Metabolic adaptations to fasting and chronic caloric restriction in heart, muscle, and liver do not include changes in AMPK activity

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AMPK activity (AMPK), a family of heterotrimeric (29, 34). The mammalian homolog of SNF1 is the AMP-}

THE ABILITY OF AN ORGANISM to survive during times of nutritional scarcity depends on its capacity to make appropriate metabolic adjustments. In yeast, withdrawal of glucose triggers the activation of the sucrose-nonfermenting kinase (SNF1), which induces the expression of genes required for catabolism of the alternative carbon sources that makes survival possible (29, 34). The mammalian homolog of SNF1 is the AMP-activated kinase (AMPK), a family of heterotrimeric (α, β, γ) serine/threonine kinases that are activated primarily as a result of increased AMP secondary to ATP depletion (12). On activation, AMPK phosphorylates multiple metabolic enzymes and transcription factors, leading to the suppression of anabolic pathways and stimulation of glucose uptake and fatty acid oxidation that restore intracellular ATP levels (11).

Although glucose deprivation elicits AMPK activation in isolated cells, whether AMPK activity is altered by acute or chronic food restriction in mammals in vivo is less clear. It is known that some in vivo stimuli, such as physical exercise, can activate AMPK in the heart, skeletal muscle, and liver in an intensity- and isoform-specific manner (6, 7, 22, 30, 33). Some indication that AMPK may play a role as a nutritional energy sensor in vivo exists, since increases in AMPK activity have been found in the rat liver after fasting (19, 31). However, in another study, fasting did not change AMPK activity in skeletal muscle (16). Although this may be due to experimental differences, it also suggests that the AMPK response to food deprivation may be organ specific.

Energy intake can also be limited chronically, as in the case of caloric restriction (CR) without malnutrition. CR provides multiple health benefits and extends life span in diverse organisms, including mammals, although the underlying mechanisms are unclear. Believed to be at the core of the beneficial effects of CR are alterations in energy metabolism pathways (35). It remains to be tested whether alterations in AMPK, an important metabolic regulator, may play a role in aging retardation by CR. Supporting this possibility is the fact that CR induces some metabolic changes similar to those associated with AMPK activation, such as increased insulin sensitivity (15, 26).

Therefore, our overall goal was to determine how both fasting and chronic CR affect AMPK activity in vivo. To accomplish this, AMPK activity (α1- and α2-isoforms) was measured in the heart, skeletal muscle, and liver of young adult mice that had been subjected to either 24 h of fasting, 4 mo of CR, both, or neither. In tissue where either fasting or CR had even a modest effect on AMPK activity, both the upstream cause and downstream consequence were explored by performing immunoblots for phosphorylated AMPK (p-AMPK) and phosphorylated acetyl-CoA carboxylase (p-ACC), respectively. A secondary goal was to determine whether fluctuations in leptin that occur during fasting and CR modulate AMPK activity. This question is of interest because leptin, a key regulator of food intake and energy homeostasis, when administered exogenously has been shown to activate AMPK (18, 20, 27). This has led to the suggestion that leptin exerts its effects...
via altering AMPK activity, at least in some tissues. However, it is unclear whether the fluctuations in endogenous plasma leptin concentration that occur in vivo alter AMPK activity.

