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Title: AMP-Activated Protein Kinase (AMPK) Action in Skeletal Muscle: Direct Phosphorylation of PGC-1〈

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Abstract

Activation of AMP-activated kinase (AMPK) in skeletal muscle increases glucose uptake, fatty acid oxidation and mitochondrial biogenesis by increasing gene expression in these pathways. However, the transcriptional components that are directly targeted by AMPK are still elusive. The peroxisome-proliferator-activated receptor-γ coactivator 1α (PGC-1α) has emerged as a master regulator of mitochondrial biogenesis; furthermore, it has been shown that PGC-1α gene expression is induced by exercise and by chemical activation of AMPK in skeletal muscle. Using primary muscle cells and mice deficient in PGC-1α, we found that the effects of AMPK on gene expression of glucose transporter 4 (GLUT4), mitochondrial genes and PGC-1α itself are almost entirely dependent on the presence of PGC-1α protein. Furthermore, AMPK phosphorylates PGC-1α directly both *in vitro* and in cells. These direct phosphorylations of the PGC-1α protein at threonine 177 and serine 538 are required for the PGC-1α-dependent induction of the PGC-1α promoter. These data indicate that AMPK phosphorylation of PGC-1α initiates many of the important gene regulatory functions of AMPK in skeletal muscle.

Introduction

Organisms at all levels of the evolutionary scale have found ways to translate changes in environmental conditions into fine metabolic adjustments. The cellular energy charge is determined by a combination of catabolic and anabolic reactions. Since the cellular concentrations of AMP changes more dramatically than that of ATP or ADP, AMP is a key monitor of the cellular energy status (1). The major molecular sensor for AMP level in cells is AMP-activated protein kinase (AMPK), an enzyme that is activated by cellular stresses that result in ATP depletion. Stimuli of AMPK are generally either processes that inhibit ATP production (e.g. metabolic poisons, hypoxia or glucose deprivation) or accelerate ATP consumption (e.g. rapid contraction in muscle) (2-5). AMPK is also activated by the adipokines leptin and adiponectin, important regulators of whole body energy metabolism (2-5). Furthermore, the finding that AMPK is an indirect target of

metformin (6-9), a widely used antidiabetic drug, has led to growing interest in AMPK as a potential target for the treatment of type 2 diabetes. The stimulation of AMPK results in the repression of many anabolic processes (such as fatty acid and cholesterol synthesis, gluconeogenesis) and activation of several catabolic processes (such as fatty acid uptake and oxidation, glucose uptake) (2-5).

Much of the progress in knowledge about the downstream targets of AMPK has come from the use of the chemical compound 5-aminoimidazole-4-carboxamide riboside (AICAR) (10), an AMP analogue. Several *in vivo* studies using AICAR to activate AMPK determined that mitochondrial enzymes (e.g. cytochrome *c*, UCP3) (11-14) and proteins involved in glucose uptake (GLUT4) (14-16) are increased at the transcriptional level in skeletal muscle. Interestingly, these genes are also downstream targets of PGC-1α (PPARγ coactivator-1α), a highly regulated coactivator of nuclear receptors and many other transcription factors outside of the nuclear receptor family (17). PGC-1α is a key player in the oxidative metabolism of brown fat and muscle by increasing mitochondrial biogenesis, and augmenting the expression of enzymes of the electron transport system and uncoupling proteins (18, 19). In addition, ectopic expression of PGC-1α in muscle cells increases expression of the GLUT4 glucose transporter, resulting in increased glucose uptake (20).

PGC-1α is preferentially expressed in oxidative muscle fibers, and transgenic mice ectopically expressing PGC-1α in muscle tissue show conversion of type IIb (glycolytic) fibers into mitochondria rich type IIa and I fibers (21). Interestingly, PGC-1α expression and PGC-1α responsive genes involved in oxidative phosphorylation are down-regulated in skeletal muscle of human type 2 diabetics (22, 23). Since PGC-1α gene expression in muscle is increased *in vivo* with exercise, AICAR and metformin treatment (24-30), these results suggest the important role of PGC-1α in whole body energy metabolism; they further suggest that PGC-1α is likely a very important downstream target of AMPK.

The mechanisms by which activated AMPK induces gene expression are not yet clear. As different subunits of AMPK are preferentially located in the nucleus (31), it has been proposed that it directly regulates gene expression by phosphorylating certain transcription factors (32). Indeed, AMPK has been shown previously to directly phosphorylate transcription factors and coactivators like p53, p300, TRIP6 and TORC2

(32-35). We show here that many effects of activated AMPK on gene expression in skeletal muscle, including the inductions of the PGC-1α, GLUT4 and mitochondrial genes, requires the presence of the coactivator PGC-1α protein. Furthermore, AMPK directly phosphorylates PGC-1α and this phosphorylation mediates the increase of the PGC-1α protein action on the PGC-1α promoter.

Results

PGC-1α protein is required for AMPK action on gene expression and on mitochondrial function. We investigated whether the presence of PGC-1α is required for the effects of AMPK activation on gene expression in catabolic pathways. Primary muscle cells isolated from wild type and PGC-1α -/- mice (36) were differentiated into myotubes and subsequently treated with either 500 μM AICAR, 1 mM metformin or vehicle for 16 hours. That AMPK was activated under these conditions in both cell types was shown by Western blotting with anti-pACC and anti-pAMPKα antibodies (Fig. 1A). These treatments induced a significant increase in the expression of mRNAs encoding PGC-1α (measured within exon 2, which is still present in the PGC-1α -/- (36)), GLUT4 and several mitochondrial target genes of AMPK, such as cytochrome c, UCP-2 and UCP-3 (Fig. 1B and C, 2A-C). Strikingly, cells lacking PGC-1α showed a complete ablation of this induction (Fig. 1B and C, 2A-C). Additionally, these increases in gene expression were almost completely blocked in cells that were pre-incubated with the AMPK inhibitor 8-Br AMP (1mM) (37) (Fig. 1B and C, 2A-C). The expression of PGC-1β did not change upon AMPK activation, showing that active AMPK specifically induces PGC-1α (Fig. 1D). This indicates that PGC-1α is absolutely required for the induction of GLUT4 and mitochondrial genes via AMPK activation.

