Energy Depletion Inhibits Phosphatidylinositol 3-Kinase/Akt Signaling and Induces Apoptosis via AMP-activated Protein Kinase-dependent Phosphorylation of IRS-1 at Ser-794*1,1

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Energy depletion activates AMP-activated protein kinase (AMPK) and inhibits cell growth via TSC2-dependent suppression of mTORC1 signaling. Long term energy depletion also induces apoptosis by mechanisms that are not well understood to date. Here we show that AMPK, activated by energy depletion, inhibited cell survival by binding to and phosphorylating IRS-1 at Ser-794. Phosphorylation of IRS-1 at this site inhibited phosphatidylinositol 3-kinase/Akt signaling, suppressed the mitochondrial membrane potential, and promoted apoptosis. Of the treatments promoting energy depletion, glucose deprivation, hypoxia, and inhibition of ATP synthesis in the mitochondrial membrane potential, and promoted apoptosis. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S10.

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2 The abbreviations used are: AMPK, AMP-activated protein kinase; CaMKKβ, Ca2+/calmodulin-dependent kinase; IRS-1, insulin receptor substrate-1; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; HIE, human embryonic kidney; IRS-1, insulin receptor substrate-1; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; MTTS, (3,4,5-trimethoxy-2,4-dihydroxyphenyl) isothiocyanate; JNK, c-Jun N-terminal kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; siRNA, short interference RNA; PBS, phosphate-buffered saline; ΔVm, membrane potential; DiIC1, 1,3,3’,3’-hexamethylindodicarbocyanine iodide; WT, wild type; ERK, extracellular signal-regulated kinase.

Energy depletion also affects the activity of the PI 3-kinase/Akt pathway (12, 13). The immediate effect of energy depletion is transient activation of the pathway due to inhibition of the mTORC1/S6K1 axis, which in turn prevents the phosphorylation of IRS-1 at inhibitory phosphorylation sites (12–14). Following its immediate activation, the pathway is repressed because of subsequent inhibitory events, which are not well understood to date. Inhibition of the PI 3-kinase/Akt pathway by prolonged energy depletion promotes apoptosis.

IRS-1 plays a central role in the activation of the PI 3-kinase/Akt pathway by insulin and insulin-like growth factor I signals. In addition to its role in PI 3-kinase/Akt activation, IRS-1 also plays an important role in a feedback loop that inhibits the pathway. The feedback loop is triggered by mTORC1/S6K1, which is activated by insulin, insulin-like growth factor I, or nutrients (12–15). The activated mTORC1/S6K1 pathway phosphorylates IRS-1 at several inhibitory sites (12–15) and inhibits PI 3-kinase/Akt signaling. In addition to mTORC1/S6K1, other kinases such as JNK and IkB kinase-β also phosphorylate IRS-1 at inhibitory sites in the course of various pathological conditions such as inflammation and diabetes (16). The importance of IRS-1 in both the activation and the inhibition of the PI 3-kinase/Akt pathway by insulin and insulin-like growth factor I raised the question whether it may contribute to the inhibition of the PI 3-kinase/Akt pathway during energy stress.

Previous studies have shown that activation of AMPK by 5-aminoimidazole-4-carboxamide riboside in C2C12 myoblasts (17) or up-regulation of the AMPK family member salt-induced kinase-2 in dexamethasone-treated 3T3-L1 adipocytes...

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Energy Depletion and PI 3-Kinase/Akt Signaling

(18) promotes the phosphorylation of IRS-1 at Ser-794. However, the natural stimuli that trigger phosphorylation of IRS-1 at Ser-794 remained unknown. Moreover, the biological significance of this phosphorylation event remained undetermined with one study suggesting that it promotes (17) and another study suggesting that it inhibits (18, 19) PI 3-kinase/Akt signaling.

The work in this study focused on the nature, regulation, and biological significance of the events leading to the inhibition of the PI 3-kinase/Akt pathway during energy stress. Our data show that energy depletion induced by glucose deprivation, hypoxia, inhibition of ATP synthesis in mitochondria, oxidative stress, and 2-deoxyglucose (2-DG) activated AMPK, which binds to and phosphorylates IRS-1 at Ser-794. Glucose deprivation, hypoxia, and inhibition of ATP synthesis stimulated phosphorylation of IRS-1 at Ser-794 primarily via the α2 subunit of AMPK in an LKB1-dependent manner, whereas oxidative stress and 2-DG stimulated phosphorylation of IRS-1 at the same site primarily via the α1 subunit perhaps in a CaMKKβ-dependent manner. Phosphorylation of IRS-1 at Ser-794 induced by energy depletion inhibited the PI 3-kinase/Akt pathway and promoted apoptosis by suppressing the mitochondrial membrane potential.

EXPERIMENTAL PROCEDURES

Cell Culture—To engineer HEK293 cells stably expressing wild-type IRS-1 or the mutants S794A and S794D, cells were transfected with pCMV<sup>his</sup>-IRS-1-WT, pCMV<sup>his</sup>-IRS-1-S794D, or pCMV<sup>his</sup>-IRS-1-S794A constructs. Transfected cells were selected for 10 days in 10 mM histidinol (Sigma). Pooled clones were analyzed from three independent transfections. Stably transfected HEK293 cells express 5 times more IRS-1 compared with non-transfected HEK293 cells (data not shown). LKB1<sup(+/-)</sup> 3T3 fibroblasts as well as the retroviral expressing vectors pBabe-LKB1-WT (wild type) and pBabe-LKB1-KD (kinase-deficient) were kindly provided by Dr. N. Bardeesy (Massachusetts General Hospital) (20). Infected cells were selected for 5 days in 2–3 μg/ml puromycin (Sigma). Pooled clones were analyzed. The myoblast cell line L6 was grown to subconfluence in DMEM containing 20% FBS and antibiotics. NIH3T3 and HEK293 cells were grown in DMEM supplemented with 10% FBS and antibiotics.

