Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells

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Wu M, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, Armistead S, Lemire K, Orrell J, Teich J, Chomicz S, Ferrick DA. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. Am J Physiol Cell Physiol 292: C125–C136, 2007. First published September 13, 2006; doi:10.1152/ajpcell.00247.2006.—Increased conversion of glucose to lactic acid associated with decreased mitochondrial respiration is a unique feature of tumors first described by Otto Warburg in the 1920s. Recent evidence suggests that the Warburg effect is caused by oncogenes and is an underlying mechanism of malignant transformation. Using a novel approach to measure cellular metabolic rates in vitro, the bioenergetic basis of this increased glycolysis and reduced mitochondrial respiration was investigated in two human cancer cell lines, H460 and A549. The bioenergetic phenotype was analyzed by measuring cellular respiration, glycolysis rate, and ATP turnover of the cells in response to various pharmacological modulators. H460 and A549 cells displayed a dependency on glycolysis and an ability to significantly upregulate this pathway when their respiration was inhibited. The converse, however, was not true. The cell lines were attenuated in oxidative phosphorylation (OXPHOS) capacity and were unable to sufficiently upregulate mitochondrial OXPHOS when glycolysis was disabled. This observed mitochondrial impairment was intimately linked to the increased dependency on glycolysis. Furthermore, it was demonstrated that H460 cells were more glycolytic, having a greater impairment of mitochondrial respiration, compared with A549 cells. Finally, the upregulation of glycolysis in response to mitochondrial ATP synthesis inhibition was dependent on AMP-activated protein kinase activity. In summary, our results demonstrate a bioenergetic phenotype of these two cancer cell lines characterized by increased rate of glycolysis and a linked attenuation in their OXPHOS capacity. These metabolic alterations provide a mechanistic explanation for the growth advantage and apoptotic resistance of tumor cells.

OXPHOS, glycolysis, tumorigenesis, mitochondrial impairment, AMPK, Warburg phenotype, mitochondrial bioenergetics, oxidative phosphorylation, ATP synthesis inhibition, AMPK, glycolysis, Warburg effect


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O2 consumption; oxidative phosphorylation; Warburg effect; real time

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tumor growth (31, 37). Most recently, attenuation of lactate dehydrogenase A expression was demonstrated to promote mitochondrial energy metabolism in murine breast cancer cell lines that likewise diminished tumorigenicity (12).

Valuable insight into the physiological state of living cells and the changes of those cells in response to experimental intervention has been gained through monitoring independently the rate of oxygen consumption using Clark electrodes (7), or the rate of extracellular acidification using the microphysiometer (28, 29), or measuring lactate concentration. Under typical in vitro cell culture conditions, extracellular acidification rate (ECAR) is dominated by lactic acid formed during glycolytic energy metabolism. In this study we employed a novel instrument that simultaneously measures the flux of oxygen (oxygen consumption rate, OCR) and protons (ECAR) of adherent cells in a microplate over time. Extracellular flux (XF) technology enables rapid, real-time detection of metabolic changes, specifically, changes in cellular respiration and glycolysis rate, due to experimental manipulation.

Despite a rapidly rising interest in cancer metabolism, the nature of the metabolic networks that give rise to metabolic phenotypes that confer tumor cells oncogenic and metastatic properties, such as increased proliferation and the ability to avoid apoptosis, are still not well understood. To gain a better understanding of energy metabolism in human tumor cells and the interplay of the two main bioenergetic pathways, oxidative phosphorylation and glycolysis, we performed a detailed bioenergetic analysis of two of the NCI60 panel of human cancer cell lines, H460 and A549. Measuring the metabolic responses of these cell lines to pharmacological modulators of cellular bioenergetic phenotype that was the result of alterations in both of the main energy producing pathways.

**MATERIALS AND METHODS**

**Cell culture.** Human non-small cell carcinoma cell line H460 and A549 were obtained from the DCTD Tumor/Cell Line Repository at the National Cancer Institute. Cells were cultured in growth medium consisting of RPMI 1640 (Invitrogen, Carlsbad, CA), 10% FBS (Hyclone, Logan, UT) and 100 units of penicillin and 100 µg/ml streptomycin (Invitrogen). The cells were maintained in either 75-cm² T flasks or 175-cm² T flasks (BD Biosciences, San Jose, CA) in an incubator (model 3100 series, Forma Scientific, Marietta, OH) controlled at 37°C, 95% humidity, and 5% CO₂. Cell culture medium was refreshed every other day. Every 2–3 days, H460 and A549 cultures were detached from the flasks using a 0.25% solution of trypsin (Invitrogen) and subcultured at an initial seeding density of 1.0 × 10⁶ cells/ml (for a 2-day culture) or 5 × 10⁶ cells/ml (for a 3-day culture) in 20 ml growth medium per T75 flask. All cultures were maintained at 80–90% confluence at the time of subculture.

