Aging Elevates Basal Adenosine Monophosphate-Activated Protein Kinase (AMPK) Activity and Eliminates Hypoxic Activation of AMPK in Mouse Liver

Jacob D. Mulligan,1 Asensio A. Gonzalez,1 Reetu Kumar,1 Ashley J. Davis,1 and Kurt W. Saupe1,2

Departments of 1Medicine and 2Physiology, University of Wisconsin, Madison.

Despite the central role of adenosine monophosphate-activated protein kinase (AMPK) in the cellular stress response, it is unknown whether age-related changes in AMPK activity play a role in the diminished stress tolerance that is characteristic of aging. To address this question, we determined in the mouse liver how normal aging affects 1) basal AMPK activity, and 2) the degree to which AMPK activity is increased by in vivo hypoxia. We found that the basal activity of AMPK α1, but not α2, was higher in livers from 24-month-old mice compared to those from 5-month-old mice. Furthermore, while hypoxia elevated AMPK α1 and α2 activities in livers from 5-month-old mice, hypoxia failed to increase the activity of either isoform of AMPK in 24-month-old mice. These findings suggest that age-associated changes in hepatic AMPK activity may play a role in the physiological changes that occur in the liver with normal aging.

One of the hallmarks of normal aging is a diminished ability of tissues to make the molecular adjustments necessary to tolerate different types of stress. For example, during normal aging there is a decline in the ability of various tissues to tolerate thermal, oxidative, pharmacological, nutritional, and hypoxic/ischemic stress (1–10). Despite the fact that this diminished stress tolerance of aged tissues is a central factor in many age-associated pathologies (see above refs), little is known regarding its biochemical basis. A key regulator of the cellular stress response in mammals is adenosine monophosphate-activated protein kinase (AMPK), which is activated by stresses that cause a decrease in cellular energy charge (decrease in [ATP]/[AMP]) such as exercise, starvation, and hypoxia/ischemia. AMPK activates ATP-producing pathways and inhibits ATP-consuming pathways by altering both enzymatic activities (11,12) and gene expression (13–15). Despite the importance of AMPK in the cellular stress response and the importance of the cellular stress response in the pathophysiology of age-associated diseases, there have been no reports investigating whether normal aging alters AMPK activity.

Predicting how AMPK activity might change with age is not straightforward; some data suggest that AMPK activity might be increased, whereas other data suggest that it might be decreased. Recently, Jin and colleagues (16) reported that aging increased the phosphorylation of AMPK protein in the kidneys of rats. Consistent with this, studies in yeast and human fibroblasts (17,18) indicate that the activity of AMPK and its yeast homologue SNF1 (sucrose non-fermenting) increase in models of in vitro aging. However, some characteristics of aging, such as poor stress tolerance and decreased insulin sensitivity, would be more consistent with a decline in AMPK activity (19–23). Further complicating our ability to predict how aging might affect AMPK activity is the fact that the exact role of AMPK in stress tolerance is unclear. For example, the poor stress tolerance associated with aged tissues could be caused by diminished basal AMPK activity or, alternately, a diminished ability to activate AMPK during acute stress.

To address these questions, basal AMPK activity, as well as the degree of AMPK activation during in vivo hypoxia, was measured in liver tissue from 5- and 24-month-old mice. Hypoxic stress was studied in the liver because: 1) hepatic hypoxic tolerance decreases with age (2,3), and 2) activation of hepatic AMPK during hypoxia/ischemia not only occurs, but is protective (24). In this study we present, to our knowledge, the first in vivo data assessing the effect of normal aging on either basal or stress-induced activation of AMPK activity.