In all experiments, cells were grown in high glucose (25 mM) DMEM (catalog number 11995-065, Invitrogen). For glucose and amino acid starvation experiments we used low glucose (5 mM) DMEM (catalog number 11855-084, Invitrogen), glucose-free DMEM (catalog number 11966-025, Invitrogen), or Hank’s balanced salt solution (amino-acid free, 5 mM glucose; catalog number 214020-17, Invitrogen). In some experiments we starved cells in Krebs-Ringer phosphate buffer (12 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, and 0.1% fatty acid-free bovine serum albumin (catalog number 126575, Calbiochem), pH 7.4) supplemented with glucose, amino acids, or both as indicated under “Results” and in the figure legends.

Expression Constructs—Site-directed mutagenesis of pCMV<sup>his</sup>-IRS-1-WT was carried out using overlap extension PCR. Amino acid numbering refers to the human isoform. To mutate the Ser-794 into aspartic acid or alanine we used the primers 5'-C-GTCTCTTTCACTGGAGCGC-3' and 5'-CGTCTCTCTTCACTGGAGCGC-3', respectively. Mutated nucleotides are underlined. The outer IRS-1 primers IRS-1 1338–1359 Forward (5'-CACACCCCCACGAGCGGGT-3') and IRS-1 3189–3195 Reverse (5'-TCCACGACAAGAAGTGGACGC-3') were designed to span two unique Bpl sites. To generate the pCMV-Myc-IRS-1-S794D and pCMV-Myc-IRS-1-S794A constructs we digested the plasmids described in the previous step with Xhol and Sall restriction enzymes, and we subcloned the fragments produced in the Xhol and HindIII sites of the previously described pCMV-Myc-IRS-1 (12). The human AMPK α1 catalytic subunit was cloned from HepG2 cells using the following primers: sense, 5'-ATATATAGTATTGCGACAGCCCGAGAAGCAGAAACACGAC-3'; and antisense, 5'-ATATATATTCTGAAGAATTATTGTGCAAAGAATTTTAATTAGATTGC-3'. The PCR product was cloned into pGEM T-vector (Promega). Following sequencing the PCR product was subcloned to a modified pCMV5-Myc vector with EcoRI and Xbal sites to generate pCMV-Myc-AMPK-α1. The pcDNA3-Myc-AMPK-α2-WT and pcDNA3-Myc-AMPK-α2-KD expression constructs were provided by Dr. M. Birnbaumn.

Short Interference RNA (siRNA) Transfection—siRNA against the human LKB1, the human AMPK α1 and α2 catalytic subunits, and the human IRS-1 were bought from Santa Cruz Biotechnology, and they were transfected using Lipofoectamine 2000 (Invitrogen). Transfections were carried out using a final siRNA concentration of 80 nM. To knock down both the α1 and the α2 subunits of AMPK simultaneously, cells were transfected with a mixture of the two siRNAs (final concentration of 40 nM for each one, 80 nM combined). Transfection efficiency, measured with the use of a fluorescein-conjugated control nonspecific siRNA (Cell Signaling Technology catalog number 6201), was higher than 90%. Cells were grown for 48–60 h before each experiment.

Antibodies—Anti-AMPK α1 and α2 antibodies were bought from Santa Cruz Biotechnology. Anti-AMPK antibody (it recognizes both the α1 and α2 catalytic subunits of AMPK) was bought from Cell Signaling Technology. Hif-1α antibody was bought from BD Transduction Laboratories. P85α antibody was bought from Upstate. All other antibodies, including secondary antibodies, were bought from Cell Signaling Technology.

Cell Lysis, Immunoprecipitation, and Western Blotting—Cells were washed in ice-cold PBS and solubilized in lysis buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1% Triton X-100, 10 mM Na<sub>2</sub>VO<sub>4</sub>, 50 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM okadaic acid supplemented with a mixture of protease inhibitors). The lysates were cleared by centrifugation for 10 min at 14,000 × g at 4 °C. For immunoprecipitation, 0.5–1 mg of a given lysate was mixed with glutathione S-transferase conjugated to protein A- or G-Sepharose beads. After extensive washing with lysis buffer, beads were resuspended in SDS sample buffer, supplemented with 5% β-mercaptoethanol, boiled for 5 min, and subjected to Western blot analysis using standard Western blotting protocols.
Measurement of IRS-1-associated PI 3-Kinase Activity—IRS-1 immune complexes, immunoprecipitated from cells lysates as described above, were washed three times with lysis buffer, once with buffer A (100 mM Tris, pH 7.4, 500 mM LiCl, 2 mM Na3VO4), once with buffer B (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 2 mM Na3VO4), and twice with kinase buffer (10 mM MgCl2, 50 mM Tris, pH 7.4). The washed beads were then mixed with 45 μl of kinase buffer, 5 μl of sonicated lipid substrate (phosphatidylinositol and phosphatidylserine (1:1) (from Avanti Polar Lipids) in a concentration of 1 mg/ml in 25 mM Hepes, pH 7.4, 1 mM EDTA)), and 20 μl of [γ-32P]ATP. The mixture was incubated for 20 min at room temperature. Reactions were stopped by the addition of 100 μl of 1 N HCl. Lipids were extracted and spotted on potassium oxalate-treated silica gel TLC plates (250-μm layer; catalog number 4420221, Whatman) and separated in chloroform, methanol, acetone, acetic acid, H2O (60:20:23:18:12, v/v/v/v/v/v). The plates were dried and exposed to x-ray film.

Measurement of Mitochondrial Membrane Potential and Annexin V Staining—To measure the mitochondrial membrane potential (ΔΨm) we used the MitoProbeTM 1,1′,3,3′,3′-hexamethylindocarbocyanine iodide (DiIC18) (5) assay kit (catalog number M34151, Molecular Probes) as instructed by the manufacturer. Briefly cells (including floating cells) grown in 12-well plates were collected following mild trypsinization. Trypsinized cells were washed once with PBS, and the cells were resuspended in 500 μl of PBS. Resuspended cells were labeled with 50 nm DiIC18 (5) (excitation/emission, 638/658 nm) at 37 °C in the dark for 20–30 min. Labeled cells were washed once in PBS, and they were analyzed by fluorescence-activated cell sorting using a CyAn (Dako Cytomation) high performance flow cytometer.

