phenylmethylsulfonyl fluoride and centrifuged at 14,000 *g* at 4°C for 30 min. The supernatants (40 μg of protein) were added to 80 μl of assay mixture containing 50 mM Tris (pH 7.8), 5 mM EDTA, 6.7 mM UDP-glucose, 10 mg/ml glycogen, 50 mM β-glycerophosphate, 50 mM sodium fluoride, and 0.1 mCi/mmol [^{14}C]UDP-glucose at 30°C, in the absence and presence of 10 mM glucose 6-phosphate (G-6-P), to measure G-6-P-independent (active form) and total GS activities, respectively. After 15 min the reaction solution was spotted onto a filter paper to terminate the reaction. The filter papers were washed extensively in 66% (vol/vol) ice-cold ethanol and then evaluated in a liquid scintillation counter for ^{14}C incorporation into glycogen. GS activity in the muscle was expressed as the active form ratio: G-6-P-independent activity ÷ total GS activity. The effect of ZMP on basal GS activity was evaluated by measurement of absolute GS activity in the presence of 6 mM ZMP, instead of 10 mM G-6-P, in the assay mixture. Absolute GS activity was expressed as ^{14}C incorporation activity (nmol·min^{-1}·mg protein^{-1}).

GP activity. Muscle GP activity was measured as described previously (15). Frozen muscles were homogenized in *buffer A*, and the supernatants were prepared as described for GS activity. Supernatants (40 μg of protein) were added to 80 μl of assay mixture containing 50 mM MES (pH 6.1), 100 mM glucose 1-phosphate, 200 mM potassium fluoride, 10 mg/ml glycogen, and 2.5 mCi/mol [^{14}C]glucose 1-phosphate, in the absence and presence of 6 mM 5′-AMP, to measure AMP-independent and total GP activities, respectively. After 15 min the reaction solution was spotted onto a filter paper to terminate the reaction. The papers were washed extensively in 66% (vol/vol) ice-cold ethanol and then evaluated in a liquid scintillation counter for ^{14}C incorporation into glycogen. GP activity in the muscle was expressed as the active form ratio: 5′-AMP-independent activity ÷ total GP activity. The effect of ZMP on basal GP activity was evaluated by measurement of absolute GP activity in the presence of 6 mM ZMP, instead of 6 mM 5′-AMP, in the assay mixture. Absolute GP activity was expressed as ^{14}C incorporation activity (μmol·min^{-1}·mg protein^{-1}).

Muscle glycogen. Frozen muscles were processed by incubation in 1 M NaOH at 85°C for 10 min. The digestates were neutralized with HCl. The glycogen in the digestates was hydrolyzed by incubation in 2 N HCl for 2 h at 85°C. The digestates were neutralized with NaOH, and the glucose released from the glycogen was measured enzymatically using a hexokinase glucose assay reagent (Sigma-Aldrich). Glycogen content was expressed as micromoles of glucosyl unit per gram wet muscle weight.

Lactate. Muscles were incubated and stimulated in KRB containing 8 mM glucose as described above. After muscle contraction for 10 min or AICAR stimulation for 40 min, aliquots of the incubation buffer were collected, and the lactate in the buffer was determined using the Determiner-LA kit (Kyowa Medex, Tokyo, Japan). The lactate released into the buffer was calculated and normalized to the wet muscle weight.

Statistical analysis. Values are means ± SE. Multiple means were compared by ANOVA. Two means were compared by Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

AICAR acutely stimulates muscle AMPK in a dose- and time-dependent manner to a level comparable to that achieved by contraction. To evaluate the effects of AICAR stimulation on rat epitrochlearis muscle, we determined the dose and time dependency of its effects on isoform-specific AMPK activities. Pharmacological stimulation with AICAR caused a two- to threefold activation of both isoforms of AMPK (Fig. 1, *A* and *B*). The effects of AICAR stimulation on AMPK activity were not observed 10 min after the start of incubation but became prominent by 30 min. The stimulatory effect of AICAR was maximal at 40 min (Fig. 1*B*) in a dose-dependent manner (Fig. 1*A*). Therefore, we judged that stimulation with 2 mM AICAR for 40 min causes maximal AMPK activation of both α-isoforms. We also compared AICAR- and contraction-stimulated AMPK activities. Both treatments caused almost equal increases in AMPK-α_1 and -α_2 activities (Fig. 1*C*).

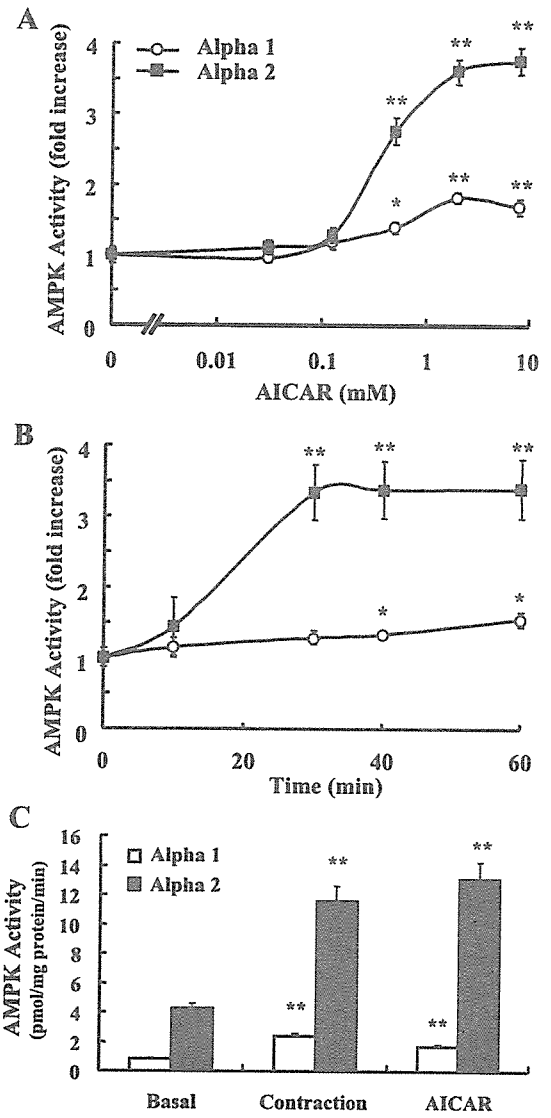

Fig. 1. Changes in α_1- and α_2-isoform-specific 5′-AMP-activated protein kinase (AMPK) activities in rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by 0.03–8 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) for 40 min (*A*), 2 mM AICAR for 10–60 min (*B*), and in vitro contraction (10 min) or 2 mM AICAR for 40 min (*C*). Values are means ± SE (n = 4–6/group). *$P < 0.05$; **$P < 0.01$ vs. basal.

Contraction and AICAR activate glucose uptake to similar levels, comparable to the level achieved by a maximally effective dose of insulin. We determined whether muscle contraction and AICAR stimulation, both of which activate AMPK to similar levels (Fig. 1), increase glucose uptake. The almost identical four- to fivefold increases in 3-MG uptake stimulated by muscle contraction and AICAR (Fig. 2) are similar to that achieved by stimulation with a maximally effective dose (1 μM) of insulin.

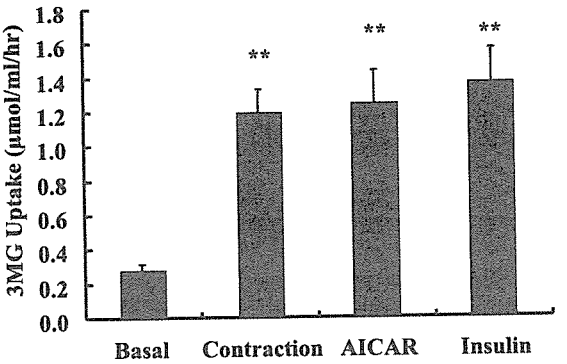

Fig. 2. 3-*O*-methylglucose (3-MG) uptake activity in rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by in vitro contraction (10 min), 2 mM AICAR for 40 min, or 1 μM insulin for 40 min. Values are means ± SE (n = 5–10/group). $^{**}P < 0.01$ vs. basal.

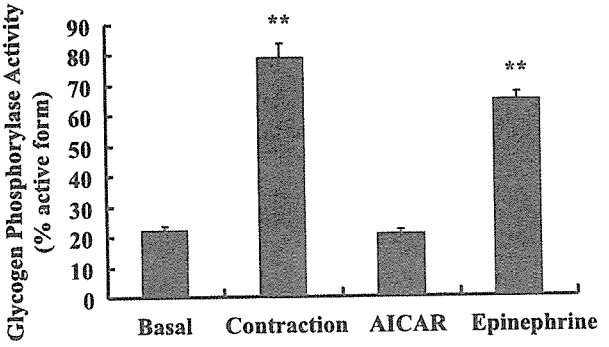

Fig. 4. Glycogen phosphorylase (GP) activity in rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by in vitro contraction (10 min), 2 mM AICAR for 40 min, or 3 μg/ml epinephrine for 15 min. Values are means ± SE (n = 6–14/group). $^{**}P < 0.01$ vs. basal.

Contraction activates, but AICAR inhibits GS activity. To determine whether the activation of AMPK affects the activity of GS, the rate-limiting enzyme of glycogen synthesis, we measured GS activity. Whereas muscle contraction caused a marked increase in GS activity, AICAR stimulation conversely decreased GS activity (Fig. 3*A*). Insulin also increased GS activity, but the effect of insulin was antagonized by the presence of AICAR [active form ratio (%) = 28.0 ± 1.0 (basal), 52.7 ± 1.5 (contraction), 24.4 ± 1.1 (AICAR), 36.1 ± 2.0 (AICAR + insulin), and 45.4 ± 1.5 (insulin); Fig. 3*A*]. AICAR-induced GS inactivation was dose dependent (Fig. 3*B*), in parallel with AMPK activity (Fig. 1*A*).

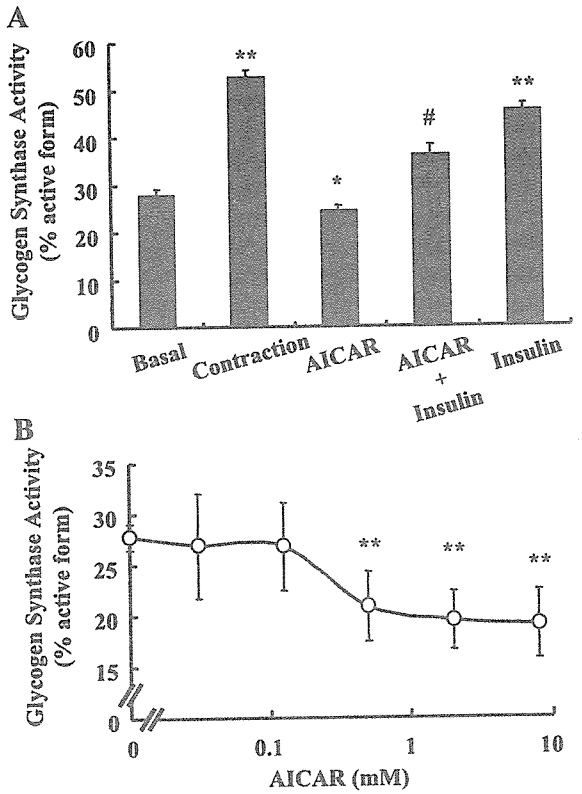

Fig. 3. Glycogen synthase (GS) activity in rat epitrochlearis muscle. *A*: isolated muscles were stimulated by in vitro contraction (10 min), 2 mM AICAR for 40 min, 2 mM AICAR + 1 μM insulin for 40 min, or 1 μM insulin. Values are means ± SE (n = 12–19/group). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. basal. $^{\#}P < 0.01$ vs. insulin. *B*: muscles were incubated and stimulated by 0.03–8 mM AICAR for 40 min. Values are means ± SE [n = 4–6/group, except basal (n = 19)]. $^{**}P < 0.01$ vs. basal.

Contraction activates, but AICAR does not alter, GP activity. To determine the effect of AMPK activation on glycogenolysis, we examined the effect of AICAR stimulation on the activity of GP, which is the rate-limiting enzyme of glycogenolysis. Whereas muscle contraction and epinephrine stimulation markedly increased GP activity, AICAR did not alter GP activity [active form ratio (%) = 22.4 ± 1.0 (basal), 78.8 ± 4.4 (contraction), 20.5 ± 1.4 (AICAR), and 64.5 ± 1.7 (epinephrine); Fig. 4].

Contraction decreases, but AICAR does not alter, muscle glycogen content. We examined the effect of AICAR-stimulated AMPK activation on the concentration of glycogen. Glycogen content was reduced in contracting and epinephrine-stimulated muscles. In contrast, glycogen was unchanged by AICAR (23.3 ± 1.3, 12.4 ± 0.9, 25.6 ± 1.3, 23.7 ± 1.2, and 13.1 ± 2.4 μmol glucosyl unit/g wet muscle wt for the basal state, contraction, AICAR, insulin, and epinephrine, respectively; Fig. 5). These results are consistent with the increase in GP activity induced by contraction or epinephrine, which was unchanged by AICAR (Fig. 4).