MATERIAL AND METHODS

Animals and dietary regimens. Male C57Bl/6J mice were housed in individual cages according to institutional protocols at the William S. Middleton Memorial Veterans Affairs Hospital, Madison, WI. After weaning, one group of 17 mice received 90% of the calories (12 kcal/day) of mice fed ad libitum, as determined in previous experiments. These mice constituted the control group. Another group of 17 mice received 65% of the calories (9 kcal/day) of mice fed ad libitum but equivalent amounts of vitamins and minerals given to control mice to avoid malnutrition. This latter group constituted the CR mice. A semipurified diet was provided to the animals in 2-day allotments on Mondays and Wednesdays and a 3-day allotment on Fridays. Details on diet composition and feeding protocols have been published elsewhere (25). Mice on both dietary regimens were killed at 5 mo of age, either 4–5 h after the last scheduled feeding (fed groups) or ~24 h afterward (fasted groups). Therefore, four groups were studied: 1) control fed, 2) control fasted, 3) CR fed, and 4) CR fasted. Because the time of completion of the last meal was not recorded, the exact length of the fasting period is not known and may have been slightly shorter than 24 h in some control mice. This is because, whereas the CR mice ate their 2-day allotment of food within 5 h of feeding, control mice occasionally had a small amount of food remaining beyond the 5 h. The control-fed and CR-fed mice had engorged stomachs at the time of tissue harvest. Tissues were homogenized in buffer A (HEPES 30 mM, EGTA 2.5 mM, EDTA 3 mM, KCl 20 mM, glycerophosphate 40 mM, sodium fluoride 40 mM, sodium pyrophosphate 4 mM, sodium vanadate 1 mM; Igepal CA-630 0.1%, glycerol 32%, and protease inhibitor cocktail, Sigma P-8340, 2%) and centrifuged. Two-hundred micrograms of supernatant protein were immunoprecipitated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and the CR fasted mice (4.3 ± 1.2 ng/ml). Plasma FFA values increased with fasting (P = 0.01) but did not significantly change by CR (P = 0.66; Fig. 1C). The increase in plasma FFA caused by fasting was more pronounced in the CR than in the control group (P = 0.03). Effects of fasting and CR on AMPK activity. Fasting for 24 h did not cause changes in AMPK activity associated with either the α1- or α2-subunits in heart or skeletal muscle (Tables 1 and 2). In the liver (Table 3), a tendency to higher activities of both AMPK during fasting and caloric restriction

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Plasma analysis of leptin and free fatty acids. For plasma analysis of leptin, a commercially available kit was used, following the instructions of the manufacturer (Crystal Chemicals, Downers Grove, IL). Free (nonesterified) fatty acids (FFAs) were analyzed with a commercial kit from Wako Chemicals (Richmond, VA).

Tissue glycogen content. Tissue samples were homogenized in 0.6% perchloric acid, and the supernatants were separated for glycogen measurement by the aminogluco side method (23). The results were expressed as nanomoles of glucosyl units per milligram wet tissue.

AMPK enzymatic activity. This solid-phase assay measures the activity of AMPK heterotrimers after isolation from other cellular constituents (8). AMPK is activated in cells both through allosteric activation by AMP and by phosphorylation of the catalytic α-subunit by upstream kinases. Because the phosphorylation state is retained during the isolation steps, the measurements reflect the intrinsic activity of AMPK within a particular metabolic state. Given that the actual AMPK activity in the tissue will depend on the intracellular concentration of AMP, both the minimal and the maximal possible AMPK activities were measured in each sample by performing the assay in the absence or presence of 0.2 mM AMP to span the possible intracellular AMP concentrations. Tissues were homogenized in buffer A (HEPES 30 mM, EGTA 2.5 mM, EDTA 3 mM, KCl 20 mM, glycerophosphate 40 mM, sodium fluoride 40 mM, sodium pyrophosphate 4 mM, sodium vanadate 1 mM; Igepal CA-630 0.1%, glycerol 32%, and protease inhibitor cocktail, Sigma P-8340, 2%) and centrifuged. Two-hundred micrograms of supernatant protein were immunoprecipitated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and the CR fasted mice (4.3 ± 1.2 ng/ml). Plasma FFA values increased with fasting (P = 0.01) but did not significantly change by CR (P = 0.66; Fig. 1C). The increase in plasma FFA caused by fasting was more pronounced in the CR than in the control group (P = 0.03).

Effects of fasting and CR on AMPK activity. Fasting for 24 h did not cause changes in AMPK activity associated with either the α1- or α2-subunits in heart or skeletal muscle (Tables 1 and 2). In the liver (Table 3), a tendency to higher activities of both
Effects of CR and fasting on phosphorylation of AMPK and ACC in the liver. AMPK activity is largely dependent on the phosphorylation of its α-subunit. Therefore, to evaluate the mechanism underlying the small (~20%) fasting-induced increase in liver AMPK activity, we measured the relative amounts of p-AMPK. As shown in Fig. 2, the intensity of the p-AMPK band was similar in all four groups of mice. Quantitation of the bands by densitometry indicated that neither fasting nor CR were associated with detectable differences in the degree of AMPK phosphorylation.

