Angiotensin II Upregulates Protein Phosphatase 2Cα and Inhibits AMP-Activated Protein Kinase Signaling and Energy Balance Leading to Skeletal Muscle Wasting

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Abstract—Congestive heart failure and chronic kidney disease are characterized by chronically elevated angiotensin II (Ang II) and muscle wasting. Ang II causes skeletal muscle wasting by reducing appetite and by enhancing catabolism. The serine/threonine kinase AMP-activated protein kinase (AMPK) functions mainly as a sensor of cellular energy status. It is energy sparing and favors ATP generation. We hypothesized that Ang II induces muscle wasting in part by inhibiting AMPK signaling and altering cellular energy balance. Our results show that Ang II infusion in mice reduced gastrocnemius muscle weight by 26% and depleted ATP by 74%. In addition, Ang II upregulated protein phosphatase 2Cα by 2.6-fold and reduced AMPK phosphorylation and signaling in muscle. Importantly, the pharmacological AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside restored AMPK activity to levels of pair-fed controls and reversed Ang II–mediated ATP depletion and muscle wasting. Moreover, 5-aminoimidazole-4-carboxamide ribonucleoside activated Akt and inhibited Ang II–induced increases in E3 ubiquitin ligase expression. These novel results demonstrate critical roles for energy depletion and AMPK inhibition in Ang II–induced skeletal muscle wasting and suggest a therapeutic potential for AMPK activators in diseases characterized by muscle wasting. (Hypertension. 2011; 58:00-00.)

Key Words: ATP □ AMPK □ AICAR □ mitochondria □ atrogin 1 □ muscle RING-finger protein 1 □ Akt

Much is known about the actions of angiotensin II (Ang II) on the vasculature, heart, and kidney, but the effects of Ang II on skeletal muscle are much less understood. We originally reported that Ang II infusion promoted loss of body weight by reducing food intake and decreasing skeletal muscle weight, effects that were pressor independent and accompanied by a marked reduction in circulating insulin-like growth factor 1.1–3 These findings were relevant to conditions such as congestive heart failure and chronic kidney disease, in which Ang II levels are elevated, and in which loss of lean body mass correlates strongly with poor prognosis.4,5 Subsequent studies demonstrated that Ang II causes skeletal muscle wasting primarily by increasing rates of protein degradation via activation of forkhead box protein (FoxO) transcription factors, caspase 3, and the ubiquitin proteasome pathway,6,7 while simultaneously decreasing protein synthesis via inhibition of the insulin-like growth factor 1/Akt/mammalian target of rapamycin signaling axis.1–3,7–12 Ang II wasting is mediated via the Ang II type 1 receptor. However, because mature skeletal muscle expresses little or no Ang II receptors (A, B, or Ang II type 2),12 these effects are likely indirect, involving inflammatory cytokines like interleukin 6,12 tumor necrosis factor-α,13,14 serum amyloid A,12 glucocorticoids,15 and reactive oxygen species.16,17

Although it has been well established that Ang II induces skeletal muscle atrophy, little is known about potential effects of Ang II on muscle metabolism and energy stores or about the potential link between these effects and Ang II wasting. AMPK is a serine-threonine kinase that plays a pivotal role in cellular and whole-body metabolism. The aims of this study were to determine whether Ang II affects skeletal muscle energy stores, whether energy depletion plays a role in skeletal muscle wasting, and to characterize the effects of Ang II on AMPK signaling in gastrocnemius muscle.

Materials and Methods

A full description of the experimental design, materials, animals, methods used, and statistical analysis can be found in the online Data Supplement. Please see http://hyper.ahajournals.org.

Results

To separate the catabolic and anorexigenic effects of Ang II on skeletal muscle wasting, 2 saline-infused control groups were included, ad libitum and pair-fed mice. Ang II–infused mice lost 12% of total body mass and 26% of gastrocnemius muscle mass compared with ad libitum controls after 4 days (Figure 1A and 1B). Ang II mice ate approximately half of what the saline-infused ad libitum–fed mice consumed.

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because of fluid retention, as we have reported previously. To ascertain the role that AMPK signaling plays in Ang II–induced muscle wasting by day 4 independent of food intake and blood pressure. A, Total body mass, B, gastrocnemius mass, C daily food intake, and D systolic blood pressure. **P<0.001 vs ad libitum. ***P<0.001 vs ad libitum. +++P<0.001 vs pair fed. \*P<0.001 vs Ang II.