AICAR increases lactate release from muscle. Because AICAR stimulation resulted in the inactivation of GS, despite a marked increase in glucose uptake, we investigated the amount of lactate released into the incubation buffer to clarify

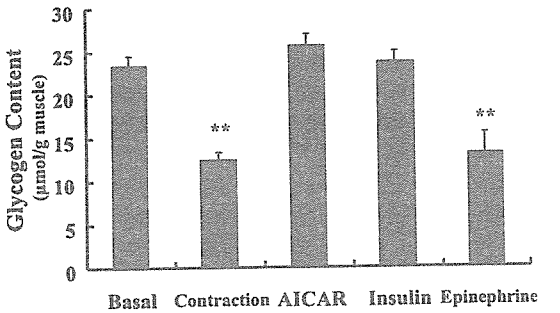

Fig. 5. Glycogen content in rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by in vitro contraction (10 min), 2 mM AICAR for 40 min, 1 μM insulin for 40 min, or 3 μg/ml epinephrine for 15 min. Values are means ± SE [n = 16–20/group, except epinephrine (n = 4)]. $^{**}P < 0.01$ vs. basal.

whether the glucose taken up into the muscle is degraded via the glycolysis pathway. The amount of lactate was measured in buffer containing glucose, instead of pyruvate. Similar to muscle contraction, AICAR caused a significant increase in lactate release (12.7 ± 0.85, 38.6 ± 2.11, and 20.1 ± 1.14 μg lactate/mg muscle for the basal state, contraction, and AICAR, respectively; Fig. 6).

ZMP stimulates GP activity, but does not affect GS activity, in vitro. When skeletal muscle is incubated with AICAR, AICAR is taken up into the muscle. It is then phosphorylated to form the AMP-like compound ZMP (17). Consequently, it is possible that increased intracellular ZMP directly modulates GS or GP activity as an AMPK-independent effect. Therefore, we measured GS and GP activities in the presence of ZMP in vitro. GS activity was unchanged in the presence of ZMP, whereas it was markedly elevated in the presence of G-6-P, a strong allosteric activator of GS (6,669 ± 446, 36,907 ± 1,085, and 6,409 ± 267 pmol·min^{-1}·mg^{-1} without G-6-P and without ZMP, with G-6-P, and with ZMP, respectively; Fig. 7*A*). GP activity was elevated in the presence of ZMP to an extent similar to that observed with AMP, a potent allosteric activator of GP (664.2 ± 36.8, 1,335 ± 61.2, and 1,416 ± 92.4 nmol·min^{-1}·mg^{-1} without AMP and without ZMP, with AMP, and with ZMP, respectively; Fig. 7*B*).

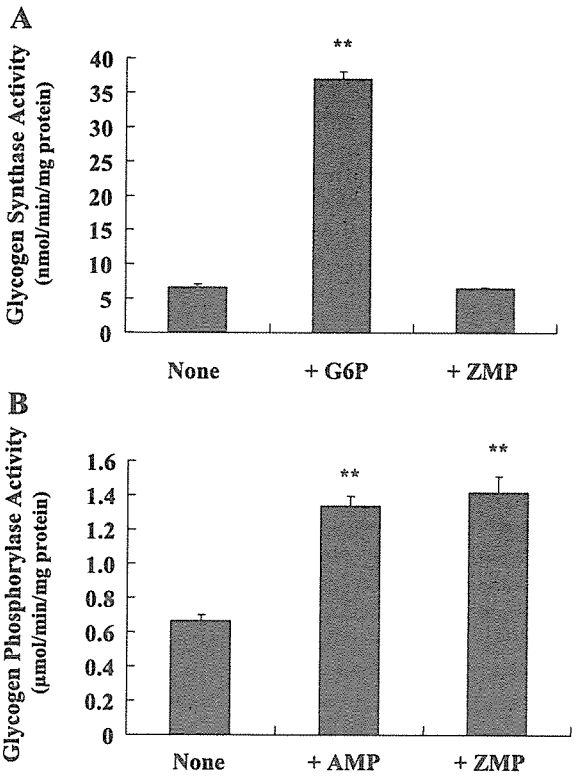

Fig. 7. Effects of ZMP on GS and GP activities in rat epitrochlearis muscle. Muscle samples in the basal state were assayed for GS activity in the presence of 10 mM glucose 6-phosphate (G-6-P) or 6 mM ZMP in vitro (*A*) and for GP activity in the presence of 6 mM AMP or 6 mM ZMP in vitro (*B*). Values are means ± SE (n = 8–9/group). $^{**}P < 0.01$ vs. none.

DISCUSSION

Studies of AICAR have provided important information about the function of acute AMPK activation in muscle glucose metabolism. The specificity of AICAR as an AMPK stimulator has been established by Mu et al. (31), who blocked AMPK activity in mouse skeletal muscle with the muscle-specific expression of a dominant-negative kinase-dead form of AMPK. In that mouse, the stimulatory effects of AICAR on glucose transport (31) and GLUT4 expression (22) were abolished completely. On the basis of findings suggesting that acute AICAR stimulation in vitro activates AMPK and glucose transport in fast-twitch muscles but has no effect on the slow-twitch soleus muscle of the rat (1, 4) and that AICAR administration also has the greatest effect on the GLUT4 content of fast-twitch muscles (7, 21, 44), we analyzed the rat epitrochlearis muscle. Differential ATPase staining of rat epitrochlearis demonstrated >80% fast-twitch and only 15% slow-twitch fibers (33, 34). In the present study, AICAR

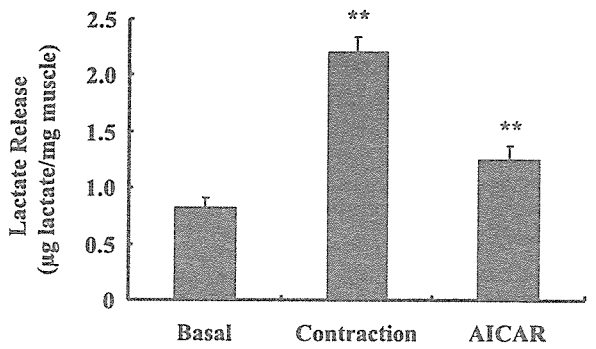

Fig. 6. Lactate release from rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by in vitro contraction (10 min) or 2 mM AICAR for 40 min in glucose-containing Krebs-Ringer bicarbonate buffer, and lactate concentrations in buffer were determined. Values are means ± SE (n = 5–6/group). $^{**}P < 0.01$ vs. basal.

treatment activated AMPK to the extent observed in skeletal muscle after contraction. AICAR (2 mM, 40 min) and tetanic contraction (10 s, 10 times) activated AMPK-α_1 and -α_2 (Fig. 1*C*), with a corresponding increase in the rate of 3-MG uptake to the level achieved by a maximally effective dose (1 μM) of insulin (Fig. 2).

We used isolated muscle incubated in vitro to eliminate the effects of systemic confounders, such as humoral factors and blood flow, because exercise in vivo evokes a number of dynamic changes, many of which can potentially alter fuel metabolism in contracting skeletal muscles (20). For example, exercise increases the blood concentration of epinephrine, a potent activator of glycogen breakdown (Fig. 4). Our method, using isolated muscle, made it possible to examine the direct effects of pharmacological manipulation and contraction on skeletal muscle metabolism.

In the present study, AICAR stimulation caused a decrease in GS activity, in contrast to muscle contraction (Fig. 3*A*). GS inactivation was dose dependent, in parallel with AMPK activation (Figs. 1*A* and 3*B*). Furthermore, insulin-stimulated GS activation was partially antagonized in the presence of AICAR (Fig. 3*A*). We eliminated the possibility of a direct inactivation of GS by ZMP, an intracellular metabolite of AICAR (Fig. 7*A*). Our observation is consistent with a previous report by Wojtaszewski et al. (45), who showed an inhibition of GS activity in rat hindlimb muscle after perfusion with AICAR. They also showed that the inactivation of GS was accompanied by a decrease in gel mobility and was abolished by protein

phosphatase treatment, indicating that AICAR stimulation causes GS phosphorylation (45). More recently, Jorgensen et al. (26) showed that AICAR treatment in vitro increases GS phosphorylation at site 2 (Ser^7) and decreases GS activity in mouse extensor digitorum longus muscle. Interestingly, however, Aschenbach et al. (3) showed that intraperitoneal administration of AICAR to a living rat inhibited GS activity in white gastrocnemius muscle, whereas AICAR administration also activated GS activity in red gastrocnemius muscle. They reported that in vitro incubation of the epitrochlearis and flexor digitorum brevis muscles with AICAR stimulated AMPK-α_2 but had no effect on GS activity (3). The reasons for these discrepancies in the responses of the red and white gastrocnemius muscles and between the in vivo and in vitro experiments are unclear. However, Aschenbach et al. speculate that these AICAR-stimulated changes in GS activity may be due to the secondary effects of glucose transport and glycogen accumulation, rather than the direct effects of AMPK on GS.

The idea that active AMPK prevents glycogen synthesis in skeletal muscle may appear to be inconsistent with the chronic accumulation of glycogen in rat skeletal muscle induced by repeated administrations of AICAR. Several reports have shown that once-a-day administration of AICAR for 5–28 days in vivo causes a marked increase in the glycogen concentration of rat muscles (7, 21, 44). The levels of GLUT4 and hexokinase proteins are also upregulated by AICAR (7, 21, 44). Furthermore, each AICAR treatment induces GLUT4 translocation and increases the rate of glucose transport into muscle cells (Fig. 2) (19, 27). Increased glucose transport and hexokinase expression may result in increased concentrations of G-6-P, a potent allosteric activator of GS (Fig. 7*A*). They may also override the effects of the inhibitory phosphorylation of GS by AMPK. Thus these combined effects of AICAR on protein expression and glucose transport may predominate and facilitate glycogen synthesis, despite the deactivation of GS by AICAR.

In the present study, epinephrine stimulation and contraction caused increases in GP activity (Fig. 4), with corresponding decreases in glycogen content (Fig. 5), whereas AICAR stimulation neither increased GP activity nor altered the glycogen content (Fig. 4). Therefore, acute AMPK activation is considered to have no significant effect on GP activity in skeletal muscle. Our findings are consistent with the report by Aschenbach et al. (3), who found that in vitro AICAR treatment had no effect on GP activity in isolated epitrochlearis or flexor digitorum brevis muscles, despite significant activation of AMPK-α_2. Interestingly, however, they also reported activation of GP in gastrocnemius muscle after intraperitoneal administration of AICAR in vivo, with a corresponding increase in AMPK-α_2 activity (3). The cause of this contradictory change in GP activity is unknown. However, Aschenbach et al. speculate that it may be due to secondary effects in response to in vivo AICAR treatment. Our findings also appear to be inconsistent with another report by Young et al. (47), who showed that GP is activated in rat soleus muscle incubated with AICAR in vitro. However, as mentioned above, AICAR stimulation has no effect on AMPK activity in rat soleus muscle (1, 4). Therefore, the GP activation observed in rat soleus muscle might not be related to AMPK activity. In support of this idea, AICAR did not alter the rate of glycogen synthesis in rat soleus in the basal state or a maximally stimulatory concentration of insulin (47). However, we found that AICAR decreased the basal and insulin-stimulated GS activity in rat epitrochlearis muscle (Fig. 3*A*), with a significant increase in AMPK activity (Fig. 1). Young et al. also demonstrated that the AICAR metabolite ZMP mimics the stimulatory effect of AMP, a known allosteric activator of GP, in extracts of rat soleus muscle. In the present study, we also found a marked elevation in GP activity when ZMP was added directly to muscle lysate to a level similar to that observed when AMP was added to the lysate (Fig. 7*B*). Longnus et al. (28) showed that AICAR activates GP in isolated rat myocardium in a dose-dependent manner, with no accompanying activation of AMPK. On the basis of these data, it seems reasonable that AICAR-induced GP activation is due to allosteric activation by ZMP, as in the rat myocardium.

On the basis of these observations, we propose that acute AMPK activation during muscle contraction antagonizes contraction-stimulated GS activity and that this effect consequently facilitates a glycolytic flux. Our proposal is consistent with the idea that AMPK acts as an energy sensor, switching off ATP-consuming pathways and switching on alternative pathways for ATP regeneration when cells sense low energy (17). The muscle glycogen accumulation induced by repeated AICAR stimulation may be due to the stimulatory effect of AMPK on glucose transport and on the expression of proteins such as GLUT4 and hexokinase. These effects may override the inhibitory action of AMPK on GS activity, resulting in enhanced glycogen synthesis in skeletal muscle. In conclusion, AMPK does not directly mediate contraction-stimulated GS or GP activation. However, AMPK may act as a metabolic regulator that leads to an increased glycolytic flux in contracting skeletal muscle.