**Test compounds.** Phloretin, 2-deoxyglucose (2-DG), sodium oxamate, 3-bromopyruvic acid, sodium iodoacetate, sodium fluoride, 2,4-dinitrophenol (2,4-DNP), rotenone and oligomycin were obtained from Sigma (St. Louis, MO). Compound C was obtained from EMD Sciences (San Diego, CA). Concentrated stocks of 2-DG, iodoacetate, fluoride, and oxamate were prepared in assay medium. Stock concentrations were 1,000 mM 2-DG, 2 mM sodium iodoacetate, 1,000 mM sodium fluoride, and 500 mM oxamate. Concentrated stocks of phloretin, 2,4-DNP, rotenone, and oligomycin were prepared in DMSO. Stock concentrations were 200 mM 2,4-DNP, 100 mM phloretin, 10 mM rotenone, and 10 mM oligomycin. 2,4-DNP was further diluted to 2 mM, followed by adjusting to pH 7.4. Phloretin was further diluted to 1 mM, followed by adjusting the pH to 7.4.

Rotenone and oligomycin were diluted to 0.01 mM. Calcein AM was purchased from Invitrogen and prepared according to manufacturer’s instructions.

**Extracellular flux (XF) analysis.** Cellular metabolism is the process of substrate uptake (oxygen, glucose, fatty acids) and energy conversion through a series of enzymatically controlled oxidation/reduction reactions. These reactions are executed through a series of intracellular biochemical processes (glycolysis, Krebs cycle, electron transport and OXPHOS) resulting in the production of ATP and the release of heat and chemical by-products (lactate and CO₂) into the extracellular environment.

The XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) is a fully integrated 24-well instrument that measures in real time the uptake and excretion of metabolic end products. Initial production versions of the XF24 Analyzer were used with XF Assay Kits (Seahorse Bioscience) to measure the extracellular flux changes of oxygen and protons in the media immediately surrounding adherent cells cultured in a XF24-well microplate (Seahorse Bioscience).

Each XF assay kit contains a disposable sensor cartridge, embedded with 24 pairs of fluorescent biosensors (oxygen and pH), which is coupled to a fiber-optic waveguide. The waveguide delivers light at various excitation wavelengths (oxygen = 532 nm, pH = 470 nm) and transmits a fluorescent signal, through optical filters (oxygen = 650 nm, pH = 530 nm) to a set of highly sensitive photodetectors. Each fluorophore is uniquely designed to measure a particular analyte. The instrument currently supports the simultaneous measurement of pH and oxygen and has capacity for two additional sensors, allowing the user to select an array of up to four sensors, specific for a particular application.

Oxygen consumption and proton extrusion cause rapid, measurable changes in oxygen tension and pH (within 3–5 min) within a transient microchamber in each of the 24 wells created by the sensor cartridge while in the measurement position (Fig. 1). During this time, analyte levels are measured every 22 s until the oxygen concentration drops ~30 mmHg and the media pH declines up to 0.4 pH units.

Each sensor cartridge is also equipped with four reagent delivery chambers per well for injecting testing agents into wells during an assay.

Prior to the start of the XF assay, the array of biosensors are each independently calibrated using an automated routine that determines a unique sensor gain based on the sensor output measured in a calibration reagent of known pH and oxygen concentration.

During the assay, baseline rates are measured twice. OCR is reported in the unit of nanomoles per minute and ECAR in milli-pH (m pH) units per minute. Testing chemical(s) or biological solution(s) are preloaded in the reagent delivery chambers of the sensor probe and are subsequently pneumatically injected into the media. After 3–5 min mixing, postexposure OCR and ECAR measurements are made multiple times. Because XF measurements are nondestructive, the metabolic rate of the same cell population can be measured repeatedly over time, while up to four different testing agents can be injected sequentially or simultaneously into each well. Upon completion of an XF assay, other types of biological assays such as cell viability can be performed on the same cell population.

**Assay medium.** A low-buffered RPMI 1640 medium containing 1 mM phosphate (Molecular Devices, Sunnyvale, CA) was used as standard medium in the XF assays and is referred to as “assay medium.” In those assays, where glucose was omitted and 10 mM glutamine was used as substrate, a low-buffered KHB buffer consisting of 110 mM NaCl, 4.7 mM KCl, 2 mM MgSO₄, and 1.2 mM Na₂HPO₄, adjusted to pH 7.4, was used.

