber type composition of the rat hindlimb. Am J Anat. 1984;171:259-72.

[14] Toyoda T, Hayashi T, Miyamoto L, Yonemitsu S, Nakano M, Tanaka S, et al. Possible involvement of the alpha1 isoform of 5'AMP-activated protein kinase in oxidative stress-stimulated glucose transport in skeletal muscle. Am J Physiol Endocrinol Metab. 2004;287:E166-73.

[15] Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, et al. Mammalian AMP-activated protein kinase subfamily. J Biol Chem. 1996;271:611-4.

[16] Young DA, Uhl JJ, Cartee GD, Holloszy JO. Activation of glucose transport in muscle by prolonged exposure to insulin. Effects of glucose and insulin concentrations. J Biol Chem. 1986;261:16049-53.

[17] Egawa T, Hamada T, Kameda N, Karaike K, Ma X, Masuda S, et al. Caffeine acutely activates 5'adenosine monophosphate-activated protein kinase and increases insulin-independent glucose transport in rat skeletal muscles. Metabolism. 2009;58:1609-17.

[18] Nakano M, Hamada T, Hayashi T, Yonemitsu S, Miyamoto L, Toyoda T, et al. alpha2 isoform-specific activation of 5'adenosine monophosphate-activated protein kinase by 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle. Metabolism. 2006;55:300-8.

[19] Davies SP, Sim AT, Hardie DG. Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. Eur J Biochem. 1990;187:183-90.

[20] Park SH, Gammon SR, Knippers JD, Paulsen SR, Rubink DS, Winder WW. Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle. J Appl Physiol. 2002;92:2475-82.

[21] Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, Goodyear LJ. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. Diabetes. 2000;49:527-31.

[22] Mu J, Brozinick JT, Jr., Valladares O, Bucan M, Birnbaum MJ. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. Mol Cell. 2001;7:1085-94.

[23] El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J Biol Chem. 2000;275:223-8.

[24] Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J. 2000;348 (Pt 3):607-14.

[25] Hardie DG. Sensing of energy and nutrients by AMP-activated protein kinase. Am J Clin Nutr. 2011;93:891S-6S.

[26] Friedrichsen M, Mortensen B, Pehmoller C, Birk JB, Wojtaszewski JF. Exercise-induced AMPK activity in skeletal muscle: role in glucose uptake and insulin sensitivity. Mol Cell Endocrinol. 2013;366:204-14.

[27] Hardie DG. Role of AMP-activated protein kinase in the metabolic syndrome and in heart disease. FEBS Lett. 2008;582:81-9.

[28] Egawa T, Tsuda S, Oshima R, Goto K, Hayashi T. Activation of 5'AMP-activated protein kinase in skeletal muscle by exercise and phytochemicals. J Phys Fitness Sports Med. 2014;3:55-64.

[29] Ma X, Iwanaka N, Masuda S, Karaike K, Egawa T, Hamada T, et al. Morus alba leaf extract stimulates 5'-AMP-activated protein kinase in isolated rat skeletal muscle. J Ethnopharmacol. 2009;122:54-9.

[30] Ma X, Egawa T, Kimura H, Karaike K, Masuda S, Iwanaka N, et al. Berberine-induced activation of 5'-adenosine monophosphate-activated protein kinase and glucose transport in rat skeletal muscles. Metabolism. 2010;59:1619-27.

[31] Ma X, Egawa T, Oshima R, Kurogi E, Tanabe H, Tsuda S, et al. Coptidis rhizoma water extract stimulates 5'-AMP-activated protein kinase in rat skeletal muscle. Chin J Nat Med. 2011;9:215-21.

[32] Tsuda S, Egawa T, Ma X, Oshima R, Kurogi E, Hayashi T. Coffee polyphenol caffeic acid but not chlorogenic acid increases 5'AMP-activated protein kinase and insulin-independent glucose transport in rat skeletal muscle. J Nutr Biochem. 2012;23:1403-9.

[33] Bikman BT, Zheng D, Kane DA, Anderson EJ, Woodlief TL, Price JW, et al. Metformin improves insulin signaling in obese rats via reduced IKKbeta action in a fiber-type specific manner. J Obes. 2010;2010: 1-8, Article ID 970865.

[34] Cheung PC, Salt IP, Davies SP, Hardie DG, Carling D. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. Biochem J. 2000;346 (Pt 3):659-69.

