Activation of a Novel Calcineurin-mediated Insulin-like Growth Factor-1 Receptor Pathway, Altered Metabolism, and Tumor Cell Invasion in Cells Subjected to Mitochondrial Respiratory Stress*

Manti Guha, Satish Srinivasan, Gopa Biswas, and Narayan G. Avadhani1

From the Department of Animal Biology and the Mari Lowe Center for Comparative Oncology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

We have previously shown that disruption of mitochondrial membrane potential by depletion of mitochondrial DNA (mtDNA) or treatment with a mitochondrial ionophore, carbonyl cyanide m-chlorophenylhydrazone, initiates a stress signaling, which causes resistance to apoptosis, and induces invasive behavior in C2C12 myocytes and A549 cells. In the present study we show that calcineurin (Cn), activated as part of this stress signaling, plays an important role in increased glucose uptake and glycolysis. Here we report that, although both insulin and insulin-like growth factor-1 receptor levels (IR and IGF1R, respectively) are increased in response to mitochondrial stress, autophosphorylation of IGF1R was selectively increased suggesting a shift in receptor pathways. Using an approach with FK506, an inhibitor of Cn, and mRNA silencing by small interference RNA we show that mitochondrial stress-activated Cn is critical for increased GLUT 4 and IGF1R expression and activation. The importance of the IGF1R pathway in cell survival under mitochondrial stress is demonstrated by increased apoptosis either by IGF1R mRNA silencing or by treatment with IGF1R inhibitors (AG1024 and picropodophyllin). This study describes a novel mechanism of mitochondrial stress-induced metabolic shift involving Cn with implications in resistance to apoptosis and tumor proliferation.

Mitochondria play important roles in energy production, cell metabolism, and both integration and execution of apoptotic signals. Dysfunctional mitochondria in mammalian cells trigger a stress signaling (1–3) analogous to the mitochondrial retrograde signaling pathway described in ρ0 yeast cells (4). A distinct difference between the yeast and mammalian cell systems is the involvement of RTG signaling pathway in the former, as against a retrograde Ca2+ signaling cascade in the latter (5). In both C2C12 myocytes, and A549 lung carcinoma cells, disruption of mitochondrial membrane potential (ΔΨm) either due to mitochondrial genetic stress (partial depletion of mtDNA) or treatment with mitochondrial ionophore, CCCP,2 caused a sustained increase in cytosolic free Ca2+ and activation of Ca2+-sensitive factors, calcineurin (Cn), NFAT, C/EBPβ (1, 2, 6), and a novel IκBβ-dependent NFκB pathway (7). In 143B osteosarcoma and other cells, mitochondrial stress resulting from mtDNA depletion or protein misfolding caused activation of CREB, and a C/EBP family factor CHOPS, respectively (8, 9). Indeed these same transcription factors have been implicated in the regulation of a number of nuclear marker genes that respond to mitochondrial respiratory stress signaling in different cells (10, 11).

Currently there is increasing evidence that mtDNA content directly reflects on the metabolic state of cells (11–13). Some of the ρ0 cells, lacking mtDNA, exhibit slow growth and higher sensitivity to apoptotic stimuli (14, 15). In contrast, mtDNA depletion induced cell proliferation and resistance to apoptosis in C2C12 and A549 cells (1, 6), 143B osteosarcoma cells (16), and SK-Hep1 cells (17). Similarly, a cybrid cell line carrying mutations in mtDNA-encoded ATPase6 gene exhibited increased tumorigenicity and reduced apoptosis (18, 19), indicating a role for mtDNA mutations in tumor progression. An increasing number of recent studies shows that proliferating tumors in both human patients and animal models contain mutated or deleted mtDNA and/or dysfunctional mitochondria (19–21). In mtDNA-depleted C2C12 and A549 cells, increased resistance to apoptosis was associated with higher expression of Bcl2 and/or increased phosphorylation of Bid, Bad, and Bax (22). Rapidly growing tumors meet their metabolic demand by increased expression of genes encoding glucose transporters and glycolytic enzymes (23). In the present study we investigated the mechanism of cell proliferation and induced glycolysis in C2C12 cells subjected to mitochondrial respiratory stress. Our results show that selective inhibition of IR autophosphorylation and Cn-dependent activation of the IGF1R pathway is the basis for increased glucose utilization and

---

1 To whom correspondence should be addressed: Dept. of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St., Philadelphia, PA 19104. Tel.: 215-898-8819; Fax: 215-573-6651; E-mail: narayan@vet.upenn.edu.

2 The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; mtDNA, mitochondrial DNA; Cn, calcineurin; IR, insulin receptor; IGF1R, insulin-like growth factor-1 receptor; GLUT4, glucose transporter 4; PPP, picropodophyllin; 2-DOG, 2-deoxyglucose; PBS, phosphate-buffered saline; FBS, fetal bovine serum; CREB, cAMP-response element-binding protein; C/EBP, CAAT/enhancer-binding protein; sIRNA, small interference RNA; AMPK, AMP kinase; CnAc, calcineurin AAc; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside.
cell proliferation. Interestingly, mitochondrial stress-induced metabolic change appears to be an important survival factor in these cells, because blocking the IGF1R function caused increased cell death. Results also show that elevated GLUT 4 and IGF1R levels are directly related to increased Cn activity, which is an essential component of the mitochondria-to-nucleus stress signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Murine C2C12 skeletal myoblasts (ATCC CRL1772), human pulmonary carcinoma A549 cells (ATTC CRL 185), mouse fibroblasts NIH 3T3 (ATCC CRL-1658), and rat H9C2 cardiac myocytes (ATCC CRL 1446) were grown in Dulbecco’s modified Eagle’s medium (Life Technology Inc.) supplemented with 10% FBS and 0.1% gentamycin. In some specified experiments charcoal-treated FBS was used. Depletion of mtDNA was carried out by EtBr treatment (100 ng/ml, for 30 passages) as described before (2). Selected clones containing ~20% mtDNA contents were grown in presence of 1 mM sodium pyruvate and 50 µM uridine. Reverted cells represent mtDNA-depleted cells (with 85% DNA depleted) grown for 30 cycles in the absence of EtBr until the mtDNA content reached 80% of control cells. The mtDNA contents were estimated by Southern hybridization using a DNA probe from cytochrome oxidase I