**XF bioenergetic assay.** H460 and A549 cells were seeded in XF 24-well cell culture microplates (Seahorse Bioscience) at 2.0–3.0 × 10⁴ cells/well (0.32 cm²) in 200–500 µl growth medium and then incubated at 37°C/5% CO₂ for 20–24 h. Assays were initiated by removing the growth medium from each well and replacing it with 600–900 µl of assay medium prewarmed to 37°C. The cells were
incubated at 37°C for 30 min to allow media temperature and pH to reach equilibrium before the first rate measurement. Prior to each rate measurement, the XF24 Analyzer gently mixed the assay media in each well for 10 min to allow the oxygen partial pressure to reach equilibrium. Following mixing, OCR and ECAR were measured simultaneously for 3–5 min to establish a baseline rate. The assay medium was then gently mixed again for 3–5 min between each rate measurement to restore normal oxygen tension and pH in the microenvironment surrounding the cells. After the baseline measurement, 75–200 μl of a testing agent prepared in assay medium was then injected into each well to reach the desired final working concentration. This was followed by mixing for 5–10 min to expedite compound exposure to cellular proteins, after which OCR and ECAR measurements were then made. Generally, two to three baseline rates and two or more response rates (after compound addition) were measured, and the average of two baseline rates or test rates was used for data analysis. For time-resolved experiments, multiple measurements as well as compound injections were made at the time points indicated. The values of OCR and ECAR reflect both the metabolic activities of the cells and the number of cells being measured. For relative measurements comparing metabolic rate after compound exposure to a pre-exposure baseline, that is, when data are expressed as a percentage of OCR or ECAR change over baseline, the number of cells present in a well is not relevant since the same cell population is assayed. In the present study, the acute responses to pharmacological modulators of energy metabolism by cancer cells were determined, and the responses were expressed as a percentage of baseline rate prior to compound exposure.

Typically, at the end of each assay cells were detached by incubating with 0.25% trypsin (Invitrogen), and the number and percentage of viable cells were determined by trypsin blue exclusion assay using a ViCell (Beckman-Coulter, Fullerton, CA). Cell viability of all treated samples was similar, compared with control cells in acute assays lasting one hour or less.

Data analysis. Sensitivity data were analyzed by nonlinear regression with GraphPad Prism 4 version 4.03 (GraphPad Software, San Diego, CA).

ATP quantification. The quantity of ATP present in the test cells in each well following compound or vehicle exposure for 30 min was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). A549 and H460 cells were seeded in white 96-well tissue culture microplates at 1.0 × 10⁴ cells/well and cultured in the 37°C incubator for 24 h prior to compound treatment. Cells were exposed to vehicle or compound for 45 min in the 37°C incubator before starting the ATP assay. The ATP assay was performed as per the manufacturer’s instruction. Luminescence intensity from each well was measured using a FLUOstar Optima plate reader (BMG Labtech, Durham, NC).

Calcein AM cell viability assay. The relative viability of the test culture following 45 min exposure to compounds or vehicle was determined using the calcein AM assay. A549 cells were seeded in black 96-well tissue culture microplates at 1.0 × 10⁴ cells/well and incubated in the 37°C incubator for 24 h in parallel with the cell seeding for ATP assay. Cells were treated with vehicle or compound at the indicated concentrations for 30 min in parallel with ATP assay. Calcein AM staining solution was prepared by dissolving calcein AM (50 μg/vial special package) in 100 μl of DMSO followed by diluting to 1 μM in Hanks’ balanced salt solution (HBSS) immediately prior to use. The assay was performed by first removing from each well the culture medium containing the compounds and then washing each well with 200 μl of HBSS. 100 μl of 1 μM calcein AM staining solution was then added to each well. After incubation at 37°C for 30 min, the fluorescent intensity of each well was measured at a wavelength of 530 nm using an excitation wavelength of 480 nm on a FLUOstar Optima plate reader (BMG Labtech).

RESULTS

Real-time perturbational profiling of bioenergetic pathways in A549 cells. To verify whether oxygen consumption rate and extracellular acidification rate reflect qualitatively the rate of mitochondrial respiration and the rate of lactic acid production via glycolysis, respectively, we measured OCR and ECAR of
A549 cells exposed sequentially to each of three well-defined small molecule modulators of mitochondrial glycolytic energy metabolism: 2,4-DNP, 2-DG and rotenone (Fig. 2A). 2,4-DNP uncouples mitochondrial respiration from ATP synthesis, 2-DG is a glucose analog that inhibits hexokinase, the first enzyme in the glycolysis pathway, which converts glucose to glucose-6-phosphate, and lastly, rotenone is an inhibitor of mitochondrial NADH dehydrogenase/complex I.

2,4-DNP administration invoked a concomitant increase in OCR and ECAR. Subsequent addition of 2-DG to the same wells elicited a rapid decrease of the 2,4-DNP-stimulated ECAR to well below the baseline rate, while OCR remained unaffected. Finally, addition of the mitochondrial respiration inhibitor rotenone, after exposure of the same cells to 2,4-DNP and 2-DG, completely diminished OCR and further reduced ECAR. These data demonstrated that the respiration rate, as measured by OCR, was stimulated by the mitochondrial uncoupler 2,4-DNP and inhibited by the respiration inhibitor rotenone, whereas ECAR was inhibited by the glycolytic inhibitor 2-DG, thus validating OCR and ECAR as indicators of cellular respiration and glycolysis, respectively.