[35] Wojtaszewski JF, Birk JB, Frosig C, Holten M, Pilegaard H, Dela F. 5'AMP activated protein kinase expression in human skeletal muscle: effects of strength training and type 2 diabetes. J Physiol. 2005;564:563-73.

[36] Toyoda T, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Sato K, et al. Low-intensity contraction activates the alpha1-isoform of 5'-AMP-activated protein kinase in rat skeletal muscle. Am J Physiol Endocrinol Metab. 2006;290:E583-90.

[37] Lessard SJ, Chen ZP, Watt MJ, Hashem M, Reid JJ, Febbraio MA, et al. Chronic rosiglitazone treatment restores AMPKalpha2 activity in insulin-resistant rat skeletal muscle. Am J Physiol Endocrinol Metab. 2006;290:E251-7.

[38] Jensen TE, Schjerling P, Viollet B, Wojtaszewski JF, Richter EA. AMPK alpha1 activation is required for stimulation of glucose uptake by twitch contraction, but not by H_2O_2, in mouse skeletal muscle. PLoS One. 2008;3:e2102, 1-9

[39] Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, et al. Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. J Biol Chem. 2004;279:1070-9.

[40] Egawa T, Hamada T, Ma X, Karaike K, Kameda N, Masuda S, et al. Caffeine activates preferentially alpha1-isoform of 5'AMP-activated protein kinase in rat skeletal muscle. Acta Physiol (Oxf). 2011;201:227-38.

[41] Egawa T, Tsuda S, Ma X, Hamada T, Hayashi T. Caffeine modulates phosphorylation of insulin receptor substrate-1 and impairs insulin signal transduction in rat skeletal muscle. J Appl Physiol. 2011;111:1629-36.

[42] Tucker GT, Casey C, Phillips PJ, Connor H, Ward JD, Woods HF. Metformin kinetics in healthy subjects and in patients with diabetes mellitus. Br J Clin Pharmacol. 1981;12:235-46.

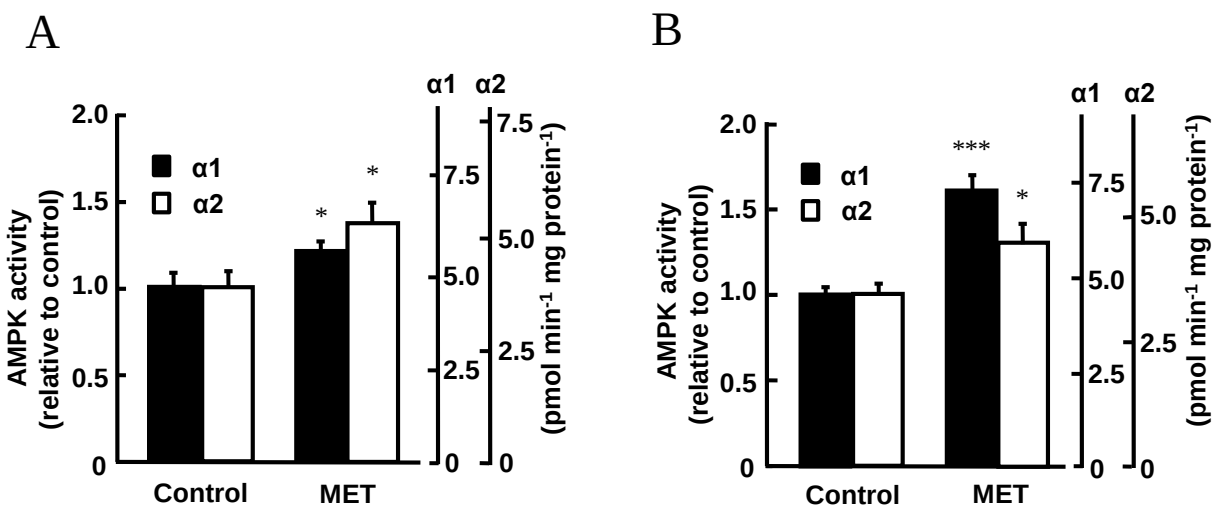
A
B
α1
α2
AMPK activity
(relative to control)
(pmol min-1 mg protein-1)
Control
MET
*
***
2.0
1.5
1.0
0.5
0
7.5
5.0
2.5