Because phosphorylation of ACC at Ser79 is thought to be accomplished exclusively by AMPK, the amount of p-ACC is often used as a downstream marker of AMPK activity (7, 19, 30, 32). Consistent with the small increase in liver AMPK activity induced by fasting, there was a slight increase in ACC phosphorylation, although this did not reach statistical significance (Fig. 3). In contrast to this slight increase in p-ACC, there was a dramatic decrease in p-ACC induced by CR. To explore this further, the protein expression of the two isoforms of ACC (ACC1 and ACC2) were measured by streptavidine binding in immunoblots. It was shown that the protein amount of ACC in the liver.

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**DISCUSSION**

Activation of AMPK plays an important role in the adaptation of cells to nutrient deprivation in vitro and to physiological (exercise) or pathological (ischemia) stresses in vivo. This link

**Table 1. Effects of CR and fasting on cardiac AMPK activity**

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<th></th>
<th>AMPKα1</th>
<th>AMPKα2</th>
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<tbody>
<tr>
<td></td>
<td>0 µM</td>
<td>200 µM</td>
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<tr>
<td>Control fed (n = 6)</td>
<td>7.6±0.4</td>
<td>16.0±1.4</td>
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<tr>
<td>Control fasted (n = 6)</td>
<td>7.9±1.1</td>
<td>15.9±2.0</td>
</tr>
<tr>
<td>CR fed (n = 6)</td>
<td>7.4±0.8</td>
<td>15.6±1.7</td>
</tr>
<tr>
<td>CR fasted (n = 6)</td>
<td>7.3±0.7</td>
<td>13.3±1.6</td>
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</tbody>
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Values are means ± SE in pmol·min⁻¹·mg protein⁻¹. AMPK, adenosine monophosphate-activated protein kinase; CR, caloric restriction. There was no significant effect of either fasting or CR.

**Table 2. Effects of CR and fasting on gastrocnemius muscle AMPK activity**

<table>
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<th>AMPKα1</th>
<th>AMPKα2</th>
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<tbody>
<tr>
<td></td>
<td>0 µM</td>
<td>200 µM</td>
</tr>
<tr>
<td>Control fed (n = 6)</td>
<td>4.2±0.6</td>
<td>7.8±0.8</td>
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<tr>
<td>Control fasted (n = 6)</td>
<td>3.8±0.7</td>
<td>6.2±0.4</td>
</tr>
<tr>
<td>CR fed (n = 6)</td>
<td>3.7±0.7</td>
<td>6.5±0.9</td>
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<tr>
<td>CR fasted (n = 6)</td>
<td>3.3±0.4</td>
<td>6.1±0.5</td>
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Values are means ± SE in pmol·min⁻¹·mg protein⁻¹. There was no significant effect of CR. The effect of fasting was P = 0.10.

**Table 3. Effects of CR and fasting on liver AMPK activity**

<table>
<thead>
<tr>
<th></th>
<th>AMPK-α1</th>
<th>AMPK-α2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 µM</td>
<td>200 µM</td>
</tr>
<tr>
<td>Control fed (n = 6)</td>
<td>20.6±1.9</td>
<td>37.4±2.8</td>
</tr>
<tr>
<td>Control fasted (n = 6)</td>
<td>25.2±2.5</td>
<td>45.6±2.3</td>
</tr>
<tr>
<td>CR fed (n = 6)</td>
<td>23.1±3.3</td>
<td>41.4±2.1</td>
</tr>
<tr>
<td>CR fasted (n = 6)</td>
<td>27.7±2.8</td>
<td>50.7±5.2</td>
</tr>
</tbody>
</table>