Calcineurin Assay, IGF1R, and IR mRNA Knockdown by siRNA—Pre-designed siRNAs for mouse CnAα (ID #292199), IGF1R (ID #159115), and IR (ID #67808) and negative controls (scrambled siRNA) were purchased from Ambion Inc. (Austin, TX). Control and mtDNA-depleted cells (106 cells/well) were transfected with pre-annealed double-stranded siRNAs at a final concentration of 30 nM by the method of reverse transfection. Transient transfections were carried out in triplicate using siPORT NeoFX reagent (Ambion Inc.). RNA was isolated 48 hr after transfections using TRIZol reagent (Invitrogen), and the level of silencing of CnAα, IGF1R, and IR mRNA was quantitated by real-time PCR as described later in this section.

Transfection with IGF1R cDNA—mtDNA-depleted cells were transfected with human IGF1R cDNA, kindly provided by Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA) (24) using FuGENE 6 by a standard method described before (1).

Uptake of 2-Deoxyglucose—Glucose uptake was measured as described previously (25). Briefly, 106 cells grown in 6-well plates were serum-starved for 6 hr, and incubated in the presence or absence of indicated levels of insulin for 30 min in glucose-free medium. 1 µCi of 2-[3H]DOG (1 mCi/0.1 mmol, American Radiolabeled Chemicals, St. Louis, MO) was added to each well containing 2 ml of medium and incubated for an additional 15 min. The transport was terminated by washing cells rapidly three times with ice-cold PBS and lysed in 1 N NaOH. Aliquots of the lysates were counted in liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Measurement of Cellular ATP Levels—Total cellular ATP levels were determined by bioluminescence using ATP-bioluminescent somatic cell assay kit (Sigma) as described before (6). Briefly, 106 cells were lysed using the “somatic cell releasing reagent,” which caused the release of cellular ATP by altering membrane permeability. ATP-dependent formation of light was measured in a Chameleon Multilabel Microplate Reader (Bioscan Inc., Washington, D. C.) with appropriate ATP standards. In these experiments, cells were treated with 25 µM CCCP and/or 25 µM oligomycin for 1 h.

Measurement of Hexokinase, Phosphofructokinase, and Pyruvate Kinase Activities—Hexokinase assay was carried out as described by Miccoli et al. (26). Glucose-6-phosphate produced in the hexokinase reaction was coupled to glucose-6-phosphate dehydrogenase. The reaction was started by the addition of 50 µg of the cell lysate, and the rate of NADPH production was measured by the increase in absorbance at 340 nm for 20 min in a Cary Varian spectrophotometer. Phosphofructokinase activity was measured spectrophotometrically by coupling the enzyme with aldolase, triosephosphate isomerase, and glycer-aldehyde-3-phosphate dehydrogenase as described before (27). The reaction was started by the addition of 50 µg of the cell lysate, and the rate of NADH metabolized over a time of 20 min was measured by the decrease in absorbance at 340 nm. Pyruvate kinase reaction was coupled to lactate dehydrogenase, and the activity was measured by the oxidation of NADH at 340 nm for 20 min as described before (28). The reaction was started by the addition of 0.15 mM phosphoenolpyruvate and 50 µg of the cell lysate.

Calcineurin Activity Assay—Calcineurin activity was assayed according to Antoni et al. (29) with the following modifications. Total serine/threonine phosphatase activity was first determined by incubating cell lysate (2 µg) from untreated, AICAR (2 mM, 2 hr)-treated, and FK506 (10 nM, 30 min)-treated cells with threonine phosphopeptide (Upstate, Cat. no. 12-219) in a final volume of 25 µl for 20 min at 30 °C in 50 mM Tris-Cl, pH 7.0, 0.1 mM CaCl2. The phosphate released was quantitated by incubating with 100 µl of malachite green and measuring the absorbance at 660 nm after 15 min. In parallel, control incubations containing phosphate inhibitor mix I (NaF, 5 mM; okadaic acid, 500 nM; orthovanadate, 100 µM; FK506, 1 nM; calyculin, 0.1 µM; and cyclosporine A, 100 µM) and phosphate inhibitors mix II (FK506, 1 nM; calyculin, 0.1 µM; and cyclosporine A, 100 µM) were also carried out. Nonspecific phosphatase activity under these conditions was subtracted from total serine/threonine phosphatase activity referred to as corrected activity. Calcineurin activity was calculated by subtracting the activity obtained from incubation with phosphate inhibitor mix II from the corrected activity.

Immunoprecipitation and Receptor Autophosphorylation—Plasma membrane fractions from control and mtDNA-depleted C2C12 cells were isolated as described (30). Autophosphorylation of the IR and IGF1R in the plasma membrane fractions was carried out at 37 °C in the presence of 10 mM MnCl2, [γ-32P]ATP (0.2 mM and 2–15 cpm/fmol), 0.1 mM insulin for 30 min. The reaction was terminated by adding 15 mM EDTA. The autophosphorylated receptors (100 µg of plasma membrane protein) were immunoprecipitated overnight at 4 °C with the IR or IGF1Rβ antibody (2 µg/ml). The immune complexes were collected on Protein A-agarose beads (Sigma) and washed extensively with PBS. The immunoprecipitates were
Mitochondrial Stress-induced IGF1R Activation

extracted from the beads with 2X Laemmli buffer devoid of β-mercaptoethanol at 95 °C for 5 min for further analysis.

Immunoblot Analysis—The protein contents were estimated by the Lowry method (31). Cytosolic, nuclear, and plasma membrane proteins (30 μg) solubilized in Laemmli sample buffer were resolved by electrophoresis on 10 or 12% SDS-polyacrylamide gels (32) and subjected to immunoblot analysis. The immunoblots were developed using SuperSignal West Femto maximum sensitivity substrate (Pierce). In each case, blots presented here are a representative of two to three independent experiments.

