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Low¹ glucose stress decreases cellular NADH and mitochondrial ATP in colonic epithelial cancer cells: Influence of mitochondrial substrates

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Abstract

In this study, we investigated how colonic epithelial cells maintained pyridine nucleotide (NADH/NAD⁺) redox homeostasis upon acute metabolic variation imposed by glucose deprivation or supplementation with mitochondrial substrates, succinate and malate/glutamate (M/G). Our results showed that low glucose caused cellular NADH/NAD⁺ redox imbalance that diminished lactate dehydrogenase (LDH) activity and resulted in lower lactate contents. The concurrent activation of malic enzyme (ME) suggested a role for malate in preserving cellular pyruvate that remained unchanged at low glucose. Mitochondrial substrates restored cellular NADH/NAD⁺ redox homeostasis at low glucose in association with specific compartmental catabolism of mitochondrial substrates. As compared with normal glucose, M/G and low glucose promoted glycolytic ATP production but inhibited mitochondrial-derived ATP generation in association with decreased glucose availability for mitochondrial respiration. At normal glucose, succinate and M/G enhanced mitochondrial respiratory activity, but had minimal impact on mitochondrial-derived ATP production. Collectively, these results are consistent with low glucose-induced NADH/NAD⁺ redox imbalance in association with decreased aerobic glycolysis that is reversed by supplementation with M/G but not succinate.

Keywords

Glucose stress; mitochondrial substrates; cellular/compartmental NADH/NAD⁺; HT29

¹**Abbreviations:** cytP450 reductase, cytochrome P450 reductase; G6PDH, glucose-6-phosphate dehydrogenase; KHB, Krebs Henseleit-bicarbonate buffer; LDH, lactate dehydrogenase; ME, malic enzyme; NAD⁺/NADH, oxidized and reduced nicotinamid adenine dinucleotide; NADP⁺/NADPH, oxidized and reduced nicotinamid adenine dinucleotide phosphate; NADK, NAD kinase; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle

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1. Introduction

Cancer cells exhibit an increased rate of glycolysis, and produce high amounts of lactate in the presence of oxygen, a process known as “Warburg effect” [1]. The increased aerobic glycolysis occurs because cancer cells transport glucose into cells at a high rate. The oxidation of glucose in the glycolytic pathway generates pyruvate, which is preferentially catalyzed by lactate dehydrogenase (LDH) to lactate within the cytosol. Lactate formation regenerates NAD^+ and enables cancer cells to produce ATP independent of mitochondrial respiration. It was initially believed that this enhanced aerobic glycolysis results from mitochondrial dysfunction [2] but now it is well recognized that most cancer cells retain functional mitochondrial metabolism [3, 4]. Therefore, pyruvate, apart from lactate formation, translocates to the mitochondria and participates in ATP generation, a process associated with NADH derived from the tricarboxylic acid cycle (TCA). In addition, mitochondrial metabolism provides substrates for the cytosolic NADPH generation through the matrix-to-cytosol export of TCA intermediates [3, 5]. Cytosolic NADPH are produced by three main reactions: (a) glucose oxidation by the pentose phosphate pathway (PPP). Glucose 6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme in the pathway is generally up-regulated in cancer cells [6, 7]; (b) malate metabolism by NADP^+ -linked malic enzyme (ME) [3]; and (c) NAD kinase (NADK)-catalyzed NAD^+ -to- NADP^+ conversion [8]

In this study, we investigated the impact of low glucose (metabolic) stress on the redox state of NAD^+ and NADH in the absence or presence of the mitochondrial substrates, succinate or malate/glutamate, in the colon carcinoma cell line HT29. We further define the metabolic relationship between a disrupted NAD^+ and NADH redox state and the activities of NADP^+ -linked metabolic enzymes, the mitochondrial O_2 dependence of pyridine nucleotide oxidation-reduction, and aerobic glycolysis. Our results show that low glucose stress impaired cellular NADH/ NAD^+ redox homeostasis in association with compromised aerobic glycolysis as reflected by the decreased LDH activity and lower lactate contents. Notably, the increase in ME activity suggested a role for malate catabolism in maintaining pyruvate, which remained unchanged despite the metabolic stress. Indeed, malate/glutamate (M/G), but not succinate, re-established the lactate at the level of normal glucose. In addition, compartmental catabolism of succinate and M/G, modulated cellular NADH and cytosolic/mitochondrial NADH/ NAD^+ , respectively, resulting in increased mitochondrial respiratory (oxidation-reduction and the oxygen consumption) activity, and ATP production, processes potentiated by glucose status.