Methods

Animals

Male C57Bl/6 mice were housed at a University of Wisconsin Animal Care Facility. All experiments were performed on mice fed ad libitum. The facilities and research protocols were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Tissue Collection

Mice aged 5 months (young) or 24 months (old) were sedated with 4% isoflurane, then intubated and ventilated at 1.1 ml × min⁻¹ × g body weight⁻¹ with 2.5% isoflurane in 100% O₂ (hypoxic, arterial blood PO₂ > 400 mmHg). The mice were then either maintained on 100% O₂ or switched to 7.9% O₂ in nitrogen (hypoxia, arterial blood PO₂ = 40 mmHg). By standardizing the amount of minute ventilation/gram body weight, arterial blood PCO₂ and pH were maintained at normal levels during the two ventilation conditions.
Basal AMPK activity was measured under hyperoxic, as opposed to normoxic (arterial blood PO2 = 100 mmHg), conditions to decrease the likelihood of some degree of ischemic activation of AMPK occurring secondary to blood loss and decreased tissue blood flow that may accompany tissue harvest. Livers were collected after 10 minutes of hyperoxia or hypoxia, freeze-clamped in liquid nitrogen, and stored at −80°C until ready for biochemical analysis.

**AMPK Activity Assay**

For AMPK analysis, tissues were homogenized in Buffer A (30 mM HEPES, 2.5 mM EGTA, 3 mM EDTA, 20 mM KCl, 40 mM glycerophosphate, 40 mM NaF, 4 mM Na3VO4, 1 mM Na2VO4, 0.1% Igepal CA-630, 32% glycerol, and 1% Sigma protease inhibitor cocktail) and centrifuged for 10 minutes at 10,000 × g. The supernatant was mixed with an equal volume of Buffer B (30 mM HEPES, 2.5 mM EGTA, 3 mM EDTA, 70 mM KCl, 20 mM glycerophosphate, 40 mM NaF, 4 mM Na3VO4, and 1 mM Na2VO4) and centrifuged for 10 minutes at 10,000 × g. Two hundred micrograms of total protein from the resulting supernatant was immunoprecipitated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and antibodies against either the α1 (cat. No. 07-350; Upstate Biotechnology, Waltham, MA) or α2 (cat. No. 07-363; Upstate Biotechnology) catalytic subunits of AMPK for 2 hours at 4°C. The beads containing the immunoprecipitated AMPK were washed, resuspended in reaction buffer (40 mM HEPES, 80 mM NaCl, 5 mM MgCl, and 1 mM DTT) with 0.2 mM SAMS peptide (25) and 0.2 mM [γ-32P]-ATP (specific activity: 1500–4000 cpm/pmol), and incubated for 10 minutes at 37°C in a thermomixer in the presence or absence of 0.2 mM AMP. After incubation, the beads were quickly pelleted and a portion of each supernatant was spotted on P-81 phosphocellulose paper, washed in 1% phosphoric acid, and dried in air. The incorporated radioactivity was counted in a TriCarb 3000 beta scintillation counter (Packard, PerkinElmer, Boston, MA). AMPK activity was expressed as pmol/min/mg protein.

**Western Blot Analysis**

Previously snap-frozen liver tissue was homogenized in 50 mM MOPS, 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 5 mM NaPPi, 1 mM Na3VO4, and 5 μl/ml protease inhibitor mixture (Sigma, St. Louis, MO). The homogenate was centrifuged at 600 × g for 10 minutes. The supernatant was mixed with an equal volume of Buffer B (30 mM HEPES, 2.5 mM EGTA, 3 mM EDTA, 70 mM KCl, 20 mM glycerophosphate, 40 mM NaF, 4 mM Na3VO4, and 1 mM Na2VO4) and centrifuged for 10 minutes at 10,000 × g. Two hundred micrograms of total protein from the resulting supernatant was immunoprecipitated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and antibodies against either the AMPK α1 (cat. No. 07-350; Upstate Biotechnology, Waltham, MA) or AMPK α2 (cat. No. 07-363; Upstate Biotechnology) catalytic subunits of AMPK for 2 hours at 4°C. The beads containing the immunoprecipitated AMPK were washed, resuspended in reaction buffer (40 mM HEPES, 80 mM NaCl, 5 mM MgCl, and 1 mM DTT) with 0.2 mM SAMS peptide (25) and 0.2 mM [γ-32P]-ATP (specific activity: 1500–4000 cpm/pmol), and incubated for 10 minutes at 37°C in a thermomixer in the presence or absence of 0.2 mM AMP. After incubation, the beads were quickly pelleted and a portion of each supernatant was spotted on phosphocellulose paper, washed in 1% phosphoric acid, and dried in air. The incorporated radioactivity was counted in a TriCarb 3000 beta scintillation counter (Packard, PerkinElmer, Boston, MA). AMPK activity was expressed as pmol/min/mg protein.