Fig. 1

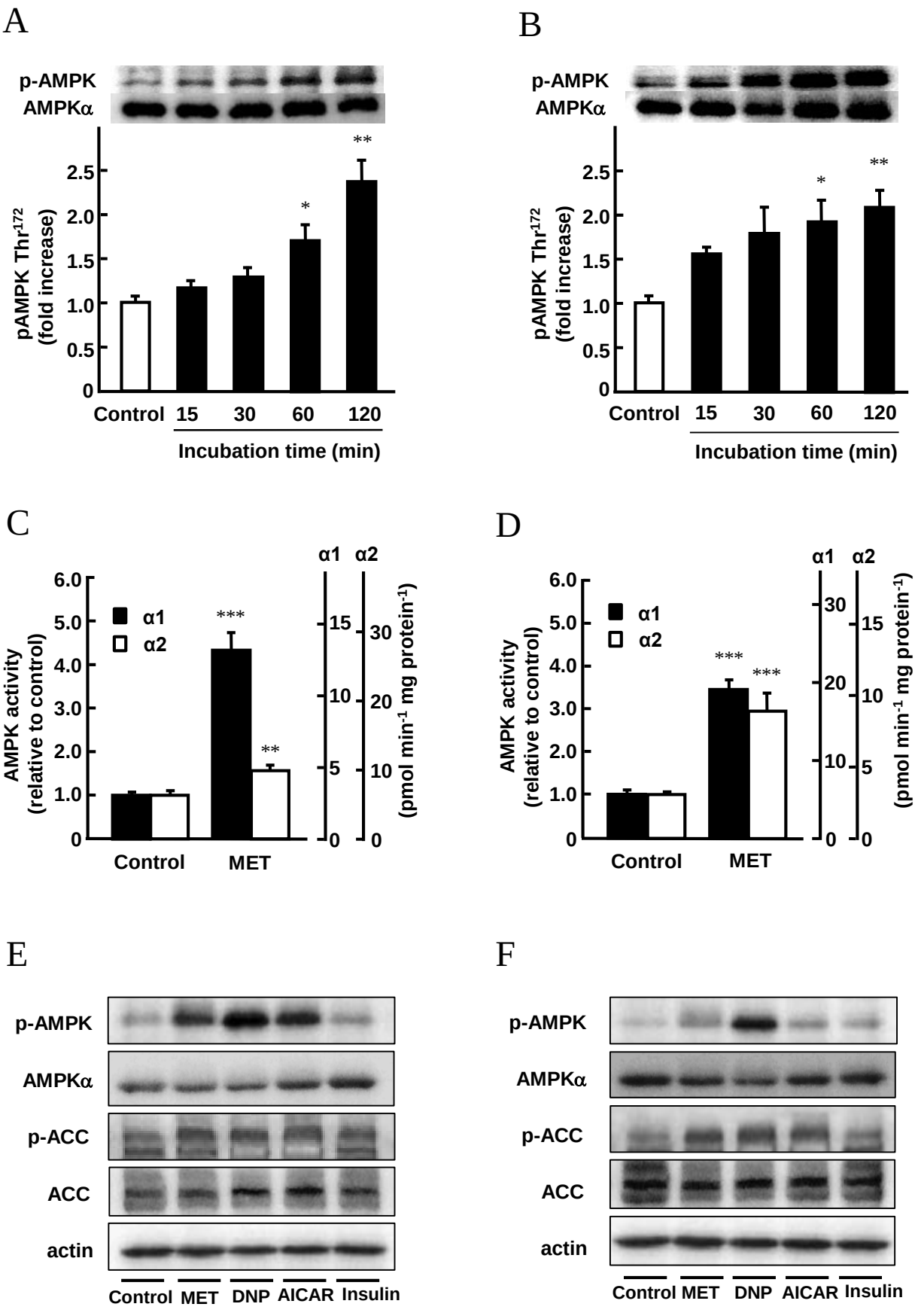
A
p-AMPK
AMPKα
pAMPK Thr172 (fold increase)
Control
15
30
60
120
Incubation time (min)
B
p-AMPK
AMPKα
pAMPK Thr172 (fold increase)
Control
15
30
60
120
Incubation time (min)
C
α1
α2
AMPK activity (relative to control)
(pmol min-1 mg protein-1)
Control
MET
D
α1
α2
AMPK activity (relative to control)
(pmol min-1 mg protein-1)
Control
MET
E
p-AMPK
AMPKα
p-ACC
ACC
actin
Control
MET
DNP
AICAR
Insulin
F
p-AMPK
AMPKα
p-ACC
ACC
actin
Control
MET
DNP
AICAR
Insulin