2. Materials and methods

2.1. Materials

The following chemicals were from Sigma Chemical Company (St. Louis, MO): lactic acid, pyruvic acid, cytochrome *c*, NAD^+ , NADH, NADP^+ , NADPH, α -ketoglutaric acid, glucose-6-phosphate, alcohol dehydrogenase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, glutamic-pyruvic transaminase, phenazine ethosulphate, Tris-HCl, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetra-zolium bromide, glutamic acid, malic acid, succinic acid, adenosine triphosphate, and proteases inhibitors. Antibiotic/antimycotic, trypsin, L-glutamine, McCoy's media were from GIBCO Corporation (Grand Isle, NY), and fetal

bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). The protein dye assay kit was purchased from BIORAD Corporation (Hercules, CA). All other chemicals were of reagent grade, and were provided by local sources.

2.2. HT29 cell culture and cell incubations

HT29 cell line, a human colon epithelial cell line derived from the adenocarcinoma of the colon of a female Caucasian [9], was purchased from American Type Culture Collection (Rockville, MD). Cells were grown in McCoy's media containing 3g/L glucose, and supplemented with: penicillin (100 units/ml), streptomycin (100 units/ml), and 10% FBS (fetal calf serum). HT29 cells were maintained at 37°C in a 5% CO₂ humidified incubator. One day prior to experimentation, confluent cells in T75 flasks were harvested and seeded at the specified densities. Unless otherwise stated, experiments were performed with cells in suspension in Krebs Henseleit-bicarbonate buffer (KHB) pH 7.4, which was previously equilibrated in a rotavapor system. The KHB contained either 3g/L or 0.2g/L glucose, which corresponded to 16.6mM or 1.1mM glucose, respectively. In some experiments, media was supplemented with 5mM of either succinate or malate/glutamate, and cells were incubated for an additional 30min.

2.3. Measurement of total (cellular) and compartmental (cytosolic and mitochondrial) pyridine nucleotide pools

Preparation of cellular and cytosolic and mitochondrial extracts—HT29 cells from confluent T75 flasks were trypsinized and resuspended (1×10^7 cells/ml) in KHB buffer, pH 7.4 containing 1.1mM or 16.6mM glucose. Upon equilibration in a rotavap system, cells were incubated for 30min with 5mM succinate or 5mM malate/glutamate [10]. For total cell extracts, 500µl aliquots (5×10^6 cells) were pipetted into 100µl PBS on top of an oil layer (silicon: mineral oil, 4:1). Then, cells were centrifuged and recovered into a 10% glycerol-KCN bottom layer. Upon sonication, the homogenate representing the total cell extract was used for HPLC measurements of pyridine nucleotides (see below).

The cytosolic and mitochondrial fractions were prepared from control cells, as previously described [11]. Briefly, 500µl aliquots (5×10^6 cells) were exposed to digitonin in order to permeabilize the plasma membrane, and then were centrifuged through an oil layer. More specifically, the Eppendorf tubes used for separation contained: a top layer of 100µl digitonin, a middle layer of silicon and mineral oil (4:1, v:v), and a bottom layer of 10% glycerol in KCN buffer. Upon centrifugation, the cytosolic and mitochondrial extracts representing the top and bottom layers, respectively, were derivatized for the pyridine nucleotide analysis (see below).

HPLC analyses—Samples (total cell extracts, or mitochondrial and cytosolic extracts) were derivatized on ice in the KCN buffer containing 0.06M KOH and 1mM bathophenanthroline-disulfonic acid to stabilize the pyridine nucleotides as nicotinamide-cyanide derivatives, as previously reported [11, 12]. Next, samples were extracted with chloroform, and DNA was removed in the lipid-free extracts by centrifugal filtration at 14,000×g (Amicon Ultra filters, Millipore Corporation, MA). The filtrates were mixed with 0.2M ammonium acetate/4% MeOH (v:v), pH 6.0, and the pyridine nucleotide derivatives

were separated on a reversed-phase C18 column (250×4.6mm), and detected at 328 nm (Gilson 118 UV/Vis detector). Pyridine nucleotides were quantified by comparison to standards, and expressed as nmol/5×10⁶ cells.

2.4. Preparation of cell extracts and assay of enzyme activities

Confluent HT29 cells were suspended (1×10⁷ cells) in KHB buffer, pH 7.4 containing 1.1mM or 16.6mM glucose, and were equilibrated at 37°C in a rotavapor system. Cells (5×10⁶ cells) were spun at 2000 rpm, then the pellets collected at this centrifugation step were taken into 500µl phosphate buffer saline (PBS), 0.1% Triton X-100, and protease inhibitors, and the cell membranes were broken by sonication. Cellular extract obtained upon centrifugation was used to measure the activity of the following cellular enzymes: NADP⁺-linked malic enzyme (ME-NADP⁺), glucose 6-phosphate dehydrogenase (G6PDH), lactic dehydrogenase (LDH), NAD⁺ kinase (NADK) and cytochrome P450 reductase using spectrophotometric enzyme-coupled assays as previously reported [11].