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REFERENCES

1. **Ai H, Ihlemann J, Hellsten Y, Lauritzen HP, Hardie DG, Galbo H, Ploug T.** Effect of fiber type and nutritional state on AICAR- and contraction-stimulated glucose transport in rat muscle. *Am J Physiol Endocrinol Metab* 282: E1291–E1300, 2002.
2. **Arad M, Benson DW, Perez-Atayde AR, McKenna WJ, Sparks EA, Kanter RJ, McGarry K, Seidman JG, Seidman CE.** Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy. *J Clin Invest* 109: 357–362, 2002.
3. **Aschenbach WG, Hirshman MF, Fujii N, Sakamoto K, Howlett KF, Goodyear LJ.** Effect of AICAR treatment on glycogen metabolism in skeletal muscle. *Diabetes* 51: 567–573, 2002.
4. **Balon TW, Jasman AP.** Acute exposure to AICAR increases glucose transport in mouse EDL and soleus muscle. *Biochem Biophys Res Commun* 282: 1008–1011, 2001.
5. **Barnes BR, Marklund S, Steiler TL, Walter M, Hjalm G, Amarger V, Mahlapuu M, Leng Y, Johansson C, Galuska D, Lindgren K, Abrink M, Stapleton D, Zierath JR, Andersson L.** The 5′-AMP-activated protein kinase γ_3-isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. *J Biol Chem* 279: 38441–38447, 2004.
6. **Bergstrom J, Hultman E.** Muscle glycogen synthesis after exercise: an enhancing factor localized to the muscle cells in man. *Nature* 210: 309–310, 1966.

7. **Buhl ES, Jessen N, Schmitz O, Pedersen SB, Pedersen O, Holman GD, Lund S.** Chronic treatment with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside increases insulin-stimulated glucose uptake and GLUT4 translocation in rat skeletal muscles in a fiber type-specific manner. *Diabetes* 50: 12–17, 2001.
8. **Carling D, Hardie DG.** The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* 1012: 81–86, 1989.
9. **Chen ZP, Stephens TJ, Murthy S, Canny BJ, Hargreaves M, Witters LA, Kemp BE, McConell GK.** Effect of exercise intensity on skeletal muscle AMPK signaling in humans. *Diabetes* 52: 2205–2212, 2003.
10. **Ciobanu D, Bastiaansen J, Malek M, Helm J, Woollard J, Plastow G, Rothschild M.** Evidence for new alleles in the protein kinase adenosine monophosphate-activated γ_3-subunit gene associated with low glycogen content in pig skeletal muscle and improved meat quality. *Genetics* 159: 1151–1162, 2001.
11. **Conlee RK, Hickson RC, Winder WW, Hagberg JM, Holloszy JO.** Regulation of glycogen resynthesis in muscles of rats following exercise. *Am J Physiol Regul Integr Comp Physiol* 235: R145–R150, 1978.
12. **Fiedler M, Zierath JR, Selen G, Wallberg-Henriksson H, Liang Y, Sakariassen KS.** 5-Aminoimidazole-4-carboxy-amide-1-β-D-ribofuranoside treatment ameliorates hyperglycaemia and hyperinsulinaemia but not dyslipidaemia in KKAy-CETP mice. *Diabetologia* 44: 2180–2186, 2001.
13. **Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Birnbaum MJ, Witters LA, Thorell A, Goodyear LJ.** Exercise induces isoform-specific increase in 5′-AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* 273: 1150–1155, 2000.
14. **Garetto LP, Richter EA, Goodman MN, Ruderman NB.** Enhanced muscle glucose metabolism after exercise in the rat: the two phases. *Am J Physiol Endocrinol Metab* 246: E471–E475, 1984.
15. **Gilboe DP, Larson KL, Nuttall FQ.** Radioactive method for the assay of glycogen phosphorylases. *Anal Biochem* 47: 20–27, 1972.
16. **Halse R, Fryer LG, McCormack JG, Carling D, Yeaman SJ.** Regulation of glycogen synthase by glucose and glycogen: a possible role for AMP-activated protein kinase. *Diabetes* 52: 9–15, 2003.
17. **Hardie DG, Carling D.** The AMP-activated protein kinase—fuel gauge of the mammalian cell? *Eur J Biochem* 246: 259–273, 1997.
18. **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* 49: 527–531, 2000.
19. **Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ.** Evidence for 5′-AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47: 1369–1373, 1998.
20. **Hayashi T, Wojtaszewski JF, Goodyear LJ.** Exercise regulation of glucose transport in skeletal muscle. *Am J Physiol Endocrinol Metab* 273: E1039–E1051, 1997.
21. **Holmes BF, Kurth-Kraczek EJ, Winder WW.** Chronic activation of 5′-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. *J Appl Physiol* 87: 1990–1995, 1999.
22. **Holmes BF, Lang DB, Birnbaum MJ, Mu J, Dohm GL.** AMP kinase is not required for the GLUT4 response to exercise and denervation in skeletal muscle. *Am J Physiol Endocrinol Metab* 287: E739–E743, 2004.
23. **Hutber CA, Hardie DG, Winder WW.** Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase. *Am J Physiol Endocrinol Metab* 272: E262–E266, 1997.
24. **Iglesias MA, Ye JM, Frangioudakis G, Saha AK, Tomas E, Ruderman NB, Cooney GJ, Kraegen EW.** AICAR administration causes an apparent enhancement of muscle and liver insulin action in insulin-resistant high-fat-fed rats. *Diabetes* 51: 2886–2894, 2002.
25. **Ivy JL, Holloszy JO.** Persistent increase in glucose uptake by rat skeletal muscle following exercise. *Am J Physiol Cell Physiol* 241: C200–C203, 1981.
26. **Jorgensen SB, Nielsen JN, Birk JB, Olsen GS, Viollet B, Andreelli F, Schjerling P, Vaulont S, Hardie DG, Hansen BF, Richter EA, Wojtaszewski JF.** The α_2-5′-AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* 53: 3074–3081, 2004.
27. **Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW.** 5′-AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* 48: 1667–1671, 1999.
28. **Longnus SL, Wambolt RB, Parsons HL, Brownsey RW, Allard MF.** 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) stimulates myocardial glycogenolysis by allosteric mechanisms. *Am J Physiol Regul Integr Comp Physiol* 284: R936–R944, 2003.
29. **Milan D, Jeon JT, Looft C, Amarger V, Robic A, Thelander M, Rogel-Gaillard C, Paul S, Iannuccelli N, Rask L, Ronne H, Lundstrom K, Reinsch N, Gellin J, Kalm E, Roy PL, Chardon P, Andersson L.** A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle. *Science* 288: 1248–1251, 2000.
30. **Mu J, Barton ER, Birnbaum MJ.** Selective suppression of AMP-activated protein kinase in skeletal muscle: update on "lazy mice." *Biochem Soc Trans* 31: 236–241, 2003.
31. **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* 7: 1085–1094, 2001.
32. **Nakano M, Hamada T, Hayashi T, Yonemitsu S, Miyamoto L, Toyoda T, Tanaka S, Masuzaki H, Ebihara K, Ogawa Y, Hosoda K, Inoue G, Yoshimasa Y, Otaka A, Fushiki T, Nakao K.** α_2-Isoform-specific activation of 5′-adenosine monophosphate-activated protein kinase by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle. *Metabolism* 55: 300–308, 2006.
33. **Nesher R, Karl IE, Kaiser KE, Kipnis DM.** Epitrochlearis muscle. I. Mechanical performance, energetics, and fiber composition. *Am J Physiol Endocrinol Metab* 239: E454–E460, 1980.
34. **Nesher R, Karl IE, Kipnis DM.** Epitrochlearis muscle. II. Metabolic effects of contraction and catecholamines. *Am J Physiol Endocrinol Metab* 239: E461–E467, 1980.
35. **Ojuka EO, Nolte LA, Holloszy JO.** Increased expression of GLUT-4 and hexokinase in rat epitrochlearis muscles exposed to AICAR in vitro. *J Appl Physiol* 88: 1072–1075, 2000.
36. **Price TB, Rothman DL, Taylor R, Avison MJ, Shulman GI, Shulman RG.** Human muscle glycogen resynthesis after exercise: insulin-dependent and -independent phases. *J Appl Physiol* 76: 104–111, 1994.
37. **Rasmussen BB, Winder WW.** Effect of exercise intensity on skeletal muscle malonyl-CoA and acetyl-CoA carboxylase. *J Appl Physiol* 83: 1104–1109, 1997.
38. **Richter EA, Garetto LP, Goodman MN, Ruderman NB.** Enhanced muscle glucose metabolism after exercise: modulation by local factors. *Am J Physiol Endocrinol Metab* 246: E476–E482, 1984.
39. **Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE.** Mammalian AMP-activated protein kinase subfamily. *J Biol Chem* 271: 611–614, 1996.
40. **Toyoda T, Hayashi T, Miyamoto L, Yonemitsu S, Nakano M, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Inoue G, Otaka A, Sato K, Fushiki T, Nakao K.** Possible involvement of the α_1-isoform of 5′-AMP-activated protein kinase in oxidative stress-stimulated glucose transport in skeletal muscle. *Am J Physiol Endocrinol Metab* 287: E166–E173, 2004.
41. **Vavvas D, Apazidis A, Saha AK, Gamble J, Patel A, Kemp BE, Witters LA, Ruderman NB.** Contraction-induced changes in acetyl-CoA carboxylase and 5′-AMP-activated kinase in skeletal muscle. *J Biol Chem* 272: 13255–13261, 1997.
42. **Winder WW.** Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. *J Appl Physiol* 91: 1017–1028, 2001.
43. **Winder WW, Hardie DG.** Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol Endocrinol Metab* 270: E299–E304, 1996.
44. **Winder WW, Holmes BF, Rubink DS, Jensen EB, Chen M, Holloszy JO.** Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *J Appl Physiol* 88: 2219–2226, 2000.
45. **Wojtaszewski JF, Jorgensen SB, Hellsten Y, Hardie DG, Richter EA.** Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle. *Diabetes* 51: 284–292, 2002.
46. **Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D.** LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* 13: 2004–2008, 2003.
47. **Young ME, Radda GK, Leighton B.** Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR—an activator of AMP-activated protein kinase. *FEBS Lett* 382: 43–47, 1996.
48. **Zheng D, MacLean PS, Pohnert SC, Knight JB, Olson AL, Winder WW, Dohm GL.** Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. *J Appl Physiol* 91: 1073–1083, 2001.

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α2 Isoform–specific activation of 5′ adenosine monophosphate–activated protein kinase by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle

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Abstract

5′ Adenosine monophosphate–activated protein kinase (AMPK) has been implicated in exercise-induced stimulation of glucose metabolism in skeletal muscle. Although skeletal muscle expresses both the α1 and α2 isoforms of AMPK, the α2 isoform is activated predominantly in response to moderate-intensity endurance exercise in human and animal muscles. The purpose of this study was to determine whether activation of α2 AMPK plays a role in increasing the rate of glucose transport, promoting glucose transporter 4 (GLUT4) expression, and enhancing insulin sensitivity in skeletal muscle. To selectively activate the α2 isoform, we used 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), which is metabolized in muscle cells and preferentially stimulates the α2 isoform. Subcutaneous administration of 250 mg/kg AICAR activated the α2 isoform for 90 minutes, but not the α1 isoform in hind limb muscles of the C57/B6J mouse. The maximal activation of the α2 isoform was observed 30 to 60 minutes after administration of AICAR and was similar to the activation induced by a 30-minute swim in a current pool. The increase in α2 activity paralleled the phosphorylation of Thr^{172}, the essential residue for full kinase activation, and the activity of acetyl-coenzyme A carboxylase β, a known substrate of AMPK in skeletal muscle. Subcutaneous injection of AICAR rapidly increased, by 30%, the rate of 2-deoxyglucose (2DG) transport into soleus muscle; 2DG transport increased within 30 minutes and remained elevated for 4 hours after administration of AICAR. Repeated intraperitoneal injection of AICAR, 3 times a day for 4 to 7 days, increased soleus GLUT4 protein by 30% concomitant with a significant 20% increase in insulin-stimulated 2DG transport. These data suggest that moderate endurance exercise promotes glucose transport, GLUT4 expression, and insulin sensitivity in skeletal muscle at least partially via activation of the α2 isoform of AMPK.

1. Introduction

Physical exercise is a potent stimulator of glucose transport and glucose transporter 4 (GLUT4) expression in skeletal muscle. An acute bout of exercise increases the rate of glucose transport into contracting muscles by inducing translocation of GLUT4 to the cell surface via an insulin-independent mechanism (contraction-stimulated glucose transport) [1]. Acute exercise also activates expression of GLUT4 protein, and the GLUT4 protein expression is elevated with repeated bouts of acute exercise [2]. The exercise-induced increase in GLUT4 is associated with improved insulin sensitivity (ie, increased rates of insulin-stimulated GLUT4 translocation and glucose transport into

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skeletal muscle) [3]. These mechanisms of enhanced glucose transport help improve glycemic control in patients with diabetes and may help prevent nondiabetic subjects from developing glucose intolerance.