To determine whether this PGC-1α mediated increase in mitochondrial genes stimulated by AMPK is reflected in mitochondrial function, we measured respiration in wild type and PGC-1α -/- myotubes. As shown in Fig. 2E, wild type cells had a 15 % increase in total respiration and a 40 % increase in uncoupled respiration upon AICAR treatment. However, total respiration did not increase with AICAR treatment of the PGC-1α -/- cells; the effect in uncoupled respiration was reduced to 24%. These data show that PGC-

1α is required for the positive effects of AMPK activation on mitochondrial function in muscle cells.

PGC-1α protein is required for AMPK action on gene expression of PGC-1α, GLUT4 and cytochrome c *in vivo*. Mice lacking PGC-1α specifically in skeletal muscle have been developed (38). To determine whether AMPK action on the expression of the PGC-1α gene and some of its key target genes *in vivo* also requires PGC-1α, wild type and skeletal muscle-specific PGC-1α -/- mice were treated with 250 mg/kg AICAR for 6 hours. AMPK was activated in both genotypes, as determined by Western blotting with anti-pACC antibodies (Fig. 3A). AICAR increased the expression of PGC-1α mRNA (exon 2) and GLUT4 mRNA about 2.5- fold in the muscle of wild type mice, while PGC-1β gene expression did not change (Fig. 3B and C). Cytochrome c mRNA increased 1.7 fold (Fig. 3B). The mice lacking PGC-1α in skeletal muscle completely failed to induce PGC-1α (exon 2), cytochrome c and GLUT4 mRNA in response to AICAR (Fig. 3B and C). Interestingly, UCP-3 mRNA was induced about 4 fold in skeletal muscle of both genotypes (Fig. 3B), indicating that this gene did not require PGC-1α for AMPK action. In addition to GLUT4, we examined two other genes involved in glucose metabolism (hexokinase and PDK4) that have been reported to be induced by AMPK (16, 26, 39). Hexokinase expression did not change with this AICAR treatment, in either WT or in the skeletal muscle-specific PGC-1α -/- mice (Fig. 3C). In contrast, gene expression of the PGC-1α target PDK4 (40, 41) was slightly induced in wild type and in the skeletal muscle-KOs (Fig. 3C). These data indicate that AMPK activation *in vivo* induces PGC-1α, GLUT4 and cytochrome c in a PGC-1α-dependent way. However an alternative pathway clearly exists *in vivo* for the AMPK-mediated induction of UCP-3 and PDK4.

AMPK directly phosphorylates PGC-1α protein on threonine 177 and serine 538. PGC-1α protein is involved in the induction of the PGC-1α gene in a feed-forward loop in skeletal muscle (42). Thus the data above suggests that AMPK could directly pactivate the PGC-1α protein , perhaps by a direct phosphorylation. We first asked whether AMPK and PGC-1α interact directly. As shown in Fig. 4A, co-immunoprecipitation

experiments indicate that AMPK and PGC-1α form a complex in cells. We next tested whether AMPK directly phosphorylates PGC-1α in cells. As shown in Fig. 4B (left panels), AICAR stimulated an increased phosphorylation of the PGC-1α protein in primary myotubes. AMPK also phosphorylates PGC-1α *in vitro,* as shown in Fig. 4C. Using full-length protein and various fragments purified from bacteria, this phosphorylation was robust and increased by the presence of AMP. Fragments of PGC-1α from aa 1-190 and 395-565 were phosphorylated, while those encoding aa 200-400 and 551-797 were not. Mass spectrometry analysis indicated that phosphorylations occurred on threonine 177 and serine 538. Mutations of these sites completely ablated these AMPK-mediated phosphorylations *in vitro* (Fig. 4D) and in primary myotubes (Fig. 4B, right panels)

Phosphorylation by AMPK increases PGC-1α–dependent activation of its own promoter. Finally we asked whether the induction of the PGC-1α promoter by AMPK requires AMPK-mediated phosphorylation of the PGC-1α protein. Fig. 4E illustrates that, as shown previously(42), the PGC-1α protein has a significant effect on the -2kb PGC-1α promoter. This effect is greatly augmented by AICAR treatment of cells. A PGC-1α protein with alanine replacements at the two direct AMPK sites (T177 and S538) is *completely* resistant to this effect of AICAR. These data strongly suggest that direct phosphorylation of the PGC-1α coactivator by AMPK initiates a cascade of gene expression that controls many mitochondrial target genes and genes of glucose and oxidative metabolism in muscle.

Discussion

AMPK and PGC-1α have both been shown to play important roles in energy homeostasis. AMPK is an important sensor of decreased energy charge in cells, and subsequently acts to increase catabolic reactions and decrease anabolic reactions. PGC-1α is a critical regulator of transcription of many genes of energy homeostasis, and is particularly involved in fuel oxidation and mitochondrial biology. A functional relationship between these two proteins is therefore not surprising. Earlier work showed

that activated AMPK increases PGC-1α gene expression in cultured muscle cells, in umbilical vein endothelial cells, and skeletal muscle (26-29, 43-49) Moreover, the use of RNAi against PGC-1α has suggested that PGC-1α mediates certain of these effects of AMPK, particularly in fatty acid oxidation (25). In this paper we demonstrate two important points: first, that AMPK requires PGC-1α for many of its most important effects on GLUT4 and mitochondrial gene expression in skeletal muscle, both in culture and *in vivo*. Second, AMPK binds to and activates PGC-1α in muscle by direct phosphorylation on two critical residues, threonine 177 and serine 538.