Throughout the experiment (Figure 1C). Although food restriction alone (pair-fed controls) resulted in significant 13% reduction in gastrocnemius muscle weight compared with ad libitum fed controls, Ang II elicited an additional highly significant 13% decrease in skeletal muscle mass, independent of food intake (Figure 1B). Of note, this catabolic effect of Ang II was not apparent from measurement of total body mass (Figure 1A) because of fluid retention, as we have reported previously. To ascertain the role that AMPK signaling plays in Ang II–induced muscle wasting, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which reliably activates AMPK, was administered via daily intraperitoneal injection. AICAR markedly blunted Ang II–induced loss in total body mass and completely reversed the 26% loss of skeletal muscle mass induced by Ang II (Figure 1A and 1B). The effects of AICAR were independent of both food intake and blood pressure (Figure 1C and 1D).

Ang II infusion or pair feeding did not alter mitochondrial content as measured by real-time PCR analysis of mitochondrial copy number relative to myofiber nuclei (Figure 2A). However, Ang II significantly reduced mitochondrial activity, specifically Ang II decreased cytochrome C oxidase activity (complex IV of the electron transport chain), by 47% (Figure 2B). Ang II also increased mitochondrial-derived superoxide, consistent with mitochondrial-derived reactive oxygen species did not directly contribute to Ang II wasting (Figure S1, available in the online Data Supplement). Ang II also reduced skeletal muscle ATP content by 74% in a food intake–independent manner (Figure 2C), and this effect was completely reversed by AICAR (Figure 2C). Of note, AICAR itself reduced ATP levels in control animals (Figure 2C).

Food restriction (pair-fed controls) increased AMPK activity in gastrocnemius muscle by 75% (assessed by Thr172 phosphorylation; Figure 3), and Ang II completely prevented this increase at 4 days (Figure 3). The inhibitory effect of Ang II on AMPK activation in response to reduced food intake was completely blocked by AICAR (Figure 3). Although caloric restriction–induced AMPK activation was not yet apparent, Ang II also inhibited AMPK phosphorylation at day 1 (Figure S2). To determine potential mechanisms by which Ang II prevented AMPK activation, we analyzed activities of the upstream AMPK kinases LKB1 and TAK1. Caloric restriction, Ang II, or AICAR had no effect on TAK1 activity (Figure S3). Conversely, there was a significant food intake–independent 56% increase in LKB1 activation with Ang II, and LKB1 activity was restored to basal levels in AICAR-treated animals (Figure S3). These findings suggest that LKB1 activation by Ang II was a compensatory response to Ang II–mediated reduction in AMPK activity. In the absence of Ang II inhibition of AMPK activating kinases, we examined the expression levels of protein phosphatase 2Cα (PP2Cα), a serine/threonine protein phosphatase known to dephosphorylate and inactivate AMPK. We detected a robust food intake–independent 2.6-fold induction of PP2Cα protein after Ang II infusion (Figure 4). These data suggest that the inhibitory effects of Ang II on AMPK activity are...
mediated by the upregulation of PP2Cα. Interestingly, AICAR completely prevented this increase in Ang II–induced PP2Cα expression (Figure 4). Neither Ang II nor AICAR altered expression of the β-isoform of PP2C (data not shown). Although Ang II increased PP2Cα expression, we detected no change in total PP2Cα activity (Figure S4).

We next examined several downstream targets of AMPK signaling, including peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and acetyl-coenzyme A carboxylase (ACC). Correlating with caloric restriction–induced AMPK activation, we observed a significant 60% increase in total PGC-1α expression in the gastrocnemius muscle of pair-fed mice, and Ang II prevented this induction of PGC-1α. AICAR completely reversed the inhibitory effect of Ang II on PGC-1α expression (Figure 5A). Correlating with AMPK inactivation, ACC phosphorylation at Ser79 was also decreased by Ang II, and the ratio of phospho-ACC/total-ACC was reduced by 41%, consistent with activation of ACC by Ang II (because ACC phosphorylation by AMPK is inhibitory). This Ang II–induced activation of ACC was not reversed by AICAR (Figure 5B). Interestingly, there was a significant 65% reduction in total ACC protein in response to Ang II (Figure S5). While having no effect on ACC activity, AICAR restored total ACC to basal levels.