Recent studies have suggested that 5′ adenosine monophosphate–activated protein kinase (AMPK) is an important signaling intermediary leading to contraction-stimulated GLUT4 translocation and glucose transport [4-9] and GLUT4 expression [10-15] in skeletal muscle. AMPK is a heterotrimeric protein composed of a catalytic α subunit and regulatory subunits, β and γ. Although the α subunit exists in different isoforms in skeletal muscle [16], α1 and α2, the α2 isoform–containing AMPK is preferentially activated in response to exercise. For example, cycle ergometer exercise at 50% of maximum energy consumption ($\dot{V}O_2$max) does not change α2 or α1 activity, and exercise at 60% to 75% of $\dot{V}O_2$max increases α2, but not α1, activity in biopsy samples of vastus lateralis muscle from healthy subjects [17-19]. Similar activation of α2 occurs in response to cycle ergometer exercise at 70% of $\dot{V}O_2$max in patients with type 2 diabetes mellitus who have similar protein expression of α isoforms as healthy subjects [20]. In contrast, both isoforms are significantly activated in response to high-intensity exercise such as sprint exercise requiring power output 2- to 3-fold greater than that attained during maximal aerobic exercise [21]. In rat skeletal muscle, voluntary treadmill running exercise increases only α2 activity, whereas high-intensity contractions, such as electrically induced tetanic contractions, increase the activities of both isoforms in isolated rat skeletal muscle [8]. These observations in human and animal muscles suggest that regulation of the α isoforms is intensity-dependent in contracting skeletal muscle, and that the α2 isoform, rather than α1, is involved in the metabolic responses to moderate-intensity endurance exercise.

We explored the physiological relevance of the predominant α2 activation in skeletal muscle, focusing particularly on glucose transport, GLUT4 expression, and insulin sensitivity by selectively activating α2 AMPK using the AMPK-stimulating agent, 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR).

2. Materials and methods

2.1. Materials

AICAR was obtained from Sigma (St Louis, MO). Phosphospecific antibody directed against AMPKα Thr^{172} was obtained from Cell Signaling Technology (Beverly, MA) and that directed against acetyl-coenzyme A carboxylase β (ACCβ) Ser^{79} from Upstate Biotechnology (Lake Placid, NY). Anti-GLUT4 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All radioactive materials were purchased from NEN Life Science Products (Boston, MA). Reagents for the protein assay were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were purchased from Sigma or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

2.2. Animals

Male C57/B6 mice, aged 7 to 10 weeks, were obtained from Shimizu Breeding Laboratories (Kyoto, Japan) and fed standard laboratory chow and water ad libitum. They were housed in plastic cages in an environmentally controlled room maintained at 23°C with a 12-hour light-dark cycle. Mice were fasted for 8 to 10 hours before the experiments, except as otherwise described. Blood samples were collected from the tail vein. All protocols for animal use and euthanasia were reviewed and approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan.

2.3. Administration of AICAR

For studies of a single administration of AICAR, AICAR was dissolved in saline (20 g/L) and injected subcutaneously or intraperitoneally without anesthesia at a dose of 250 mg/kg body weight. Mice were then killed by cervical dislocation at the indicated time points, and either hind limb muscles (gastrocnemius, soleus, tibialis anterior, and extensor digitorum longus [EDL] muscles) or soleus and EDL muscles were dissected. For studies of repeated injections of AICAR, 250 mg/kg of AICAR was dissolved in saline (20 g/L) and injected into fed mice intraperitoneally 3 times a day for up to 8 days. Mice were killed by cervical dislocation 12 to 16 hours after the last injection, and the hind limb or soleus and EDL muscles were collected. The muscles were either processed fresh to measure 2-deoxyglucose (2DG) transport or frozen and stored in liquid nitrogen for later assays. Saline was injected as a control condition in the studies using the single and repeated administration of AICAR.

2.4. Swimming exercise

Mice swam in groups of 6 or less at a time at ~60% of $\dot{V}O_2$max (5 L/min flow rate) for 30 minutes during the dark cycle as described previously [22]. A large adjustable-current pool (90 × 45 × 45 cm) filled to a depth of 38 cm [22] allowed each mouse to swim without interference with other mice. A constant current was generated by circulating water with a pump, and the flow was monitored by a water flow meter, which was used to adjust the strength of the current. The temperature of the water was maintained at 34°C with a water heater and thermostat. For studies involving a single bout of exercise, mice were killed by cervical dislocation immediately after swimming, the hind limb muscles were dissected, and the muscles were frozen and stored in liquid nitrogen. For studies involving repeated bouts of exercise, fed mice swam for 30 minutes during the dark cycle twice a day for up to 7 days. Twelve to 16 hours after the last exercise session, the mice were killed and muscle samples were dissected, frozen, and stored in liquid nitrogen.

2.5. Intraperitoneal glucose test and insulin tolerance test

The intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed as described [23], with modifications. For the GTT, glucose (2.0 g/kg body weight) was administered intraperitoneally to conscious animals 12 to 16 hours after the last injection of AICAR or saline, or swimming exercise. For the ITT, human recombinant insulin (Eli-Lilly, Indianapolis, IN) (1.2 U/kg body weight diluted with saline) was injected intraperitoneally to fed conscious mice.

2.6. Isoform-specific AMPK activity

Isoform-specific AMPK activity was determined as described [24], with modifications. Frozen muscles were weighed and then homogenized in ice-cold lysis buffer (1:100 wt/vol) containing 20 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 50 mmol/L NaCl, 250 mmol/L sucrose, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 2 mmol/L dithiothreitol, 4 mg/L leupeptin, 50 mg/L soybean trypsin inhibitor, 0.1 mmol/L benzamidine, and 0.5 mmol/L phenylmethylsulfonyl fluoride, and centrifuged at 14000*g* for 30 minutes at 4°C. Supernatants (200 μg protein) were immunoprecipitated with specific antibodies against the α1 or α2 catalytic subunit [24] and protein A/G agarose beads (Pierce, Rockford, IL). Immunoprecipitates were washed twice in lysis buffer and twice in wash buffer containing 240 mmol/L HEPES (pH 7.0) and 480 mmol/L NaCl. The kinase reaction, which was started by adding 0.1 mmol/L SAMS peptide with the sequence HMRSAMSGLHLVKRR, contained 40 mmol/L HEPES (pH 7.0), 0.2 mmol/L AMP, 80 mmol/L NaCl, 0.8 mmol/L dithiothreitol, 5 mmol/L $MgCl_2$, and 0.2 mmol/L ATP [2 μCi (γ-^{32}P)ATP] at 30°C for 20 minutes in a final volume of 40 μL. At the end of the reaction, a 15-μL aliquot was removed and spotted onto Whatman P81 paper (Whatman International, Maidstone, UK). The papers were washed 6 times in 1% phosphoric acid and once in acetone. ^{32}P incorporation was quantified with a scintillation counter, and kinase activity was expressed as fold increases relative to basal levels.

2.7. 2-Deoxyglucose transport activity

The amount of 2DG transport was determined as described [25], with modifications. Tendons from both ends of dissected soleus and EDL muscles were tied with sutures (silk 3-0, Natsume Seisakusho, Tokyo, Japan), and the muscles were mounted on an incubation apparatus to maintain resting length. To measure 2DG transport after a single injection of AICAR, muscles were incubated for 10 minutes in 7 mL of incubation buffer containing Krebs-Ringer bicarbonate (KRB) buffer (117 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 2.4 mmol/L KH_2PO_4, 2.4 mmol/L $MgSO_4$, and 24.6 mmol/L $NaHCO_3$) with 2 mmol/L pyruvate and gassed continuously with 95% O_2 and 5% CO_2. Muscles were then transferred to 2 mL of transport buffer containing KRB buffer with 1 mmol/L 2-deoxy-D-[^{3}H]glucose (1.5 mCi/L) and 7 mmol/L D-[^{14}C]mannitol (0.45 mCi/L) at 30°C and incubated for 10 minutes. To measure basal- and insulin-stimulated 2DG transport after repeated AICAR treatment, dissected muscles were preincubated in the incubation buffer for 40 minutes and then incubated in the incubation buffer with or without 5000 mU/L insulin for 40 minutes. Muscles were then transferred to 2 mL of the transport buffer with or without 5000 mU/L insulin and incubated for 10 minutes. Transport was terminated by dipping muscles in KRB at 4°C, and the muscles were frozen in liquid nitrogen. Frozen muscles were weighed and then processed by incubating in 300 μL of 1 mol/L NaOH at 80°C for 10 minutes. Digestates were neutralized with 300 μL of 1 mol/L HCl. Radioactivity in aliquots of the digestates was determined by liquid scintillation counting for dual labels, and the extracellular and intracellular spaces were calculated.

2.8. Muscle glycogen content

Glycogen content was assayed as described [26], with modifications. Frozen muscles were weighed and digested in 1 mol/L NaOH (1:9 wt/vol) at 85°C for 10 minutes. At the end of the incubation, tubes were shaken by hand to facilitate digestion. After cooling to room temperature, digestates were neutralized with 1 mol/L HCl (1:9 wt/vol), and then 5 mol/L HCl was added to obtain a final concentration of 2 mol/L HCl. The digestates were incubated again at 85°C for 2 hours and then neutralized with 5 mol/L NaOH. The concentration of hydrolyzed glucose residues was measured enzymatically using the hexokinase glucose assay reagent (Sigma). Glycogen content was expressed as micromoles of glucose units per gram (wet weight) of muscle.

2.9. Glycogen synthase activity

Glycogen synthase activity was assayed as described [26], with modifications. Frozen muscles were homogenized in buffer containing 20 mmol/L HEPES (pH 7.4), 1% Triton X-100, 50 mmol/L NaCl, 2 mmol/L EGTA, 50 mol/L NaF, 50 mol/L β-glycerophosphate, 10 mg/L aprotinin, 3 mol/L benzamidine, 4 mg/L leupeptin, and 0.5 mol/L phenylmethylsulfonyl fluoride, and centrifuged at 14000*g* for 30 minutes at 4°C. The supernatants (40 μg of protein) were added to 80 μL of reaction solution containing 50 mmol/L Tris-HCl (pH 7.8), 5 mol/L EDTA, 6.7 mmol/L UDP-[^{14}C]glucose (100 μCi/mmol/L), 10 g/L glycogen, 50 mol/L β-glycerophosphate, and 50 mmol/L NaF in the presence or absence of 6.7 mmol/L glucose-6-phosphate at 30°C to measure the glucose-6-phosphate–independent (I-form) and the total glycogen synthase activities, respectively. The reaction was terminated after 15 minutes by spotting the reaction mixture on filter papers; after extensive washing with 66% (vol/vol) ethanol, the samples were counted in a scintillation counter to measure ^{14}C incorporated into glycogen. The enzyme activity was calculated as the ratio of the I-form activity to total activity.

2.10. Immunoblotting

Immunoblotting was performed as described [26], with modifications. Frozen muscles were homogenized in 10 volumes (1:10, wt/vol) of a solution containing 20 mmol/L HEPES (pH 7.4), 50 mmol/L β-glycerophosphate, 2mmol/L EGTA, 1% Triton X-100, 10% glycerol, 1 mmol/L dithiothreitol, 3 mmol/L benzamidine, 1 mmol/L $NaVO_4$, 0.5 mmol/L phenylmethylsulfonyl fluoride, 200 mg/L of soybean trypsin inhibitor, 10 mg/L aprotinin, and 10 mg/L leupeptin. The homogenates were centrifuged at 14000*g* at 4°C for 30 minutes. The supernatants were then diluted with water and Laemmli buffer and boiled at 80°C for 2 minutes. Denatured lysates (20-30 μg protein) were separated on a 10% polyacrylamide gel. Proteins were then transferred to a polyvinylidene difluoride membrane (PolyScreen; Perkin-Elmer, Boston, MA) at 100 V for 1 hour. The membranes were blocked with Block Ace (Yukijirushi Nyugyo, Sapporo, Japan) and left to incubate overnight with antibodies. The membranes were then washed, reacted with antirabbit immunoglobulin G coupled to peroxidase (Santa Cruz Biotechnology), and developed with an enhanced chemiluminescence reagent (Hyperfilm) according to the manufacturer's instructions (Amersham, Uppsala, Sweden). The signal on the blot was detected and quantified with a Lumino-Image Analyzer LAS-1000 System (Fuji Photo Film, Tokyo, Japan). Data were expressed relative to control values.

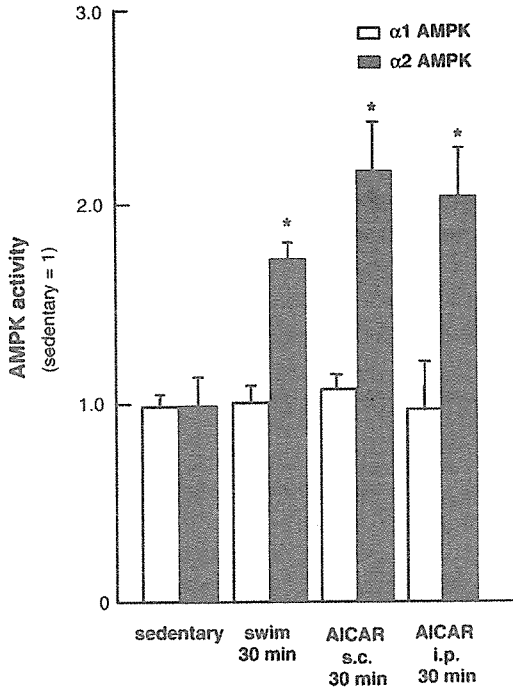

Fig. 1. Effects of exercise and AICAR on α1 and α2 AMPK activities in hind limb muscles. After a 30-minute bout of swimming or 30 minutes after subcutaneous (s.c.) or intraperitoneal (i.p.) injection of 250 mg/kg AICAR, hind limb muscles (gastrocnemius, soleus, tibialis anterior, and EDL) were removed, and isoform-specific AMPK activities were determined. Results are means ± SE (n = 7-10 per group). *P < .05 compared with muscles from sedentary animals.