Previouswork has demonstrated that PGC-1α can function as a regulator of its own gene expression in muscle, in a feed-forward loop (42). Therefore, it was possible that the AMPK-phosphorylated PGC-1α was involved in the induction of the PGC-1α gene, with many subsequent effects of AMPK secondary to this induction of PGC-1α. As shown in Fig. 4, work with the isolated PGC-1α promoter supports this idea. AMPK robustly increases the action of PGC-1α on the 2 kb promoter. Mutation of the two AMPK phosphorylation sites in the PGC-1α protein completely ablated the effect of AICAR on this promoter. These data also suggest that the phosphorylation of PGC-1α will likely affect the action of this protein on other promoters, either positively or negatively. It is worth noting that PGC-1α is a potent activator of gluconeogenic gene expression in the liver, while AMPK activators like AICAR and metformin suppress gluconeogenic gene expression. It has been shown in liver that activated AMPK prevents the nuclear import of TORC2, the transcriptional coactivator of CREB, and therefore blocks the fasting induced induction of PGC-1α (35); it remains to be determined whether the phosphorylation at threonine 177 and serine 538 occurs in liver and whether these phosphorylations are activating or inhibiting in this tissue.

Mechanistically, these AMPK-mediated phosphorylations could modulate the ability of PGC-1α to dock on certain transcription factors or affect the binding or function of other cofactors in the PGC-1α coactivator complex. The modulation of PGC-1α docking on certain transcription factors by AMPK might provide a simple explanation by which this enzyme could activate certain PGC-1α functions in muscle (such as GLUT4 gene expression), while inhibiting PGC-1α functions in liver (such as gluconeogenic gene

expression). More specific research into mechanisms affected by the AMPK-mediated phosphorylation of PGC-1α is warranted.

Materials and Methods

Reagents. AICAR (5-aminoimidazole-4-carboxamide 1-ß-D-ribofuranoside) was obtained from Calbiochem (animal experiments) or Toronto Chemicals (cell experiments). 8-Bromo AMP and metformin were purchased from Sigma. bFGF (basic Fibroblast Growth Factor) was obtained from Invitrogen. Antibodies against pACC, pAMPK, AMPK, and γ-tubulin were purchased from Cell Signaling. Active AMPK was obtained from Upstate Biotechnology. The 2 kb PGC-1α promoter has been previously described (42). PGC-1α mutant constructs encoding PGC-1α T177A S538A were generated using a Quick Change Site Directed Mutagenesis Kit (Stratagene)

Cell Culture, transfection, reporter gene assays, and co-immunoprecipitations. C2C12 myotubes were grown in DMEM supplemented with 10% FBS and differentiated into myotubes in DMEM supplemented with 2% HS. Primary muscle cells were isolated from PGC-1α wild type and -/- mice as described previously (50). Myoblast were cultured in F10-HAM medium supplemented with 20% FBS and bFGF. For differentiation into myotubes, cells were shifted to DMEM supplemented with 5% HS for two days. The myotubes were treated with 500 μM AICAR or 1 mM metformin in DMEM supplemented with 0.5% BSA for 16 hs. For the inhibitor studies, the myotubes were pre-treated for 30 min with 1 mM 8-Bromo AMP. Reporter gene assays were performed in a C2C12 muscle cell line. Myoblast were transfected with SuperFect (Qiagen) and subsequently differentiated for 36 hours before treatment with 500 μM AICAR for 7.5 hours. Firefly luciferase activity was measured and normalized to Renilla luciferase expression (Dual Luciferase Reporter Assay System, Promega). Empty pGL3basic reporter gene vector and pCDNA.3 vector served as the control for the PGC-1α 2 kb promoter and PGC-1α constructs, respectively. For co-immunoprecipitations experiments, cells were transformed with the corresponding plasmids (pCMV-myc-AMPKα2 gift of P. Sanz (34) and pCMV-Flag-PGC-1α) with Superfect (Quiagen). 48 hrs after transfection cells were lysed (50 mM Tris-HCl ph 7.8, 137 mM NaCl, 1 mM EDTA, 0.2% Sarkosyl, 1% Triton-X100, 1 mM DTT, 10% Glycerol) and 500 μM total protein was subjected to immunoprecipitation with an M2 agarose anti-FLAG resin (Sigma) for 2 hrs at 4 °C. Proteins were separated by SDS/PAGE and transferred to

PVDF membrane. PGC-1α was detected with anti-PGC-1α antibodies (51) and the Myc-AMPKα2 was detected with anti-C-Myc (A 14) antbodies (Santa Cruz).

Phosphorylation Analyses *In Vitro* and *In Vivo*. GST-PGC-1α fragments wild type and mutants were expressed in bacteria (BL21 strain, Invitrogene) and purified using glutathione sepharose beads (GE Healthcare Bio-Sciences AB). Recombinant proteins were used as a substrate for *in vitro* phosphorylation reaction with activated AMPK, as per manufacturer's instructions (Upstate Biotechnology). After the phosphorylation reactions, the glutathione beads were extensively washed and eluted proteins were analyzed by SDS-PAGE and autoradiography. Protein levels were monitored by Coomassie blue staining. For *in vivo* phosphorylation, primary PGC-1α -/- myotubes were infected with retroviruses expressing a Flag PGC-1α wild-type or mutant protein, respectively. Stable cell lines were established by selection with puromycin (pMSCVpuro Clontech). Cells were differentiated and treated with 250 μM AICAR and labeled for 1 hr with [^{32}P] ortophosphate (Amersham Biosciences). Cells were harvested and lysed ((50 mM Tris-HCl ph 7.8, 137 mM NaCl, 1 mM EDTA, 0.2% Sarkosyl, 1% Triton-X100, 1 mM DTT, 10% Glycerol) and subjected to immunoprecipitation with an M2 agarose anti-FLAG resin (Sigma) for 2 hrs at 4 °C. Immunprecipitates were subjected to SDS-PAGE and transferred to a PVDF membrane: ^{32}P incorporation was visualized by autoradiography. PGC-1α protein levels were analyzed by Western blot using M2 anti-FLAG antibodies (Sigma).