Values are means ± SE in pmol·min⁻¹·mg protein⁻¹. There was no significant effect of CR. The effect of fasting was P = 0.10.
between nutritional status and AMPK activity has raised the possibility that AMPK may play a role in regulating food intake or in the metabolic adaptation to CR (1). However, to date, the effects of dietary manipulations on AMPK activity in vivo have not been systematically evaluated. To address this, we determined the effects of fasting and CR on AMPK activity in heart, skeletal muscle, and liver. We came to three surprising conclusions. First, neither fasting nor CR significantly altered AMPK activity in these tissues. Second, an eightfold change in plasma leptin concentration (a putative activator of AMPK) did not alter AMPK activity. Third, CR dramatically decreased the amount of ACC phosphorylated at Ser79. This effect of CR was due to a decrease in the expression of both ACC1 and ACC2 protein, not to changes in ACC phosphorylation.

Effect of fasting. The effect of fasting on AMPK activity was measured to determine whether AMPK participates in nutritional energy sensing in vivo as it does in vitro (12). In the CR mice, fasting caused the expected changes in body weight and in plasma concentrations of leptin and FFA, but it did not alter cardiac or skeletal muscle AMPK activity. In the control mice, fasting decreased leptin but did not affect FFA concentration or body weight, indicating differences in either the extent of or the response to the fast in the two groups. Nevertheless, as was the case in the CR mice, fasting had no effect on cardiac or skeletal muscle AMPK activity in the control mice. Thus activation of AMPK is not an obligatory component of the complex metabolic response to fasting in these tissues. Although this finding is somewhat surprising in light of the robust activation of skeletal muscle AMPK that occurs during glucose withdrawal in vitro, it is consistent with a previous study in rats where overnight fasting failed to significantly increase soleus muscle AMPK activity (16). The most likely explanation for this result is that the hypoglycemia induced by a 24-h fast is not severe enough to cause the fall in intracellular ATP-to-AMP ratio to increase AMPK activity. This possibility is supported by data indicating that ATP is kept within normal levels in rat muscle during fasting at the cost of the creatine phosphate pool (24). Because the heart has a much higher metabolic rate than skeletal muscle, and thus a larger possibility of developing the type of energetic imbalance that would activate AMPK, it might be expected that fasting would increase cardiac muscle

Fig. 2. A: effects of fasting and CR on phosphorylated adenosine monophosphate-activated protein kinase (p-AMPK) in the liver. B: representative immunoblot showing AMPK phosphorylation in liver proteins from control (fed and fasted) and CR (fed and fasted) mice. OD, optical density. Note the lack of an effect of either fasting or CR on the amount of p-AMPK.

Fig. 3. A: effects of fasting and CR on phosphorylated acetyl-CoA carboxylase (p-ACC) in the liver. B: representative immunoblot showing ACC phosphorylation in liver proteins from control (fed and fasted) and CR (fed and fasted) mice. Note the significant decrease in p-ACC with CR. *Significant effect of CR.

Fig. 4. A: effects of CR on the amounts of ACC1 and ACC2 proteins. CR significantly decreased the amounts of both proteins. *Significant effect of CR. B: representative immunoblot showing ACC1 and ACC2 in both groups (n = 4/group).
AMPK activity. However, similar to what we observed in skeletal muscle, fasting did not activate AMPK in cardiac muscle. Our data illustrate that energetic challenges more severe than a 24-h fast are needed to cause in vivo activation of AMPK in heart or skeletal muscle.

In contrast to our findings in cardiac and skeletal muscle, there was some tendency in the liver for both α1- and α2-AMPK activities to increase after 24 h of fasting, with a similar trend to increase ACC phosphorylation. Although not statistically significant, this may be attributable to our small sample size and the limited sensitivity of immunoblots. Additionally, 24 h may not be enough fasting time to induce robust activation of liver AMPK, even though ACC activity may be independently affected (9). Munday et al. (19) demonstrated that 48 h of fasting significantly increases AMPK activity with a concomitant fall in ACC activity, as would be expected with increased p-ACC. Together our results suggest that more sensitive mechanisms for AMPK activation in vivo may exist in the liver than in heart or muscle in response to fasting. This is perhaps not surprising, owing to the central role of the liver in whole body metabolism and the abundance of AMPK in this organ. It is worth noting that glucose-sensing mechanisms that activate AMPK in perportal regions of the liver have been reported (5, 31).