Plasma Membrane Lawn Assay—The level of plasma membrane GLUT 4 was determined using the plasma membrane lawn assay as described earlier (33). Cells grown on poly-L-lysine-coated coverslips were treated with or without insulin (100 nM for 30 min). The coverslips were rinsed in PBS and subjected to hypotonic shock with three washes (10 s each) in membrane buffer (70 mM KCl, 5 mM MgCl2, 3 mM EGTA, 1 mM dithiothreitol, 30 mM HEPES, pH 7.2). The coverslips were submerged in membrane buffer and sonicated using a Probe sonicator, to generate a lawn of plasma membrane fragments that remained attached to the coverslip. The fragments were then immunolabeled with anti-GLUT 4 polyclonal antibody (1:100, U.S. Biologicals, Swampscott, MA) and Alexa 488 goat anti-rabbit secondary antibody (Molecular Probes, 1:300). The membrane fragments were viewed through an immunofluorescence microscope. At least six fields were examined within any one experiment for each condition.

mRNA Quantitation by Real-time PCR—Total RNA was isolated using TRIzol reagent as per the manufacturer’s protocol (Invitrogen). cDNA was generated from 5 μg of RNA using the cDNA Archive kit from Applied Biosystems (Foster City, CA), and 50 ng of this cDNA was used as template per reaction. Relative quantification of GLUT 4, IGF1R, and IR mRNA by real-time PCR was done using a TaqMan assay probe (Applied Biosystems), and CnAα mRNA was quantified using SYBR Green (Applied Biosystems) in an ABI PRISM 7300 sequence detection system (Applied Biosystems). Data were normalized to 18 S RNA (for TaqMan assay) and β-actin contents (for SYBR Green) as endogenous controls.

Annexin V Assay—The assay for cells undergoing apoptosis was performed using a Nexin Kit (Guava Technologies, Hayward, CA) according to the manufacturer’s suggested protocol. Cells (106 each) treated without or with inhibitors (Genistein, 0.1 mg/ml; AG1024, 10 μM; and PPP, 2.5 μM) for 2 h were washed with 1 ml of 1X Nexin Buffer (Guava Technologies) and resuspended in the same buffer. After labeling with Annexin V-PE, the percentage of apoptotic cells was quantified using the Personal Cytometer (Guava Technologies). In the case of the siRNA experiments, cells were harvested 48 h after the siRNA transfections and counted as described above.

Matrigel Invasion Assay—The in vitro invasion assays were carried out as described previously (34). The Matrigel invasion chambers were prepared at 1:2 dilution of Matrigel (BD Biosciences, Belford, MA) as described before (1). Equal numbers of viable cells (4 × 105) were seeded on top of the Matrigel layer. After incubation for 24 h at 37 °C, non-invading cells in the Matrigel layer were quantitatively removed, and the micro-porous membrane containing invaded cells was stained and viewed under a Olympus BX 61 bright field microscope as described before (22). At least six fields were examined within any one experiment for each condition.

Statistical Analysis—Data on enzyme activity, glucose uptake, mRNA quantitation, and immunoblot analysis have been presented as means ± S.D. of three to five independent measurements. Differences between paired variables were determined using two-way analysis of variance. p values <0.05 were considered statistically significant, and p values <0.01 were highly significant.

RESULTS

Increased Glucose Uptake by mtDNA-depleted Cells—Initially, we evaluated glucose uptake by control and mtDNA-depleted C2C12 cells containing 20% of control cell mtDNA content, and reverted cells with mtDNA content restored to ~80% of control cells (Fig. 1A, left panel). Fig. 1A (right panel) shows...
Mitochondrial Stress-induced IGF1R Activation

Fig. 2. Increased GLUT 4 protein and mRNA levels in mtDNA-depleted C2C12 cells. A, C2C12 cells were treated with AG1024 (10 μM, 2 h), CCCP (25 μM, 2 h), or FK506 (10 nM, 2 h) as indicated. Plasma membrane fractions (30 μg each) were subjected to immunoblot analysis, and the blots were co-developed with antibody to GLUT 4 (1:1000 dilution) and Na+/K+-ATPase (1:1000 dilution). The latter was used as a loading control. B, RT-PCR analysis of total RNA (50 ng each of RT product) from control, mtDNA-depleted, and reverted C2C12 cells. C, lawn assay for GLUT 4 contents of plasma membrane fractions from control and mtDNA-depleted cells before or after treatment with insulin (100 nM, 30 min). Details of immunoblot analysis, real-time PCR, and the lawn assay are as described under "Experimental Procedures." *p ≤ 0.05; **p ≤ 0.001. C, real-time PCR analysis of total RNA (50 ng each of RT product) from C2C12 cells transfected with 2 μg of IGF1R cDNA for 48 h, and total RNA was isolated. Real-time PCR analysis of GLUT 4 mRNA was done using a TaqMan probe as described under "Experimental Procedures." *p ≤ 0.05; **p ≤ 0.001. D, lawn assay for GLUT 4 contents of plasma membrane fractions from control and mtDNA-depleted cells before or after treatment with insulin (100 nM, 30 min). Details of immunoblot analysis, real-time PCR, and the lawn assay are as described under "Experimental Procedures."
Mitochondrial Stress-induced IGF1R Activation

mtDNA-depleted cells as compared with control cells. Furthermore, insulin treatment increased the plasma membrane contents of GLUT 4 in both cell types. These results are consistent with GLUT4 mRNA and protein levels in mtDNA-depleted cells.