Finally, we examined effects of Ang II and AICAR on the expression of the E3 ubiquitin ligases muscle atrophy F-box protein 1 (atrogin 1) and muscle RING-finger protein 1 in gastrocnemius after 24 hours of treatment. As we have reported previously,3,18 Ang II significantly upregulated both atrogin 1 and muscle RING-finger protein 1 mRNA expression (8.2-fold and 6.2-fold, respectively). Intriguingly, AICAR completely prevented their upregulation, without affecting basal expression of these E3 ligases (Figure 6A and 6B). Because FoxO transcription factors regulate E3 ligase downregulation, AICAR increased inhibitory phosphorylation at Thr172 on FoxO1 by 96% (Figure S6).

**Discussion**

We report for the first time that Ang II caused marked ATP depletion in skeletal muscle independent of food intake and blocked caloric restriction–induced AMPK activation, probably via increased expression of the protein phosphatase...
Importantly, our data also demonstrated that the AMPK activator AICAR restores ATP levels to that of controls and reverses Ang II–induced loss of gastrocnemius muscle mass. In addition, AICAR blocked Ang II–induced increases in E3 ubiquitin ligase expression via Akt activation, thereby contributing to its anticatabolic effects. These novel findings suggest that AMPK activators may have a therapeutic potential in congestive heart failure and chronic kidney disease, 2 disease states characterized by elevated levels of Ang II and wasting.

AMPK activation by AICAR reversed Ang II–induced skeletal muscle wasting independent of food intake and blood pressure. Of note, AMPK activation can increase appetite; however, our experiment was designed to minimize the orexigenic effect of AICAR to focus on its direct signaling effects in skeletal muscle. To accomplish this, food was provided only during nocturnal hours. Because AICAR was injected in the morning, food was unavailable for 12 hours after administration, and food intake was unaltered in AICAR mice. Also, although AICAR has been reported to reduce blood pressure, this occurred at a much higher dose and longer duration of administration (7 weeks) than was used in our study.

Neither caloric restriction nor Ang II altered mitochondrial content or protein expression of electron transport chain components. These results contrast with that reported previously in C2C12 myotubes and C57Bl/6 mice. In that study, Ang II, infused for 10 days at a subpressor dose, reduced both mitochondrial content and protein levels of the electron transport chain complexes IV and V. Strain, dosage, and time point differences might have contributed to these differences. In addition, it is possible that meal-interval training of our mice preconditioned them to states of metabolic stress, a protective effect of AMPK activation observed in a variety of cell types and animal models. As such, mitochondrial biogenesis could have been activated in the days before the start of the experiment, protecting against Ang II–induced reductions in mitochondrial content. In any case, Ang II

**Figure 4.** Angiotensin II (Ang II) increased expression of protein phosphatase 2Cα (PP2Cα) in gastrocnemius independent of food intake, whereas 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) blocked this Ang II–mediated increase. +P<0.05 vs pair fed. αααP<0.001 vs Ang II. αP<0.05 vs Ang II.

**Figure 5.** Angiotensin II (Ang II) inhibited AMP-activated protein kinase (AMPK) signaling, inhibited peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression, and activated acetyl-coenzyme A carboxylase (ACC). A, PGC-1α expression and (B) ACC phosphorylation. *P<0.05 vs ad libitum. **P<0.01 vs ad libitum. +P<0.01 vs pair fed. αP<0.05 vs Ang II.
reduced cytochrome C oxidase activity, and this mitochondrial dysfunction led to reduced ATP content in gastrocnemius muscles of Ang II–infused mice. Despite reduced caloric intake, the reduction in gastrocnemius weight in pair-fed controls was not accompanied by reduced ATP, suggesting that increased AMPK activity was able to maintain normal ATP levels and indicating that there is not a direct correlation between ATP and muscle weight. This is further reflected in the low ATP in gastrocnemius muscles of AICAR-treated control animals (pair fed and ad libitum), in which there was no wasting. Ang II mice were unable to respond to the initial energy imbalance induced by Ang II, leading to prolonged ATP depletion, which potentially contributed to the wasting phenotype. This lack of an appropriate adaptive response in Ang II mice was reflected in gastrocnemius AMPK activity, which was increased by pair feeding but failed to increase with Ang II. Despite severe energy depletion, Ang II inhibited the ability of AMPK to sense the low fuel state and signal accordingly to correct the imbalance. Direct stimulation of AMPK by AICAR was able to override the inhibitory effect of Ang II and to normalize Ang II–induced energy depletion by 4 days. The mechanism by which Ang II enhances AMPK dephosphorylation and inactivation appears to be via upregulation of PP2C expression in muscle. A similar mechanism of AMPK inhibition has been described previously in skeletal muscle insulin resistance induced by tumor necrosis factor-α. AICAR returned PP2C expression to basal levels in Ang II–infused mice, and this reduction in expression of the competing phosphatase might have contributed to the ability of AICAR to prevent Ang II–mediated reduction in AMPK phosphorylation.