Oxygen Consumption Measurements. Primary muscle cells from PGC-1α wild type and -/- mice were differentiated for two days in DMEM supplemented with 5% HS with or without 500 μM AICAR. Myotubes were treated for another 16 hours with AICAR in DMEM supplemented with 0.5% BSA. Four days after differentiation, cells were washed with PBS at room temperature and trypsinized from the plates. After centrifugation, cells were resuspended in PBS and transferred to a 1 ml Clark-type oxygen electrode chamber. After recording the basal respiration rate, the uncoupled respiration was measured in the presence of the ATP synthase inhibitor, oligomycin (2.5 μg/ml). Rates of oxygen consumption were normalized to cell counts.

Analysis of gene expression. Total RNA was isolated from cells using Trizol (Invitrogen). For real time PCR analysis, RNA was treated with DNAse and subsequently reverse transcribed using iSCRIPT (Bio-Rad). Using semiquantitative PCR (Applied Biosystems) and SYBRGreen (Applied Biosystems), mRNA levels were first normalized to TBP mRNA, and then relative mRNA levels were determined using the ΔΔCt.

Animal experiments. All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee. Mice with a muscle-specific mutation in PGC-1α are described elsewhere (38). 5-7 weeks old female mice were injected intraperitoneally with 250 mg/kg AICAR in sterile 0.9% NaCl or with 0.9% NaCl. Mice were sacrificed 6 hours later, and the gastrocnemius muscle was harvested for RNA analysis and Western analysis.

Statistical analysis. Results are expressed as +/-SD for cell experiments and +/- SEM for animal experiments. Two-tailed student's t-tests were used to determine p values.

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References:

1. Hardie, D. G. & Hawley, S. A. (2001) *Bioessays* **23,** 1112-1119.
2. Hardie, D. G., Hawley, S. A., & Scott, J. W. (2006) *The Journal of physiology* **574,** 7-15.
3. Carling, D. (2004) *Trends Biochem Sci* **29,** 18-24.
4. Kahn, B. B., Alquier, T., Carling, D., & Hardie, D. G. (2005) *Cell Metabolism* **1,** 15-25.
5. Fryer, L. G. & Carling, D. (2005) *Biochemical Society transactions* **33,** 362-366.
6. Musi, N., Hirshman, M. F., Nygren, J., Svanfeldt, M., Bavenholm, P., Rooyackers, O., Zhou, G., Williamson, J. M., Ljunqvist, O., Efendic, S.*, et al.* (2002) *Diabetes* **51,** 2074-2081.
7. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N.*, et al.* (2001) *J Clin Invest* **108,** 1167-1174.
8. Fryer, L. G., Parbu-Patel, A., & Carling, D. (2002) *The Journal of biological chemistry* **277,** 25226-25232.
9. Hawley, S. A., Gadalla, A. E., Olsen, G. S., & Hardie, D. G. (2002) *Diabetes* **51,** 2420-2425.

10. Corton, J. M., Gillespie, J. G., Hawley, S. A., & Hardie, D. G. (1995) *Eur J Biochem* **229,** 558-565.
11. Winder, W. W., Holmes, B. F., Rubink, D. S., Jensen, E. B., Chen, M., & Holloszy, J. O. (2000) *J Appl Physiol* **88,** 2219-2226.
12. Zhou, M., Lin, B. Z., Coughlin, S., Vallega, G., & Pilch, P. F. (2000) *Am J Physiol Endocrinol Metab* **279,** E622-629.
13. Putman, C. T., Kiricsi, M., Pearcey, J., MacLean, I. M., Bamford, J. A., Murdoch, G. K., Dixon, W. T., & Pette, D. (2003) *J Physiol* **551,** 169-178.
14. Jorgensen, S. B., Treebak, J. T., Viollet, B., Schjerling, P., Vaulont, S., Wojtaszewski, J. F., & Richter, E. A. (2007) *Am J Physiol Endocrinol Metab* **292,** E331-339.
15. Zheng, D., MacLean, P. S., Pohnert, S. C., Knight, J. B., Olson, A. L., Winder, W. W., & Dohm, G. L. (2001) *J Appl Physiol* **91,** 1073-1083.
16. Holmes, B. F., Kurth-Kraczek, E. J., & Winder, W. W. (1999) *J Appl Physiol* **87,** 1990-1995.
17. Lin, J., Handschin, C., & Spiegelman, B. M. (2005) *Cell Metab* **1,** 361-370.
18. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C*., et al.* (1999) *Cell* **98,** 115-124.
19. Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., & Spiegelman, B. M. (1998) *Cell* **92,** 829-839.
20. Michael, L. F., Wu, Z., Cheatham, R. B., Puigserver, P., Adelmant, G., Lehman, J. J., Kelly, D. P., & Spiegelman, B. M. (2001) *Proceedings of the National Academy of Sciences of the United States of America* **98,** 3820-3825.
21. Lin, J., Wu, H., Tarr, P. T., Zhang, C. Y., Wu, Z., Boss, O., Michael, L. F., Puigserver, P., Isotani, E., Olson, E. N*., et al.* (2002) *Nature* **418,** 797-801.
22. Mootha, V. K., Lindgren, C. M., Eriksson, K. F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstrale, M., Laurila, E*., et al.* (2003) *Nat Genet* **34,** 267-273.
23. Patti, M. E., Butte, A. J., Crunkhorn, S., Cusi, K., Berria, R., Kashyap, S., Miyazaki, Y., Kohane, I., Costello, M., Saccone, R*., et al.* (2003) *Proceedings of the National Academy of Sciences of the United States of America* **100,** 8466-8471.
24. Hood, D. A., Irrcher, I., Ljubicic, V., & Joseph, A. M. (2006) *J Exp Biol* **209,** 2265-2275.
25. Lee, W. J., Kim, M., Park, H. S., Kim, H. S., Jeon, M. J., Oh, K. S., Koh, E. H., Won, J. C., Kim, M. S., Oh, G. T*., et al.* (2006) *Biochem Biophys Res Commun* **340,** 291-295.
26. Jorgensen, S. B., Wojtaszewski, J. F., Viollet, B., Andreelli, F., Birk, J. B., Hellsten, Y., Schjerling, P., Vaulont, S., Neufer, P. D., Richter, E. A*., et al.* (2005) *Faseb J* **19,** 1146-1148.
27. Terada, S., Goto, M., Kato, M., Kawanaka, K., Shimokawa, T., & Tabata, I. (2002) *Biochem Biophys Res Commun* **296,** 350-354.
28. Terada, S. & Tabata, I. (2003) *Am J Physiol Endocrinol Metab*.
29. Terada, S. & Tabata, I. (2004) *Am J Physiol Endocrinol Metab* **286,** E208-216.
30. Suwa, M., Egashira, T., Nakano, H., Sasaki, H., & Kumagai, S. (2006) *J Appl Physiol* **101,** 1685-1692.