Fig. 2

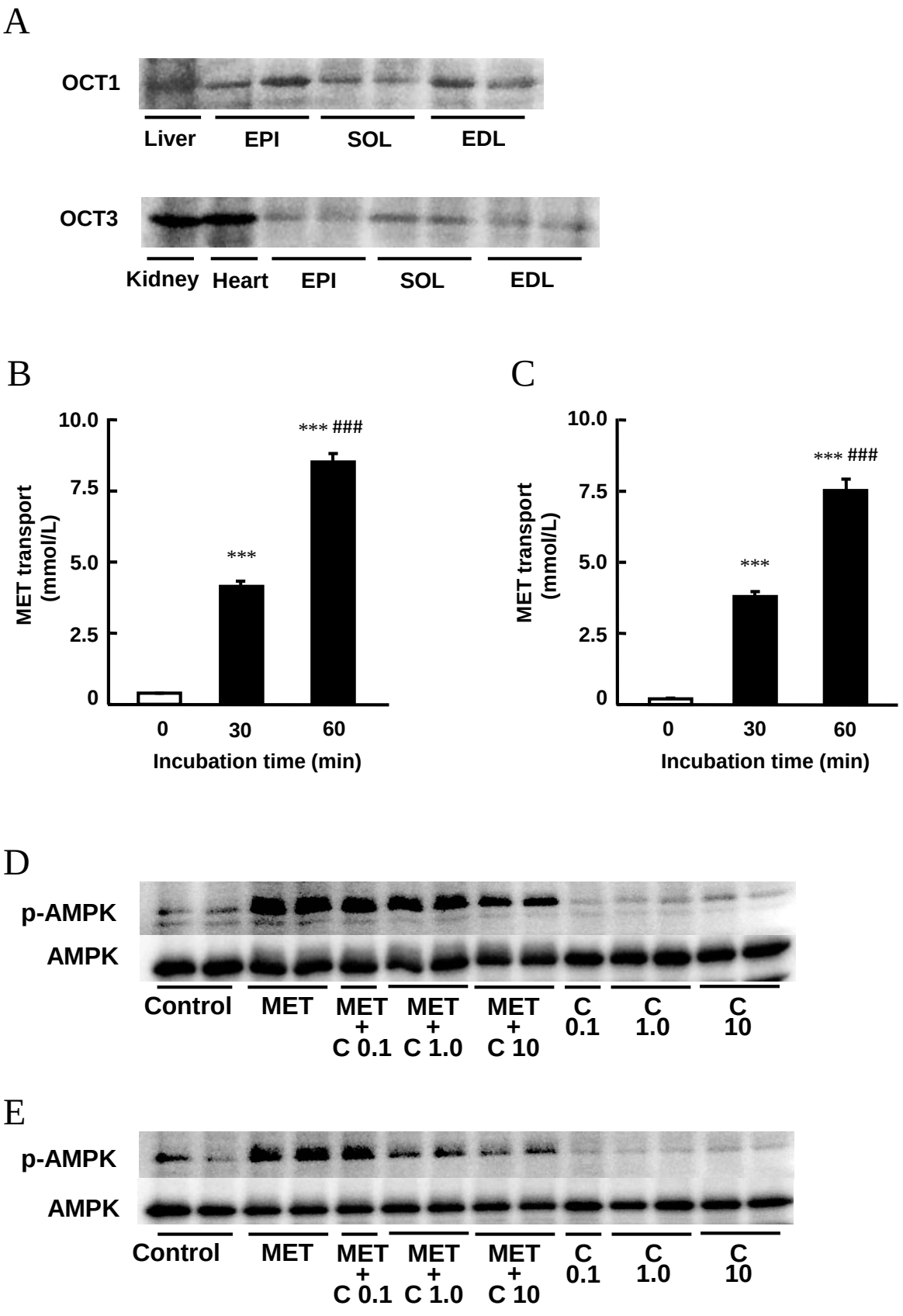
A
OCT1
Liver
EPI
SOL
EDL
OCT3
Kidney
Heart
EPI
SOL
EDL
B
MET transport (mmol/L)
10.0
7.5
5.0
2.5
0
*** ###
***
0
30
60
Incubation time (min)
C
MET transport (mmol/L)
10.0
7.5
5.0
2.5
0
*** ###
***
0
30
60
Incubation time (min)
D
p-AMPK
AMPK
Control
MET
MET + C 0.1
MET + C 1.0
MET + C 10
C 0.1
C 1.0
C 10
E
p-AMPK
AMPK
Control
MET
MET + C 0.1
MET + C 1.0
MET + C 10
C 0.1
C 1.0
C 10