Increased Expression of GLUT 4 and IGF1R mRNAs and Activation of IGF1R in mtDNA-depleted Cells—IR and IGF1R both activate GLUT 4 and glucose uptake in C2C12 cells. To gain insight into the mechanism of increased glucose uptake and induced expression of GLUT 4 in mtDNA-depleted cells, we studied the effects of specific inhibitors of receptor pathways that regulate glucose uptake. Fig. 3A shows that both wortmannin and LY294002, general inhibitors of the phosphatidylinositol 3-kinase pathway (38, 39), inhibited glucose uptake both in control and mtDNA-depleted cells by ~70–85%. Genistein, a known inhibitor of IR (40) inhibited glucose uptake by ~30% in control cells and by 15% in mtDNA-depleted cells. AG1024, which inhibits IGF1R more preferentially at the concentration used (10 μM), had no significant effect on glucose uptake in control cells, whereas it inhibited glucose uptake by nearly 85% in mtDNA-depleted cells (Fig. 3A). PPP, a specific inhibitor of IGF1R (36, 37), is known to specifically inhibit the phosphorylation of Tyr(1136), which is critical for the activation of IGF1 receptor, but has no effect on IR activation (41). PPP inhibited glucose uptake to ~46% in control cells and 88% in mtDNA-depleted cells. The differential effects of these inhibitors suggest that insulin receptor plays a more prominent role in glucose uptake in control cells, whereas IGF1R plays a more prominent role in mtDNA-depleted cells.

We then evaluated IR and IGF1R levels and their autophosphorylation in these cells. The immunoblot in Fig. 3B shows that the IR level was increased by ~1.5-fold in mtDNA-depleted cells, which was back to near control cell level in reverted cells. Treatment with FK506 had no significant effect on IR protein level in control and reverted cells, whereas it inhibited the level by ~50% in mtDNA-depleted cells. The autoradiogram in Fig. 3C shows the level of autophosphorylation of IR in control, depleted, and reverted cells. Results show high levels of autophosphorylation in control and reverted cells but a markedly lower level in mtDNA-depleted cells. These levels were not significantly affected by FK506, suggesting marked differences in the level of IR activation in control and mtDNA-depleted cells.

The immunoblot in Fig. 3D shows that the IGF1R level in mtDNA-depleted cells is ~4.5-fold higher than in control cells. Reverted cells, on the other hand, contained a IGF1R level closer to the control cells. CCCP treatment in control cells induced the IGF1R level by ~3-fold. The level of IGF1R protein in mtDNA-depleted cells was reduced by treatment with FK506 suggesting a possible role of Cn in increased expression or translocation of receptor protein. The autoradiogram in Fig. 3E shows that the receptor is autophosphorylated at low levels in control and reverted cells, which is not significantly affected by FK506. In mtDNA-depleted cells, however, the level of autophosphorylation was markedly higher (~6-fold) and was sensitive to FK506. Consistent with the IGF1R protein levels in different cell types, real-time PCR data in Fig. 3F shows that the IGF1R mRNA level was increased 7-fold in mtDNA-depleted cells, and the level returned to near control cell level in reverted cells. The results collectively suggest that increased glucose uptake in mtDNA-depleted cells was probably due to increased expression and/or increased autophosphorylation of IGF1R. Measurement of IGF1 in culture medium showed a
small increase in IGF1 level in mtDNA-depleted cells (∼75 ng/ml) compared with control cells (∼50 ng/ml). To evaluate if the marked difference in the receptor autophosphorylation was due to extracellular or intracellular factors, we cultured the cells in medium containing charcoal pre-adsorbed FBS. As shown in Fig. 3G, the IGF1R autophosphorylation was ∼3-fold higher in mtDNA-depleted cells and only marginally higher in control cells grown in charcoal-treated FBS. Similarly, the level of IR autophosphorylation was reduced marginally in mtDNA-depleted cells in comparison to control cells.

Possible Role of AMPK in Increased Glucose Uptake in mtDNA-depleted Cells—AMP-activated protein kinase is known to play an important role in insulin signaling, glucose uptake, and energy homeostasis (42). Recently AMPK has also been implicated to be involved in PGC1α-mediated mitochondrial biogenesis (43). For these reasons we assessed the role of AMPK in the respiratory stress-mediated activation of Cn and glucose uptake in mtDNA-depleted C2C12 cells. We evaluated the effects of AICAR, a well known activator of AMPK (42), and FK506, an inhibitor of Cn, on the calcineurin activity in cell extracts. As shown in Fig. 4A, AICAR had no effect on the Cn activity in mtDNA-depleted cells, whereas FK506 inhibited the activity. As seen from Fig. 4B, AICAR marginally increased glucose uptake by mtDNA-depleted cells. Similarly, AICAR was able to partially restore FK506-mediated inhibition of glucose uptake. These results suggest that, although AMPK is activated in response to respiratory stress, it does not have a role in increased Cn activity in mtDNA-depleted cells. Additionally, the contribution of AMPK to increased glucose uptake in mtDNA-depleted cells appears to be only marginal. These results also suggest that the two pathways may not be dependent on each other.

Activation of IGF1R and Glucose Uptake Is a General Phenomenon in Cells Subjected to Mitochondrial Respiratory Stress—The generality of increased glucose uptake and elevated levels of GLUT 4 and IGF1R mRNA observed in mtDNA-depleted C2C12 cells was investigated using three other cell lines. For this purpose we chose mtDNA-depleted A549 cells (6) and H9C2 cardiomyocytes, both of which are transformed cells and also non-transformed 3T3 cells, all of which express GLUT 4 and are insulin-responsive. In the latter two cell types, CCCP treatment was used to induce mitochondrial stress. Fig. 5A shows that in all three cell lines mitochondrial stress either due to mtDNA depletion or CCCP treatment caused 2- to 3-fold increased 2-DOG uptake. As shown for mtDNA-depleted C2C12 cells, 2-DOG uptake in H9C2 cells was inhibited by FK506 (Fig. 5A). Although not shown, a similar pattern of inhibition was seen in 3T3 and A549 cells. Immunoblots in Fig. 5B show that IGF1R protein levels were 2- to 5-fold higher in all three cells either in response to mtDNA depletion or CCCP treatment. Similarly, GLUT 4 protein levels (Fig. 5C) were increased 2.0- to 2.5-fold in A549 and 3T3 cells in response to added CCCP. Consistent with increased protein levels, Fig. 5D shows that GLUT 4 and IGF1R mRNA levels were increased by ∼2.5- to 3.5-fold in H9C2 cells in response to CCCP treatment. The CCCP-mediated increase of both GLUT 4 and IGF1R mRNAs was sensitive to FK506 confirming the role of Cn. These results collectively show that activation of IGF1R, increased GLUT 4, and glucose uptake are general features of mitochondrial stress response in many cell types.