2.5. Lactate and pyruvate assays

Cellular levels of lactate and pyruvate were measured in neutralized perchloric acid supernatants by spectrophotometric methods adapted from Lowry and Passoneau [13]. Briefly, HT29 cells from confluent T75 flasks were suspended (1×10⁷ cells) in KHB buffer, pH 7.4 containing either 1.1mM or 16.6mM glucose; in some experiments, media was supplemented with 5mM succinate or malate/glutamate. At specific times, 100µl aliquots (1×10⁶ cells) were acidified with an equal volume of 12% perchloric acid (PCA) to precipitate proteins. The clear supernatants obtained upon neutralization of acidified extracts with 30% KOH were used for lactate and pyruvate measurements. Cellular metabolites were determined by an enzyme-coupled assay at 340nm using a Hitachi U2000 UV/Vis dual wavelength spectrophotometer [13]. The protein pellets obtained after PCA addition were solubilized in 0.1M NaOH, and used for protein quantification. Lactate and pyruvate concentrations were quantified by comparison with known standards, and were expressed as nmoles/mg protein.

2.6. ATP determination

Cellular ATP levels were measured using a luciferin/ luciferase bioluminescence Promega kit according to the manufacturer's instructions. Briefly, HT29 cells from confluent T75 flasks were trypsinized and plated (1×10⁴) in 96 well plates. The next day, media was replaced with KHB buffer pH 7.4 containing either 1.1mM or 16.6mM glucose, or supplemented with 5mM of either succinate or malate/glutamate, and cells were incubated for 30min prior to ATP measurements. Glycolytic-derived ATP was measured in the presence of antimycin A (2µM) and sodium cyanide (250µM), specific inhibitors of the mitochondrial electron transport chain. The difference between cellular ATP and the glycolytic ATP represented the mitochondria-derived ATP. The level of glycolytic ATP was also verified by the treatment of HT29 cells with 2-deoxy glucose (at 3.5× glucose concentration). After 30min, the incubation media was removed, and equal volumes of lysis buffer containing the luciferase reagent were added to the cell monolayers. After 20min incubation, luminescence signals proportional to the ATP levels were quantified by using a BMG-Fluostar Optima plate reader. All measurements were performed in four-to-eight

replicates, and were conducted for three separate experiments. ATP contents are expressed as percent relative to control (100%).

2.7. Oxygen dependence of oxidation-reduction of pyridine nucleotides

HT29 cells from confluent T75 flasks were suspended (5×10^6 /ml) in modified Gey's buffer containing 1.1mM or 16.6mM glucose. Whenever present, succinate and malate/glutamate were added to final concentrations of 5mM, and cells were pre-incubated for 30min prior to the measurements. Oxidation-reduction of cellular pyridine nucleotides was performed by dual-wavelength spectrophotometry in an AMINCO DW-2000 spectrophotometer, as previously described [14]. Briefly, cells in 5-ml buffer were kept in suspension by gentle stirring, and solution O_2 was measured using a Clark-type O_2 electrode calibrated with respect to air (21% O_2) and 100% O_2 . At the beginning of experiments, the cells were rendered anaerobic by purging them with prepurified argon, and then known volumes of 21% O_2 or 100%-saturated buffer were added using a Hamilton syringe to obtain the desired O_2 concentrations in solutions. Changes in oxidation-reduction of pyridine nucleotides from anaerobic-to-aerobic transitions were measured at the wavelength pair 340-375nm, and expressed as P_{50} values, defined as the O_2 concentrations at half maximal oxidation. The rates of O_2 consumption at $[O_2]$ at P_{50} and at saturating $[O_2]$ levels for the different metabolic conditions were calculated from the oxidation-reduction tracings, and were expressed in $nmolO_2/min/10^6$ cells.

2.8. Protein assay

Protein was measured using Bio-Rad Assay kit according to the manufacturer's protocol.

2.9. Statistical analysis

Results are expressed as mean $\pm$ SEM. Unless otherwise specified, all data was analyzed by one-way ANOVA statistical analysis with Student Newman-Keuls posttest for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cellular and mitochondrial/ cytosolic NADH/NAD⁺ redox status in HT29 cells at different glucose states and supplementation with mitochondrial substrates

