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# Diquat-induced cellular pyridine nucleotide redox changes and alteration of metabolic enzyme activities in colonic carcinoma cells

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ABSTRACT

Previously we have shown that the redox cycler menadione (MQ) induced cellular pyridine nucleotide redox imbalance that was linked to a decrease in aerobic glycolysis and perturbation of the mitochondrial respiratory activity due to the redox cycling of the compound; these processes were potentiated by low glucose. In this study, we investigated how colonic epithelial cells maintained pyridine nucleotide (NAD$^+$/NADH and NADP$^+$/NADPH) redox homeostasis upon acute metabolic variation and exposure to the redox cycling diquat (DQ). Our results show that DQ challenge disrupted cellular NADH/NAD$^+$ redox status and enhanced cellular NADPH generation. Notably, DQ-induced NADH decrease was associated with enhanced lactate production, a process that was potentiated by glucose availability, but not by the mitochondrial substrates, succinate or malate/glutamate. In addition, DQ increased glucose 6-phoshate dehydrogenase (G6PDH) activity consistent with glucose diversion towards pentose phosphate pathway. As a consequence, steady-state NADPH levels were maintained during MQ challenge at normal glucose. In contrast and despite increased G6PDH and malic enzyme (ME) activities, DQ induced cellular NADPH-to-NADP$^+$ shift at low glucose, a situation that was reversed by mitochondrial substrates. Collectively, these results are consistent with increased aerobic glycolysis by DQ and specific metabolic changes leading to enhanced NADPH generation upon oxidative challenge.



## 1. Introduction

Cellular pyridine nucleotides comprising reduced and oxidized nicotinamide adenine dinucleotide (NADH/NAD$^+$) and reduced and oxidized nicotinamide adenine dinucleotide phosphate (NADPH/NADP$^+$) participate in redox-dependent processes associated with ATP (adenosine triphosphate) production and biosynthetic and detoxification reactions, respectively. As such, the NADH/NAD$^+$ couple transfers electrons to the mitochondrial respiratory chain supporting ATP production, while the NADPH/NADP$^+$ couple supplies reducing equivalents for cellular biosynthesis or for the redox pathways containing glutathione (GSH) or thioredoxins [1,2]. Based on the rates of electron transfer, the electron transfer via NADH-utilizing mitochondrial respiratory chain is regarded as a high-flux electron transfer pathway [3]. In comparison, the NADPH-utilizing redox pathway can transfer electrons at much lower rates being viewed as a low-flux circuit [3]. Notably, perturbation in the cellular NADH or NADPH status under different conditions is predicted to disrupt cellular ATP generation and the NADPH-dependent reactions involved in cellular biosynthesis or detoxification processes that are associated with the high-flux (NADH) or the low-flux (NADPH) electron transfer circuits, respectively [3].

In this study, we investigated how cellular pyridine nucleotide redox homeostasis is maintained in colonic epithelial cells

*Abbreviations:* ATP, adenosine triphosphate; ASC, alanine, serine, cysteine, sodium dependent transport system; cytP450R, cytochrome P450 reductase; DQ, diquat dibromide or 1,1'-ethylene-2,2'-bipyridylum; G6PDH, glucose-6-phosphate dehydrogenase; Glc, glucose; GSH, glutathione; HPLC, high performance liquid chromatography; KHB, Krebs Henseleit-bicarbonate buffer; KCN, potassium cyanide; KOH, potassium hydroxide; LDH, lactate dehydrogenase; M/G, malate/glutamate; ME, malic enzyme; MeOH, methanol; MQ, menadione; NAD$^+$/NADH, oxidized and reduced nicotinamid adenine dinucleotide; NADP$^+$/NADPH, oxidized and reduced nicotinamid adenine dinucleotide phosphate; NADK, NAD kinase; NaOH, sodium hydroxide; $O_2^{\cdot-}$, superoxide radical; PCA, perchloric acid; PPP, pentose phosphate pathway; PBS, phosphate buffer saline; S, succinate; TCA, tricarboxylic acid cycle.