Ang II did not activate PP2C enzymatic activity in addition to increasing PP2Cα expression in gastrocnemius. However, our assay could not discriminate between PP2Cα and PP2Cβ activities. In addition, the specific mechanism of Ang II–induced PP2Cα upregulation remains to be elucidated, although we observed no increase in gastrocnemius redox status (Figure S7), suggesting that reactive oxygen species may not be directly involved. However, it remains possible that other types of reactive oxygen species may mediate the effect (i.e., NADPH oxidase–derived superoxides, which are important in Ang II wasting). Although our data suggest

Figure 6. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) blocked angiotensin II (Ang II)–induced increases in E3 ligase expression via Akt activation. A, Atrogin 1, (B) muscle RING-finger protein-1 (MuRF-1) mRNA, (C) Akt phosphorylation at Ser473, and (D) Akt phosphorylation at Thr308. *P<0.05 vs ad libitum. **P<0.001 vs pair fed. ***P<0.001 vs ad libitum. ○P<0.05 vs Ang II. ○○○P<0.001 vs Ang II.
that upregulation of PP2Cα by Ang II mediates its inhibitory effects on AMPK phosphorylation and energy balance, further experiments are required to establish a causal link. In particular, it will be necessary to demonstrate that inhibition of PP2Cα in vivo can mimic the rescue effects of AICAR.

ACC phosphorylation status and PGC-1α expression correlated closely with levels of AMPK phosphorylation. As AMPK is activated, PGC-1α transcription is turned on, leading to increased expression of mitochondrial genes and mitochondrial biogenesis in skeletal muscle. Caloric restriction induced AMPK activity with a corresponding increase in PGC-1α levels at 4 days. By blocking AMPK activation, Ang II also likely blocked activation of PGC-1α transcription, and, as such, protein levels of PGC-1α remained unchanged. AICAR maintained AMPK activation in the presence of Ang II and blocked the inhibitory effect of Ang II on PGC-1α expression. Consistent with its inhibitory effect on AMPK activity, Ang II reduced ACC phosphorylation. As AMPK is activated, it phosphorylates and inactivates ACC, in effect turning off fatty acid synthesis. Active ACC catalyzes the synthesis of malonyl-coenzyme A, which, in turn, blocks carnitine palmitoyltransferase-1 function via allosteric inhibition. Phosphorylation and inactivation of ACC relieves this inhibition of carnitine palmitoyltransferase 1 and allows for the transporter to facilitate mobilization of fatty acids to mitochondria, where they can be β-oxidized for acute ATP production in times of metabolic stress. Therefore, Ang II–induced reduction of ACC phosphorylation is consistent with activation of ACC and would be a maladaptive response, because long chain fatty acid synthesis (which is energy consuming) would continue, whereas β-oxidization (which is ATP generating) would be inhibited. Of note, Ang II reduced total ACC at 4 days, which may be a compensatory response. AICAR restored total ACC to basal levels but did not alter ACC activity.

Ang II upregulates the E3 ubiquitin ligases atrogin 1 and muscle RING-finger protein 1 in skeletal muscle, and these are essential to the wasting process. Interestingly, AICAR blocked this upregulation, providing a potential additional mechanism whereby AICAR treatment prevents Ang II–induced wasting. AICAR induced Akt activation and inhibitory phosphorylation of FoxO1, explaining the ability of AICAR to abrogate Ang II–mediated upregulation of E3 ubiquitin ligases. The predominant effects of AMPK–mediated phosphorylation of FoxO are believed to be activating. Therefore, its plausible that Akt–mediated inhibitory phosphorylation of FoxO is dominant over any direct activating effects of AMPK.