Distinctive Patterns of Metabolic Shifts in Response to Mitochondrial Stress—With a view to understanding the significance of IGF1R activation and increased glucose uptake, we...
assayed the levels of the three irreversible and regulated steps of glycolysis. Table 1 shows that the hexokinase, phosphofructokinase, and pyruvate kinase activities were increased 2.5- to 4.5-fold in CCCP-treated cells, except that the unit increase of PFK in mtDNA-depleted C2C12 and A549 cells was only marginal. The activities of these enzymes were also increased 2.5- to 4.5-fold in mtDNA-depleted C2C12 and A549 cells was only marginal. The activities of these enzymes were also increased 2.5- to 4.5-fold in CCCP-treated cells. The level of increase in CCCP-treated H9C2 cells was significantly lower. In particular, the PK activity was marginally reduced, which is not statistically significant. Although the reason for this difference remains unknown, it may be related to cell-specific differences in the inducibility of glycolytic pathways. Consistent with induced glycolytic pathways, results in Table 2 show that the levels of oligomycin-insensitive ATP synthesis is increased nearly 2-fold in cells subjected to mitochondrial genetic (mtDNA-depleted) and metabolic (CCCP-treated) stress. These results collectively show that mitochondrial respiratory stress induces a shift in cellular metabolic pattern.

Effects of Calcineurin Aa mRNA Silencing on GLUT 4, IGF1R, and IR mRNA Levels—To confirm the role of increased Cn activity on increased steady-state GLUT 4 and IGF1R mRNA levels in cells subjected to mitochondrial stress, we used an siRNA approach to knock down CnAa mRNA in C2C12 cells. Fig. 6A shows that transient expression of siRNA reduced CnAa mRNA levels by ~60% in control cells and nearly 80% in mtDNA-depleted cells compared with mock transfections. Results in Fig. 6 (B and C) show that Cn mRNA silencing in control cells did not have a significant effect on either IGF1R or GLUT 4 mRNA levels. However, in mtDNA-depleted cells, Cn mRNA silencing had a profound effect, because both IGF1R and GLUT 4 mRNA levels were reduced by 75-85%. Further, Fig. 6D shows that Cn mRNA silencing had only a marginal effect on IR mRNA levels. These results provide confirmatory evidence that Cn and its target factors are involved in the regulation of IGF1R and GLUT 4 genes. Results also suggest that, in control cells, basal levels of expression of these two genes are not regulated by Cn.
IGF1R Inactivation Induces Apoptosis and Inhibits Invasive Property of mtDNA-depleted C2C12 Cells—Previously we showed that mitochondrial stress in otherwise non-tumorigenic C2C12 cells renders them highly invasive (2, 6). Here we ascertained the role of IGF1R activation on increased glucose transport and invasive property of cells by mRNA silencing. Fig. 7A shows that transfection with siRNA constructs reduced IR and IGF1R mRNA contents in mtDNA-depleted C2C12 cells by 80 and 60%, respectively, of mock transfected cells (p < 0.001). As shown in Fig. 7B, in control cells IR mRNA silencing reduced 2-DOG uptake by ~50%, but IGF1R mRNA silencing had only a marginal effect. In mtDNA-depleted cells, IR mRNA silencing reduced 2-DOG uptake only marginally (~20%), whereas IGF1R mRNA silencing was more effective by inhibiting 2-DOG uptake by ~60% (p < 0.001). This level of inhibition was consistent with the level of mRNA silencing (see Fig. 7A). As seen from Fig. 7C, both IR and IGF1R mRNA silencing in control cells marginally affected GLUT 4 mRNA levels. In mtDNA-depleted cells, IR mRNA silencing reduced GLUT 4 mRNA levels by ~20%. IGF1R mRNA silencing in mtDNA-depleted cells, however, had a profound effect in that GLUT 4 mRNA levels were reduced by ~75%. These results show that IGF1R activation as part of mitochondrial respiratory stress plays a direct role in increased GLUT 4 mRNA and increased glucose uptake.

Because IGF1R activation is associated with tumorigenic property of cells, we tested the effects of mRNA silencing on in vitro invasive property and rate of apoptosis. Fig. 7D shows that IR mRNA silencing had no significant effect on the ability of mtDNA-depleted cells to invade through the Matrigel membrane, whereas IGF1R mRNA silencing reduced the number of invading cells by ~85%. An opposite effect was observed on the propensity of cells to undergo apoptosis. The number of cells undergoing apoptosis was measured using the Nexin assay, which utilizes annexin V-PE to detect phosphatidylserine on the plasma membrane signifying apoptotic cells. The Nexin assay in Fig. 7E showed only a marginal increase in apoptosis by IR mRNA silencing, whereas IGF1R mRNA silencing caused a vastly increased 50% apoptosis.

We also assessed the effects of known inhibitors of IGF1R on the invasive property and rate of apoptosis on control, mtDNA-depleted, and reverted C2C12 cells. The cell distribution pattern in Fig. 8A and the quantitation in Fig. 8B show that control, depleted, and reverted cells contained only 8–10% apoptotic cells. Treatment with AG1024 and PPP, specific inhibitors of IGF1R, however induced apoptosis in mtDNA-depleted cells to ~28% and 40%, respectively (p < 0.001), whereas these agents had no significant effect on control cells. In reverted cells the
Mitochondrial Stress-induced IGF1R Activation

A

Untreated + Genistein + AG1024 + PPP

Cont Dep Cont + PPP

B

Control Depleted Reverted

% Apoptotic

C

Cont Cont + PPP

FIGURE 8. Increased sensitivity of mtDNA-depleted C2C12 cells to AG1024- and PPP-mediated apoptosis. Treatment of cells with various inhibitors was as described under “Experimental Procedures” and in Fig. 3A. A, cells undergoing early apoptosis were detected by using a Guava Nexin assay kit for cell labeling. B, results from four independent assays as in A were used for calculating average ± S.D. *, increased cell death (p ≤ 0.05); **, p ≤ 0.001. C, Matrigel invasion assay with control and mtDNA-depleted C2C12 cells treated with or without PPP (2.5 μM, 2 h).

number of cells undergoing apoptosis in response to added AG1024 and PPP increased to 15–17% (p < 0.05), further supporting the possible role of mitochondrial stress signaling in altered metabolic state of cells. Genistein had only marginal effects on all three cell types. These results therefore indicate that increased glucose utilization induced by mitochondrial stress functions as a survival factor in mtDNA-depleted cells.