31. Salt, I., Celler, J. W., Hawley, S. A., Prescott, A., Woods, A., Carling, D., & Hardie, D. G. (1998) *Biochem J* **334 (Pt 1),** 177-187.
32. Leff, T. (2003) *Biochemical Society transactions* **31,** 224-227.
33. Imamura, K., Ogura, T., Kishimoto, A., Kaminishi, M., & Esumi, H. (2001) *Biochem Biophys Res Commun* **287,** 562-567.
34. Solaz-Fuster, M. C., Gimeno-Alcaniz, J. V., Casado, M., & Sanz, P. (2006) *Cell Signal* **18,** 1702-1712.
35. Shaw, R. J., Lamia, K. A., Vasquez, D., Koo, S. H., Bardeesy, N., Depinho, R. A., Montminy, M., & Cantley, L. C. (2005) *Science* **310,** 1642-1646.
36. Lin, J., Wu, P. H., Tarr, P. T., Lindenberg, K. S., St-Pierre, J., Zhang, C. Y., Mootha, V. K., Jäger, S., Vianna, C. R., Reznick, R. M*., et al.* (2004) *Cell* **119,** 121-135.
37. Musi, N., Hayashi, T., Fujii, N., Hirshman, M. F., Witters, L. A., & Goodyear, L. J. (2001) *Am J Physiol Endocrinol Metab* **280,** E677-684.
38. Handschin, C., Kobayashi, Y. M., Chin, S., Seale, P., Campbell, K. C., & Spiegelman, B. M. (2007) *Genes and Development* **21**.
39. Stoppani, J., Hildebrandt, A. L., Sakamoto, K., Cameron-Smith, D., Goodyear, L. J., & Neufer, P. D. (2002) *Am J Physiol Endocrinol Metab* **283,** E1239-E1248.
40. Wende, A. R., Huss, J. M., Schaeffer, P. J., Giguere, V., & Kelly, D. P. (2005) *Mol Cell Biol* **25,** 10684-10694.
41. Araki, M. & Motojima, K. (2006) *Febs J* **273,** 1669-1680.
42. Handschin, C., Rhee, J., Lin, J., Tarr, P. T., & Spiegelman, B. M. (2003) *Proceedings of the National Academy of Sciences of the United States of America* **100,** 7111-7116.
43. Suwa, M., Nakano, H., & Kumagai, S. (2003) *J Appl Physiol* **95,** 960-968.
44. Winder, W. W., Taylor, E. B., & Thomson, D. M. (2006) *Med Sci Sports Exerc* **38,** 1945-1949.
45. Kuhl, J. E., Ruderman, N. B., Musi, N., Goodyear, L. J., Patti, M. E., Crunkhorn, S., Dronamraju, D., Thorell, A., Nygren, J., Ljungkvist, O*., et al.* (2006) *Am J Physiol Endocrinol Metab* **290,** E1296-1303.
46. Sriwijitkamol, A., Ivy, J. L., Christ-Roberts, C., DeFronzo, R. A., Mandarino, L. J., & Musi, N. (2006) *Am J Physiol Endocrinol Metab* **290,** E925-932.
47. Ojuka, E. O. (2004) *Proc Nutr Soc* **63,** 275-278.
48. Kukidome, D., Nishikawa, T., Sonoda, K., Imoto, K., Fujisawa, K., Yano, M., Motoshima, H., Taguchi, T., Matsumura, T., & Araki, E. (2006) *Diabetes* **55,** 120-127.
49. Taylor, E. B., Lamb, J. D., Hurst, R. W., Chesser, D. G., Ellingson, W. J., Greenwood, L. J., Porter, B. B., Herway, S. T., & Winder, W. W. (2005) *Am J Physiol Endocrinol Metab* **289,** E960-968.
50. Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E., & Rudnicki, M. A. (1996) *Genes Dev* **10,** 1173-1183.
51. Puigserver, P., Adelmant, G., Wu, Z., Fan, M., Xu, J., O'Malley, B., & Spiegelman, B. M. (1999) *Science* **286,** 1368-1371.

Fig. 1. AMPK-driven increase in PGC-1α and GLUT4 gene expression requires PGC-1α protein. A) AMPK is activated in AICAR and metformin-treated primary myotubes. Comparison of AMPKα protein phosphorylated at threonine 172 (pT172) to total levels of AMPKα protein in WT and PGC-1α KO primary myotubes treated with vehicle, AICAR or Metformin for 1h. A also shows the levels of ACC protein phosphorylated at serine 79: loading control (γ-tubulin) B) AICAR and metformin treatments elevate the expression of PGC-1α in WT but not in PGC-1α -/- cells. The inhibitor of AMPK, 8-Br AMP blocks this increase. C) mRNA levels of glucose transporter 4 (GLUT4), B) the relative gene expression of PGC-1β does not change under the same conditions. Primary myotubes were treated with vehicle, 500 μM AICAR, 1 mM metformin, 1mM 8-BrAMP, 8-Br AMP/AICAR and 8-Br AMP/metformin in DMEM supplemented with 0.5% BSA for 16hs. The relative PGC-1α mRNA levels were determined with primers in exon2, which is present in WT and PGC-1α -/- cells, using semiquantitative PCR.