To measure apoptosis we used the Annexin V fluorescein staining kit (catalog number A13199, Molecular Probes) as instructed by the manufacturer. Cells treated as described in the previous paragraph were collected following mild trypsinization. Trypsinized cells were washed once with PBS, and they were resuspended in 100 μl of annexin binding buffer mixed with 5 μl of Annexin V fluorescein conjugate (excitation/emission, 494/518 nm). Resuspended cells were incubated at room temperature in the dark for 15 min. Labeled cells were analyzed by fluorescence-activated cell sorting. All flow cytometry data were analyzed using Summit Version 3.1 software.

Cell Proliferation Assay—To measure changes in the number of live cells, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT; catalog number M5655, Sigma) assay. HEK293 cells stably transfected with the wild-type IRS-1 or the S794A IRS-1 mutant were seeded at 104 cells/well in 24-well tissue culture plates. Sixteen hours later, cells were cultured in glucose- or serum-free media for an additional 48 h period as indicated under “Results” and in the figure legends. Then cells were placed in complete medium containing MTT at a final concentration of 500 μg/ml for an additional 2-h period. Absorbance was measured with a Bio-Rad Benchmark microplate reader at 490 nm.

Statistical Analysis—Results are expressed as means ± S.E. Differences between two groups were assessed using unpaired two-tailed t tests. Western blot band densitometry was done with IGOR Pro software (Wavemetrics, Inc.).

RESULTS

Energy Depletion Induces Phosphorylation of IRS-1 at Ser-794 and Down-regulates IRS-1 Expression—Energy depletion is defined as an increase in the AMP:ATP ratio, and it can be caused by a variety of factors such as glucose deprivation, hypoxia, inhibition of ATP synthesis in the mitochondria, oxidative stress, and 2-DG. Fig. 1A demonstrates that in L6 myoblasts glucose deprivation induced phosphorylation of the AMPK kinase on Thr-172 and inhibited the mTORC1 pathway. In addition, glucose deprivation stimulated phosphorylation of IRS-1 at Ser-794 with kinetics similar to the kinetics of phosphorylation of AMPK. Phosphorylation in response to glucose deprivation was not affected by insulin stimulation (supplemental Fig. S1) and was amino acid-independent (supplemental Fig. S1 and data not shown). Long term glucose deprivation also down-regulated the expression of endogenous IRS-1 whose levels decreased with a half-life of ∼9 h (Fig. 1A), whereas amino acid deprivation alone did not affect IRS-1 expression (data not shown). Therefore, in L6 myoblasts a glucose-dependent pathway regulates both the phosphorylation and expression of IRS-1.

To explore the mechanism of IRS-1 phosphorylation at Ser-794 and to determine its functional significance we needed to establish a system in which glucose deprivation would not induce the down-regulation of IRS-1. To this end, we engineered HEK293 cells stably expressing IRS-1 under the constitutively active cytomegalovirus promoter. HEK293 cells normally express IRS-1 at very low levels. Fig. 1B shows that glucose deprivation did not down-regulate the ectopically expressed IRS-1. This suggests that IRS-1 down-regulation following glucose deprivation (Fig. 1A) may be regulated transcriptionally possibly by a mechanism that involves suppression of mTORC1 signaling and mitochondria dysfunction as reported previously (15, 21). HEK293 cells stably expressing IRS-1 allowed us to measure the stoichiometry of phosphorylation of IRS-1 over time in cells cultured in media with decreasing concentrations of glucose. The results show that, upon glucose deprivation, IRS-1 phosphorylation at Ser-794 took place as early as 10 min from the start of the starvation period and persisted for 9 h similarly to phosphorylation of AMPK at Thr-172 (Fig. 1B). Moreover phosphorylation of IRS-1 at Ser-794 occurred within physiological glucose concentrations (4–6 mM glucose; supplemental Fig. S2) in a manner similar to phosphorylation of AMPK at Thr-172.

Hypoxia increases the AMP:ATP ratio and activates AMPK (22). Fig. 1C shows that hypoxia, in addition of stabilizing Hif-1α, stimulated phosphorylation of IRS-1 at Ser-794 in HEK293 cells stably expressing IRS-1 with kinetics that were similar to the kinetics of AMPK phosphorylation at Thr-172. Similarly the hypoxia-mimicking agent, cobalt chloride (CoCl2), also stimulated AMPK and IRS-1 phosphorylation at these sites (Fig. 1D). Lastly short term insulin stimulation did not affect hypoxia-induced phosphorylation of IRS-1 at Ser-794 (supplemental Fig. S3).
FIGURE 1. Energy depletion and oxidative stress stimulate phosphorylation of IRS-1 at Ser-794. L6 myoblasts (A) and HEK293 cells (B) stably transfected with IRS-1 were serum-starved for 1 h and then cultured in media lacking glucose. Whole cell lysates were harvested at the indicated time points and were analyzed by Western blotting. C and D, HEK293 cells stably expressing IRS-1 were either cultured in a hypoxic environment (C) or in cobalt chloride-containing media for the indicated period of time (D). Whole cell lysates were harvested at the indicated time points and were analyzed by Western blotting. E, HEK293 cells stably expressing IRS-1 were treated with increasing concentrations of oligomycin or CCCP for 30 min. Whole cell lysates were analyzed by Western blotting. F, HEK293 cells stably expressing IRS-1 were treated with increasing concentrations of hydrogen peroxide for 15 min as indicated. Whole cell lysates were analyzed by Western blotting. G, L6 myoblasts were treated with hydrogen peroxide (500 μM). Whole cell lysates harvested at the indicated time points were analyzed by Western blotting.
Mitochondrial oxidative phosphorylation produces the majority of cellular ATP. To directly address the role of mitochondrial dysfunction in the regulation of IRS-1 phosphorylation at Ser-794 we examined the phosphorylation of IRS-1 at this site in cells treated with oligomycin and carbonyl cyanide 3-chloroperoxybenzoic acid (CCCP). Oligomycin and CCCP inhibit ATP production via inhibition of the F1F0 ATP synthase and via irreversible dissipation of the electrochemical gradient, respectively. Fig. 1E demonstrates that inhibition of ATP synthesis in the mitochondria by both oligomycin and CCCP activated AMPK and stimulated IRS-1 phosphorylation at Ser-794.