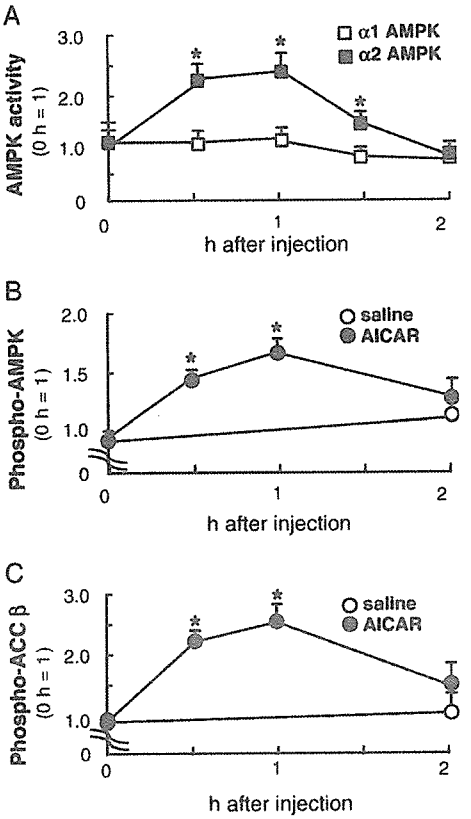

Fig. 2. Time course of changes in isoform-specific AMPK activity (A), AMPK phosphorylation (B), and ACCβ phosphorylation (C) in hind limb muscles after subcutaneous AICAR injection (250 mg/kg). Results are means ± SE (n = 7-10 per group). *P < .05 compared with basal levels.

2.11. Blood lactate, insulin, and glucose concentrations

Blood lactate concentration was measured by the lactate oxidase method using an automated analyzer (Lactate Pro; Arcray, Kyoto, Japan). Serum insulin concentration was determined using an Insulin ELISA kit (Morinaga Institute of Biological Sciences, Yokohama, Japan). Blood glucose concentration was measured by the glucose oxidase method with an automated blood glucose analyzer (Glutest Ace, Sanwa Kagaku, Nagoya, Japan).

2.12. Statistical analysis

Results are presented as means ± SE. Two means were compared by the unpaired Student *t* test. Multiple means were compared by analysis of variance followed by post hoc comparison using the Fisher protected least-significant difference method. P < .05 was considered statistically significant.

3. Results

3.1. Moderate-intensity exercise and AICAR activated predominantly α2 AMPK to a similar extent

After 30 minutes of moderate-intensity swimming exercise, α2 AMPK activity increased by 80%, but α1

AMPK activity did not change significantly in the hind limb muscles (Fig. 1). Similarly, subcutaneous and intraperitoneal injection of AICAR (250 mg/kg) activated α2 AMPK by 110% and 100%, respectively, but did not activate α1 AMPK (Fig. 1). The stimulation of α2 AMPK activity by exercise did not differ significantly from that induced by AICAR injection. The exercise-stimulated activation of α2 was abolished within 2 hours after exercise.

3.2. AICAR increased α2 AMPK activity, AMPK phosphorylation, and ACCβ phosphorylation in skeletal muscle

α2 AMPK activity was significantly higher 30, 60, and 90 minutes after subcutaneous injection of AICAR and returned to baseline within 2 hours after injection in the hind limb muscles (Fig. 2A). α1 AMPK activity did not change at any time point examined (Fig. 2A). Phosphorylation of Thr[172], an essential residue for full kinase activity [27], increased significantly in parallel with α2 AMPK activation (Fig. 2B). Phosphorylation of ACCβ, a known substrate of AMPK [28], also displayed a similar

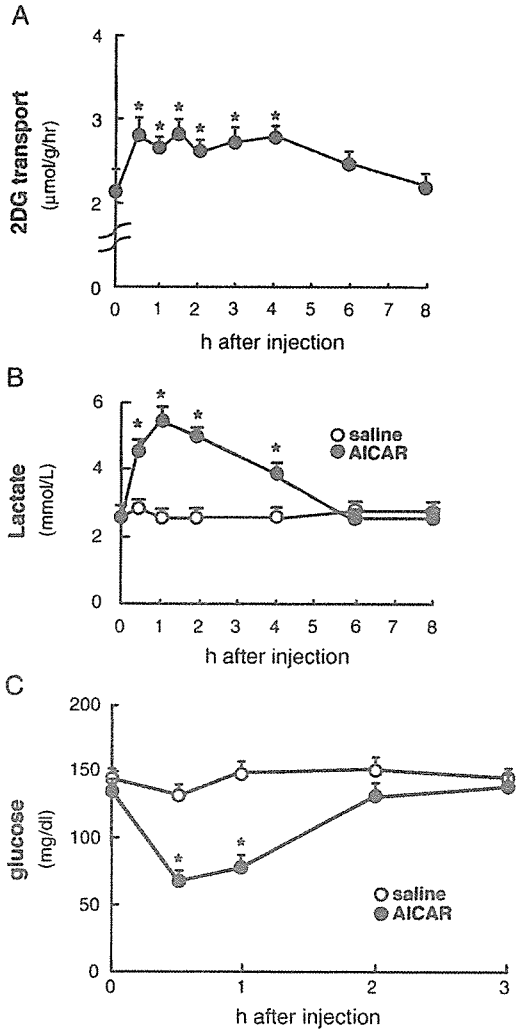

Fig. 3. Time course of 2DG transport activity in soleus muscle (A), blood lactate concentration (B), and blood glucose concentration (C) after subcutaneous injection of AICAR (250 mg/kg). Results are means ± SE (n = 7 to 10 per group). *P < .05 compared with basal levels.

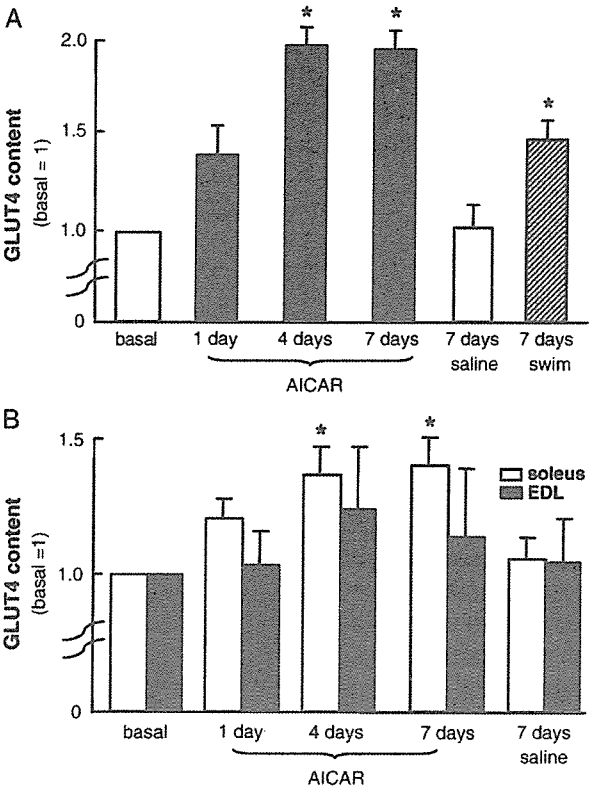

Fig. 4. Glucose transporter 4 protein content in hind limb muscles (A), and soleus and EDL muscles (B). After repeated intraperitoneal injection of AICAR (250 mg/kg) or saline 3 times a day for up to 7 days, or exercise swim training for 7 days, either hind limb or soleus and EDL muscles were isolated, and GLUT4 content was determined with immunoblotting. Results are means ± SE (n = 10 per group). *P < .05 compared with basal levels.

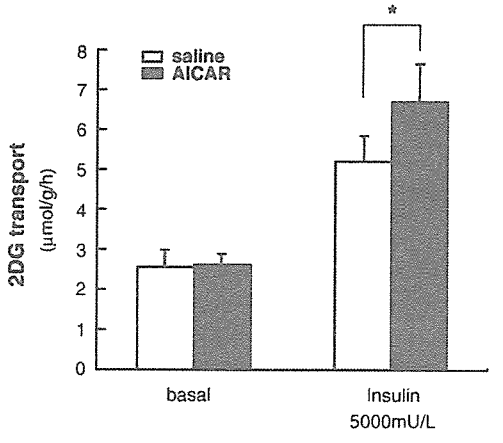

Fig. 5. Basal and insulin-stimulated 2DG transport activity in isolated soleus muscle after repeated intraperitoneal injection of AICAR or saline for 7 days. Soleus muscle was isolated 12 to 16 hours after the last injection, and 2DG transport activity was determined in the absence or presence of 5000 mU/L insulin. Results are means ± SE (n = 7-10 per group). *P < .05 compared with the saline group.

pattern as α2 AMPK activity (Fig. 2C). The α2 AMPK activity was significantly elevated for 4 hours after a single intraperitoneal injection of AICAR, whereas the α1 AMPK activity did not change.

3.3. The AICAR-induced increase in 2DG transport activity into skeletal muscle was accompanied by an increase in blood lactate concentration and decrease in blood glucose concentration

In the soleus muscle, a single subcutaneous injection of AICAR increased the rate of 2DG transport by 30%, and this elevated activity was maintained for 4 hours (Fig. 3A). Neither glycogen concentration nor glycogen synthase activity was altered (glycogen: baseline, 36.3 ± 1.6 μmol/g; 0.5 hour, 36.6 ± 0.6 μmol/g; 1.0 hour, 37.9 ± 1.9 μmol/g; 2.0 hours, 38.4 ± 1.3 μmol/g; n = 7-8 per group; glycogen synthase: baseline, 14.9% ± 0.2%; 0.5 hour, 15.2% ± 0.3%; 1.0 hour, 14.4% ± 0.4%; 2.0 hours, 14.6% ± 0.3%; n = 8 per group). In the EDL muscle, 2DG transport did not increase significantly (baseline, 2.0 ± 0.1 μmol/g per hour; 0.5 hour, 2.2 ± 0.1 μmol/g per hour; n = 8 per group, $P < .10$). Blood lactate concentration, an indicator of nonoxidative glucose utilization, was also elevated for 4 hours after injection (Fig. 3B). Blood glucose concentration decreased after a single AICAR injection, and this reduction was abolished within 2 hours after injection (Fig. 3C). Plasma insulin concentration was unchanged at the time points studied (baseline, 1.3 ± 0.3 μg/L; 0.5 hour, 1.3 ± 0.1 μg/L; 1.0 hour, 1.3 ± 0.3 μg/L; 2.0 hours, 1.3 ± 0.3 μg/L; n = 7 per group).

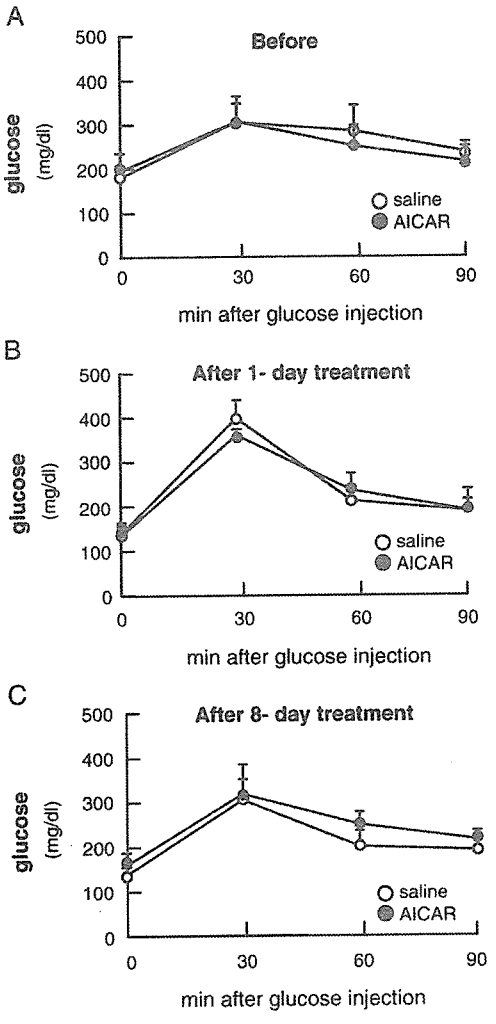

Fig. 6. Glucose tolerance in AICAR- and saline-treated mice. The intraperitoneal GTT was performed before (A) and after repeated intraperitoneal injection of AICAR (250 mg/kg) or saline 3 times a day for 1 day (B) and 8 days (C). Glucose (2.0 g/kg body weight) was administered by intraperitoneal injection 12 to 16 hours after the last injection of AICAR. Results are means ± SE (n = 7-10 per group).