Fig. 3

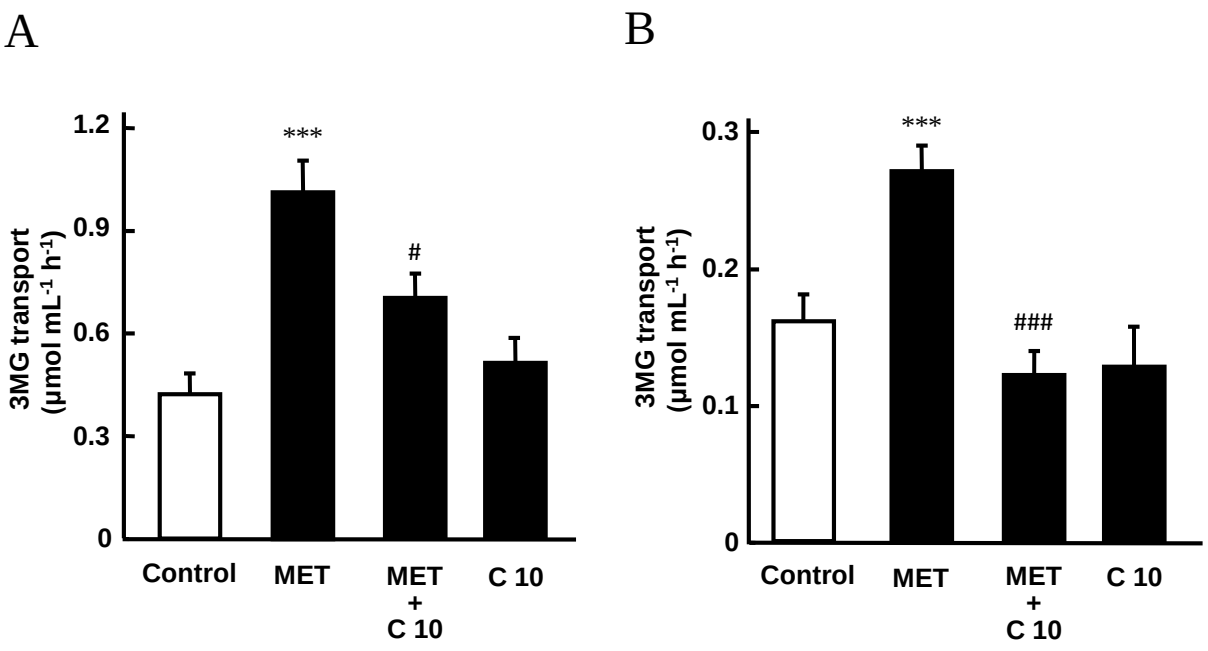
A
B
3MG transport
(µmol mL-1 h-1)
1.2
0.9
0.6
0.3
0
***
#
Control
MET
MET
+
C 10
C 10
0.3
0.2
0.1
0
***
###
Control
MET
MET
+
C 10
C 10