Hydrogen peroxide-induced oxidative stress acutely activates AMPK by increasing the cellular AMP:ATP ratio (8, 9, 11, 23). In HEK293 cells and L6 myoblasts hydrogen peroxide stimulated AMPK and IRS-1 phosphorylation at Thr-172 and Ser-794, respectively, in a dose-dependent manner within 2 min from the start of the treatment (Figs. 1, A and B, and 4C and Refs. 15 and 21). Of note, energy depletion caused by all tested treatments completely eliminated previously described nutrient-sensitive mTORC1/S6K1-mediated phosphorylations of IRS-1 at inhibitory sites (data not shown and Refs. 12–14).

**IRS-1 Phosphorylation at Ser-794 in Response to Glucose Deprivation, Hypoxia, and Oxidative Stress Depends on the AMPK Catalytic Subunit α**—To determine whether IRS-1 phosphorylation at Ser-794 in response to energy depletion is AMPK-dependent we either overexpressed or knocked down the catalytic subunit of AMPK, and we examined the effects of these treatments on IRS-1 phosphorylation at this site. Fig. 2A demonstrates that overexpression of the wild-type form of the α2 (or the α1, not shown) catalytic subunit of AMPK significantly enhanced IRS-1 phosphorylation at Ser-794 following glucose deprivation, whereas overexpression of the kinase-deficient mutant of AMPK did not. Next we used siRNA that specifically knocked down the α1 and α2 catalytic subunits of the AMPK in HEK293 cells stably transfected with IRS-1. HEK293 cells express both the α1 and α2 catalytic subunits of AMPK with the α1 subunit being more abundant (5, 24). Fig. 2B shows that knocking down either subunit efficiently suppressed the phosphorylation of IRS-1 at Ser-794 in response to glucose deprivation. Simultaneous knockdown of both subunits, using half the amount of siRNA for each subunit (see “Experimental Procedures”), completely eliminated basal as well glucose deprivation-induced phosphorylation of IRS-1 at Ser-794.

Next we addressed the role of AMPK in the hypoxia-induced phosphorylation of IRS-1 at Ser-794. Fig. 2C shows that overexpression of the wild-type form of AMPK enhanced the hypoxia-induced phosphorylation of IRS-1 at Ser-794, whereas the use of compound C, a specific AMPK inhibitor (25), reversed the effect of AMPK. Knocking down either of the two AMPK catalytic subunits suppressed hypoxia-induced Ser-794 phosphorylation, although simultaneous knockdown of both subunits had a synergetic effect, suggesting that both isoforms phosphorylate IRS-1 at Ser-794 in response to hypoxic stress (Fig. 2D).

Data presented in Fig. 1E suggest that inhibition of ATP synthesis in the mitochondria by oligomycin or CCCP induced phosphorylation of IRS-1 at Ser-794. Fig. 2E shows that compound C suppressed oligomycin- and CCCP-induced IRS-1 phosphorylation at Ser-794. Similarly siRNA against AMPK suppressed oligomycin- and CCCP-induced phosphorylation of IRS-1 at Ser-794 (data not shown) suggesting an important role for AMPK in mediating these effects.

To dissect the signaling pathway that mediates the phosphorylation of IRS-1 at Ser-794 in response to oxidative stress we used an array of known kinase inhibitors. Supplemental Fig. S5 shows that compound C suppressed IRS-1 phosphorylation induced by hydrogen peroxide treatment in a dose-dependent manner, whereas JNK, ERK, and PI 3-kinase/Akt inhibitors did not interfere with this event. Consistent with these data, knocking down the α1 catalytic subunit of AMPK suppressed hydrogen peroxide-induced IRS-1 phosphorylation at Ser-794 (Fig. 2F). However, knocking down the α2 subunit did not, suggesting that whereas both the α1 and the α2 subunits contribute to the phosphorylation of IRS-1 at Ser-794 in response to glucose deprivation and hypoxia, it is the α1 subunit that is primarily responsible for the phosphorylation of IRS-1 at this site in response to oxidative stress. This is in agreement with previous reports showing that hydrogen peroxide activates preferentially the AMPK α1, as opposed to the α2, catalytic subunit in vitro (23). Indeed knocking down the α1 subunit lowered the stoichiometry of AMPK phosphorylation at Thr-172, whereas knocking down the α2 subunit did not (Fig. 2F). Lastly supplemental Fig. S6 shows that siRNA against the AMPK α1 catalytic subunit also suppressed 2-DG-induced IRS-1 phosphorylation at Ser-794.

Previous studies have shown that energy depletion induces the phosphorylation of TSC2 by AMPK via a mechanism that involves direct interaction between AMPK and TSC2 (3). To this end we performed co-immunoprecipitation experiments to address whether AMPK, which is required for the phosphorylation of IRS-1 at Ser-794 upon energy depletion, interacts with IRS-1 and whether such an interaction can be induced by energy depletion. For these experiments we used HEK293 cells stably expressing IRS-1 to avoid its down-regulation upon AMPK knockdown (5, 24), we conclude that the α2 catalytic subunit is the one that primarily phosphorylates IRS-1 in glucose- or oxygen-deprived cells.
Energy Depletion and PI 3-Kinase/Akt Signaling

A.  

|                | 5 mM Glucose | Glucose free |
|----------------|-------------|--------------|
| Myc-AMPK α2 WT | -           | +            |
| Myc-AMPK α2 KD | -           | +            |

B.  

|                | siGFP        | siAMPKα1     | siAMPKα2     | siAMPKα1α2 |
|----------------|--------------|--------------|--------------|------------|
| Glucose        | +            | -            | -            | -          |

C.  

|                | Hypoxia (0.2% O₂) |
|----------------|-------------------|
| Myc-AMPK α2 WT | -                 |
| AMPK Inh (10 µM)| -                 |

D.  

|                | Hypoxia (0.2% O₂) |
|----------------|-------------------|
| siGFP          | -                 |

E.  

|                | AMPK Inh (10 µM) |
|----------------|-----------------|
| CCCP (50 µM)   | -               |
| Oligomycin (1 µM) | -          |

F.  