Fig. 2. AMPK-driven increase in expression of mitochondrial genes and in respiration requires PGC-1α. Primary myotubes were treated as in Figure 1 and mRNA levels of A) cytochrome c, B) uncoupling protein 3 (UCP-3), and C) uncoupling protein 2 (UCP-2) were determined using semiquantitative PCR. D) WT and PGC-1 α -/- primary myotubes were treated with AICAR in DMEM supplemented with 5%HS for two days. At day three, cells were shifted to DMEM 0.5% BSA and treated for an additional 16 hrs before oxygen consumption was measured as described in *Materials and Methods*.

Fig. 3. AMPK-driven increase in PGC-10α, GLUT4 and cytochrome c gene expression requires PGC-1α protein *in vivo*. A) AMPK is activated in skeletal muscle of AICAR injected mice. The upper blot shows the levels of ACC protein phosphorylated at S79 in gastrocnemeus muscle from WT and PGC-1α muscle-specific KO mice, injected with saline or AICAR. B) Injection of AICAR induces the mRNA expression of PGC-1α, cytochrome c but not PGC-1β in the skeletal muscle of wild type mice (*$p < 0.01$); this induction does not occur in the skeletal muscle of the muscle-specific PGC-1α -/- mice. UCP-3 gene expression is also increased in the muscle-specific PGC-1α -/- mice (** $p <$

0.05). C) Injection of AICAR induces the mRNA expression of GLUT4 (* $p < 0.01$), PDK4 (** $p < 0.1$) but not of hexokinase in the skeletal muscle of wild type mice. PDK4 gene expression increases also in the muscle-specific PGC-1α -/- mice: Female mice were injected with either saline or 250 mg/kg AICAR. Skeletal muscle was harvested after 6 hrs, and gene expression was measured using semiquantitative PCR (n = 5-7).

Fig. 4. AMPK phosphorylates PGC-1α at threonine residue 177 and serine residue 538 *in vitro* and in cells. A) PGC-1α interacts with AMPKα2 in cells. Expression vectors for Flag-PGC-1α and Myc-AMPKα2 were transfected into BOSC cells, as indicated. Co-immunoprecipitation was performed as described in *Materials and Methods*. B) Primary PGC-1α -/- myotubes stably expressing PGC-1α and PGC-1α T177A S538A, respectively, were treated with vehicle or AICAR for 1hr in the presence of ^{32}P. C) Purified recombinant GST-PGC-1α fragments (full length 1-797, 1-190, 200-400, 395-565, 551-797) were incubated with purified AMPK and phosphorylation was determined by incorporation of γ-^{32}P ATP. D) Mass spectrometry identified threonine 177 and serine 538 as phosphorylated residues. The GST-PGC-1α fragment containing amino acid 1-190 T177A and the GST-PGC-1α fragment containing amino acids 395-565 S538A are not phosphorylated by AMPK. E) The phosphorylation of PGC-1α protein by AMPK is required for elevated PGC-1α dependent activity of the PGC-1α promoter. C2C12 muscle cells were transfected with a 2 kb PGC-1α promoter construct and expression plasmids for PGC-1α or PGC-1α T177A S538A, respectively. After transfection, cells were differentiated for one day and treated with AICAR for 7.5 hrs before reporter-gene levels were determined (* $p < 0.01$).

Figure 1

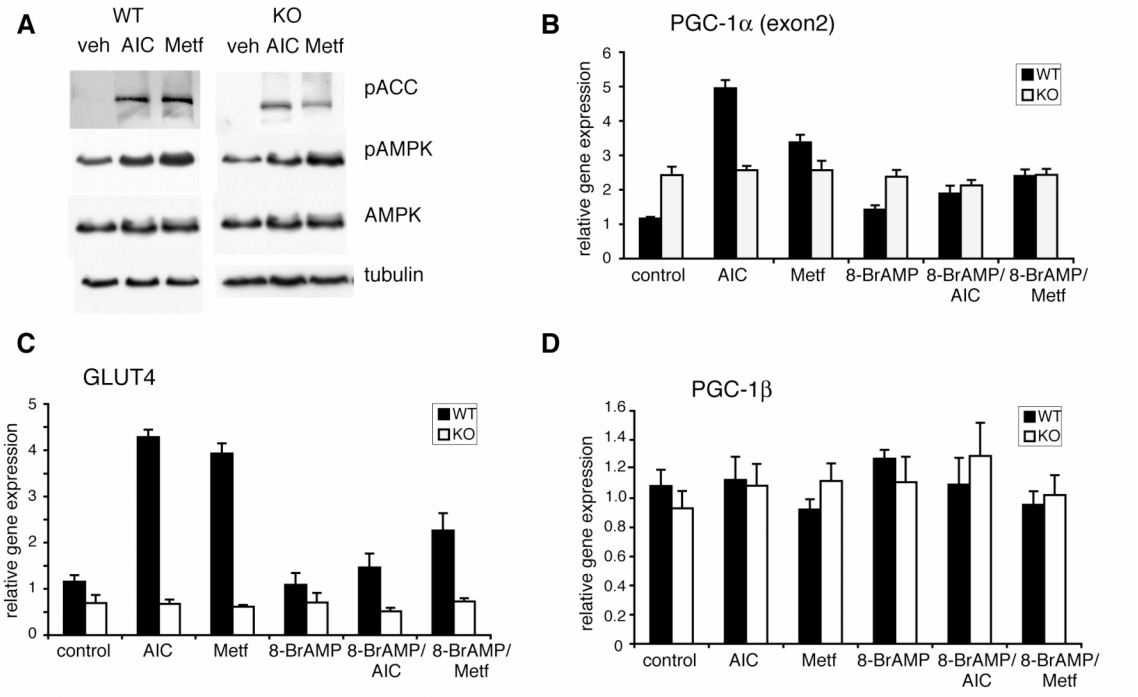

A
WT
veh AIC Metf
KO
veh AIC Metf
pACC
pAMPK
AMPK
tubulin
B
PGC-1α (exon2)
relative gene expression
WT
KO
control
AIC
Metf
8-BrAMP
8-BrAMP/ AIC
8-BrAMP/ Metf
C
GLUT4
relative gene expression
WT
KO
control
AIC
Metf
8-BrAMP
8-BrAMP/ AIC
8-BrAMP/ Metf
D
PGC-1β
relative gene expression
WT
KO
control
AIC
Metf
8-BrAMP
8-BrAMP/ AIC
8-BrAMP/ Metf