Effect of CR. CR extends life span and protects against many aging-related diseases through mechanisms that remain largely unknown (28). Because the benefits of CR are intimately related to the quantitative decrease in energy intake rather than to the quality of the caloric source, the current belief is that the protective effects of CR are linked to energy-related biochemical pathways (14, 35), and we hypothesized that AMPK may be involved in the beneficial effects of CR. However, our results do not support this view. Despite the fact that our mice demonstrated the expected metabolic characteristics of CR (lower body weight, plasma leptin, and liver glycogen content), neither heart, skeletal muscle, nor liver showed any evidence of increased activity of either the α1- or α2-isofrom of AMPK (10). This was true whether the CR mice were studied in the fed or fasted state. As was the case during the 24-h fast, the most likely explanation for a lack of an effect of CR on AMPK activity is that the energetic challenge presented by CR was not severe enough to cause the changes in ATP and AMP to activate AMPK.

We cannot rule out that a more severe CR would have led to AMPK activation. However, since the present CR conditions lead to life span extension in mice, AMPK activation at an early age does not seem to account for the health benefits associated with CR (25). A more complete conclusion on the relationship between AMPK and CR will come from studies in old CR animals.

Even though CR did not alter AMPK activity, CR decreased the amount of p-ACC by ~75% in the liver. This is surprising, since it has been suggested that Ser79 on ACC is phosphorylated exclusively by AMPK. Reconciling this dilemma is our finding that the decrease in phosphorylated ACC is secondary not to changes in AMPK but instead to decreases in the amounts of ACC1 and ACC2 proteins. This novel finding that chronic CR drastically decreases ACC protein amounts represents an extension of earlier work by several investigators showing that acute fasting decreases the amount of liver ACC mRNA and protein (21). Neither the mechanisms by which CR decreases the expression of ACC protein nor the role that this decrease in ACC plays in the health benefits caused by CR are known.

Endogenous leptin and AMPK. Leptin, a hormone that regulates energy homeostasis in rodents and humans, promotes energy expenditure over storage (3). Although its mechanism(s) of action is incompletely understood, exogenous leptin has been reported to stimulate fatty acid oxidation by activating AMPK in skeletal muscle but not in heart, liver, or brain (1, 2, 17, 18, 27). However, it is unclear whether the fluctuations that occur physiologically in plasma leptin concentration in vivo influence AMPK activity. In other words, although it is well established that plasma leptin declines during fasting and weight-reducing regimens, the effect of diet-induced changes in leptin on AMPK activity is unknown (13). Our results indicate that profound changes in plasma leptin during fasting and/or CR are not associated with changes in AMPK activity in heart, muscle, or liver. Specifically, our control fed group had plasma leptin levels ~8 times higher than the CR fasted mice, but the AMPK activities in all organs from the two groups were still similar.

There are several probable explanations for this lack of relationship between leptin and AMPK activity in our study. First, any effects of leptin on AMPK may be transient and thus not observable in a study like ours. This would be consistent with the idea that increases in leptin acutely activate AMPK, but once AMPK has effected appropriate metabolic adjustments, AMPK activity returns to baseline. A related possibility is that any leptin-induced activation of AMPK in vivo is balanced by neuroendocrine inhibition. In this respect, the orexigenic hormone ghrelin, which is released after a meal, is known to antagonize leptin in vivo (4). Supporting this possibility is a report where leptin and ghrelin were found to elicit opposed AMPK responses in the hypothalamus (1). A final possibility is that the magnitude of the changes in plasma leptin that occur in vivo may not be sufficient to induce measurable effects on AMPK activity.

In summary, although activation of AMPK plays an important role in the adaptation of cells to nutrient deprivation in vitro and to physiological or pathophysiological stresses in vivo, AMPK is not activated in the tissues we studied by the metabolic challenges imposed by either a 24-h fast or 4 mo of CR in young mice. Thus, while the metabolic adaptations to fasting and CR include large changes in plasma leptin concentration and p-ACC, these effects occur without apparent changes in AMPK activity.

GRANTS

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