Cancer cells reside on higher rate of glucose utilization to support their metabolic needs. How these cells adapt to acute variation in metabolic conditions is not completely understood. Here we used the changes in the NAD⁺ and NADH redox state to establish how colonic carcinoma cells respond to 30min fluctuations in glucose. The results in Figure 1A show that, compared with normal glucose (16.6mM), low glucose (1.1mM) significantly decreased cellular NADH, and significantly increased cellular NAD⁺ resulting in cellular NADH-to-NAD⁺ loss. Except for cytosolic NADH and NADH-to-NAD⁺ decrease, low glucose minimally changed compartmental pyridine nucleotide levels (Figure 1A). In addition, there is a minimal change in markers of mitochondrial respiratory activity, namely, the O_2 dependence of oxidation reduction of pyridine nucleotides (NAD⁺/NADH, Figure 1B) and the O_2 consumption rates (Figure 1C) in the two glucose states. Figure 1D illustrates the changes in cellular, mitochondria-derived and glycolysis-derived ATP at both

glucose states. Treatment of control cells with antimycin A and cyanide revealed that the glycolytic-derived ATP accounted for ~65% and 75% of cellular ATP at normal and low glucose, respectively, thus indicating that glycolysis is a major pathway of ATP production in HT29 cells (Figure 1D). Interestingly, previous studies have shown that the inhibition of the electron transport chain by antimycin A or cyanide readily leads to the increase in the rate of glycolysis [15, 16]. However, this process is cell-type specific [17]. Our results show that, compare with normal glucose, low glucose induced 15-20% increases in baseline glycolysis-derived ATP. In addition, basal mitochondria-derived ATP contents were consistently reduced at low glucose, i.e. 20% (Figure 1D). Collectively, these results indicate that both cytosolic as well as mitochondrial ATP are modulated by cellular glucose status.

3.2. Cellular and mitochondrial/ cytosolic NADH/NAD⁺ redox status in HT29 cells at different glucose states and supplementation with mitochondrial substrates

Since cellular/cytosolic pyridine nucleotide redox status was compromised by low glucose, we evaluated whether supplementation with mitochondrial substrates, succinate and malate/ glutamate, can prevent the cellular pyridine nucleotide loss. The results in Figure 2A show that, at normal glucose, 5mM succinate significantly decreased cellular NADH level, whereas malate/glutamate (M/G, 5mM each) increased cellular NAD⁺ concentration; there was no change in cellular NADH-to-NAD⁺ upon supplementation (Figure 2A). In addition, succinate minimally changed compartmental pyridine nucleotide contents (Figure 2B and 2C). In contrast, M/G significantly increased cytosolic and mitochondrial NADH generation resulting in an increase in NADH-to-NAD⁺ ratio in both cellular compartments (Figure 2B and 2C). Although dicarboxylic acids do not readily enter most cells [18], our results are indicative of a direct effect of supplementation on the status of cytosolic and mitochondrial pyridine nucleotides. Normally, the uptake of mitochondrial substrates at the plasma membrane occurs via sodium-dependent X_{AG}⁻ system, a process that leads to the intracellular accumulation of succinate and M/G within minutes [19, 20].

A metabolic stress (lower glucose, 1.1mM) impaired cellular NADH/NAD⁺ redox status that was partially reversed by the mitochondrial substrates (Figure 2A). Notably, 5mM succinate increased cellular NADH content and cellular NADH/NAD⁺ ratio, whereas 5mM M/G reduced cellular NAD⁺ level, minimally changing NADH level resulting in cellular NAD⁺-to-NADH increase (Figure 2A). Mitochondrial substrates had similar effects on compartmental pyridine nucleotide changes as in normal glucose (Figure 2B and 2C). However, under low glucose conditions, M/G elicited lower cytosolic but not mitochondrial NADH contents, resulting in a significantly lower NADH/NAD⁺ ratio as compared with normal glucose (Figure 2C). Collectively, these results indicate that compartmental catabolism of mitochondrial substrates result in quantitative changes in pyridine nucleotide contents.

3.3. Low glucose modulates NAD(P)⁺-dependent enzyme activities and potentiates lactate generation

To identify cellular pathways of pyridine nucleotide utilization in HT29 cells, we measured the activities of NAD(P)⁺-linked metabolic enzymes and the cellular lactate and pyruvate levels under different metabolic conditions. The results showed that 1.1mM glucose had

minimal effect on basal G6PDH, NADK, and cytP450 activities (Table 1). In exchange, LDH activity was significantly decreased whereas NADP⁺-dependent ME activity was significantly increased at low glucose (Table 1).

The results in Table 2 showed that cellular pyruvate levels were unchanged by acute metabolic stress (low glucose, 1.1mM). In comparison, lactate levels were significantly decreased at low glucose. Notably, 5mM succinate did not prevent lactate decrease associated with metabolic stress, whereas M/G (5mM) reestablished cellular lactate at the level of normal glucose (Table 2). Collectively, these results suggest a role for malate catabolism in compensating for the metabolic stress-induced decreased aerobic glycolysis in HT29 cells.