Our findings are relevant to wasting conditions in which the renin-angiotensin system is activated. Patients with congestive heart failure have 2- to 4-fold increases in plasma Ang II levels, in many cases even in the presence of angiotensin-converting enzyme inhibitor therapy. There is also evidence for 5-fold increases in circulating Ang II levels in chronic kidney disease patients. Infusion of 1000 ng/kg per minute of Ang II in our model yields a 2.8-fold increase in plasma Ang II, which is well within this range. Furthermore, infusion of low-dose Ang II (80 ng/kg per minute) for 28 days into rats also caused loss of lean body mass with no reduction in food intake, clearly indicating catabolic activation with Ang II. Our finding that AMPK activation reverses Ang II–induced catabolic effects may offer new therapeutic strategies for the treatment of skeletal muscle wasting.

Perspectives
Our data show that Ang II–mediated skeletal muscle wasting is characterized by mitochondrial dysfunction-induced skeletal muscle ATP depletion and inhibition of the normal physiological response to energy depletion. Specifically, Ang II prevented AMPK activation, likely via upregulation of the inactivating phosphatase PP2Cα, thereby preventing normalization of muscle energy balance. In addition, Ang II upregulated E3 ubiquitin ligase expression. The AMPK activator AICAR essentially reversed these effects and restored muscle mass. Thus, our studies describe the mechanisms underlying Ang II–induced skeletal muscle wasting and suggest a therapeutic potential for AMPK activators in congestive heart failure and chronic kidney disease, disease states characterized by chronic activation of the renin-angiotensin system and muscle wasting.

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Disclosures
None.

References


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ANGIOTENSIN II UPREGULATES PP2Ca AND INHIBITS AMPK SIGNALING AND ENERGY BALANCE LEADING TO SKELETAL MUSCLE WASTING

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Running Title: Ang II Inhibits AMPK Signaling

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EXPANDED MATERIALS AND METHODS

Animals and Experimental Design
Male, 8-10 week old FVB mice (Charles River) were meal-interval trained\textsuperscript{1-3} for 7 days, during which time normal chow was available during the 12h dark cycle and removed during the 12h light cycle each day. All mice were implanted subcutaneously under ketamine/xylazine anesthesia with Alzet micro-osmotic pump model 1007D which continuously infused either sterile saline (ad-libitum fed and pair-fed groups) or 1000ng/kg/min Ang II (Phoenix Pharmaceuticals) for 4 days (or 1 day) with continued dark-cycle feeding. Ad-libitum and Ang II mice were allowed free dark-cycle access to chow, while pair-fed animals were individually matched to Ang II mice and fed only what their counterpart consumed over the previous day. Starting 1 day prior to minipump implantation and continuing until day 4, sterile saline or 100mg/kg/day AICAR (Toronto Research Chemicals) was administered via once daily intraperitoneal injection. Systolic blood pressures were measured following 7 days of training via tail-cuff at day 0 and at day 3 with the Visitech BP-2000 (Visitech System, Apex, NC). For the MitoTEMPO\textsuperscript{4} experiment, minipumps infused either sterile saline (ad-libitum fed group), 0.4 μg/kg/min MitoTEMPO (ad-libitum + MitoTEMPO group, Alexis Corp., San Diego, CA), 1000ng/kg/min Ang II (Ang II group), or 1000ng/kg/min Ang II + 0.4 μg/kg/min MitoTEMPO (co-infused, Ang II + MitoTEMPO group) for 7 days. The animal protocols were approved by the Tulane University Institutional Animal Care and Use Committee.

Mitochondrial Quantification by Real Time PCR
Mitochondria were quantified as previously described\textsuperscript{5}. Briefly, muscle tissue digestes were prepared using Proteinase K buffer followed by Phenol-Chloroform DNA extractions and real-time PCR amplification of 3ng template using 16S (mitochondrial DNA specific) and Hexokinase2 (genomic) primers with SYBR-Green dye. The ratio of 16S:Hex2 provides a measure of mitochondrial copy number relative to genomic DNA copy number.

Cytochrome C Oxidase Activity
Mitochondria were isolated using differential centrifugation following homogenization in mitochondrial isolation buffer (Mitochondria Isolation Kit for Tissue and Cultured Cells, BioChain) then cytochrome C oxidase activity was determined (Mitochondria Activity Assay Kit, BioChain) via the addition of ferrocytochrome C followed by immediate measurement of kinetic absorbance readings at 550nm over 1 minute for each sample. All data were normalized to protein load (BCA Protein Assay Kit, Pierce) then expressed in Units per mL, where one unit oxidizes one micromole of ferrocytochrome c per minute at 25°C, pH 7.0.