|                | H₂O₂ (500 µM) |
|----------------|--------------|
| siGFP          | -            |

G.  

|                | +Glu | -Glu | +Glu | -Glu |
|----------------|------|------|------|------|
| Myc-LKB1       | -    | +    | -    | +    |
| Myc-AMPK α1    | -    | +    | -    | +    |
| Myc-AMPK α2    | -    | +    | -    | +    |

IP: Myc
LKB1 Is Required for the Phosphorylation of IRS-1 at Ser-794 upon Glucose Deprivation, Hypoxia, and Inhibition of ATP Synthesis in the Mitochondria—The LKB1 kinase, in complex with the STE20-related adapter (STRAD)-α/β and MO25-α/β proteins, has been identified as an upstream AMPK kinase (7). Overexpression of wild-type LKB1 did not stimulate Ser-794 phosphorylation (data not shown). However, overexpression of the kinase-deficient mutant of LKB1 weakly inhibited IRS-1 phosphorylation at this site in glucose- or oxygen-deprived cells (data not shown). This observation prompted us to further address the role of LKB1 in LKB1−/− knock-out 3T3 fibroblasts reconstituted with the wild-type or the kinase-deficient form of LKB1. The results show that the kinase activity of LKB1 was required for AMPK activation and IRS-1 phosphorylation at Ser-794 following glucose deprivation (Fig. 3A), hypoxia (Fig. 3B), and inhibition of ATP synthesis in the mitochondria (Fig. 3C). On the contrary, hydrogen peroxide and 2-DG continued to partially activate AMPK and to induce phosphorylation of IRS-1 at Ser-794 in LKB1−/− fibroblasts. Knocking down LKB1 in HEK293 cells stably expressing IRS-1 confirmed that LKB1 is selectively required for the phosphorylation of IRS-1 at Ser-794 following glucose deprivation or inhibition of mitochondrial oxidative phosphorylation but not following exposure to hydrogen peroxide or 2-DG (Fig. 3E). These findings suggest that glucose deprivation, hypoxia, and the inhibition of ATP synthesis in the mitochondria may transmit via LKB1 a mitochondrially derived signal that activates AMPK. In addition, the findings suggest that hydrogen peroxide and 2-DG may also activate AMPK and may stimulate phosphorylation of IRS-1 at Ser-794 via a kinase other than LKB1. Further studies indeed showed that hydrogen peroxide-induced phosphorylation of IRS-1 at Ser-794 in LKB1 knock-out fibroblasts was sensitive to STO-609 (Fig. 3F), suggesting that phosphorylation depends on the activity of the CaMKKβ as reported previously (8–11). Overall the data presented in Fig. 3 support the conclusion that energy depletion due to glucose deprivation, hypoxia, and inhibition of ATP synthesis in the mitochondria stimulate phosphorylation of IRS-1 at Ser-794 in an LKB1-dependent manner, whereas hydrogen peroxide and 2-DG stimulate phosphorylation of IRS-1 at the same site via CaMKKβ and to a lesser extent LKB1.

Energy Depletion and Oxidative Stress Inhibit IRS-1-associ- ated PI 3-Kinase/Akt Signaling—Next we sought to determine the effect of energy depletion on IRS-1-associated PI 3-kinase/Akt signaling. Fig. 4A shows that long term glucose deprivation (16 h) stimulated phosphorylation of IRS-1 at Ser-794 and suppressed insulin-induced IRS-1-associated PI 3-kinase activity (data not shown) and Akt phosphorylation (Fig. 4A) in L6 myoblasts. However, the observed inhibitory effect of chronic glucose removal on Akt phosphorylation in these cells was largely due to down-regulation of endogenous IRS-1 (Figs. 1A and 4A).

To determine the effects of IRS-1 phosphorylation at Ser-794 in the absence of IRS-1 down-regulation, we performed the same experiments in HEK293 cells stably transfected with IRS-1. Prior to that, we examined whether IRS-1 is required for Akt activation by insulin in HEK293 cells. Supplemental Fig. S8 shows that knocking down endogenous IRS-1 interfered with Akt phosphorylation at Thr-308 in response to insulin, suggesting that insulin-induced activation of Akt in HEK293 cells depends on IRS-1. Next Fig. 4B shows that long term glucose deprivation induced robust phosphorylation of IRS-1 at Ser-794 and suppressed insulin-stimulated IRS-1-associated PI 3-kinase activity and Akt phosphorylation in the absence of IRS-1 down-regulation. The inverse correlation between IRS-1 phosphorylation at Ser-794 and Akt phosphorylation suggests an inhibitory effect of this phosphorylation on PI 3-kinase signaling.

Next we examined the effect of hypoxia and oxidative stress on IRS-1-associated PI 3-kinase/Akt activity. Long term hypoxia in both L6 myoblasts and HEK293 cells ectopically expressing IRS-1 induced Ser-794 phosphorylation and inhibited serum-induced IRS-1-associated PI 3-kinase activity (Fig. 4C and D, left panel). Similarly hydrogen peroxide significantly suppressed basal as well as insulin-stimulated IRS-1-associated PI 3-kinase activity (Fig. 4E, right panel) and Akt phosphorylation (not shown) in L6 myoblasts, an effect that correlates with Ser-794 phosphorylation (Fig. 4E, left panel). Overall the data presented in Fig. 4 suggest that energy depletion due to glucose deprivation, hypoxia, and oxidative stress suppresses insulin-stimulated IRS-1-associated PI 3-kinase/Akt signaling.