**Statistics**

All data are expressed as mean ± SEM. Statistical significance was determined using a two-tailed Student’s t test assuming equal variance or unequal variance where appropriate, with statistical significance defined as p < .05.

**RESULTS**

**Basal AMPK α1 Activity Is Increased With Aging in Liver**

AMPK activity has been shown to be increased with senescence in yeast and cultured human fibroblasts (17,26). However, no data exist regarding the effect of aging on AMPK activity in vivo. To test whether aging affects basal AMPK activity in the liver, AMPK α1 and α2 isoforms were individually isolated from livers of young (5-month-old) and old (24-month-old) mice, and AMPK activity was measured. Activity of the AMPK α1 isoform was 2.0-fold higher in livers from old mice relative to young mice (Figure 1A, p = .018). In contrast to AMPK α1, the activity of AMPK α2 did not differ between livers from young and old mice (Figure 1B).

The allosteric activation of AMPK by AMP is well established. However, the effect of aging on this process is unknown. To test whether aging affects the intrinsic ability of AMPK to be activated by AMP, the AMPK activity assay was performed in the presence and absence of 200 μM AMP. AMPK activity was approximately doubled with the addition of AMP in both young and old livers, regardless of isoform (Figures 1A and 1B; Table 1).

**Increase in AMPK Activity in Liver With Aging Is Due to Increased Phosphorylation of AMPK**

Because endogenous AMP is effectively removed during the isolation of AMPK prior to the activity assay, the observed increase in AMPK α1 activity with aging can be due to increases in protein levels and/or phosphorylation state of AMPK α1. To examine the relative contributions of
these factors, we performed Western blot analyses on liver proteins from young and old mice. A 62% increase was observed in the phosphorylated form of AMPK (combined \(\alpha_1\) and \(\alpha_2\)) in old liver relative to young liver (Figure 2A, \(p = .039\)). Although AMPK protein had a tendency to be higher in the old livers as determined by an antibody against pan-AMPK \(\alpha\), the difference was not significant (Figure 2B, \(p = .151\)). Furthermore, when AMPK protein levels were determined by antibodies against either the \(\alpha_1\) or \(\alpha_2\) isoform specifically, no difference was observed between the young and old livers (Figure 2B).

**Increased Basal AMPK Activity Does Not Result in Increased ACC Phosphorylation**

To examine whether an increase in basal AMPK activity with aging leads to an increase in the basal degree of phosphorylation of a known AMPK target, Western blot analysis for phosphorylated ACC was performed. Surprising

**Hypoxic Response of AMPK \(\alpha_1\) and \(\alpha_2\) in Liver Is Eliminated With Aging**

Aging is known to be associated with a decrease in the ability to tolerate hypoxic stress (2–4,6–8). Whether AMPK is involved in this decreased hypoxic tolerance is unknown. To examine how aging affects hypoxic activation of AMPK in liver, young and old mice were subjected to 10 minutes of hypoxia prior to tissue collection. In livers from young mice, hypoxia increased AMPK \(\alpha_1\) and \(\alpha_2\) activities by 48% and 47%, respectively (Figures 4A and 5A, left bars, \(\alpha_1\): \(p \leq .001\), \(\alpha_2\): \(p = .034\)).

In contrast to the young mice, 10 minutes of hypoxia did not significantly alter either AMPK \(\alpha_1\) or \(\alpha_2\) activity in livers from old mice (Figures 4A and 5A, right bars). It is interesting that old mice were less able to tolerate prolonged hypoxia; over half of the old mice studied died near the end of a 30-minute hypoxic period. As a result, we were not able to assess the effect of 30 minutes of hypoxia on AMPK activity in old mice. In the young mice, 30 minutes of hypoxia did not further increase AMPK activity of either isoform (data not shown). None of the young mice died during 30 minutes of hypoxia.