To correlate these observed changes in OCR and ECAR directly to ATP production, we measured cellular ATP concentration in an equivalently treated set of cells exposed for 45 min to: 1) vehicle, 2) 2,4-DNP, 3) 2,4-DNP plus 2-DG, and 4) 2,4-DNP plus 2-DG plus rotenone (Fig. 2B). We observed that the 2,4-DNP treatment reduced ATP content by ~40% compared with cells due to the uncoupling of ATP synthesis from respiration. 2-DG in combination with 2,4-DNP caused a dramatic decrease in ATP turnover to less than 10% of the control cells as glycolytic ATP production was now also blocked. And finally, the combination of the mitochondrial respiration inhibitor rotenone, along with the glycolysis inhibitor, 2-DG, and uncoupler, 2,4-DNP, effectively depleted the cells of their ATP content. Since the ATP concentration of a cell population is determined by both the number of viable cells present and their relative metabolic rates, the cell viability of each population must be determined to ensure similar viability of control and testing cells if the changes in ATP concentration are to be attributed to alterations in their metabolic rate. Therefore, cell viability was measured in a parallel experiment with identically treated A549 cells by using calcein AM staining. The cell viability remained essentially constant among all the experimental samples (Fig. 2B), thus indicating that the reduced ATP levels in the A549 cells were due to metabolic changes and not cell loss as a result of compound toxicity.

Contribution of mitochondrial respiration to total cellular oxygen consumption rates and glycolysis to total extracellular acidification rate in H460 and A549 cells. To assess these parameters, the basal cellular oxygen consumption rate and extracellular acidification rate were determined initially for H460 and A549 cells. The cellular respiration rate was similar for both cell lines: 1.8 ± 0.2 nmol/min per 10⁶ cells for H460 and 1.6 ± 0.1 nmol/min per 10⁶ cells for A549 (Fig. 3A). The cellular respiration rates correspond well with measurements made in several cancer cell lines using a Clarke electrode (12, 37). The observed extracellular acidification rate of H460 cells was significantly higher than that of A549 cells (Fig. 3B), suggesting that the former is more glycolytic than the latter. Both cell lines are known to be metastatic in animal models (23).

Subsequently, the fraction of mitochondrial respiration that contributes to total cellular oxygen consumption was determined by using an inhibitor of the mitochondrial respiratory chain, rotenone. Mitochondrial respiration in general accounts for ~90% of cellular oxygen consumption (23). The remaining 10% is comprised of nonmitochondrial respiration, including substrate oxidation and cell surface oxygen consumption (23, 18). The rotenone-sensitive oxygen consumption rate specifically identifies respiration in the mitochondria, while the rotenone-resistant rate reflects the nonmitochondrial respiration rate. In the presence of 1 μM rotenone, the OCR of H460 and A549 was reduced to about 15% and 25% of their respective baseline rates, suggesting that mitochondrial respiration accounted for 85% and 75% of their total cellular respiration, respectively (Fig. 3C). Likewise, the contribution of lactic acid production from glycolysis to ECAR was assessed by using oxamate, an inhibitor of lactate dehydrogenase, which catalyzes the conversion of pyruvate to lactic acid during the last step of glycolysis. The oxamate-sensitive ECAR reflects the glycolysis rate and the oxamate-insensitive ECAR is due to nonglycolytic acidification, mostly acidification by carbon dioxide [the end product of the tricarboxylic acid cycle (TCA) in mitochondria], which can be converted to bicarbonate and hence contribute to extracellular acidification. The ECAR of H460 and A549 cells was decreased to 20% of their respective baseline rates after exposure to oxamate, indicating that glycolysis accounts for about 80% of their total ECAR (Fig. 3D).
Perturbational profiling of the glycolytic pathway of H460 and A549 cells. We determined the relative sensitivity of H460 and A549 cells to an inhibitor of the glucose transporter, GLU-1, and four inhibitors selective for various enzymes along the glycolytic pathway. The cell lines were exposed for 10 min to various concentrations of each of the following inhibitors before the response rates were measured: phloretin, 3-bromopyruvic acid, iodoacetate, fluoride or oxamate. The glycolytic enzymes that these small molecules perturb are illustrated in Fig. 4. The changes in rates of cellular respiration and glycolysis of the two cell lines in response to the glycolysis inhibitors are expressed as a percentage of ECAR and OCR relative to their respective baseline rates that are measured prior to compound addition (Fig. 4). Increasing doses of 3-bromopyruvic acid, iodoacetate, fluoride and oxamate incrementally decreased ECAR in both H460 and A549 cells, indicating a dose-dependent inhibition of glycolysis. In an inverse manner, mitochondrial respiration was stimulated as the glycolysis rates declined for both cell lines with one exception. Although phloretin decreased glycolysis in a manner similar to the other four glycolytic inhibitors, mitochondrial respiration rate was also decreased, instead of the expected increase. This apparent discrepancy is due to the fact that phloretin not only inhibits GLUT-1 but also has a second target in the mitochondria. Thus, the decreased respiration was probably due to phloretin's direct inhibitory effect on mitochondrial respiration, which prevented the compensatory increase in respiration which would otherwise have occurred in response to the glycolytic block created by a lack of glucose uptake. The extent of respiratory increase was enzyme dependent. Inhibition of hexokinase by 3-bromopyruvic acid and glyceraldehyde-3-phosphate dehydrogenase by iodoacetate caused the largest increases in respiration (~200% of baseline OCR in A549 cells). The maximum cellular respiration increase in H460 cells, regardless of glycolysis inhibitor, was lower than the corresponding increase in A549 cells (Fig. 4).