Phosphorylation of IRS-1 at Ser-794 Inhibits PI 3-Kinase/Akt Signaling—To directly address the role of Ser-794 phosphorylation on PI 3-kinase/Akt signaling we engineered HEK293 cells stably expressing equal levels of wild-type IRS-1 or the IRS-1 mutants S794D and S794A. We then cultured these cells in media containing serum and physiological concentration of glucose (5 mm), and we asked whether IRS-1 undergoes phosphorylation at Ser-794 and whether phosphorylation at

**FIGURE 2. IRS-1 phosphorylation at Ser-794 in response to glucose deprivation, hypoxia, and oxidative stress depends on the AMPK catalytic subunit**  
A, HEK293 cells engineered to stably express IRS-1 were transiently transfected with the wild-type or the kinase-deficient form of the AMPK α2 catalytic subunit. Twenty-four hours later, whole cell lysates were analyzed by Western blotting. B, HEK293 cells engineered to stably express IRS-1 were transiently transfected with siRNA against the AMPK α1 or α2 catalytic subunits or both as described under “Experimental Procedures.” Forty-eight hours later, whole cell lysates were analyzed by Western blotting. C, HEK293 cells engineered to stably express IRS-1 were transiently transfected with the wild-type form of the AMPK α2 catalytic subunit. Twenty-four hours later, whole cell lysates were analyzed by Western blotting. D, the same cells shown in C were transfected with siRNA against the AMPK α1 or α2 catalytic subunits or both. Forty-eight hours later, whole cell lysates were analyzed by Western blotting. E, HEK293 cells stably expressing IRS-1 were transiently transfected with siRNA against the AMPK α1 or α2 catalytic subunits or both. Forty-eight hours later, whole cell lysates were analyzed by Western blotting. F, HEK293 cells stably expressing IRS-1 were transiently transfected with siRNA against the AMPK α1 or α2 catalytic subunits or both. Forty-eight hours later, whole cell lysates were analyzed by Western blotting. G, HEK293 cells stably expressing IRS-1 were transfected with the indicated cDNAs. Twenty-four hours later, whole cell lysates were analyzed by Western blotting. GFP, green fluorescent protein; IP, immunoprecipitate.
Energy Depletion and PI 3-Kinase/Akt Signaling

A. Glucose

- pBabe
- pBabe-LKB1 WT
- pBabe-LKB1 KD

| Glucose | + | - | + | - | + | - |
|---------|---|---|---|---|---|---|
| IRS-1 Ser794 |
| IRS-1 |
| AMPK Thr172 |
| AMPK |
| LKB1 |

B. Hypoxia (0.2% O₂)

- pBabe LKB1 WT
- pBabe LKB1 KD

| Hypoxia (0.2% O₂) |
|-------------------|
| + | - | + | - | + | - |
| IRS-1 Ser794 |
| IRS-1 |
| AMPK Thr172 |
| AMPK |
| LKB1 |

C. CCCP (50μM)

- pBabe
- pBabe-LKB1 WT
- pBabe-LKB1 KD

| CCCP (50μM) | - | + | - | + | - | + |
|-------------|---|---|---|---|---|---|
| IRS-1 Ser794 |
| IRS-1 |
| AMPK Thr172 |
| AMPK |
| LKB1 |

D. H₂O₂ (500μM)

- pBabe
- pBabe-LKB1 WT
- pBabe-LKB1 KD

| H₂O₂ (500μM) | - | + | - | + | - | + |
|--------------|---|---|---|---|---|---|
| IRS-1 Ser794 |
| IRS-1 |
| AMPK Thr172 |
| AMPK |
| LKB1 |

E. siLKB1

| siLKB1 | - | + | - | + | - | + |
|--------|---|---|---|---|---|---|
| Long exp. |
| Short exp. |

| IRS-1 Ser794 |
| IRS-1 |
| LKB1 |
| Non specific |

F. STO-609 (1μg/ml)

- pBabe
- H₂O₂ (500μM)

| STO-609 (1μg/ml) | - | + |
|-------------------|---|---|
| IRS-1 Ser794 |
| IRS-1 |
that mTOR phosphorylation at Ser-2448 was impaired in cells expressing the IRS-1 S794D mutant but not in HEK293 cells expressing wild-type IRS-1 or IRS-1 S794A.

Phosphorylation of IRS-1 at Ser-794 in HEK293 Cells Growing under Energetically Unfavorable Conditions Promotes Apoptosis—Inhibition of Akt activation by external signals may result in apoptosis (30–32). We therefore hypothesized that apoptosis induced by energy depletion may depend on the inhibition of Akt via IRS-1 phosphorylation at Ser-794. To address this question, we seeded an equal number of HEK293 cells stably expressing the wild-type or the S794A mutant of IRS-1 and cultured them in media containing serum and either low (1 mM) or high (25 mM) glucose concentrations. After 2 days we measured cell viability with the MTT assay (see "Experimental Procedures"). Fig. 6A shows that, in the presence of glucose, serum deprivation reduced the number of live HEK293 cells expressing wild-type IRS-1 to levels ~5.8-fold lower than the levels of live control cells growing in the presence of glucose (25 mM). Interestingly glucose deprivation reduced the number of live HEK293 cells expressing the S794A IRS-1 mutant to levels only 1.5-fold lower than the levels of live control cells growing in the presence of glucose (25 mM). Therefore, the IRS-1-1-expressing and the IRS-1 S794A-expressing cells were equally sensitive to serum withdrawal (data not shown). Therefore, phosphorylation of IRS-1 at Ser-794 selectively regulates survival following glucose, but not serum, depletion.

To determine whether the decrease in the number of live cells in wild-type IRS-1-expressing HEK293 cells undergoing phosphorylation of IRS-1 at Ser-794 in response to energy depletion is due to apoptosis, we stained the cells with Annexin V, which specifically labels apoptotic cells. Supplemental Fig. S10 shows that HEK293 cells stably expressing wild-type IRS-1 and growing in media containing low concentrations of glucose (1 mM) exhibit a statistically significant increase in Annexin V staining compared with HEK293 cells stably expressing the S794A IRS-1 mutant. Consistently Western blot analysis of lysates of energy-depleted cells revealed that energy depletion induced caspase-3 cleavage only in HEK293 cells expressing wild-type IRS-1 but not the S794A IRS-1 mutant (Fig. 6B). Therefore, the IRS-1 S794A mutant protects HEK293 cells from apoptosis induced by glucose deprivation.