Fig. 8C shows the patterns of in vitro invasion in a Matrigel invasion assay system. It is seen that PPP, a specific inhibitor of IGF1R had no significant effect on the rate of invasion of control cells. As reported before, mtDNA-depleted cells showed vastly increased invasion, which was effectively inhibited by PPP. These results are consistent with the view that activation of the IGF1R pathway as part of mitochondrial stress signaling provides survival advantage to mtDNA-depleted cells as they become more resistant to apoptosis and gain the invasive ability.

DISCUSSION

The IR and IGF1R family of receptors bears both structural and functional similarities (44), and IGF1R can functionally replace IR in inducing glucose uptake and utilization in many cell types. Although IGF1 is the preferred ligand, insulin also binds to and activates IGF1R, at a lower efficiency than IR (45). Both IR- and IGF1R-mediated signaling involves autophosphorylation of tandem activation loop Tyr residues at the kinase domain of the receptor facing the cytoplasmic side, which is critical for the activation of signaling cascades through cytosolic IRS1 or IRS2 kinase systems (44). In both cases the signaling is propagated through activation of phosphatidylinositol 3-kinase, Akt, and protein kinase C, ultimately leading to transcriptional activation of many genes involved in glucose uptake and utilization. Induction and activation of IGF1R pathways through a number of external and internal factors in many tumor cell models have been described (46, 47). In this study we describe a novel mechanism of mitochondrial respiratory stress-induced switching from IR to IGF1R pathway leading to induced glucose uptake and resistance to apoptosis in C2C12 rhabdomyocytes. Mitochondrial stress, due to reduced mtDNA content and/or treatment with respiratory inhibitors (2), is the major contributing factor in this switch, because restoration of mtDNA content and thus the ΔΨm effectively reverses the level of glucose uptake and the entire signaling cascade.

Our results show that both GLUT 4 and IGF1R genes are overexpressed and/or activated in mtDNA-depleted cells, and the levels are reduced to control cell level in reverted cells suggesting the role of mitochondrial stress in these changes. Three separate lines of experiments provide supporting evidence for this possibility: 1) The mitochondrial ionophore, CCCP, which is known to induce mitochondrial stress signaling, also induced the steady-state levels of these two proteins and glucose uptake in four different cell lines. 2) FK506, an inhibitor of Cn, not only inhibited stress-induced glucose uptake but also reduced the steady-state levels of IGF1R and GLUT 4 proteins as well as mRNAs. 3) Further confirmatory evidence on the role of Cn comes from the mRNA silencing experiments, which show that knockdown of Cnα mRNA by ~80% also caused reduced steady-state levels of GLUT 4 and IGF1R mRNAs by a similar magnitude in mtDNA-depleted cells.

Results on overexpression of IGF1R in FK506-treated cells suggest that IGF1R plays a direct role in GLUT4 mRNA expression. Thus, Cn appears to exert a multimodal effect on GLUT 4 gene expression, namely through activation of IGF1R and through activation of other Cn target factors. Unpublished studies in our laboratory show that GLUT 4 expression is transcriptionally up-regulated by Cn-activated NFκB, C/EBPβ, CREB, and NFAT, in addition to IGF1R. It is likely that yet unknown IGF1R-activated factors cooperatively interact with other Cn-activated factors in GLUT 4 expression.

The steady-state levels of both IR and IGF1R proteins in the plasma membrane compartment were increased in mtDNA-depleted cells, although only the latter remained functional probably because of selective inhibition of autophosphorylation of IR (Fig. 3). A possible receptor switch and the role of IGF1R pathway are further confirmed by results showing that IGF1R mRNA silencing selectively reduced the glucose uptake, reduced the cell invasion through Matrigel membrane, and increased the rate of apoptosis. IR mRNA silencing affected all these cellular parameters, although at a vastly reduced level. These results provide firm evidence for the Cn-mediated recep-
tor switch for the observed invasive behavior and resistance to apoptosis in mtDNA-depleted cells. The molecular basis of preferentially higher autophosphorylation of IGF1R currently remains unclear. Preliminary results, however, suggest that increased PTP1B activity and possible post-translational modification of this enzyme in mtDNA-depleted cells may be responsible for this selectivity.

Consistent with the observed receptor switch, cells subjected to mitochondrial stress also show a distinct metabolic shift. An increasing number of studies suggest that mitochondrial mutations and/or dysfunction are closely associated with a wide variety of neuromuscular, retinal degenerative disease, diabetes, ischemic injury, cancer, and aging (19, 48, 49). In a large majority of these cases, the heteroplasmic nature of mutations with an age-dependent increase in the ratio of mutated to wild-type mtDNA is implicated as an important factor. In this regard, partial mtDNA depletion used in this study reflects the steady decline of functional mtDNA levels in many diseases (50). The respiratory stress-induced metabolic shift with induced glycolysis is not restricted to either mtDNA depletion or C2C12 cells. We show that mitochondrial stress induced either by mtDNA depletion in A549 cells or disruption of ΔΨm by CCCP treatment in H9C2 cardiomyocytes and 3T3 fibroblasts causes increased IGF1R activity, increased GLUT 4 mRNA levels, and induced glucose uptake. The relative increase in activities of different glycolytic enzymes in the four cell types varied markedly suggesting cell-specific effects. Further, although the precise molecular mechanism of this increase remains unclear, unpublished results with hexokinase promoter suggests transcriptional activation as the main factor. Additionally, the level of oligomycin-insensitive ATP synthesis, which probably reflects the contribution of the glycolytic pathway, is increased >2-fold in all cases, confirming the metabolic switch. Based on these observations we propose that stress-mediated activation of the IGF1R pathway is closely linked to the higher resistance to apoptosis and invasive properties of mtDNA-depleted C2C12 and A549 cells.