It is now well established that cancer cells display an elevated expression of glycolysis enzymes as well as expression of alternative isoenzymes (10, 26). For example, the high affinity lactate dehydrogenases, 4 and 5 (LDH4 and LDH5), which consist of three subunits of LDH-A and one subunit of LDH-B vs. four subunits of LDH A, respectively, are two of five LDH isoenzymes which can promote anaerobic glycolysis by favoring rapid conversion of very low concentrations of pyruvate to lactate in skeletal muscles. High LDH5 levels in tumor cells constitute a phenotype linked to aggressive disease in colorectal adenocarcinomas (22). Both cell lines in this study displayed high glycolytic activity as indicated by the drastic decrease in glycolysis rates invoked by all glycolysis inhibitors used. All four inhibitors consistently reduced ECAR to ~20% of their respective baseline rates for each cell line at saturating concentrations. The greatest difference in sensitivity to these inhibitors between H460 and A549 cells was observed with oxamate (Fig. 4).

IC50 values for 3-bromopyruvic acid, iodoacetate, fluoride and oxamate were determined for both cell lines (Fig. 5). To a varying degree, we observed a higher sensitivity to all four inhibitors in the highly metastatic H460 cells relative to the A549 cells. The most significant difference between the two cell lines was once again their sensitivity to oxamate. The IC50 value for oxamate in the H460 cells was approximately six times higher than that of A549 cells, suggesting the increased expression of alternative lactate dehydrogenase isoenzymes (Fig. 5D). Altogether, the observed increases in glycolysis rates may reflect elevated activity of glycolysis enzymes and/or alternative isoenzyme expression in these cells. In fact, it was very recently reported that knocking down LDH-A resulted in
stimulation of mitochondrial respiration and diminished tumori-
genicty in murine breast cancer cells (12).

Dependency on glycolysis for bioenergetics. We next inves-
tigated the bioenergetic dependency on glycolysis of the two
cell lines. Changes in cellular respiration and glycolysis rate
after administration of 2-DG, which blocks the first step of the
glycolytic pathway, were determined. Indeed, the glycolysis
rates declined while the respiration rates climbed in response to
increasing concentrations of 2-DG in both H460 and A549
cells (Fig. 6). Interestingly, the cellular ATP turnover in both
cell lines was markedly decreased in parallel with the declining
glycolytic rates, even though there appeared to be a compen-
satory rise in cellular respiration (Fig. 6). This suggests that
both H460 and A549 cells are extremely dependent on glyco-
lytic generation of ATP, as they were unable to maintain
their ATP balance in the presence of 2-DG. The fact that
cellular ATP concentrations could not be maintained despite
the observed increase in the respiration rate also suggests that
OXPHOS may be impaired as well. Lastly, compared with
A549, H460 cells appeared to rely even more heavily on
glycolysis as their ATP turnover declined to a greater degree at
maximal inhibitory 2-DG concentrations (Fig. 6).

Attenuated mitochondrial respiration capacity and inability
to upregulate OXPHOS in H460 and A549 cells. As described
earlier, we observed a consistently smaller degree of respira-
tion stimulation in H460 cells, compared with that of A549

Fig. 4. Perturbational profiling of bioener-
getics of H460 and A549 cells: phloretin (A),
3-bromopyruvic acid (3BrPA) (B), iodoac-
etate (C), fluoride (D), and oxamate (E).
Data are expressed as percent change in
oxygen consumption rate (OCR) and ECAR
relative to baseline rate in response to com-
ponent exposure. The specific steps perturbed
by the inhibitor of the glycolysis pathway
are as depicted. Note: phloretin is known to
inhibit mitochondrial respiration, in addition
to the glucose transporter GLUT-1. Each
data point represents mean ± SE; n = 4.
cells, triggered by the four glycolysis inhibitors (Fig. 4). However, the basal respiration rates were very similar between the two cell lines (Fig. 3A). We hypothesized that the mitochondrial respiration capacity of H460 cells might be more impaired and therefore less able to upregulate mitochondrial respiration to compensate for ATP depletion caused by the pharmacological inhibition of glycolysis. To test this hypothesis, we determined the mitochondrial respiration capacity of the two cell lines using 2,4-DNP, which uncouples mitochondrial respiration from ATP synthesis, thus stimulating maximal mitochondrial respiration by dissipating the mitochondrial proton gradient. Indeed, we found once again that the ability of H460 cells to increase their respiration by 2,4-DNP was highly compromised and considerably less than that of A549 cells (Fig. 7A). This confirmed that H460 cells have a more attenuated mitochondrial respiration capacity and therefore probably lack plasticity in switching from glycolysis to mitochondrial respiration when glycolytic ATP production is abolished.