3.4. The O₂ dependence of oxidation-reduction of pyridine nucleotides: effect of low glucose and mitochondrial substrates

To evaluate how different metabolic conditions impact cellular bioenergetics in HT29 cells, we assessed markers of mitochondrial respiratory activity, namely, the O₂ dependence of oxidation-reduction of pyridine nucleotides (NAD⁺/NADH) and the O₂ consumption rates. The results in Figure 3A showed that at normal glucose the P₅₀ value, the O₂ concentration at half maximal oxidation, was 5.2μM. M/G (5mM) minimally changed the O₂ dependence tracing at normal glucose; whereas, succinate (5mM) shifted the O₂ dependence curve to the right with a P₅₀ value of 11.5μM, which is significantly higher than glucose alone. Notably, when compared with glucose alone or M/G, the O₂ dependence response by succinate elicited significance at [O₂] between 2-33μM and 7-20μM O₂, respectively (Figure 3A). The O₂ consumption was unchanged at the P₅₀ value in all conditions. However, supplementation significantly increased O₂ consumption at saturating [O₂], indicative of enhanced mitochondrial respiration by mitochondrial substrates (Figure 3B).

Surprisingly, the P₅₀ at 1.1mM glucose was 5.6μM, thus not different from that at normal glucose (Figure 3C). Neither succinate (5mM) nor M/G (5mM) mediated a significant difference in the O₂ dependence profile or in the O₂ consumption at low glucose (Figure 3C and 3D). These results indicate that mitochondrial respiratory activity is minimally affected by supplementation at low glucose.

3.5. Cellular ATP levels: the effect of glucose level and mitochondrial substrates

Figures 4A and 4B show that regardless of glucose status, mitochondrial substrates minimally changed cellular ATP levels. Notably, basal glycolytic-derived ATP contents were 10%, 15% and 27% higher at low glucose and succinate, or M/G supplementation, versus similar condition at normal glucose (Figure 4B). In contrast to glycolytic ATP, basal mitochondria-derived ATP contents were reduced at low glucose, and succinate and M/G supplementation, i.e. 15% and 51%, as compared with similar condition at normal glucose (Figure 4B). Although M/G promoted an ~40% increase in mitochondria-derived ATP, due to the high variability this increase was not significantly higher compared with normal glucose; in exchange, it reached significance versus M/G at low glucose (Figure 4A). Collectively these results are consistent with potentiation of glycolytic-and mitochondrial-dependent ATP generation by M/G at low and normal glucose, respectively.

4. Discussions

In the present study, we use the redox status of pyridine nucleotides to identify metabolic responses in HT29 colon carcinoma cells to acute changes in substrates availability. We showed that low glucose disrupted cellular NADH/NAD⁺ redox status in association with impaired glucose flux through aerobic glycolysis as reflected by decreased LDH activity and lower lactate levels and minimal change in mitochondrial respiration. While mitochondrial substrates alleviated low glucose-induced cellular pyridine nucleotide redox imbalance and potentiated aerobic glycolysis, their compartmental catabolism contributed to cellular NAD(P)H generation and stimulation of mitochondrial function at normal glucose suggesting enhanced reducing and bioenergetic capacity by the available substrates. Taken together, these results underscore the metabolic changes in colonic cancer cells to combat pyridine nucleotide redox imbalance and acute glucose deprivation-induced aerobic glycolysis impairment (Figure 5).

Cancer cells uptake high amounts of glucose from the surrounding media, and catabolized it, preferentially through aerobic glycolysis (Warburg effect) instead of mitochondrial respiration [1, 2]. The initial observation made by Warburg that cancer cells have dysfunctional mitochondria has been challenged by recent evidence that shows that many types of cancer cells preserve mitochondrial function [11], or have an enhanced mitochondrial oxidative phosphorylation compared to normal cells [21]. Our results in colonic cancer cells show that cellular NADH/NAD⁺ redox status is a function of glucose catabolism through aerobic glycolysis and mitochondrial respiration. The finding that metabolic stress impairs cellular NADH/NAD⁺ redox state, and results in decreased aerobic glycolysis with minimal effect on mitochondrial respiratory activity underscores the centrality of cytosolic glucose metabolism in cancer cells. This reduced glucose flux through aerobic glycolysis led to decreased LDH activity concurrent with lower cellular lactate, but not pyruvate levels. In addition, ME activity was stimulated by glucose stress suggesting a contribution of malate catabolism to cytosolic pyruvate and NADPH generation under reduced glucose conditions. Recently, it was shown that ME is part of an enzyme network that can contribute to formation of TCA intermediates (e.g. acetyl CoA and oxalacetate) from glutamine in glucose deprived conditions [22]. However, in our study, M/G and not succinate alleviated lactate loss at low glucose, suggesting that cytosolic catabolism of malate contributed to aerobic glycolysis in glucose depletion state. Studies from the literature show that the activities of metabolic enzymes, including LDH, pyruvate kinase in cancer cells, are modulated by phosphorylation/dephosphorylation processes [23, 24]. For example, phosphorylation of LDH-A via FGFR1-dependent tyrosine phosphorylation enhanced the enzymatic activity and promoted lactate generation in 293T cells [23]. How the activities of metabolic enzymes are modulated by glucose decrease in colon carcinoma cells remains to be established. However, we anticipate that this fast response in modulating the enzyme activities may represent a metabolic rearrangement to prevent cancer cell death if glucose depletion persists. Indeed, previous studies show that supra-physiological levels of phospho-tyrosine signaling activation associated with focal adhesion occurred within hours upon glucose withdrawal in glioblastoma, melanoma, and sarcoma cells; this signaling activation preceded ROS-mediated cell death at 24h [25].