AMPK activity in all groups was doubled with the addition of 200 \(\mu M\) AMP (Figures 4B and 5B; Table 1), demonstrating that the lack of activation of AMPK with aging was not due to decreased allosteric activation by AMP.

**DISCUSSION**

AMPK plays a protective role in response to many types of stress (21,22,24,27). Despite the evidence that aging is associated with a decline in the ability to tolerate stress (1–10), there have been no studies assessing whether normal aging in vivo alters AMPK activity in the basal state or in response to acute stress. In this study, we present two novel findings in this regard. First, we show that basal activity of AMPK \(\alpha_1\), but not \(\alpha_2\), is 2-fold higher in livers from old mice than in those from young mice (Figure 1). Elevated basal AMPK activity with aging is consistent with in vitro data in yeast and human fibroblasts (17,26) and AMPK protein analysis in kidneys of aged rats (16). Second, we show that aging eliminates the hypoxia-induced activation

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**Table 1. Effect of Age and Hypoxia on Allosteric Activation of AMPK by 200 \(\mu M\) AMP**

<table>
<thead>
<tr>
<th></th>
<th>AMPK (\alpha_1)</th>
<th>AMPK (\alpha_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 (\mu M)/</td>
<td>10-Minute</td>
<td>10-Minute</td>
</tr>
<tr>
<td>0 (\mu M)</td>
<td>Hyperoxia</td>
<td>Hyperoxia</td>
</tr>
<tr>
<td>5-month</td>
<td>1.77 ± 0.03</td>
<td>1.85 ± 0.07</td>
</tr>
<tr>
<td>24-month</td>
<td>1.79 ± 0.04</td>
<td>1.92 ± 0.12</td>
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Notes: Data from Figures 1, 4, and 5 are expressed here in terms of fold increase in AMPK activity when measured in the presence of 200 \(\mu M\) as compared to 0 \(\mu M\) AMP.

AMPK = adenosine monophosphate-activated protein kinase.
of AMPK α1 and α2 (Figures 4 and 5), possibly contributing to the poor hepatic tolerance to hypoxia (and perhaps other stresses) with aging. This finding is supported by the elimination of oxidative stress-induced expression of phosphorylated AMPK protein in aged rat kidneys (16).

Possible Mechanisms Underlying Age-Associated Increase in Basal AMPK Activity

There are numerous mechanisms by which aging may increase basal AMPK activity, such as increasing the levels of intracellular AMP, AMPK protein, or phosphorylated AMPK. Direct allosteric activation by AMP could not account for our finding because endogenous AMP is washed out prior to the AMPK activity assay. We found that AMPK α1 and α2 protein levels were not altered with age (Figure 2B), suggesting that the observed increase in AMPK α1 activity was not caused by an increase in the synthesis or stability of AMPK mRNA or protein. We did, however, observe an increase in the phosphorylated form of AMPK with aging (Figure 2A), indicating a likely mechanism by which aging may increase AMPK α1 activity. The phosphorylation of AMPK by the upstream AMPK kinase, LKB1, is known to be facilitated by an increased [AMP]/[ATP] ratio (11). The data regarding alterations in AMP and ATP levels in the liver with aging are inconclusive (28–32); however, the age-related increase in AMPK activity noted by Wang and colleagues (26) in human fibroblasts was accompanied by an increase in the [AMP]/[ATP] ratio. To our knowledge, the effect of aging on LKB1 expression and activity has not been studied in any system.

It is interesting that we observed that aging increased the basal activity of AMPK in an isoform-specific manner. Therefore, it is likely that aging creates or takes advantage of some
difference between the two AMPK α isoforms, possibly causing AMPK α1 and α2 to be spatially separated from one another or to be differently modified. It is worth noting that an isoform-specific effect of aging has also been shown for SNF1, the homologue of AMPK in yeast; the age-dependent elevation in SNF1 activity was mediated by one isoform of the β subunit specifically, and resulted in the subcellular re-location of SNF1 (33). Given the above findings, it would be of interest to study the possible effects of aging on the different isoforms of the noncatalytic β and γ subunits of AMPK.