Mitochondrial Superoxide Quantification
Slides were prepared with two 10 μm gastrocnemius cross-sections per slide. One section of each slide was pre-incubated with 20 μl of 2.6 U/μl PEG-SOD (Sigma-Aldrich, St. Louis, MO) and the other section with 20 μl of PBS for 30 minutes at 37°C. 1 μl of DAPI (1 ng/μl final concentration) and 1 μl of MitoSOX\textsuperscript{TM} (2 μM final concentration, Invitrogen, Carlsbad, CA) was then added to each section and incubated for another 30 minutes at 37°C. Fluorescent microscopy images were analyzed with ImagePro Plus (Media Cybernetics, Bethesda, MD).
**ATP Quantification**

Tissue was homogenized in hypotonic cell lysis buffer (25mM HEPES, 5mM MgCl₂, 5mM EDTA, 5mM DTT, protease inhibitor cocktail tablets (Roche), pH 7.5) then diluted in Nucleotide Releasing Buffer (ApoSensor ADP/ATP Ratio Assay Kit, BioVision) and ATP was immediately quantified by addition of ATP monitoring enzyme (BioVision, luciferin + luciferase). Light emission was quantified in a luminometer following a 30s delay, integrated over 12s. All data were normalized to protein load.

**Immunoblotting**

Tissue was homogenized by bead beating in 10 volumes of RIPA + protease inhibitors (protease inhibitor cocktail tablet, Roche) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail, Thermo Scientific). Homogenates were clarified by centrifugation at 12,000 rpm for 5 min at 4°C, and supernatants were collected. Following determination of protein concentration via BCA Protein Assay (Pierce), samples were run according to standard SDS-PAGE and Western blotting protocols with chemiluminescent detection (Pierce ECL). Band width and density was quantified with ImageJ software (NIH) and data are expressed as change relative to saline infused, ad-libitum fed controls. Primary antibodies utilized were as follows: Rabbit monoclonal to phospho-AMPKα (Thr172), AMPKα, phospho-LKB1 (Ser428), LKB1, phospho-TAK1 (Ser412), TAK1, phospho-ACC (Ser79), ACC, phospho-Akt (Thr308), phospho-Akt (Ser473), Akt, phospho-Fox01 (Thr24), and Fox01 from Cell Signaling Technology, rabbit polyclonal to PGC1α and PP2C alpha + PP2C beta from Abcam, and MitoProfile Total OXPHOS Rodent WB Antibody Cocktail from MitoSciences.

**PP2C Activity**

Gastrocnemius muscle was homogenized in hypotonic cell lysis buffer (HCLB, 25mM HEPES, 5mM MgCl₂, 5mM EDTA, 5mM DTT, protease inhibitor cocktail tablets (Roche), pH 7.5) then diluted in assay buffer and PP2C activity was quantified via the Malachite Green Phosphate Detection Kit (R&D Systems) as previously described with minor modifications. Briefly, 100μM synthetic phosphopeptide serine/threonine phosphatase substrate (R&D Systems) was added to assay wells to measure PP2C activity in the presence of magnesium (which is required for PP2C activity, 5mM, contained in HCLB/assay buffer), +/- 100nM okadaic acid (which inhibits PP2A, PP1, and PP2B activities, but not PP2C activity), and +/- 100μM oleic acid (which activates PP2C while inhibiting PP2B and PP1). After addition of Malachite Green reagents, samples were incubated for 20 minutes at room temperature and absorbance was measured at 620nm. All data were normalized to protein load.

**Gene Expression by Real-Time PCR**

mRNA was isolated from tissues by TriPure/chloroform extraction followed by purification with RNeasy mini columns (Qiagen). Concentration and purity was determined using a Nanodrop spectrophotometer. cDNA was prepared using 1ug total RNA and RT2 first strand synthesis kit (SA Biosciences) and real-time PCR was performed using SYBR-green dye and SuperArray optimized primers for atrogin-1, MuRF-1, PP2Cα, and HPRT1 (housekeeping gene). Calculated fold changes in gene expression were compared to Ad-libitum controls.