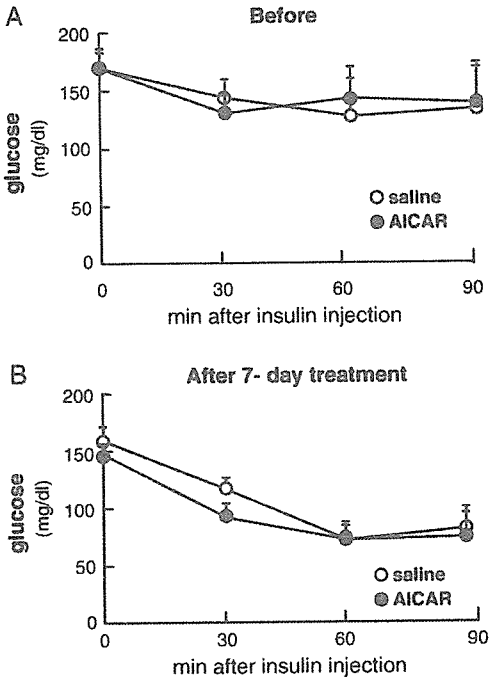

Fig. 7. Insulin tolerance in AICAR- and saline-treated mice. The intraperitoneal ITT was performed before (A) and after (B) repeated intraperitoneal injections of AICAR (250 mg/kg) or saline 3 times a day for 7 days. Glucose (1.2 U/kg body weight) was administered by intraperitoneal injection 12 to 16 hours after the last injection of AICAR. Results are means ± SE (n = 7-10 per group).

3.4. Repeated AICAR injection increased GLUT4 content and insulin-stimulated glucose transport in skeletal muscle

Glucose transporter 4 content in the hind limb muscles increased by 50% after 7 consecutive days of swimming (Fig. 4A). Similarly, repeated intraperitoneal AICAR injection 3 times a day for 4 to 7 days increased GLUT4 content in the hind limb muscles by 90% (Fig. 4A) and in soleus muscle by 40% (Fig. 4B). The increase in GLUT4 in EDL muscle was not significant (Fig. 4B). To determine whether the increased GLUT4 content was associated with enhanced insulin-stimulated glucose transport, we measured 2DG transport activity in soleus muscle treated with AICAR and saline for 7 days. As shown in Fig. 5, the baseline rate of 2DG transport was not affected by AICAR, whereas the

insulin-stimulated rate of 2DG transport activity was 20% higher in AICAR-treated than in saline-treated soleus muscle. In soleus muscle, glycogen synthase activity (% I-form) was not affected (baseline, 14.1% ± 0.3%; 7 days, 14.8% ± 0.8%; n = 8), and glycogen content did not change in response to AICAR treatment (baseline, 39.2 ± 3.1 μmol/g; 7 days, 44.0 ± 3.5 μmol/g; n = 10).

3.5. Whole-body glucose tolerance and insulin tolerance were not affected by repeated AICAR injection

The intraperitoneal GTT (Fig. 6) and ITT (Fig. 7) were performed to determine the effects of repeated AICAR injection on whole-body glucose metabolism. The GTT was performed before (Fig. 6A), after 1 day (Fig. 6B), and after 8 days (Fig. 6C) of administration of AICAR or saline. Glucose concentration did not differ between the AICAR- and saline-treated groups at any time point. Fasting insulin concentration was not affected by the AICAR treatment (baseline, 1.3 ± 0.1; 4 days, 1.2 ± 0.2; 7 days, 1.2 ± 0.2; n = 7). The ITT was performed before (Fig. 7A) and after 7 days (Fig. 7B) of repeated intraperitoneal injection of AICAR or saline. Similar to the results of the GTT, the response to the ITT did not differ significantly between the AICAR- and saline-treated groups. The responses to the GTT (Fig. 8A) and ITT (Fig. 8B) did not differ between sedentary animals and those exercised for 7 days. Body weight was unchanged after AICAR administration (baseline, 24.2 ± 0.6 g; 7 days, 24.4 ± 0.4 g; n = 10).

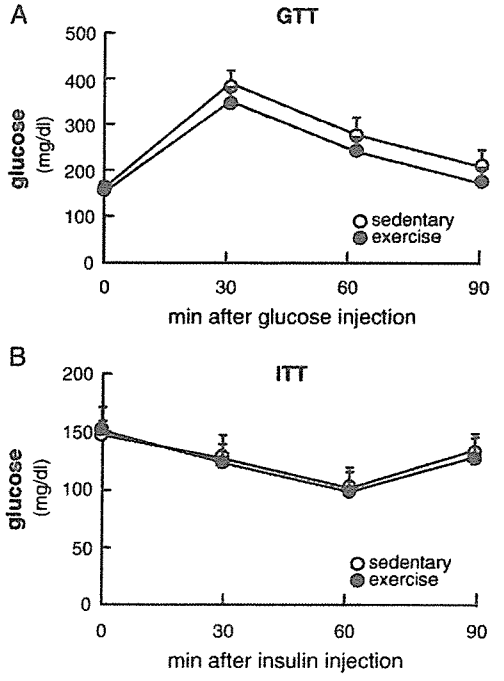

Fig. 8. Glucose tolerance and insulin tolerance in sedentary and exercise-trained mice. The exercise group swam for 30 minutes twice a day for 7 days. The intraperitoneal GTT (A) and intraperitoneal ITT (B) were performed 16 hours after the last bout of exercise. Results are means ± SE (n = 7-10 per group).

4. Discussion

Endurance exercise activates predominantly α2 AMPK in human and animal skeletal muscles. Fujii et al [17] reported a 2-fold increase in α2 AMPK activity in human vastus lateralis muscle after 60-minute cycle exercise at 70% $\dot{V}O_2$max, but no change in α1 AMPK after exercise. Similarly, Wojtaszewski et al [18] reported a 3-fold activation of α2 AMPK in human vastus lateralis after cycle exercise at 75% $\dot{V}O_2$max for 55 minutes followed by 90% $\dot{V}O_2$max for 5 minutes. Musi et al [8] showed that α2 AMPK is activated by 50% to 100% after treadmill running (18-32 m/min at 10% grade for 1 hour) and that this activation is accompanied by a significant increase in 3-*O*-methylglucose transport in rat epitrochlearis muscle. Our observations of a 2-fold increase in α2 AMPK activity after 30-minute swimming at ~60% $\dot{V}O_2$max in mouse skeletal muscle, but no increase in α1 AMPK activity, are consistent with these previous findings.

We administered 250 mg/kg AICAR subcutaneously or intraperitoneally to activate α2 AMPK to the same extent as the activation observed in skeletal muscle after exercise. AICAR is taken up into skeletal muscle and metabolized by adenosine kinase to form ZMP, a monophosphorylated derivative that mimics the effects of AMP on AMPK without changing the intracellular levels of AMP or ATP [29]. The concentration of intracellular AMP and the AMP/ATP ratio are both important determinants of AMPK activity; α2 AMPK has greater dependence on AMP than the α1 isoform in both the allosteric activation by AMP and the covalent activation by upstream kinase [27,30]. In our study, both types of injections activated α2 AMPK in skeletal muscle, but the intraperitoneal injection produced a longer-lasting activation than the subcutaneous injection. α2 AMPK activity increased for at least 4 hours after the intraperitoneal injection, but the enzyme activity returned to baseline within 2 hours after subcutaneous injection. Although the precise mechanism by which AICAR activates α2 AMPK is unknown, the site of injection may have a substantial influence on the rate of absorption, and the time course and intensity of activation.

Although AICAR is not strictly specific for AMPK [31-33], recent studies with AICAR have provided important information about the function of AMPK in muscle glucose transport and GLUT4 expression. Mu et al [7] selectively blocked AMPK in mouse skeletal muscle with muscle-specific expression of a dominant-negative, kinase-dead form of α2 AMPK. In this mouse, the stimulatory effects of AICAR on glucose transport [7] and GLUT4 expression [15] were blocked completely. In addition, the AICAR-stimulated glucose transport was abolished in skeletal muscles from whole-body α2 knockout mouse, but not in muscles from whole-body α1 knockout mouse [9]. Furthermore, incubating isolated animal muscles in the presence of AICAR increased glucose transport [4,34] and GLUT4 protein expression [11]. These results strongly

indicate that the metabolic effects of AICAR on skeletal muscle involve α2 AMPK-dependent signaling events, which can occur independent of changes in systemic factors. Interestingly, in mice with muscle-specific expression of a dominant-negative, kinase-dead AMPK, glucose transport is only partially reduced in response to electrically stimulated contractions of hind limb muscles [7], and muscle GLUT4 messenger RNA increases after endurance exercise (two 3-hour bouts of treadmill running) [15]. Similarly, the rate of glucose transport increases after electrical stimulation of isolated muscles from the transgenic mouse expressing the dominant-negative α2 AMPK [9]. Although the previous studies did not examine the effects of moderate-intensity exercise, there may be additional signaling mechanisms, other than α2 AMPK, leading to exercise-induced metabolic events in skeletal muscle.

We found that the α2 AMPK activity decreased within 2 hours after subcutaneous injection of AICAR (Fig. 2A), whereas 2DG transport remained elevated for at least 4 hours (Fig. 3A). Musi et al [8] previously demonstrated that the time course of AMPK is dissociated from the glucose transport activities in isolated rat epitrochlearis muscle during in vitro electrical stimulation of muscle contractions. AMPK activity decreased rapidly after the cessation of tetanic contractions ($t_{1/2}$ = 8 minutes), whereas the rate of decrease in 3-*O*-methylglucose transport was much slower and had decreased by only 48% after 60 minutes. This previous report and our findings suggest that, although AMPK may be involved in stimulating glucose transport, sustained AMPK activity is not required to maintain transport activity.

Activation of AMPK and its effects vary by muscle fiber type. In the studies using rat skeletal muscle, long-term AICAR administration has the greatest effects on GLUT4 and glycogen content in fast-twitch muscles [10,12,35]. Daily subcutaneous injections of AICAR at a dose of 1 g/kg body weight for 4 weeks increased GLUT4 and glycogen content in the red and white quadriceps, but not in the soleus muscle in rats [35]. Moreover, acute AICAR exposure stimulates glucose transport in white muscles, but has no effect in rat soleus muscle [34,36]. In contrast, incubation with AICAR markedly increases glucose transport in both soleus and EDL muscles in the mouse [34]. We also found significant increases in 2DG transport activity and GLUT4 content ($P < .05$) in mouse soleus muscle. This discrepancy in the effects of AICAR between rat and mouse soleus muscles may be due to a greater percentage of fast-twitch muscle fibers within mouse soleus (ie, the mouse soleus has proportionately more fast-twitch fibers than the rat soleus) [34]. Because of the nonspecific stimulation by AICAR in mouse skeletal muscle, we believe that most skeletal muscles responded to the subcutaneous and intraperitoneal AICAR administration in our study.

The concept that a large number of skeletal muscles are stimulated by AICAR in mouse is indirectly supported by our observation that AICAR treatment caused a marked increase in blood lactate concentration and reduction in blood glucose concentration, with a corresponding increase in glucose transport activity (Fig. 3). The hypoglycemic effect of AICAR is blunted in mice with muscle-specific expression of the dominant negative AMPK [7], emphasizing the pivotal role of muscle AMPK in AICAR-induced hypoglycemia. However, it has also been reported that, after the conversion into ZMP, AICAR exerts a dose-dependent inhibition of fructose-1,6-bisphosphatase, which inhibits gluconeogenesis and enhances lactate production in the liver [32,33]. Thus, the effects on both skeletal muscle and other tissues, including liver, may contribute to the hypoglycemia and elevated lactate concentrations caused by AICAR.

The contribution of increased muscle GLUT4 to glucose tolerance and insulin sensitivity has been clearly documented in studies using transgenic mice with muscle-specific overexpression of GLUT4 [37,38]. However, we found that AICAR treatment had no effect on blood glucose excursions during the GTT and ITT compared with the saline treatment (Figs. 6 and 7). Similarly, swimming exercise for 7 days did not affect glucose excursions compared with sedentary mice (Fig. 8). Although the underlying mechanism is unclear, the effects of long-term AICAR treatment and swimming exercise may be below the detectable limit of the GTT or ITT because we used the metabolically normal mouse (C57/B6). This concept is consistent with the observation that metabolic improvements occurred after 7 days of treatment with AICAR and were detected by the oral GTT (3 mg/kg) and ITT (10 U/kg) in KKA^y-CETP mice, a model of insulin-resistant type 2 diabetes mellitus [39]. Because we performed ITTs on fed animals, the food consumption of the animals coming into the test could affect the response, and there may have been a different response at 15-minute time point that returned to control at 30 minutes in GTTs.