Next, we examined the effect of inhibition of mitochondrial respiration vs. uncoupling respiration from OXPHOS on intracellular ATP levels. H460 and A549 cells were incubated with 2,4-DNP and rotenone, respectively, for 45 min before measuring their ATP content. Interestingly, the cellular ATP levels remained nearly unchanged by rotenone, suggesting that glycolytic ATP synthesis was sufficient to maintain the energy balance (Fig. 7B). However, ATP content of H460 and A549 cells showed a differential sensitivity to 2,4-DNP. Intracellular ATP content of A549 cells was lowered by 40% in the presence of 2,4-DNP compared with the untreated control, whereas that of H460 cells was not significantly altered (Fig. 7B). It is known that in response to dissipating proton gradient, in this case caused by 2,4-DNP, mitochondrial ATP synthase can be reversed to hydrolyze ATP to maintain the mitochondrial membrane potential (27). Unless rapidly replenished by glycolysis, the cytoplasmic pool of ATP will be depleted under this condition. Clearly, A549 cells lack the ability to sufficiently upregulate glycolysis to counter the depletion of cytoplasmic ATP by 2,4-DNP. In contrast, the lack of ATP diminution in the H460 cells indicates a sufficient upregulation of glycolysis to meet the increased energy demand. Once again, this demonstrates the extremely high glycolysis capacity and dependency of H460 cells relative to A549 cells. To investigate the possible mechanism responsible for attenuation of mitochondrial bioenergetics in these cancer cell lines, we exposed them to an inhibitor of the F1F0-ATP synthase, oligomycin, which acts to inhibit OXPHOS. In the presence of increasing doses of oligomycin, the cellular respiration rate was reduced, while the glycolysis rate was simultaneously increased (Fig. 8,
A and B). The observed increase in the glycolytic rates was more pronounced in H460 cells than in A549 cells, consistent with their observed higher glycolytic capacity (Fig. 6). Interestingly, unlike the previously described inhibition of glycolysis with 2-DG, but similar to inhibition of mitochondrial respiration by rotenone, the cellular ATP level was effectively maintained after 45 min exposure to oligomycin. In this instance, there must be an effective compensatory upregulation of glycolysis to sustain ATP levels.

To prove that an increase in glycolytic ATP turnover compensated for the inhibition of OXPHOS by oligomycin, we performed the same set of metabolic measurements in glucose-deprived medium supplemented with 10 mM glutamine, thus forcing the cells to rely solely on glutaminolysis through OXPHOS for ATP synthesis with no possibility of glycolytic ATP generation from exogenous glucose.

In contrast to the cells assayed in glucose medium, the cellular ATP level of the glucose-deprived cells became sensitive to oligomycin and were markedly reduced (~60%) in H460 and (~40%) A549 cells (Fig. 8, C and D). These results indicate that OXPHOS is functional in these cell lines and contributes to ATP turnover; clearly, the OXPHOS capacity is compromised. This attenuation only becomes evident when the cells are under energy stress imposed by inhibition of glycolysis or glucose deprivation. These results also illustrate the essential role of glycolysis in meeting the energy requirements of these cancer cell lines. It is noteworthy that the basal ECAR level was very low, at or less than 10 μmH/min (and noisy) in the glucose-deprived medium compared with 60–80 μmH/min in glucose-supplemented medium with similar numbers of cells per well, further verifying that in these experiments the primary contribution to ECAR is lactic acid produced through glycolysis.

It is interesting to note that although dramatically reduced, the cellular ATP was not completely diminished by oligomycin in the glucose-deprived medium (Fig. 8, C and D). One possible explanation is that endogenous glycogen is mobilized...
to provide substrate for glycolytic energy production to compensate for the ATP loss caused by inhibition of OXPHOS and lack of exogenous glucose.

Since glycogenolysis converts glycogen into glucose-6-phosphate, which subsequently enters the glycolysis pathway yielding ATP, we tested this possibility by blocking glycogen synthesis downstream of glucose-6-phosphate with iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, which catalyzes nonoxidative, glycolytic ATP synthesis. The cells were incubated for 45 min in glucose-deprived medium containing 200 μM iodoacetate and 1 μM oligomycin. Indeed, under these extreme conditions of exogenous and endogenous glucose substrate deprivation and OXPHOS inhibition, intracellular ATP was completely diminished (data not shown). In fact, cells maintained in glucose-deprived medium in the presence of oligomycin died after 20 h (data not shown). This suggested that the cells eventually depleted their glycogen and thus the ATP pools, rendering them nonviable. In addition, the larger loss of ATP in H460 cells suggested that they had less glycogenolytic activity or smaller glycogen stores than A549 cells.