**8-isoprostane Quantification**
Tissue 8-isoprostane levels were quantified in triplicate via the 8-isoprostane EIA kit (Cayman Chemical). Gastrocnemius muscles were homogenized in 500ul MES buffer (0.4M 2-(N-morpholino-ethanesulphonic acid), 0.1M phosphate, 2mM EDTA) per half gastrocnemius and homogenates were centrifuged 15 minutes at 10,000g, 4°C. Supernatants were collected and following development, absorbance was read at 405nm. Protein concentration was determined via BCA protein assay. Data were normalized to protein load and expressed as pg/mL 8-isoprostane per 50ug protein.

**Oxidized Glutathione Ratio Quantification**
Gastrocnemius muscle was homogenized in 500ul MES buffer (0.4M 2-(N-morpholino-ethanesulphonic acid), 0.1M phosphate, 2mM EDTA) per half gastrocnemius by bead beating at 4°C for 5 minutes. The homogenate was then centrifuged for 15 minutes at 10,000g, 4°C, and the supernatant was collected. An aliquot was removed and assayed for protein concentration via BCA assay and the remainder of the supernatant was deproteinized by addition of 13% 5-sulfosalicylic acid in 10mM HCl (10% v/v, 1.3% final) followed by 5 minute incubation at room temperature and a 5 minute centrifugation at 10,000g, 4°C. The supernatant was then removed without disturbing the protein pellet and was stored on ice. An aliquot of deproteinated sample was removed, to which 1% v/v of 1M 2-vinylpyridine (in ethanol) was added. Samples were mixed by briefly vortexing then incubated for 1 hour at room temperature (these samples containing 2-vinylpiridine were used for derivation of GSSG, exclusive of GSH, while total glutathione, GSHt, was determined in samples without 2-vinylpiridine). 50μl of each sample and standard (prepared +/- 2-vinylpyridine) were added to a 96-well plate in duplicate. Then 150μl assay cocktail (MES buffer, Cofactor mixture, Enzyme mixture, water, and freshly reconstituted Ellman’s reagent, DTNB) was added to each well. Absorbance was then read at 405nm at 5 minute intervals over 30 minutes, with shaking prior to each measurement. GSSG/GSH ratio was calculated according to the equation: GSSG/GSH = GSHt-2GSSG/GSSG.

**Statistical Analysis**
All data are presented as means +/- standard errors (SEM). GraphPad software (Version 4.0 for Windows, Graphpad Software, San Diego, CA) was used to perform the statistical analysis. Differences between groups were determined by one-way or two-way ANOVA, as appropriate, followed by Tukey or Bonferroni post tests, respectively. A value of p<0.05 was considered statistically significant.
REFERENCES


SUPPLEMENTAL DATA

Figure S1. Ang II increases mitochondrial superoxides, but mitochondrial ROS do not contribute to Ang II wasting as determined by failure of MitoTEMPO to rescue against Ang II wasting. (A) Mitochondrial superoxides, AFU - arbitrary fluorescence units (B) gastrocnemius mass (C) total body mass. * P<0.05 vs. Ad-Libitum. ** P<0.01 vs. Ad-Libitum. *** P<0.001 vs. Ad-Libitum. + P<0.05 vs. Pair-fed. Φ P<0.05 vs. Ad-Libitum + MitoTEMPO.
Figure S2. Ang II inhibits the activity of AMPK and AICAR activates AMPK at Day 1. * P<0.05 vs. Ad-Libitum.
Figure S3. Ang II does not inhibit the activity of the upstream AMPK kinases: LKB1 and TAK1. (A) LKB1 phosphorylation and expression (B) TAK1 phosphorylation. + P<0.05 vs. Pair-fed. o P<0.05 vs. Ang II.
Figure S4. Ang II does not alter enzymatic activity of PP2C.
Figure S5. Effects of caloric restriction, Ang II, and AICAR on protein expression of ACC. * P<0.05 vs. Ad-Libitum. *** P<0.001 vs. Ad-Libitum. o P<0.05 vs. Ang II
Figure S6. AICAR increases inhibitory Thr24 phosphorylation of FoxO1.
Figure S7. Caloric restriction, Ang II, and AICAR had no effect on indices of oxidative stress in gastrocnemius muscle.  (A) 8-isoprostane and (B) oxidized glutathione ratio in gastrocnemius.