In summary, we found that pharmacological activation of α2AMPK by AICAR at a physiological level led to a short-term increase in glucose transport and that long-term activation of the isoform increased GLUT4 protein and enhanced insulin-stimulated glucose transport in mouse skeletal muscle. These results strongly suggest that activation of α2 AMPK during moderate exercise plays pivotal roles in exercise-stimulated glucose uptake and utilization in skeletal muscle. Our data also support the hypothesis that α2 AMPK can be a target of pharmacological manipulation aiming to improve glucose metabolism in skeletal muscle.

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References

[1] Hayashi T, Wojtaszewski JF, Goodyear LJ. Exercise regulation of glucose transport in skeletal muscle. Am J Physiol 1997;273: E1039-51.

[2] MacLean PS, Zheng D, Dohm GL. Muscle glucose transporter (GLUT 4) gene expression during exercise. Exerc Sport Sci Rev 2000;28:148-52.

[3] Etgen Jr GJ, Jensen J, Wilson CM, et al. Exercise training reverses insulin resistance in muscle by enhanced recruitment of GLUT-4 to the cell surface. Am J Physiol 1997;272:E864-9.

[4] Hayashi T, Hirshman MF, Kurth EJ, et al. Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. Diabetes 1998;47:1369-73.

[5] Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, et al. 5′AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes 1999;48:1667-71.

[6] Hayashi T, Hirshman MF, Fujii N, et al. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. Diabetes 2000;49:527-31.

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

[8] Musi N, Hayashi T, Fujii N, et al. AMP-activated protein kinase activity and glucose uptake in rat skeletal muscle. Am J Physiol Endocrinol Metab 2001;280:E677-84.

[9] Jorgensen SB, Viollet B, Andreelli F, 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.

[10] Holmes BF, Kurth-Kraczek EJ, Winder WW. Chronic activation of 5′-AMP–activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. J Appl Physiol 1999;87:1990-5.

[11] Ojuka EO, Nolte LA, Holloszy JO. Increased expression of GLUT-4 and hexokinase in rat epitrochlearis muscles exposed to AICAR in vitro. J Appl Physiol 2000;88:1072-5.

[12] Buhl ES, Jessen N, Schmitz O, et al. Chronic treatment with 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside increases insulin-stimulated glucose uptake and GLUT4 translocation in rat skeletal muscles in a fiber type–specific manner. Diabetes 2001;50:12-7.

[13] Zheng D, MacLean PS, Pohnert SC, et al. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J Appl Physiol 2001;91:1073-83.

[14] MacLean PS, Zheng D, Jones JP, et al. Exercise-induced transcription of the muscle glucose transporter (GLUT 4) gene. Biochem Biophys Res Commun 2002;292:409-14.

[15] Holmes BF, Lang DB, Birnbaum MJ, et al. AMP kinase is not required for the GLUT4 response to exercise and denervation in skeletal muscle. Am J Physiol Endocrinol Metab 2004;287:E739-43.

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

[17] Fujii N, Hayashi T, Hirshman MF, et al. Exercise induces isoform-specific increase in 5′AMP-activated protein kinase activity in human skeletal muscle. Biochem Biophys Res Commun 2000;273:1150-5.

[18] Wojtaszewski JF, Nielsen P, Hansen BF, et al. Isoform-specific and exercise intensity-dependent activation of 5′-AMP–activated protein kinase in human skeletal muscle. J Physiol 2000;528(Pt 1):221-6.

[19] Stephens TJ, Chen ZP, Canny BJ, et al. Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. Am J Physiol Endocrinol Metab 2002;282:E688-94.

[20] Musi N, Fujii N, Hirshman MF, et al. AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. Diabetes 2001;50:921-7.

[21] Chen ZP, McConell GK, Michell BJ, et al. AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. Am J Physiol Endocrinol Metab 2000;279:E1202-6.

[22] Matsumoto K, Ishihara K, Tanaka K, et al. An adjustable-current swimming pool for the evaluation of endurance capacity of mice. J Appl Physiol 1996;81:1843-9.

[23] Masuzaki H, Ogawa Y, Aizawa-Abe M, et al. Glucose metabolism and insulin sensitivity in transgenic mice overexpressing leptin with lethal yellow agouti mutation: usefulness of leptin for the treatment of obesity-associated diabetes. Diabetes 1999;48:1615-22.

[24] Toyoda T, Hayashi T, Miyamoto L, 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.

[25] Bruning JC, Michael MD, Winnay JN, et al. A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. Mol Cell 1998;2:559-69.

[26] Hayashi T, Hirshman MF, Dufresne SD, et al. Skeletal muscle contractile activity in vitro stimulates mitogen-activated protein kinase signaling. Am J Physiol 1999;277:C701-7.

[27] Stein SC, Woods A, Jones NA, et al. The regulation of AMP-activated protein kinase by phosphorylation. Biochem J 2000;3:437-43.

[28] 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.

[29] Hardie DG, Carling D. The AMP-activated protein kinase—fuel gauge of the mammalian cell? Eur J Biochem 1997;246:259-73.

[30] Salt I, Celler JW, Hawley SA, et al. AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. Biochem J 1998;334 (Pt 1):177-87.

[31] Young ME, Radda GK, Leighton B. Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR—an activator of AMP-activated protein kinase. FEBS Lett 1996;382:43-7.

[32] Vincent MF, Erion MD, Gruber HE, et al. Hypoglycaemic effect of AICAriboside in mice. Diabetologia 1996;39:1148-55.

[33] Vincent MF, Marangos PJ, Gruber HE, et al. Inhibition by AICA riboside of gluconeogenesis in isolated rat hepatocytes. Diabetes 1991;40:1259-66.

[34] Balon TW, Jasman AP. Acute exposure to AICAR increases glucose transport in mouse EDL and soleus muscle. Biochem Biophys Res Commun 2001;282:1008-11.

[35] Winder WW, Holmes BF, Rubink DS, et al. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. J Appl Physiol 2000;88:2219-26.

[36] Ai H, Ihlemann J, Hellsten Y, et al. Effect of fiber type and nutritional state on AICAR- and contraction-stimulated glucose transport in rat muscle. Am J Physiol Endocrinol Metab 2002;282: E1291-300.

[37] Leturque A, Loizeau M, Vaulont S, et al. Improvement of insulin action in diabetic transgenic mice selectively overexpressing GLUT4 in skeletal muscle. Diabetes 1996;45:23-7.

[38] Tsao TS, Burcelin R, Katz EB, et al. Enhanced insulin action due to targeted GLUT4 overexpression exclusively in muscle. Diabetes 1996;45:28-36.

[39] Fiedler M, Zierath JR, Selen G, et al. 5-Aminoimidazole-4-carboxyamide-1-beta-D-ribofuranoside treatment ameliorates hyperglycaemia and hyperinsulinaemia but not dyslipidaemia in KKAy-CETP mice. Diabetologia 2001;44:2180-6.

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メタボリックシンドロームの第1次予防
——一無・二少・三多の健康習慣—

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要　旨：一無(無煙)，二少(少食・少酒)，三多(多動・多休・多接)の6項目の健康習慣の実践がメタボリックシンドローム(MS)の第1次予防として有効であるかを検証した。平均実践数は3.5種類であった実践数の増加に比例して，ウエスト周囲径，血圧，中性脂肪，血糖の減少，HDL-Cの上昇を認め，4種類以上の実践群では3種類以下の実践群に比べ有意に動脈硬化が軽度であった。一無・二少・三多の健康習慣はMSの予防に有益であると考えられた。
(J Jpn Coll Angiol, 2006, 46: 341–344)

Key words: metabolic syndrome, life-style, atherosclerosis, prevention

はじめに

メタボリックシンドローム(metabolic syndrome: MS)は，生活習慣病の一つと考えられる[1]。世間において，Aという疾病の予防については，これこれの生活習慣がよい，Bという疾病の予防については別の生活習慣が効果的である，などといったさまざまな情報が国民に向けて発信されている。疾病別にリスクファクターが異なるのは医学的にいたしかたないことではある。しかし，基本的でしかも広範囲な疾病予防に有効な生活習慣，つまり健康的な生活習慣とは何であるかを端的に国民に発信することも，きわめて大切なことである。生活習慣は，身体のみならず，不安定な社会情勢によって増加しているストレスやこころの病についても配慮したものでなければならないと考えている。われわれは基本的健康習慣として，「一無・二少・三多」を提唱してきた。この健康習慣がMSの予防に有効であるかについて，これまでに発表された健康習慣を交えて考察する。

Breslowの7つの健康習慣

どのような生活習慣が生活習慣病予防に有効かについての研究は，Breslowらによって1965年という日本に比べてかなり早い時期に始まった。生活習慣病予防の視点から，喫煙，飲酒，身体的運動量，体重管理，睡眠，朝食，間食といった基本的な日常生活習慣に注目した内容である。

Belloc[2]は，Breslowとともに7つの健康習慣の実践が死亡率に関係するか否かの前向き調査(**Table 1**)を地域住民に行った。健康習慣をより多く行っている集団と，少ない集団とを9年間追跡した結果，死亡率に数倍の差があること，また若年者ほど，健康習慣の死亡率への関与が強いことを実証した。このような結果から，Breslowの7つの健康習慣は日本でも広く紹介され，平成9年版厚生白書にも掲載されている。しかし，これを含めほとんどが，原文に忠実でない翻訳で日本に紹介されている問題点が指摘されている。

Breslowの7つの健康習慣は発表されてから40年が経過し，いくつかの問題点が指摘されている。たとえば，多くの疾病の発病にストレスが間接的に関与しているが，Breslowの7つの健康習慣には，ストレスに関する項目が含まれていない。疲労回復に必要な睡眠時間には個人差がある。4～6時間睡眠者より8時間睡眠者のほうが死亡率が高く，睡眠が短いと感じている人が健康のために無理に長時間眠ろうとすることは，か

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Table 1 Belloc and Breslow's seven physical health status

1. Never smoked cigarettes
2. Drink not more than four drinks at a time
3. Often or sometimes engage in active sport, swim or take long walk, or often garden or do physical exercise
4. Report weight within the range of 5% under and 19.99% over the desirable standard for weight for men, or not more than 9.99% over for women
5. Usual hours of sleep 7 or 8
6. Eat breakfast almost every day
7. Eat between meals once in a while, rarely or never

Table 2 Questions about healthy habits in "give up one, reduce two, and increase three"

Do you smoke?	no smoking (1)	yes (0)
Do you think you eat too much?	no (1)	yes (0)
Do you drink alcohol less than 150 g per week?	yes (1)	no (0)
Do you regularly do vigorous exercise for 1 hour or more per week?	yes (1)	no (0)
Days off per month	six days and over (1)	less than six days (0)
Do you have enjoyable activities out of business?	yes (1)	no (0)

えって睡眠の質を低下させることが明らかになってきた[3]。このようなことを考慮すると，睡眠時間は7～8時間必要であるというBreslowの提言は適切とはいえなくなってきている。加えて日本人を対象にした研究で，Breslowの健康習慣の実践の有無にかかわらず，死亡率には関連がないという報告[4]がある。以上の結果より，Breslowの健康習慣を日本国民に示し続けることには問題があると考えられる。

池田の一無・二少・三多

池田義雄は，1991年「一無・二少・三多」という健康習慣の標語を提唱した[5]。一無とは無煙，二少とは少食・少酒，三多とは多動，多休，多接である。朝鮮半島では古くから一少(少食)，三多(多動，多休，多接)が用いられてきた。池田はこれに一無(無煙)と少酒を加えて一無・二少・三多という健康標語を創案した。一無とは煙が無い，つまりたばこを吸わないことである。少食，少酒は，食事量と飲酒量を少なめにすることに心がけることである。少食は腹八分目に相当する。多動とは，体をできるだけ動かすことである。多休は，休息や睡眠を十分にとり，体をリフレッシュさせることである。多休は，Breslowの睡眠時間とは異なり，休憩，仕事をしない休日の日数が十分であることをも含む。多接は，趣味などを通じて多くの物や人に接し，ストレスを発散し創造的な人生を送ることである。悩みを一人で抱え込まないことによりメンタルトラブルの予防にも有効である。

「一無・二少・三多」は一般大衆が覚えやすい語句になっていることも特徴である。

一無・二少・三多の実践に関する調査

東京慈恵会医科大学附属病院健康医学センターで，人間ドック受診の際に行った自記式問診票を用いて一無・二少・三多に関する調査を行った。内容は，一無・二少・三多をより具現化したものとして調査している(Table 2)。対象者の平均年齢は46歳で，ほとんどが勤労者であるため，多休は月間休日で評価しているが，高齢者を多く含む調査では，多休を「仕事・家事に追われて休息や睡眠が不十分ですか」という設問に変更してもよいと考えている。

一無・二少・三多とウエスト周囲径

動脈硬化性疾患の危険因子として，近年MSが注目されている。内臓脂肪型肥満はMSの中心的存在である。そこでまず，一無・二少・三多の生活習慣の実践と肥満の関連を検証した[6]。6つの健康習慣のうち，実践数

Table 3　Correlation of waist circumference (cm) with practicing number of six healthy habits

Practicing number	0–2	3–4	5–6	p value
	(n = 1299)	(n = 3539)	(n = 1153)	
Male	85.8 ± 8.1	84.4 ± 7.9	82.6 ± 7.9	< 0.0001*
Female	76.9 ± 9.1	76.0 ± 9.4	73.9 ± 8.2	< 0.0001**

*: There are significant differences among three groups
**: There are significant differences between 3–4 and 5–6 group, and between 0–2 and 5–6 group

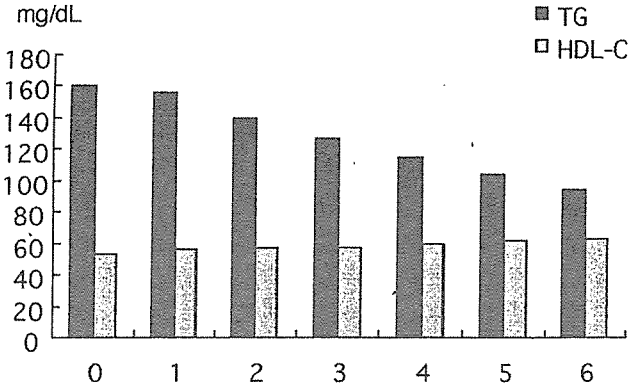

Figure 1　Triglyceride (TG) and HDL-cholesterol (HDL-C) according to number of practicing six health habits "give up one, reduce two, and increase three."
Multiple regression analysis was applied, adjusted age and gender. Significant level was $p < 0.05$.