As proposed by Warburg (51), the capacity to sustain high rates of glycolysis under aerobic conditions appears to be an important feature of rapidly growing tumors. The Warburg effect has been observed in many solid tumors, and accordingly increased expression of genes encoding glucose transporter genes and glycolytic enzymes has been suggested as the basis for an altered metabolic profile of tumor cells (52). Other studies show that the high metabolic activity of tumor cells is associated with activation of Akt, an Ser/Thr kinase (53, 54). Akt has been shown to induce anabolic metabolism and suppress apoptosis by multiple mechanisms (55), thus providing a molecular basis for an altered metabolic state of proliferating tumors. Warburg also predicted that impaired mitochondrial oxidative function may be a factor in increased glucose utilization of tumor cells. Our results show that mtDNA depletion and other types of mitochondrial respiratory dysfunction induce stress signaling, which culminates in altered glucose uptake and metabolism. In this regard our results provide a potential mechanistic explanation for the Warburg hypothesis.

Cn is known to play critical roles in many physiological processes, including T-cell differentiation, myogenesis, switch of muscle fiber types, and muscle contraction/glucose homeostasis (56–58). Here we show that mitochondrial stress-activated Cn plays a direct role in the altered metabolic property of tumor cells through an induced IGF1R pathway and increased expression of GLUT 4. In summary, we describe a novel Cn-mediated IGF1R activation pathway that is likely to play an important role in tumor progression and tumor invasion.

Acknowledgments—We are thankful to the members of the Avadhani laboratory for useful discussions and suggestions. We are also thankful to Drs. Morris J. Birnbaum and Kendra Bence for their useful comments and suggestions on the manuscript. We also thank Dr. Renato Baserga for providing the IGF1R expression cDNA construct.

REFERENCES

1. Amuthan, G., Biswas, G., Zhang, S. Y., Klein-Szanto, A., Vijayasathy, C., and Avadhani, N. G. (2001) EMBO J. 20, 1910–1920
2. Biswas, G., Abdelanjo, O. A., Freedman, B. D., Anandatheerthavarada, H. K., Vijayasathy, C., Zaidi, M., Kotlikoff, M., and Avadhani, N. G. (1999) EMBO J. 18, 522–533
3. van Waveren, C., Sun, Y., Cheung, H. S., and Moraes, C. T. (2006) Carcinogenesis 27, 409–418
4. Parikh, V. S., Morgan, M. M., Scott, R., Clements, L. S., and Butow, R. A. (1987) Science 235, 576–580
5. Butow, R. A., and Avadhani, N. G. (2004) Mol. Cell 14, 1–15
6. Amuthan, G., Biswas, G., Anandatheerthavarada, H. K., Vijayasathy, C., Shephard, H. M., and Avadhani, N. G. (2002) Oncogene 21, 7839–7849
7. Biswas, G., Anandatheerthavarada, H. K., Zaidi, M., and Avadhani, N. G. (2003) J. Cell Biol. 161, 507–519
8. Arnauld, T., Vankoningloon, S., Renard, P., Houbion, A., Ninane, N., Demay, C., Remacle, J., and Raes, M. (2002) EMBO J. 21, 53–63
9. Zhao, Q., Wang, J., Levichkin, I. V., Stasinopoulos, S., Ryan, M. T., and Hoogenraad, N. J. (2002) EMBO J. 21, 4411–4419
10. Biswas, G., Guha, M., and Avadhani, N. G. (2005) Gene (Amst.) 354, 132–139
11. Singh, K. K., Kuhalwice, M., Still, I., Desouki, M. M., Geradts, J., and Matsu, S. (2005) Gene (Amst.) 354, 140–146
12. Meierhofer, D., Mayr, J. A., Foetschtl, U., Berger, A., Fink, K., Schmeller, N., Hacker, G. W., Hauser-Kronberger, C., Kofler, B., and Sperl, W. (2004) Carcinogenesis 25, 1005–1010
13. Saada, A., Shaag, A., and Elpeleg, O. (2003) Mol. Genet. Metab. 79, 1–5
14. Liu, C. Y., Lee, C. F., Hong, C. H., and Wei, Y. H. (2004) Ann. N. Y. Acad. Sci. 1011, 133–145
15. Wang, J., Silva, J. P., Gustafsson, C. M., Rustin, P., and Larsson, N. G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4038–4043
16. Dey, R., and Moraes, C. T. (2000) J. Biol. Chem. 275, 7087–7094
17. Kim, J. Y., Kim, Y. H., Chang, I., Kim, S., Pak, Y. K., Oh, B. H., Yagita, H., Jung, Y. K., Oh, Y. J., and Lee, M. S. (2002) Oncogene 21, 3139–3148
18. Petros, J. A., Baumann, A. K., Ruiz-Pesini, E., Amin, M. B., Sun, C. Q., Hall, J., Lim, S., Issa, M. M., Flanders, W. D., Hosseini, S. H., Marshall, F. F., and Wallace, D. C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 719–724
19. Shidara, Y., Yamagata, K., Kanamori, T., Nakano, K., Kwong, J. Q., Manfredi, G., Oda, H., and Ohta, S. (2005) Cancer Res. 65, 1655–1663
20. Cottrell, D. A., Blakely, E. L., Johnson, M. A., Borthwick, G. M., Ince, P. I., and Turnbull, D. M. (2001) Novartis Found. Symp. 235, 234–243
21. Parello, P., Xiao, Y., Flink, M., Sanchez-Cespedes, M., Mazzarelli, P., Rinaldi, M., Nicol, T., Gabrielson, E., Cuomo, C., Cohen, D., Pandit, S., Spencer, M., Rabitti, C., Fazio, V. M., and Sidransky, D. (2001) Cancer Res. 61, 7623–7626
22. Biswas, G., Anandatheerthavarada, H. K., and Avadhani, N. G. (2005) Cell Death Differ. 12, 266–278
23. Mazurek, S., and Eigenbrodt, E. (2003) Anticancer Res. 23, 1149–1154
Mitochondrial Stress-induced IGF1R Activation