Mitochondrial proton leak. It is well established that mitochondrial respiration comprises coupled and uncoupled respiration. Coupled respiration represents the fraction that is used for ATP synthesis, while uncoupled respiration represents the fraction of mitochondrial respiration that is used to drive the futile cycle of proton pumping and proton leak back across the inner mitochondrial membrane. Cells or tissues derived across animal species in vitro spend up to 20% of their basal mitochondrial respiration rate to drive proton leak and the remaining 80% is coupled to ATP turnover (6). The proposed physiological function of proton leak includes heat production and prevention of oxidative stress caused by reactive oxygen species (5, 6). Uncoupled and coupled respiration was calculated for the cell lines in this study from the data in Figs. 3C, 8A, and 8B. In Fig. 3C, mitochondrial respiration of H460 and A549 cells account for ~85% and 75% of total cellular respiration, respectively. The coupled respiration and proton leak were determined using the ATP synthase inhibitor oligomycin as shown in Fig. 8. The oligomycin-sensitive respiration was ~70% and ~60% of the total cellular respiration rate in H460 and A549 cells, respectively (Fig. 8, A and B). Thus, H460 and A549 cells devoted ~82.5% and ~80%, respectively, of their mitochondrial respiration for ATP turnover and ~17.5% and 20%, respectively, for proton leak. A similar distribution of ATP turnover and proton leak was maintained in the glucose-deprived medium (Fig. 8, C and D), assuming that the nonmitochondrial respiration was similar in both glucose-containing and -deprived medium. The precise role and mechanism of proton leak in cancer cells remains elusive, although there is some evidence that an uncoupling protein (UCP)-like protein is downregulated, resulting in less proton leak and hence elevated mitochondrial membrane potential, which is a common phenomenon in cancer cells (41).

AMPK-mediated upregulation of glycolysis due to OXPHOS inhibition. Finally, we investigated the mechanism by which inhibition of mitochondrial respiration and OXPHOS may stimulate glycolysis in A549 cells. Little is known about how tumor cells maintain their energy balance under acute metabolic stress. It is well appreciated that AMP-activated protein kinase (AMPK) is a key cellular sensor of reduced energy supply and as such is activated by increases in the intracellular ratio of AMP/ATP, either by inhibiting ATP production or by accelerating ATP consumption. AMPK has been shown to be a critical mediator of increased glucose uptake and glycolysis in “glycolic” skeletal muscle in mice and astrocytes in rats (1, 2, 44). To test if AMPK was involved in stimulating glycolysis in A549 cells, we measured their glycolytic response to 2,4-DNP in the presence of the AMPK inhibitor compound C (44). Indeed, stimulation of glycolysis was prevented by preincubation for 30 min with compound C (Fig. 9). In contrast, the increase in mitochondrial respiration due to 2,4-DNP was present, albeit slightly blunted (data not shown). It should also be noted that the cells appeared to maintain a constitutive AMPK activity as preincubation of compound C also suppressed baseline respiration and glycolysis rates (before 2,4-DNP injection) by about 20% (data not shown). Taken together, these results strongly suggest that activation of AMPK is required for upregulation of glycolysis in response to metabolic stress.

DISCUSSION

Otto Warburg postulated that cancer might be caused by an increased rate of glycolysis and a concomitant impairment of mitochondrial respiration (41, 42). In this study, we examined and extended this hypothesis by clearly demonstrating bioenergetic alterations of two human cancer cell lines perturbed by pharmacological modulators of cellular energy metabolism. Cellular bioenergetics was assessed using a novel approach that enabled simultaneous monitoring of cellular respiration rate and glycolysis rate in parallel with ATP concentration determination. Our results show a correlation, in H460 and A549 cell lines, between increased glycolysis rate and attenuated mitochondrial respiration capacity. This is in agreement with the original observation made by Warburg (41) and extends this hypothesis by demonstrating a bioenergetic phenotype, characterized by a high rate of glycolysis and attenuated mitochondrial respiration capacity. The consequence of this phenotype is manifested by an inability to invoke
OXPHOS to meet cellular energy demand upon abrogation of glycolysis. In response to inhibition of mitochondrial OXPHOS, both cancer cell lines displayed a tremendous glycolytic capacity and dependency that enabled them to sustain their intracellular ATP turnover. This is consistent with a metabolic strategy that would allow cancer cells to proliferate under adverse conditions such as hypoxia. In fact, the only way that we could completely deplete intracellular ATP within a short period of time (an hour) was by depriving the cells of exogenous and endogenous glucose in the presence of OXPHOS inhibitors (Fig. 8, C and D and data not shown). This is a good example of how malignant transformation can lead to cellular addiction for a particular pathway(s), or more specifically in this case, dependency on glucose as the primary substrate for these two tumor cell lines.

The observed upregulation of glycolysis in these cells appeared to be mediated by the AMPK pathway. In addition to its reported activation in response to acute decreases in intracellular AMP/ATP ratios, AMPK has also been shown to be directly activated via phosphorylation, at residue Thr-172, by the upstream tumor suppressor LKB1 (38). LKB1 ablation studies suggested that AMPK activity is critical to meet the increased demand for energy during malignant transformation. Our results (Fig. 9) imply a role for AMPK in the upregulation of glycolysis, when cells are placed under short-term energy stress due to inhibition of mitochondrial ATP synthesis. Our findings along with the published LKB1 studies point to a central role for AMPK in both malignant transformation and tumor maintenance.