Our results show that mitochondrial substrates had minimal effect on cellular lactate levels at normal glucose and, thereby, on aerobic glycolysis. The fact that M/G enhanced cytosolic and mitochondrial NADH/NAD⁺ redox state and increased O₂ consumption is consistent with an enhanced mitochondrial bioenergetic capacity due compartmental catabolism of substrates. Indeed, M/G had a tendency to increase mitochondria-derived ATP at normal glucose and this increase in ATP production might subsequently drive cellular biosynthesis, a response expected in glucose and substrates plentiful conditions. Although, succinate at normal glucose switched the oxidation-reduction of pyridine nucleotides to the right, and enhanced the O₂ consumption at saturating O₂ levels, it did not increase the cellular ATP production. These results indicate that succinate promoted mitochondrial respiratory activity without a corresponding increase in ATP generation, an effect that might be associated with a reduced activity of succinate dehydrogenase as it was previously reported for these cells [26]. Indeed, mitochondria isolated from glucose⁺ (Glc⁺) HT29 cells had a lower respiratory capacity with different substrates like glutamate, glutamine, malate, succinate, 2-oxoglutarate, than mitochondria from Glc⁻ HT29 cells, differentiated colon cancer cells, that was associated with lower succinate dehydrogenase and glutamate dehydrogenase activities observed in the mitochondria of the Glc⁺HT29 cells [26].

In contrast to normal glucose, M/G and low glucose caused a significantly lower cytosolic NADH and NADH/NAD⁺ ratio that were associated with an increase in aerobic glycolysis. Moreover, the glycolytic ATP in M/G and low glucose was significantly higher than similar condition at normal glucose whereas mitochondria-derived ATP was significantly lower as compare with normal glucose and M/G. These results indicate that a predominantly cytosolic catabolism of mitochondrial substrates may spare glucose for aerobic glycolysis but not for mitochondrial respiration. Notably, base-line level of aerobic glycolysis was higher at low glucose and/or mitochondrial substrates as compared with similar condition at normal glucose, indicating that glucose deficiency impairs mitochondrial metabolism and enhances cytosolic aerobic glycolysis.

Collectively, our results link cellular changes in pyridine nucleotides with metabolic alterations employed by colonic carcinoma cells to alleviate acute metabolic stress effects. The findings that available mitochondrial substrates are directed in a glucose-dependent way in three different ways either towards supplementing aerobic glycolysis at low glucose or through enhancing mitochondrial bioenergetics, or cellular reductive pathways at normal glucose demonstrate that tumor cells have enzyme-regulatory mechanisms to respond to acute variation in glucose or substrate availability. Additional studies are necessary to unravel the metabolic regulation of cancer cells to challenging conditions, such as: acute variation of metabolic substrates or the presence of anti-cancer drugs or oxidants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Glucose deprivation impairs redox status of cellular pyridine nucleotides
- Glucose stress enhances cytosolic aerobic glycolysis but not mitochondrial activity
- The increase in malic enzyme activity provides pyruvate under low glucose condition
- Cytosolic catabolism of mitochondrial substrates spares glucose for aerobic glycolysis
- Colonic carcinoma cells employ metabolic alterations to alleviate glucose stress

Figure 1. Cellular and compartmental (cytosolic and mitochondrial) NADH and NAD⁺ contents in HT29 cells: effect of glucose availability