が0〜2つの場合を少実践群，3〜4つの場合を中実践群，5つ以上実行している場合を多実践群とした。健康習慣の実践数が多いほどウエスト周囲径は低値を示した(Table 3)。男性において，ウエスト周囲径85cm以上を示す率は，少実践群55%，中実践群48%，多実践群38%であった。女性においてウエスト周囲径90cm以上を示す率は，少実践群11%，中実践群7%，多実践群3%であり，これも実践数が多くなるにつれ，異常を示す率は減少を示した。以上より，一無・二少・三多の健康習慣を多く実践することは，21世紀最大の課題となるであろうMS予防に有用な健康標語になると思われる。

一無・二少・三多とMS

2005年4月に日本で発表されたMSの診断基準は，上記のウエスト周囲径の基準値を超え，かつ，中性脂肪高値あるいはHDLコレステロール(HDL-C)低値，血圧高値，高血糖の3項目のうち2項目以上を満たした場合をいう。

Fig. 1に一無・二少・三多の6種類の生活習慣の実践数とトリグリセリド(TG)，HDL-Cとの関係を示す。性と年齢で補正したlinear regression解析の結果，実践数の増加に比例して中性脂肪は有意な低値を，HDL-Cの有意な高値を認めた。TGにおいてはすべての実践数間で，またHDL-Cは実践数0と1の間および実践数5と6を除いたすべての群間で統計学的有意($p < 0.05$)な差を認めた。同様に血圧[7]，血糖[8]についても一無・二少・三多の健康習慣の実践数に比例して有意に低値を示すことが確認されている。ただし，これは断面調査であるので，因果関係について述べることには限界がある。

一無・二少・三多と動脈硬化

MSの概念の誕生は，動脈硬化予防が目的である。よって一無・二少・三多の健康習慣も動脈硬化を予防することができてこそ意義がある。これまでの調査から実践数の平均は3.5種類であることから，超音波断層法により計測した頸動脈硬化のプラーク指数を4種類以上実践群と3種類以下の実践群で比較した。4種類以上実践している群のプラーク指数は4.9 ± 4.2で，3種類以下の実践群の7.6 ± 5.8に比べ有意に($p < 0.001$)低かった[9]。ただしこれも断面調査であるので，因果関係について述べることには限界がある。

総　括

まずMSとは何か，そしてMSが動脈硬化の重要なリスクファクターであることを国民に知ってもらう必要がある。そこで，smoking, metabolic syndrome, LDLコレステロールを動脈硬化の新御三家とした。これらの頭文字，S・M・Lはちょうど洋服のサイズになるので覚えやすい。そして「一無・二少・三多」の健康習慣の

実践は，MSの元凶である内臓脂肪蓄積を予防し，また高血圧，脂質代謝異常，高血糖を抑制して，動脈硬化の進行を遅らせることができる可能性を明らかにした。この簡潔な健康標語を国民に広く浸透させることに力を注いでいきたい。

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文　献

1) Urashima M, Wada T, Fukumoto T et al: Prevalence of metabolic syndrome in a 22,892 Japanese population and its associations with life style. JMAJ, 2005, **48**: 441–450.
2) Belloc NB, Breslow L: Relationship of physical health status and health practices. Prev Med, 1972, **1**: 409–421.
3) Kripke DF, Garfinkel L, Wingard DL et al: Mortality associated with sleep duration and insomnia. Arch Gen Psychiatry, 2002, **59**: 131–136.
4) 川田智之：日本人男女の健康習慣と死亡．厚生の指標，2001，**48**：8–13.
5) 池田義雄：自己管理．別冊プラクティス．医歯薬出版，東京，1991，119–128.
6) 和田高士，福元　耕，常喜真理他：一無・二少・三多の健康習慣とBMI，ウエスト周囲径，体脂肪率の関係．肥満研，2003，**9**：348–352.
7) 和田高士，福元　耕，藤代健太郎：日本人のための健康習慣「一無・二少・三多」．循環器専門医，2004，**12**：235–240.
8) Wada T, Fukumoto T, Joki M et al: Effective prevention of diabetes mellitus: a motto for healthy habits—"none of one, less of two, more of three." Intern Med, 2005, **44**: 413–416.
9) Wada T, Fujishiro K, Fukumoto T et al: Effective prevention of carotid artery atherosclerosis by following this motto for healthy habits: "give up one, reduce two, and increase three." Methods Inf Med, 2005, **44**: 319–322.

The First Strategy for Metabolic Syndrome: Healthy Habits "Give up One, Reduce Two, and Increase Three"

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Key words: metabolic syndrome, life-style, atherosclerosis, prevention

"Give up one, reduce two, and increase three" is our motto in promoting healthy habits. The one thing to give up is smoking. The two things to reduce are the intake of food and alcohol. The three things to increase are exercise, rest, and enjoyable activities. We conducted a survey to determine the relationship between metabolic syndrome and compliance with the motto. Waist circumference, blood pressure, plasma glucose and triglyceride decreased significantly in relation to the number of healthy habits practiced. On the other hand, HDL-cholesterol increased significantly with the number of healthy habits practiced. Mean practicing number was 3.5. Plaque score in the carotid arteries detected using ultrasound was significantly higher in the unfavorable (practicing four to six habits) group (7.6 ± 5.8) than in the optimal (practicing zero to three habits) group (4.9 ± 4.2) ($p < 0.001$). The healthy habits contributed to prevention of atherosclerosis. This easy-to-remember motto of "give up one, reduce two, and increase three" appears to aid in preventing atherosclerotic lesions developed by metabolic syndrome. Since these results of the cross-sectional study have limitations, further studies are required to determine the effectiveness of the strategy. (J Jpn Coll Angiol, 2006, **46**: 341–344)

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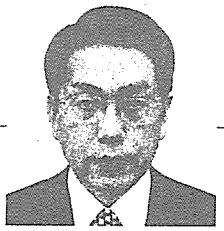
特集 食後の代謝異常


和田 高士

6．健康診断・人間ドックと耐糖能異常のスクリーニング

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はじめに

血糖検査が手軽に行えなかった時代には，糖尿病のスクリーニングは尿糖検査で行われていた．尿糖排泄閾値の存在から，尿糖は血糖値が160 mg/dL以上にならないと陽性に至らないため，少なくとも空腹時での尿糖検査では糖尿病の早期診断は不可能であった．しかし，尿糖検査は費用がかからず，極めて簡便であるので，食後高血糖を知る手段として尿糖検査を活用することには十分な意義があるといえる．その長所を利用して，家庭でもチェックできるデジタル尿糖計（タニタ：UG-102）が発売されている．従来の定性的な試験紙法では詳細な変化が捕捉しにくかったが，定量測定すること，特に食後2時間目の尿糖を計測することで，境界型～軽症な糖尿病型のセルフコントロールに役立つ．

健康診断で行われる血液検査は空腹時血糖，ヘモグロビンA_{1C}（HbA_{1C}）が代表である[1]．グリコアルブミン，1.5AGは，糖尿病治療中の人を対象とした診療の場で用いられている．病態を詳しく知ることができる検査は，経口ブドウ糖負荷試験である．しかし，検査時間だけで2時間を要し，何よりも健康診断は健康保険を使えないために，インスリン検査を含めると相当の費用がかかる．このため公的な健康診断では実施が困難である．人間ドックにおいても，後述する2日人間ドック（1泊2日）あるいは人間ドックのオプション検査のみで行われている程度である．

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表1　法律で定められている健康診断

	老人保健法	労働安全衛生法
施行者	自治体	事業主
対　象	40歳以上の地域住民	勤務している労働者
実施回数	年1回	雇入れ時・年1回以上
強制力	任　意	強　制
検査内容	血　糖 ヘモグロビンA_{1C} 尿　糖	血　糖 ヘモグロビンA_{1C} 尿　糖

健康診断・人間ドックの種類

成人を対象とした健康診断には，老人保健法による基本健康診査，労働安全衛生法に基づく雇入れ時健康診断ならびに定期健康診断がある（表1）．

老人保健法による健康診査は自治体が地域住民を対象に行うもので，保健所，開業医で行われる．新聞折り込みなどを利用した広報を通じて案内が配布され，1年間に1回の受診ができる機会があり，受診するかしないかは任意である．

労働安全衛生法による健康診断は，事業主が労働者を対象に行う健康診断である．雇入れ時ならびに年に1回（労働状況により年2回の場合もある）実施される．

人間ドックにおける検査

人間ドックは任意の健康診断であるため，実施施設によって検査内容は様々である．また，1日人間ドッ

表2　老人保健法による基本健康診査結果判定基準(平成14年4月改訂)

	空腹時血糖	随時血糖	ヘモグロビンA_{1C}
異常認めず	110 mg/dL未満	140 mg/dL未満	5.5%未満
要指導	110～125 mg/dL	140～199 mg/dL	5.5～6.0%
要医療	126 mg/dL以上	200 mg/dL以上	6.1%以上

注）ヘモグロビンA_{1C}は血糖値が要指導の者を対象に実施される．
(文献3より引用改変)

表3　日本人間ドック学会による判定基準

	空腹時血糖	ヘモグロビンA_{1C}
異常なし	～109 mg/dL	～5.8%
軽度異常	110～115 mg/dL	
要経過観察・生活改善	116～125 mg/dL	5.9～6.1%
要医療	126 mg/dL～	6.2%～

(文献2より引用)

表4　ヘモグロビンA_{1C}の判定上の注意すべき病態

偽高値	偽低値
腎不全	肝硬変
異常ヘモグロビン	溶　血
アルコール多飲者	悪性貧血
高ビリルビン血症	妊　娠

(文献4より引用)

表5　平成20年度からの健康診断での耐糖能異常者の指導方針

	空腹時血糖	随時血糖	ヘモグロビンA_{1C}
保健指導	100～125 mg/dL	140～179 mg/dL	5.5～6.0%
受診勧奨	126 mg/dL以上	180 mg/dL以上	6.1%以上

(文献5より引用)

ク，2日人間ドック，長期人間ドックなど期間もいろいろであるが，近年の傾向としては1日人間ドック受診者数が増加し，2日人間ドックは減少傾向にある．この2つで人間ドック受診者全体の大半を占める．日本人間ドック学会は，この1日人間ドックと2日人間ドックでの耐糖能検査に関する基本検査項目として，前者では空腹時血糖とヘモグロビンA_{1C}を，後者では経口ブドウ糖負荷試験を採用している[2]．空腹時に行う必要がある上部消化管バリウム検査とブドウ糖負荷検査は同時に行えず，両方を実施するには2日に分けて行う必要があるため，ブドウ糖負荷検査は1日ドックには含まれていない．しかしながら，ヘモグロビンA_{1C}検査により，後述するが，大まかな耐糖能異常がわかるようになったため，ヘモグロビンA_{1C}検査と空腹時血糖との組み合わせが，1日人間ドックの基本検査項目として採用されている．

判定方法

老人保健法に基づく基本健康診査[3]と日本人間ドック学会が定めている判定方法[2]には差異がある．前者については表2，後者については表3に示す．

ヘモグロビンA_{1C}検査による判定上の注意

ヘモグロビンA_{1C}は，食後採血でも耐糖能異常を診断できるという長所をもっている．一方，ヘモグロビンA_{1C}値は耐糖能異常以外の病態でも上下することがあるため，判定には注意を要する(表4)[4]．

平成20年度からの健康診断の改正

平成17年4月にメタボリックシンドロームの診断基準が発表され，内臓脂肪型肥満の簡易指標として腹囲の測定意義，有用性が明らかにされた．これを受けて厚生労働省は，平成20年度からの健康診断の内容を抜本的に改変する予定である(表5)[5]．本稿は，その案が作成されている段階での執筆であるため，今後変わることがあり得ることをご了承いただきたい．

1．検査項目

老人保健法の健康診査と，労働安全衛生法の健康診