24. Pietrzkowski, Z., Lammers, R., Carpenter, G., Soderquist, A. M., Limardo, M., Philips, P. D., and Baserga, R. (1992) Cell Growth & Differ. 3, 199–205
25. Frevert, E. U., and Kahn, B. B. (1997) Mol. Cell. Biol. 17, 190–198
26. Miccoli, L., Oudard, S., Sureau, F., Poirson, F., Dutrillaux, B., and Poupon, M. F. (1996) Biochem. J. 313, 957–962
27. Liu, Y. Q., Tornheim, K., and Leahy, J. L. (1998) J. Clin. Invest. 101, 1870–1875
28. Devin, A., Nogueira, V., Leverve, X., Guerin, B., and Rigoulet, M. (2001) Eur. J. Biochem. 268, 3943–3949
29. Antony, F. A., Shipston, M. J., and Smith, S. M. (1993) Biochem. Biophys. Res. Commun. 194, 226–233
30. Nishimura, H., Saltis, J., Habberfield, A. D., Garty, N. B., Greenberg, A. S., Cushman, S. W., Londos, C., and Simpson, I. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11500–11504
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
32. Laemmli, U. K. (1970) Nature 227, 680–685
33. MaCaulay, S. L., Stoichevska, V., Grusovin, J., Gough, K. H., Castelli, L. A., and Ward, C. W. (2003) Biochem. J. 376, 123–134
34. Yagel, S., Warner, A. H., Nellans, H. N., Lala, P. K., Waghorne, C., and Denhardt, D. T. (1989) Cancer Res. 49, 3553–3557
35. Watson, R. T., and Pessin, J. E. (2001) Recent Prog. Horm. Res. 56, 175–194
36. Girnita, A., All-Ericsson, C., Economou, M. A., Astron, K., Axelsson, M., Seregard, S., Larsson, O., and Girnita, L. (2006) Clin. Cancer Res. 12, 1383–1391
37. Stromberg, T., Ekman, S., Girnita, L., Dimberg, L. Y., Larsson, O., Axelsson, M., Lennartsson, J., Hellman, U., Carlson, K., Osterborg, A., Vanderkerken, K., Nilsson, K., and Jernberg-Wiklund, H. (2006) Blood 107, 669–678
38. Hu, Y., Yao, J., Liu, Z., Liu, X., Fu, H., and Ye, K. (2005) EMBO J. 24, 3543–3554
39. Walker, E. H., Pacold, M. E., Perisic, O., Stephens, L., Hawkins, P. T., Wymann, M. P., and Williams, R. L. (2000) Mol. Cell 6, 909–919
40. Smith, R. M., Tiesinga, J. J., Shah, N., Smith, J. A., and Jarett, L. (1993) Arch. Biochem. Biophys. 300, 238–246
41. Vasilcanu, D., Girnita, A., Girnita, L., Vasilcanu, R., Axelsson, M., and Larsson, O. (2004) Oncogene 23, 7854–7862
42. Jenssen, N., Pold, R., Buhl, E. S., Jensen, L. S., Schmitz, O., and Lund, S. (2003) J. Appl. Physiol. 94, 1373–1379
43. Zong, H., Ren, J. M., Young, L. H., Pypaert, M., Mu, J., Birnbaum, M. J., and Shulman, G. I. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15983–15987
44. Lee, J., and Pilch, P. F. (1994) Am. J. Physiol. 266, C319–C334
45. Shefi-Friedman, L., Wertheimer, E., Shen, S., Bak, A., Accili, D., and Sampson, S. R. (2001) Am. J. Physiol. 281, E16–E24
46. Larsson, O., Girnita, A., and Girnita, L. (2005) Br. J. Cancer 92, 2097–2101
47. Peruzzi, F., Prisco, M., Dew, M., Salomoni, P., Grasselli, E., Romano, G., Calabretta, B., and Baserga, R. (1999) Mol. Cell. Biol. 19, 7203–7215
48. Loeb, L. A., Wallace, D. C., and Martin, G. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 18769–18770
49. Park, S. Y., Choi, B., Cheon, H., Pak, Y. K., Kula, M., Singh, K. K., and Lee, M. S. (2000) Biochem. Biophys. Res. Commun. 275, 1399–1405
50. Zeviani, M., and Antozzi, C. (1997) Mol. Hum. Reprod. 3, 133–148
51. Warburg, O. (1956) Science 123, 309–314
52. Semenza, G. L., Artemov, D., Bedi, A., Bhuja, Z., Chiles, K., Feldser, D., Laughner, E., Ravi, E., Simon, S., and Zhong, H. (2001) N. Vartis Found. Symp. 240, 251–260
53. Bae, S. S., Cho, H., Mu, J., and Birnbaum, M. J. (2003) J. Biol. Chem. 278, 49530–49536
54. Buzzai, M., Bauer, D. E., Jones, R. G., Deberardinis, R. J., Hatzivassiliou, G., Elstrom, R. L., and Thompson, C. B. (2005) Oncogene 24, 4165–4173
55. Whiteman, E. L., Cho, H., and Birnbaum, M. J. (2002) Trends Endocr. Metab. 13, 444–451
56. Ryder, J. W., Long, Y. C., Nilsson, E., Mahlapuu, M., and Zierath, J. R. (2005) J. Physiol. 567, 379–386
57. Clipstone, N. A., and Crabtree, G. R. (1992) Nature 357, 695–697
58. Molkenstj, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) Cell 93, 215–228