Even though Warburg observed impaired mitochondrial respiration in tumor cells and postulated it as the cause of cancer over 80 years ago, the role of mitochondria in malignant transformation is still not well understood. However, studies are rapidly emerging that show active involvement of mitochondria in malignant transformation. For example, it has been shown that mitochondrial β-F1-ATPase was downregulated in human liver, colon, renal, and breast tumors and significantly correlated with disease prognosis (8, 9, 19). In addition, reduced expression of respiratory complexes, including II, III, and IV, and deficient complex I, were demonstrated in renal cell carcinomas (39, 40). Mitochondria of HeLa cells, as well, have been shown to adapt their respiration capacity to meet their energy demands (35). The attenuated capacity for mitochondrial respiration and OXPHOS observed in this study corroborates and extends several reports demonstrating decreased expression of mitochondrial respiratory and OXPHOS enzymes in a wide spectrum of human tumor specimens (8, 9, 39, 40). We believe that this is most likely a metabolic reflection of either overall reduced enzyme activities of respiratory complexes of the mitochondria and ATP synthase, or a reduced number of mitochondria in cancer cells.

It has been suggested that reduced OXPHOS is the mechanism underlying ROS-mediated apoptotic resistance of highly glycolytic cancer cells induced by staurosporine (36). Reduced mitochondrial OXPHOS limits reverse ATP synthase-supported increases in mitochondrial potential and, consequently, reduced ROS production, leading to a decrease in apoptotic sensitivity. We would speculate that the more attenuated mitochondrial OXPHOS of H460 cell line would be more resistant to ROS-mediated apoptotic induction. Therefore, the bioenergetic properties of increased glycolysis, and attenuated respiration and OXPHOS, identified in this study, provide a metabolic basis for both tumor cell proliferation and resistance to apoptosis.

The consequences of a reduced OXPHOS capacity only became evident when the cells were put under acute energy stress. Uncoupling OXPHOS with protonophores such as 2,4-DNP not only abolishes mitochondrial ATP synthesis by uncoupling respiration from OXPHOS, but also dissipates the proton gradient, resulting in hydrolysis of glycolytic ATP via reversal of ATP synthase. This dual effect of 2,4-DNP, in contrast to the singular effect of either the ATP synthase inhibitor oligomycin, or the respiration inhibitor rotenone, leads to a rapid depletion of the cytoplasmic ATP pool unless it is countered by a substantial increase in glycolysis.

In the context of this study, the use of protonophore 2,4-DNP as a perturbing agent revealed two essential alterations of energy metabolism in the cancer cell lines studied. First, the differential respiration capacity of the two cell lines was only directly observed when the overall activity of the enzyme complexes within the mitochondrial respiratory chain was allowed to function at a maximum (Fig. 7A). This suggests that respiratory capacity, as measured by 2,4-DNP, is a more discriminative parameter of mitochondrial respiration than the basal respiration rate, which was shown to be very similar for the two cell lines (Fig. 3A). Second, the close link between attenuated mitochondrial OXPHOS and increased glycolysis was uncovered by assessing the effect of 2,4-DNP on ATP turnover. The glycolytic capacity of H460 cells was large enough for them to sustain the intracellular ATP level, and most likely their mitochondrial membrane potential as well, even in the face of rapid ATP depletion as a consequence of both abolished mitochondrial ATP synthesis and hydrolysis of cytoplasmic ATP mediated by 2,4-DNP. This was not true for A549 cells.

We believe that expanding this type of bioenergetic analysis to the entire NCI60 panel of tumor cell lines, to assess their glycolytic dependency, would allow establishment of patterns of altered cellular bioenergetics in these human cancer cell lines with diverse tissue origins. These patterns, like those demonstrated in this study, could be the basis for identifying effective anticancer compounds that target tumor-specific, metabolic changes caused by cellular or genetic alterations. In addition, our results show that cancer cells are insensitive to inhibition of mitochondrial energy metabolism alone; however, abrogation of glycolysis either pharmacologically by 2-DG or by substrate glucose deprivation sensitizes them to inhibition of mitochondrial respiration and OXPHOS. 2-DG has been used in clinical settings to sensitize tumors to radiation therapy and chemotherapy. The mechanism(s) that underlie its therapeutic effect are not well defined, although several mechanisms have been suggested, including inhibition of DNA damage and promotion of apoptosis (24). The findings of this study suggest a mechanistic explanation for 2-DG-induced sensitization to radiation therapy and chemotherapy.

In summary, we have demonstrated a unique metabolic phenotype of two human cancer cell lines by simultaneously measuring respiration and glycolysis rates as well as cellular ATP concentration in response to bioenergetic pathway per-
turbation. The bioenergetic phenotype defined in this study is characterized by an increased glycolytic rate and capacity that are intimately linked to an attenuated mitochondrial function. These metabolic alterations may confer on tumor cells the ability to promote growth, survival, invasion, and metastasis. In light of the most recent reports indicating that the tumor suppressor p53 gene regulates the balance between glycolysis and mitochondrial respiration (25), and LDH-A attenuation stimulates mitochondrial function (12), along with a rapidly growing body of evidence linking oncogenes and tumor suppressor genes to cellular energy metabolism, this type of bioenergetic analysis may prove essential to deciphering mechanisms of tumor evolution leading to the identification of tractable targets for therapeutic intervention.

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REFERENCES