Figure 2

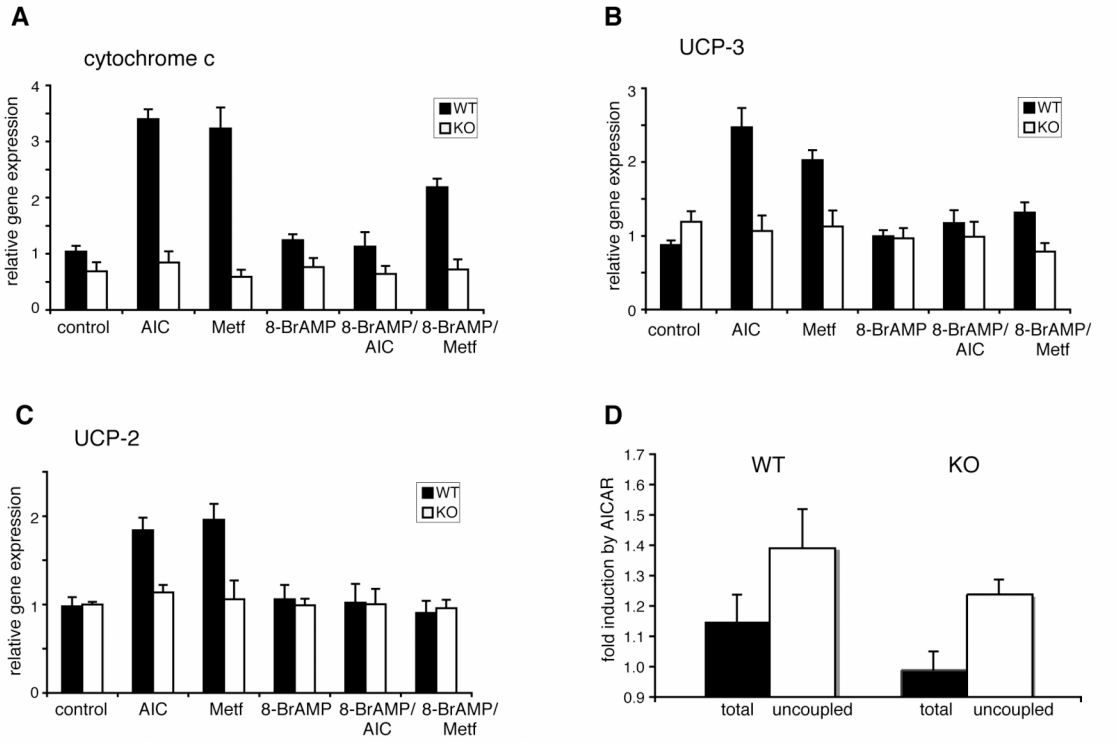

A
cytochrome c
relative gene expression
WT
KO
control
AIC
Metf
8-BrAMP
8-BrAMP/ AIC
8-BrAMP/ Metf
B
UCP-3
C
UCP-2
D
fold induction by AICAR
WT
KO
total
uncoupled