**Chronic Activation of AMPK**

Whether the age-dependent elevation in basal AMPK α1 activity in the liver is adaptive or maladaptive is difficult to determine; there is evidence in the literature for both possibilities. Some of the beneficial effects of acute AMPK activation, such as increased glucose uptake and β-oxidation of fatty acids in skeletal muscle, have recently been extended to chronic pharmacological activation of AMPK (20,34,35). Furthermore, chronic AMPK activation has been shown to decrease blood pressure, plasma triglycerides, and intra-abdominal fat, while increasing high-density lipoprotein (HDL) cholesterol in fa/fa rats (36). In rat hepatocytes, chronic activation of AMPK has been shown to block induction of lipogenic genes (37).

However, chronic AMPK activation has also been shown to lead to deleterious phenotypes that are often associated with aging. For example, chronic activation was shown to cause decreases in β-cell insulin secretion (38) and in the stabilization of proliferative genes (18), and was also shown to induce apoptosis in liver cells (39) and pancreatic β cells (40,41). Chronic activation of AMPK has also been associated with cardiac hypertrophy and arrhythmias (42,43).
In the present study, we observed that, despite increased basal AMPK activity, an AMPK target, ACC was not more phosphorylated in the aged livers. This may represent another difference between acute and chronic AMPK activation and suggests that perhaps not all pathways regulated by acute AMPK activation will be significantly altered by chronic AMPK activation with aging. Therefore, the effect of elevated basal AMPK activity on the aged organism is likely to be complex, requiring more information before any positive and negative aspects can be distinguished.

**Effect of Age on Hypoxic Response of AMPK**

One notable effect of aging is a decrease in the ability of numerous tissues, including the liver, to tolerate hypoxic stress. Although there are many possible causes, it has been suggested that this phenomenon may be due to the reduction in hypoxia-inducible factor-1 (HIF-1) activity with age (44). Importantly, AMPK is activated by hypoxia and, in turn, activates HIF-1 (45). The reduction in HIF-1 activity and inability to tolerate hypoxic stress provide a reason to believe that AMPK activity may be decreased with aging. However, all available data, including the results presented here, indicate that AMPK activity is increased with age.

It is interesting that we found that, while AMPK activity remains higher in livers from old mice relative to young mice following hypoxic stress, the actual response to hypoxia is negligible in the old mice (Figures 4 and 5). It is reasonable to expect that a chronic elevation in AMPK activity may be necessary to deal with the basal level of stresses faced by the aging organism. Therefore, the ability of hypoxia to elicit an acute increase in AMPK activity may be more crucial to the tolerance of hypoxia than is an elevated basal level of AMPK activity. This raises the possibility that poor tolerance to hypoxia with aging may be caused by a decreased hypoxic response of AMPK.

One possible mechanism for the poor hypoxic response with aging is an age-dependent decrease in the degree to which AMP can allosterically activate AMPK. Indeed, it has been reported that aging decreases the ability of AMP to activate rat muscle phosphofructokinase (46). However, we found that aging did not affect the activation of AMPK by AMP under basal or hypoxic conditions (Figures 1, 4, and 5; Table 1). It is interesting that the effect of aging on the hypoxic response of AMPK is not isofrom specific, unlike the effect of aging on basal AMPK activity, which was specific for the z1 isofrom. Therefore, it is probable that aging blunts the hypoxia-mediated activation of AMPK by a mechanism that is independent from the mechanism by which aging increases basal AMPK z1.

**Summary**

Our data demonstrate that aging alters basal AMPK activity and the response of AMPK to hypoxia in vivo. It remains unknown whether these effects of aging on AMPK are beneficial or harmful. On the one hand, experiments in yeast and human fibroblasts have revealed that activation of AMPK is sufficient to cause a senescent phenotype. On the other hand, AMPK activity has been shown to increase insulin sensitivity and stress tolerance, both of which diminish with age. The multifaceted relationship between aging and various AMPK-regulated pathways indicates that changes in the AMPK system will likely be an important component of the aging process.

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Address correspondence to Kurt W. Saupe, 1630 Medical Sciences Center, 1300 University Ave., Madison, WI 53706. E-mail: kws@medicine.wisc.edu

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