Fig. 4

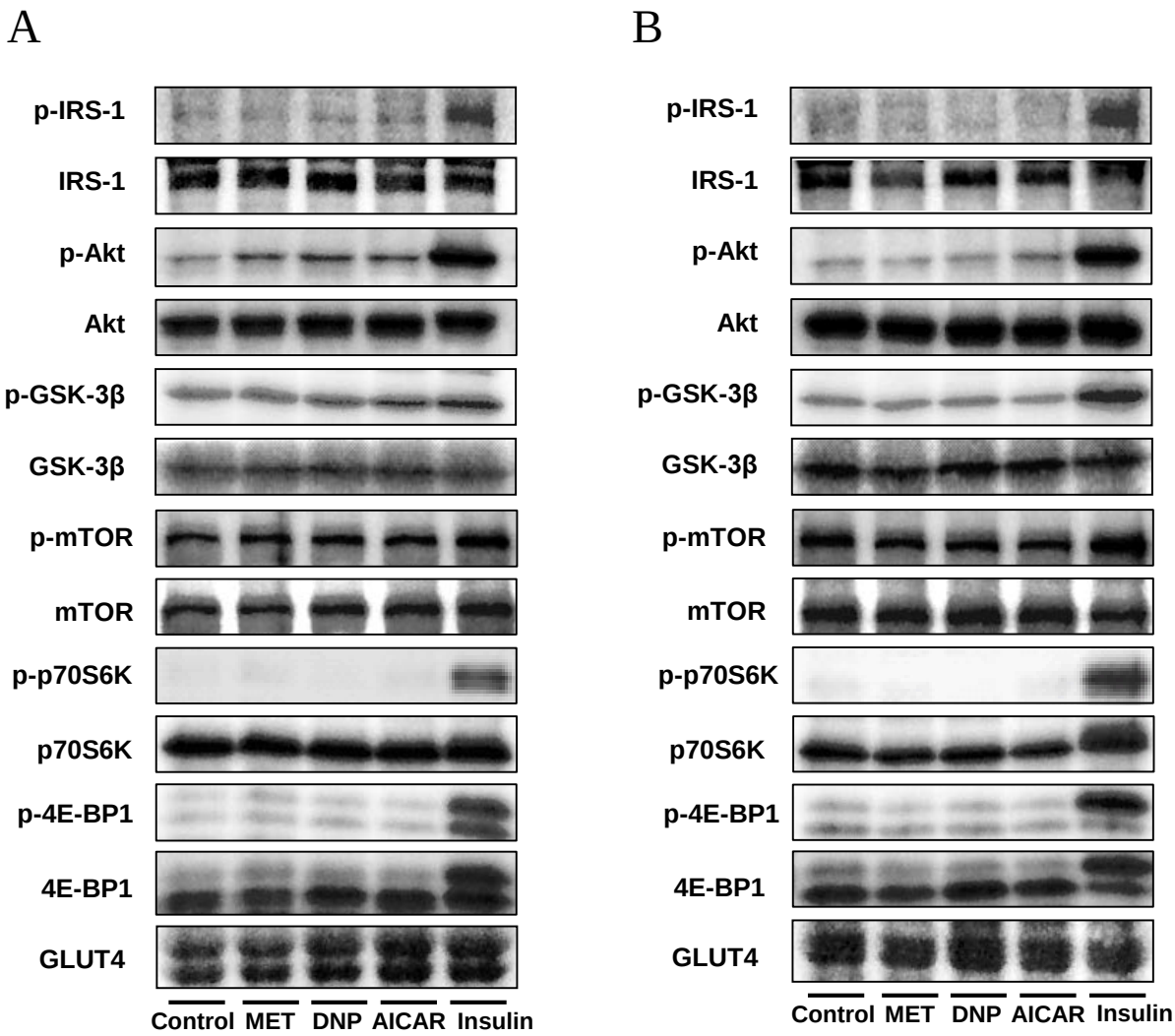
A
p-IRS-1
IRS-1
p-Akt
Akt
p-GSK-3β
GSK-3β
p-mTOR
mTOR
p-p70S6K
p70S6K
p-4E-BP1
4E-BP1
GLUT4
Control MET DNP AICAR Insulin
B
p-IRS-1
IRS-1
p-Akt
Akt
p-GSK-3β
GSK-3β
p-mTOR
mTOR
p-p70S6K
p70S6K
p-4E-BP1
4E-BP1
GLUT4
Control MET DNP AICAR Insulin

Fig. 5

		Control	MET
EPI	ATP	6.50±0.38	4.34±0.45**
	PCr	12.0±0.50	4.31±0.87***
	Glycogen	19.2±1.62	7.34±1.22***
SOL	ATP	1.65±0.11	1.13±0.20*
	PCr	6.24±0.27	5.04±0.31**
	Glycogen	15.2±0.56	13.6±0.60*

Table 1