Figure 3

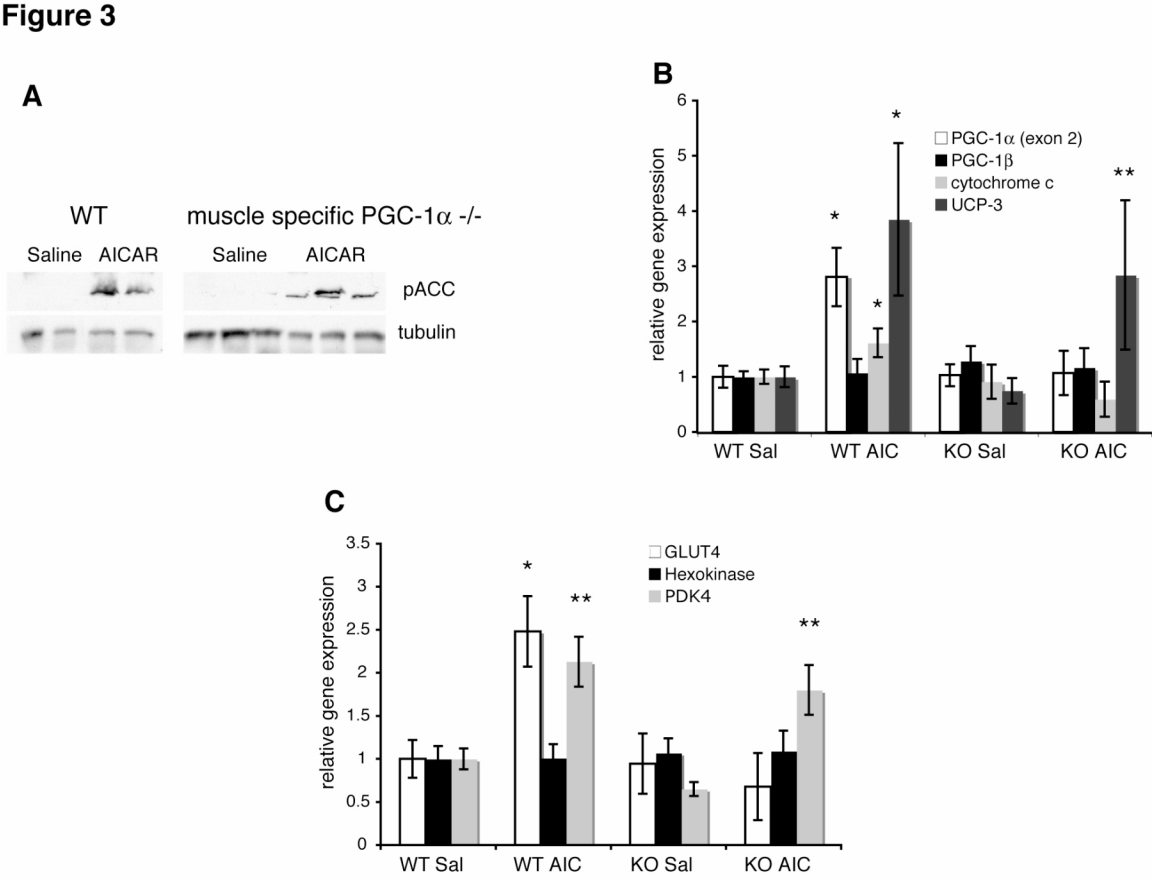
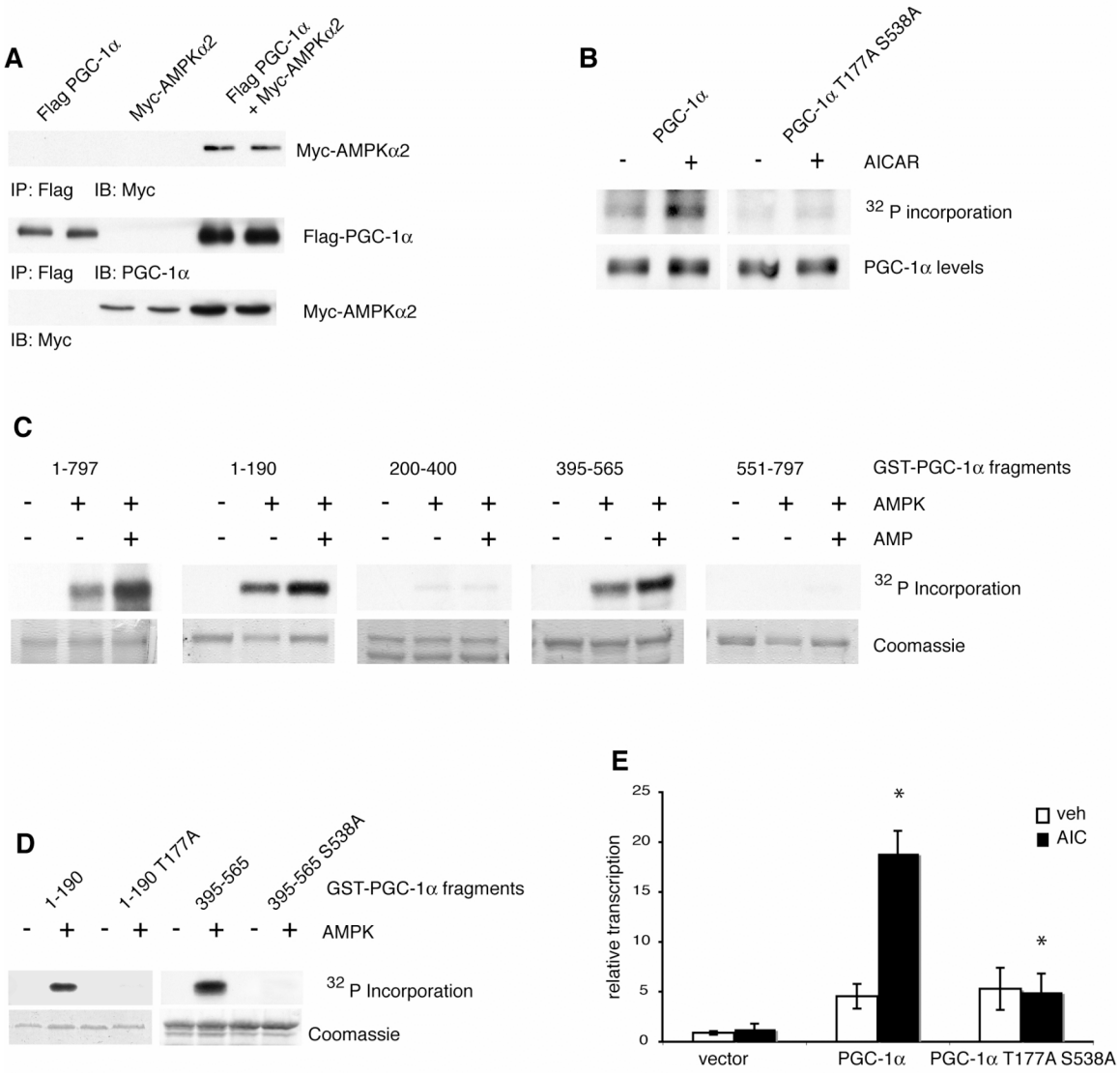

A
WT
muscle specific PGC-1α -/-
Saline
AICAR
Saline
AICAR
pACC
tubulin
B
relative gene expression
PGC-1α (exon 2)
PGC-1β
cytochrome c
UCP-3
WT Sal
WT AIC
KO Sal
KO AIC
C
relative gene expression
GLUT4
Hexokinase
PDK4
WT Sal
WT AIC
KO Sal
KO AIC

Figure 4


A
Flag PGC-1α
Myc-AMPKα2
Flag PGC-1α + Myc-AMPKα2
Myc-AMPKα2
IP: Flag IB: Myc
Flag-PGC-1α
IP: Flag IB: PGC-1α
Myc-AMPKα2
IB: Myc
B
PGC-1α
PGC-1α T177A S538A
- + - +
AICAR
32 P incorporation
PGC-1α levels
C
1-797
1-190
200-400
395-565
551-797
GST-PGC-1α fragments
AMPK
AMP
32 P Incorporation
Coomassie
D
1-190
1-190 T177A
395-565
395-565 S538A
GST-PGC-1α fragments
AMPK
32 P Incorporation
Coomassie
E
relative transcription
25
20
15
10
5
0
veh
AIC
vector
PGC-1α
PGC-1α T177A S538A