Mitochondrial ΔΨm is often used as an indicator of cellular viability, and its disruption has been implicated in the initiation of the intrinsic pathway of apoptosis that culminates with the cleavage of caspase-3 (33). To measure ΔΨm we used the membrane potential-sensitive cyanine dye Mitoprobe DiIC1₅ (5). DiIC₁₅ (5) localizes in mitochondria in a ΔΨm-dependent manner. The intensity of fluorescence reflects the integrity of mitochondrial function in living cells. Fig. 6C shows that HEK293 cells ectopically expressing the S794A mutant of IRS-1 and grown in the presence of serum and a low (1 mM) concentration

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** LKB1 is selectively required for IRS-1 phosphorylation at Ser-794 upon glucose deprivation, hypoxia, and inhibition of ATP synthesis in the mitochondria. LKB1 knock-out fibroblast reconstituted with the wild-type or the kinase-deficient (KD) form of LKB1 were cultured in serum-free media in the presence or absence of glucose for 8 h (A) or in serum-free, glucose-containing media in a normoxic or hypoxic environment for 16 h (B). C and D, the cells as in A and B were cultured in serum-free media, and they were treated either with CCCP for 30 min (C) or hydrogen peroxide for 15 min (D). Whole cell lysates were analyzed by Western blotting. E, HEK293 cells stably transfected with IRS-1 were treated with LKB1 siRNA. Forty-eight hours later, cells were treated as indicated in the figure. Whole cell lysates were analyzed by Western blotting. F, LKB1 knock-out fibroblast were pretreated with the CaMKKβ inhibitor STO-609 for 1 h. Subsequently they were treated with hydrogen peroxide for an additional 15-min period. Whole cell lysates were analyzed by Western blotting, exp., exposure.
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A. Glucose (mM) - 2 4 6 - 2 4 6
   Insulin - - - + + + +
   IRS-1 Ser794
   IRS-1
   S6K1 Thr389
   Akt Thr308
   Akt
   AMPK Thr172
   AMPK

B. +Glu +Glu
   Insulin - + +
   IRS-1 Ser794
   IRS-1
   Lysate
   TLC

C. Hypoxia (0.2% O2)
   Days 0 1 2
   IRS-1 Ser794
   IRS-1
   S6K1 Thr389
   Akt Thr308
   Akt
   AMPK Thr172
   AMPK α2
   AMPK α1
   tubulin

D. normoxia 0.2% O2
   serum - + +
   IRS-1 Ser794
   IRS-1
   Lysate
   IP: IRS-1

E. Insulin (1μU)
   H2O2 (500μM)
   IRS-1 Ser794
   IRS-1
   TLC
   origin
   p85α
   IgG
   IRS-1
   IP: IRS-1
of glucose exhibited intact mitochondrial membrane potential even after 3 days of treatment. On the contrary, the mitochondrial membrane potential of HEK293 cells expressing wild-type IRS-1 was gradually reduced under similar conditions.

Specifically Fig. 6D shows that HEK293 cells expressing the wild-type IRS-1 and grown for 2 days in the presence of serum and low (1 mM) glucose concentrations exhibited a statistically significant drop both in the mean (1.6 times) and peak (3.1 times) fluorescence intensities compared with HEK293 cells expressing the S794A mutant of IRS-1.

**DISCUSSION**

Data presented in this report show that energy depletion activated AMPK, which bound to and phosphorylated IRS-1 at Ser-794. The phosphorylation of IRS-1 at Ser-794 provides an entry into a novel and previously unsuspected mechanism by which glucose deprivation and hypoxia mediate their physiological effects via regulation of IRS-1-associated PI 3-kinase/Akt signaling. Indeed there has been no evidence to date that glucose availability, oxygen tension, and oxidative stress regulate the phosphorylation of IRS-1 at any site. Although the phosphorylation of IRS-1 at Ser-794 has been reported previously following 5-aminimidazole-4-carboxamide riboside (17) or dexamethasone (18) treatment its biological significance has been a matter of controversy because it has been correlated both with inhibition (18, 19) and activation (17) of PI 3-kinase/Akt signaling by insulin. The studies reported here settled this controversy. By using L6 myoblasts and HEK293 cells engineered to stably express IRS-1 as model systems we demonstrated that energetically unfavorable conditions stimulate IRS-1 phosphorylation at Ser-794. In addition, we showed that the same conditions suppress IRS-1-associated PI 3-kinase activity and Akt phosphorylation in response to insulin (Fig. 4).

**FIGURE 4. Energy depletion and oxidative stress inhibit IRS-1-associated PI 3-kinase/Akt signaling.** A, L6 myoblasts cultured in media containing different concentrations of glucose were serum-starved. Sixteen hours later, the cells were stimulated with insulin for 15 min. Whole cell lysates were analyzed by Western blotting. B, HEK293 cells stably transfected with IRS-1 were maintained in serum-free media in the absence or presence of glucose. Twenty-four hours later, they were stimulated with insulin for an additional 15-min period. IRS-1 was immunoprecipitated, and the PI 3-kinase regulatory subunit p85α bound to IRS-1 was measured by probing the immunoprecipitates with an antibody specific for this subunit. The IRS-1-associated PI 3-kinase activity was measured by TLC (right panel). C, HEK293 cells were transfected with the indicated IRS-1 constructs, serum-starved for 16 h in media containing different glucose concentrations as indicated in the figure, and stimulated with insulin for an additional 15-min period. Western blot of cell lysates was probed with the indicated antibodies. The bottom panel shows the fold induction of Akt phosphorylation at Thr-308 upon insulin stimulation normalized to the total Akt level. This experiment was repeated four times with similar results. The panel shows a representative experiment. D, cumulative data from four independent experiments, including the experiment in C, showing the effects of the IRS-1 Ser-794 mutation on the fold induction of Akt phosphorylation at Thr-308 upon insulin stimulation of serum-starved cells growing in media containing 4 mM glucose. Fold induction was normalized to the total Akt levels (error bars indicate mean values ± S.E.). The asterisk indicates a p value of <0.005. IP, immunoprecipitate.
By using IRS-1 mutants at Ser-794, we demonstrated that the S794D mutant inhibits insulin-stimulated Akt phosphorylation, whereas the IRS-1 S794A mutant does not (Fig. 5). Moreover expression of IRS-1 mutant S794A in HEK293 cells prevented the loss of mitochondrial membrane potential and suppressed apoptosis following energy depletion (Fig. 6). Based on these findings we conclude that IRS-1 phosphorylation at Ser-794 upon energy depletion inhibits PI 3-kinase/Akt signaling and induces apoptosis (Fig. 7).