(A) Cellular, cytosolic and mitochondrial levels of NAD⁺ and NADH contents and NADH/NAD⁺ ratio in HT29 cells at normal (16.6mM) and low glucose (1.1mM) glucose. Cells (1 × 10⁷ cells/ml) were maintained in KHB containing either 16.6mM glucose or 1.1mM glucose. Aliquots (5 × 10⁶ cells) were removed, total cell lysates were prepared, and cellular contents of NADH and NAD⁺ were determined by HPLC. Cytosolic and mitochondrial fractions were prepared by digitonin fractionation method, as presented in the Method section. Next, cytosolic and mitochondrial fractions were derivatized with KCN buffer to stabilize the pyridine nucleotides as nicotinamide-cyanide derivatives and these derivatives were separated by HPLC. Pyridine nucleotide levels were quantified by comparison with known standards. Results are mean ± SEM for 5 cell preparations. *p < 0.05 16.6mM glucose versus 1.1mM glucose. **Changes in the O₂ dependence of oxidation-reduction of pyridine nucleotides (B), O₂ consumption (C) and cellular ATP levels (D) in HT29 cells.** (B) O₂ dependence of NADH/NAD⁺ was determined in HT29 cells (5 × 10⁶ cells) in Gey's buffer containing 16.6mM glucose or 1.1mM glucose. Cells were maintained in suspension by gentle stirring, and changes in the oxidation-reduction of NADH/NAD⁺ were measured by dual-wavelength pairs of 340-375nm at different O₂ concentrations. Results are expressed as percent oxidation calculated with respect to aerobic cells, and, from the oxidation-reduction plots, a P₅₀ value representing half-maximal oxidation was calculated. Results are mean ± SEM of at least 5 cell preparations. Data was analyzed by two-way ANOVA with Bonferroni's post-test for multiple comparisons. (C) O₂ consumption rates were obtained at conditions of saturating [O₂] and at [O₂] at half-maximal oxidation (P₅₀) values. Results are

expressed as nmol/min/ 10^6 cells and are mean $\pm$ SEM of at least 5 cell preparations. **(D)** For *cellular ATP* HT29 cells (1×10^4) were seeded in 96-well plates and incubated overnight at 37°C. The next day, media was changed to KHB containing 16.6mM glucose or 1.1mM glucose. The contribution of glycolytic-derived ATP to total ATP level was determined in the presence of antimycin A (2 μ M) and sodium cyanide (250 μ M), selective inhibitors of mitochondrial respiration. Glycolytic-derived ATP was calculated by subtracting glycolytic-derived ATP from cellular contents. ATP was measured with a luciferin/ luciferase bioluminescence Promega kit according to the manufacturer's protocol. Each experiment was performed in triplicates, and ATP contents were expressed as percent relative to untreated controls (100%). *p<0.05 16.6mM glucose versus 1.1mM glucose.

Figure 2. Acute metabolic variations-induced pyridine nucleotide changes in HT29 cells
 Effect of mitochondrial substrates, and glucose states on cellular (A), cytosolic (B) and mitochondria (C) NADH/NAD⁺ redox homeostasis in HT29 cells. Cells (1×10^7 cells/ml) were maintained in KHB containing either 16.6mM glucose or 1.1mM glucose in some experiments media was supplemented with 5mM succinate (S) or 5mM malate/glutamate (M/G). After 30min incubation, aliquots (5×10^6 cells) were removed, total cell lysates were prepared, and cellular contents of NADH and NAD⁺ were determined by HPLC. Cytosolic and mitochondrial fractions were prepared by digitonin fractionation method, as presented in the Method section. Next, cytosolic and mitochondrial fractions were derivatized with KCN buffer to stabilize the pyridine nucleotides as nicotinamide-cyanide derivatives and these derivatives were separated by HPLC. Pyridine nucleotide levels were quantified by comparison with known standards. Results are mean $\pm$ SEM for 5 cell preparations. * $p < 0.05$ glucose alone versus supplementation, # $p < 0.05$ 16.6mM glucose + M/G versus 1.1mM glucose + M/G.

Figure 3. Changes in the O_2 dependence of oxidation-reduction of pyridine nucleotides and O_2 consumption in HT29 cells

O_2 dependence of $NADH/NAD^+$ was determined in HT29 cells (5×10^6 cells) in Gey's buffer containing 16.6mM glucose (**A**) or 1.1mM glucose (**C**); in some experiments 5mM succinate (S) or 5mM malate/glutamate (M/G) were added to the media. Cells were maintained in suspension by gentle stirring, and changes in the oxidation-reduction of $NADH/NAD^+$ were measured by dual-wavelength pairs of 340-375nm at different O_2 concentrations. Results are expressed as percent oxidation calculated with respect to aerobic cells, and, from the oxidation-reduction plots, a P_{50} value representing half-maximal oxidation was calculated. Results are mean $\pm$ SEM of at least 5 cell preparations. Data were analyzed by two-way ANOVA with Bonferroni's post-test for multiple comparisons.

* $p < 0.05$ glucose versus S supplementation; + $p < 0.05$ 16.6mM S versus M/G

supplementation. *Changes in O_2 consumption rates in normal (B) and low glucose (D).* The O_2 consumption rates were calculated from the oxidation-reduction tracings of mitochondrial pyridine nucleotides. O_2 consumption rates were obtained at conditions of saturating $[O_2]$ and at $[O_2]$ at half-maximal oxidation (P_{50}) values. Results are expressed as nmol/min/ 10^6 cells, and are mean $\pm$ SEM of at least 5 cell preparations. * $p < 0.05$ glucose versus + S or M/G supplementation.