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exposed to acute metabolic variations and to diquat, a redox cycling compound. Recent studies from our laboratory found that the redox cycler menadione disrupted cellular pyridine nucleotide redox status in association with the impairment of cellular bioenergetics and the decrease of NADPH-dependent reducing capacity in HT29 cells; all these processes were exaggerated at low glucose status [4]. In this study, we used diquat (1,1'-ethylene-2,2'-bipyridylum) (Fig. 1A), a herbicide and an environmental pollutant that, as opposed to MQ, is minimally eliminated by intracellular catabolism [5,6]. Cellular NAD(P)H-dependent reductases, such as, microsomal cytochrome P450 reductase (cytP450R), catalyze one electron reduction of DQ, leading to the formation of DQ radical. In the presence of oxygen, DQ radical forms superoxide anion and regenerates the parent compound (Fig. 1B) [7,8]. Therefore, DQ-induced redox cycling activity would impose a constant demand for cellular NAD(P)H generation or for $NADP^+$ reduction. Intracellularly, the pentose phosphate pathway and the first enzyme, glucose 6-phosphate dehydrogenase, are major contributors to cellular NADPH from glucose. Other cellular mechanisms responsible for NADPH generation are the malate catabolism via malic enzyme and the cytosolic phosphorylation of NAD(H) by NAD kinase, NADK (Fig. 1B) [9]. Cellular catabolism of glucose also generates NADH that can be oxidized by enzymes like lactate dehydrogenase, LDH, a central reaction essential for aerobic glycolysis and ATP production in cancer cells [10,11].

Our results show that an oxidative stress imposed by DQ mediated cellular $NAD^+$/NADH redox imbalance and switched glucose utilization towards NADPH generating processes. Disruption of cellular $NAD^+$/NADH redox occurred in conjunction with enhanced lactate production, a process that was dependent on glucose availability but not on mitochondrial substrates. The increased G6PDH activity that maintained steady-state NAPDH levels upon DQ challenge at normal glucose level but not at low glucose is consistent with enhanced glucose-dependent NADPH generation via PPP, pentose phosphate pathway. Despite an additional increase in ME activity, DQ promoted cellular NADPH-to-$NADP^+$ oxidation at low glucose, a situation reversed by mitochondrial substrates. These collective results showed that DQ potentiated aerobic glycolysis and indicated that colonic carcinoma cells employed specific metabolic changes to alleviate oxidant-induced NADPH decrease.

## 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, diquat dibromide, 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, and 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

Colonic intestinal cells are good models for studying the effects of oxidant challenge since intestinal epithelium is often exposed to the environmental toxicants or to the chemotherapy drugs. In this study, we used HT29 cell line (American Type Culture Collection, Rockville, MD), a human colon epithelial cell line derived from the adenocarcinoma of the colon of a female Caucasian [12]. 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. 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 that was previously aerated 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, and defined as normal and low glucose, respectively. In some experiments, media was supplemented with 5 mM of either succinate (S) or malate/glutamate (M/G), and cells were incubated for 30min prior to DQ addition. In all experiments, DQ was used at a final concentration of 50 μM, and cells were incubated for various periods of time.

### 2.3. DQ treatment for measurement of total (cellular) pyridine nucleotide pools

HT29 cells from confluent T75 flasks were trypsinized and resuspended ($1 \times 10^7$ 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 exposed either directly to 50 μM DQ or were pre-incubated for 30min with 5 mM succinate or 5 mM malate/glutamate prior to DQ treatment [13]. Our rotavap system consisted of a rotary evaporator (Buchi RSB/40-50-5, Flawil, Switzerland) equipped with an "udder" attachment, and adjusted to rotate through a heated water bath (Precision Water Bath Model 184, Precision Scientific Incorporated, Chicago, IL). The samples were placed in round bottom flasks and attached to the spider attachment. At designated times, 500 μl aliquots ($5 \times 10^6$ 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 (high performance liquid chromatography) measurements of cellular pyridine nucleotides (see below).

*HPLC analyses.* As previously reported, samples were derivatized on ice in the KCN buffer containing 0.06M KOH and 1 mM bathophenanthroline-disulfonic acid to stabilize the pyridine nucleotides as nicotinamide-cyanide derivatives [4,14]. 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 (methanol) (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). The mobile phase consisted of 0.2M ammonium acetate and HPLC-grade MeOH at a flow rate of 1 ml/min. Cellular 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

HT29 cells ($1 \times 10^7$ cells) were suspended 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 were exposed to DQ for 30min, and 500 μl aliquots ($5 \times 10^6$ cells) were spun at 2000 rpm to remove the media containing DQ. 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