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Low 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.

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

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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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, McCoys' 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 3 g/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% CO_2 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 3 g/L or 0.2 g/L glucose, which corresponded to 16.6 mM or 1.1 mM glucose, respectively. In some experiments, media was supplemented with 5 mM 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 1.1 mM or 16.6 mM glucose. Upon equilibration in a rotavap system, cells were incubated for 30min with 5 mM succinate or 5 mM malate/glutamate [10]. For total cell extracts, 500 μl aliquots (5×10^6 cells) were pipetted into 100 μl 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, 500 μl 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 100 μl 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 0.06 M KOH and 1 mM 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 0.2 M ammonium acetate/4% MeOH (v:v), pH 6.0, and the pyridine nucleotide derivatives were separated on a reversed-phase C18 column (250 × 4.6 mm), and detected at 328 nm (Gilson 118 UV/Vis detector). Pyridine nucleotides were quantified by comparison to standards, and expressed as nmol/$5*10^6$ cells.

2.4. Preparation of cell extracts and assay of enzyme activities

Confluent HT29 cells were suspended (1×10^7 cells) in KHB buffer, pH 7.4 containing 1.1 mM or 16.6 mM glucose, and were equilibrated at 37 °C in a rotavapor system. Cells (5×10^6 cells) were spun at 2000 rpm, then the pellets collected at this centrifugation step were taken into 500 μl 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 (1×10^7 cells) in KHB buffer, pH 7.4 containing either 1.1 mM or 16.6 mM glucose; in some experiments, media was supplemented with 5 mM succinate or malate/glutamate. At specific times, 100 μl aliquots (1×10^6 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 340 nm using a Hitachi U2000 UV/Vis dual wavelength spectrophotometer [13]. The protein pellets obtained after PCA addition were solubilized in 0.1 M 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 (1×10^4) in 96 well plates. The next day,