Figure 4. Cellular ATP in HT29 cells at normal glucose (A) and low glucose level (B)
 HT29 cells (1×10^4) were seeded in 96-well plates, and incubated overnight at 37°C. The next day, media was changed to KHB containing 16.6mM glucose or 1.1mM glucose; whenever present, succinate and malate/glutamate were added at a final concentration of 5mM, and cells were incubated for 30min. The contribution of glycolytic-derived ATP to total ATP level was determined in the presence of antimycin A (2μM) and sodium cyanide (250μM), selective inhibitors of mitochondrial respiration. Glycolytic-derived ATP was calculated by subtracting glycolytic-derived ATP from cellular contents. ATP was measured with a luciferin/ luciferase bioluminescence Promega kit according to the manufacturer's protocol. Each experiment was performed in triplicates and ATP contents were expressed as percent relative to untreated controls (100%). * $p < 0.05$ low glucose+ M/G versus normal glucose + M/G.

Figure 5. Acute metabolic variations-induced pyridine nucleotide changes in HT29 cells
(A) At normal glucose (16.6mM), cellular NADH/NAD⁺ redox state is a function of glucose flux through mitochondrial respiration and aerobic glycolysis. Succinate (S) increases mitochondrial NADH and promotes mitochondrial respiration resulting in cellular NADH/NAD⁺ decrease. Malate/glutamate (M/G) enhances cytosolic and mitochondrial NADH and stimulates cytosolic aerobic glycolysis causing cellular NADH/NAD⁺ redox imbalance (Figure 5A). **(B)** Effect of mitochondrial substrates and low glucose on cytosolic and mitochondrial NADH/NAD⁺ redox state in HT29 cells. Compared with normal glucose, low glucose (1.1mM) and M/G potentiate mitochondrial NADH without a corresponding increase in mitochondrial respiration fact that enhances cellular NADH/NAD⁺ levels as compared with low glucose. Malate/glutamate (M/G) increases cytosolic and mitochondrial NADH promoting aerobic glycolysis and restores NADH/NAD⁺ at low glucose (Figure 5B).

Table 1**Effect of glucose exposure on activities of NAD(P)⁺-linked metabolic enzymes**

Cells (5×10^6 cells) were maintained in KHB containing either 16.6mM glucose or 1.1mM glucose. Total cell lysate was prepared, and the enzyme activities were measured, as described in the Method section. The enzyme activities are expressed as the amount of NAD⁺ or NADPH produced per minute per milligram protein. Results represent means $\pm$ SEM (n=5);

Metabolic enzymes	16.6mM Glucose	1.1mM Glucose
Lactate dehydrogenase ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	1.15 ± 0.06	$0.85 \pm 0.04^*$
Glucose 6-phosphate dehydrogenase ($\text{nmol}/\text{min}/\text{mg}$ protein)	124.4 ± 4.7	115.9 ± 4.3
NADP ⁺ -linked Malic Enzyme ($\text{nmol}/\text{min}/\text{mg}$ protein)	111.4 ± 8.4	$149.3 \pm 7.6^*$
NADPH-linked Cytochrome P450 reductase ($\text{nmol}/\text{min}/\text{mg}$ protein)	2.07 ± 0.11	1.81 ± 0.06
NAD Kinase ($\text{pmol}/\text{min}/\text{mg}$ protein)	27.6 ± 2.3	20.8 ± 1.9

*
p<0.05, 16.6mM glucose versus 1.1mM glucose.

Table 2
Effect of glucose and mitochondrial substrates on cellular pyruvate and lactate levels

HT29 cells (1×10^6 cells) were maintained for 30 min in KHB containing 16.6 mM glucose or 1.1 mM glucose; whenever present, succinate and malate/ glutamate were added at a final concentration of 5 mM. Cellular levels of lactate and pyruvate were measured as described in the Method section. Results are expressed as nmol/mg protein, and are mean $\pm$ SEM of 5 cell preparations;

Cellular metabolites (nmol/mg protein)	16.6mM Glucose +/- substrates				1.1mM Glucose +/- substrates			
	Basal	Succinate	Malate/ Glutamate		Basal	Succinate	Malate/ Glutamate	
Pyruvate	36.9 $\pm$ 2.5	36.9 $\pm$ 2.7	32.2 $\pm$ 1.1		29.8 $\pm$ 1.8	32.5 $\pm$ 1.1	33 $\pm$ 1.5	
Lactate	162.8 $\pm$ 7.6	185.5 $\pm$ 12.6	172.2 $\pm$ 10		124.2 $\pm$ 7.5 *	137.4 $\pm$ 14.1 *	148.7 $\pm$ 10	

* p<0.05 16.6mM glucose versus 1.1mM glucose.