Previous studies have suggested that Akt requires glucose to exert its antia apoptotic function via regulation of the mitochondrial hexokinase (30–32), the enzyme that controls the first step in glycolysis. The data presented here suggest that another reason for the glucose dependence of Akt survival signals may be the glucose-mediated suppression of IRS-1 phosphorylation at Ser-794, which is required for the activation of Akt by hormonal and growth factor signals.

Another interesting finding of this study is that phosphorylation of IRS-1 at Ser-794 by AMPK depended on the α1 or the α2 isoforms of the AMPK catalytic subunit in a stimulus-specific manner. The α2 subunit, which is the main subunit expressed in metabolically relevant tissues, was preferentially used to stimulate phosphorylation of IRS-1 at Ser-794 following glucose deprivation and hypoxia, whereas the α1 subunit exclusively mediated the effect of oxidative stress. The signal specificity of AMPK isoform utilization appears to reflect the specificity by which various energy depletion signals regulate the upstream kinases involved in AMPK activation. Thus, in accordance with earlier studies showing that the LKB1/AMPK

![FIGURE 6. IRS-1 S794A enhances the survival of energy-depleted HEK293 cells. A, 10⁴ HEK293 cells stably expressing wild-type IRS-1 or the IRS-1 mutant S794A were seeded in 24-well plates, and they were cultured in media containing FBS and low (1 mM) or high (25 mM) glucose concentrations as indicated. Forty-eight hours later, live cells were measured with the MTT assay (see "Experimental Procedures"). This was repeated in three independent experiments, all done in quadruplicate. The bar graphs are based on the combined data from all three experiments, and they show percent changes in the number of live cells (mean values ± S.E.). The asterisk indicates a p value of <0.039. B, HEK293 cells stably expressing wild-type IRS-1 or the IRS-1 mutant S794A were cultured in media supplemented with serum in the presence or absence of glucose as indicated for 36 h. Whole cell lysates were analyzed by Western blotting. C, HEK293 cells stably expressing wild-type IRS-1 or the IRS-1 mutant S794A were cultured in media containing FBS and high (25 mM) or low (1 mM) glucose concentrations as indicated. Mitochondrial membrane potential was measured at the indicated times as described under "Experimental Procedures." A representative experiment is shown. D, cumulative data from four independent experiments, including the one in C, showing the percent changes of the mean (left) and peak (right) fluorescence intensities (mitochondrial membrane potential) (error bars indicate mean values ± S.E.) after 2 days of treatment. The asterisk indicates a p value of <0.037 for the mean and <0.012 for the peak fluorescence intensities.

![FIGURE 7. Energy depletion inhibits survival via AMPK-dependent phosphorylation of IRS-1 at Ser-794. AMPK is activated via AMP binding and upstream kinase signals. AMPK activated by energy depletion signals phosphorylates IRS-1 at Ser-794. Phosphorylation of IRS-1 at this site inhibits the activity of PI 3-kinase and Akt and promotes apoptosis.](https://example.com/fig7.png)
α2 axis plays a prominent role in the regulation of metabolic homeostatic mechanisms (5, 34–42), our study showed that the phosphorylation of IRS-1 at Ser-794 in glucose- or oxygen-deprived cells depends on the LKB1/AMPK α2 axis. On the other hand, oxidative stress and 2-DG, which induced IRS-1 phosphorylation at Ser-794 via an α1 catalytic subunit-dependent manner, depend on a kinase other than LKB1. Because the phosphorylation of IRS-1 at Ser-794 in LKB1−/− fibroblasts treated with hydrogen peroxide was inhibited by STO-609, we conclude that the upstream kinase activated by these treatments is CaMKKβ (8, 9, 11).

Oxidative stress, induced by reactive oxygen species (ROS), is causally involved in mitochondrial dysfunction and aging as well as insulin resistance (43). ROS-induced insulin resistance has been attributed to the inhibitory effects of ROS on the activation of Akt by insulin (43). However, the molecular mechanism for this effect remains unknown. Our data show that phosphorylation of IRS-1 at Ser-794 was sensitive to oxidative stress and provide a molecular basis for the previously described inhibitory effect of oxidative stress on Akt activity in vitro and in vivo (43). Moreover our data show that this phosphorylation was preferentially mediated by the AMPK α1 catalytic subunit, suggesting a possible role for this isoform of AMPK in the pathogenesis of oxidative stress-induced insulin resistance.

ROS can be induced by numerous physiological and stress stimuli, including those produced by growth factors, cytokines, glucocorticoids, and phorbol esters. In the context of these stimuli, ROS contribute to signal transduction by functioning as second messenger molecules. ROS produced by any of these stimuli should be expected to promote the phosphorylation of IRS-1 at Ser-794. Indeed insulin, platelet-derived growth factor, stimuli should be expected to promote the phosphorylation of second messenger molecules. ROS produced by any of these glucocorticoids, and phorbol esters. In the context of these resistance.

The preceding studies focused on various immortalized but not transformed cell lines to show that energy depletion induced by glucose or oxygen deprivation and other insults leads to IRS-1 phosphorylation at Ser-794, which in turn inhibits Akt and cell survival. Cancer cells defy the homeostatic limits of normal cells and grow even in the presence of insufficient nutrient and oxygen supplies because they are specifically selected to survive these insults. It is therefore logical to expect that cancer cells may accumulate mutations that either prevent phosphorylation of IRS-1 at Ser-794 in response to such insults or suppress the downstream effects of this phosphorylation event once it takes place. In agreement with this prediction, loss of functional LKB1 has been observed in various neoplasms including lung and breast cancer (7, 44). Moreover germ line mutations of LKB1 have been linked to the development of a dominantly inherited cancer predisposition syndrome, the Peutz-Jeghers syndrome (7, 44). Finally consistent with our data previous studies have shown that long term pharmacological activation of AMPK suppresses proliferation of cancer cells by suppressing Akt activity (45–47). Deciphering the molecular circuitry that couples cellular energy status with cell growth and proliferation may provide new insights into the biology of cancer cells and into strategies for the translation of these insights into novel therapeutics.

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