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Preferential utilization of NADPH as the endogenous electron donor for NAD(P)H:quinone oxidoreductase 1 (NQO1) in intact pulmonary arterial endothelial cells

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Abstract

The goal was to determine whether endogenous cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1) preferentially utilizes NADPH or NADH in intact pulmonary arterial endothelial cells in culture. The approach was to manipulate the redox status of the NADH/NAD^+ and NADPH/$NADP^+$ redox pairs in the cytosolic compartment using treatment conditions targeting glycolysis and the pentose phosphate pathway alone or with lactate, and to evaluate the impact on the intact cell NQO1 activity. Cells were treated with 2-deoxyglucose (2-DG), iodoacetate (IOA) or epiandrosterone (EPI) in the absence or presence of lactate, NQO1 activity was measured in intact cells using duroquinone (DQ) as the electron acceptor and pyridine nucleotide redox status was measured in total cell KOH extracts by high performance liquid chromatography (HPLC). 2-DG decreased the NADH/NAD^+ and NADPH/$NADP^+$ ratios, by 59% and 50%, respectively, and intact cell NQO1 activity by 74%; lactate restored NADH/NAD^+, but not NADPH/$NADP^+$ or NQO1 activity. IOA decreased NADH/NAD^+ but had no detectable effect on NADPH/$NADP^+$ or NQO1 activity. EPI decreased NQO1 activity by 67%, and while EPI alone did not alter NADPH/$NADP^+$ or NADH/NAD^+, when the NQO1 electron acceptor DQ was also present, NADPH/$NADP^+$ decreased by 84% with no impact on NADH/NAD^+. DQ alone also decreased NADPH/$NADP^+$ but not NADH/NAD^+. The results suggest that NQO1 activity is more tightly coupled with the redox status of the NADPH/$NADP^+$ than NADH/NAD^+ redox pair, and that NADPH is the endogenous NQO1 electron donor. Parallel studies of pulmonary endothelial transplasma membrane electron transport (TPMET), another redox process that draws reducing equivalents from the cytosol, confirmed previous observations of a correlation with the NADH/NAD^+ ratio.

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Keywords

pyridine nucleotides; pulmonary endothelial cells; lung; quinone; NAD(P)H:quinone oxidoreductase; HPLC; phase II enzymes

Introduction

NAD(P)H:quinone oxidoreductase (NQO1) is a predominately cytosolic (>90%) two-electron quinone reductase ubiquitously distributed in a wide range of tissue and cell types including the pulmonary endothelium and other lung cell types [1–11]. NQO1 catalyzes reduction of a broad range of physiological, pharmacological and toxicological quinones and other redox active substances, wherein it may promote either protective or toxic effects, depending on the chemical properties of the substrate and product formed [2,7,11]. NQO1 has been implicated in protection from carcinogenesis, superoxide scavenging, modulation of intracellular redox status, cellular metabolism and cancer cell growth, signaling mediated by tumor necrosis factor and other inflammatory mediators, regeneration of antioxidants and other processes [6,7,12–19].

Given its role in protection and activation of quinones and other redox active compounds, the relatively high NQO1 expression in endothelium and epithelium is perhaps not surprising given that they represent cell types in direct contact with airborne or blood-borne xenobiotics [10]. In this regard, the pulmonary endothelium is of particular importance because of its large surface area and position in the circulation. These characteristics confer on pulmonary endothelial and other lung cell enzymes the ability to influence the chemical composition of the plasma, including the redox status and disposition of redox active compounds, during passage of the blood from the venous to the systemic arterial circulations [5,20–23]. For example, when duroquinone (DQ) is introduced into the pulmonary arterial inflow of the perfused rodent lung, or into the extracellular medium of pulmonary arterial endothelial cells in culture, durohydroquinone (DQH_2) is generated in the perfusate and extracellular medium, respectively, with the effect in this case attributable predominately to NQO1 [4,5,9,24]. The implication is that pulmonary endothelial NQO1 provides a means for altering the bioactivity of cell membrane permeant NQO1 substrates not only within the lung, but also within the blood and downstream vessels and organs. Another NQO1 function with potential particular significance to the pulmonary endothelium, given its high sensitivity to oxidative stress, is its superoxide scavenging activity [6,12].

Despite the attention that has been focused on identification of NQO1 substrates and products in various cells and tissues, and the fact that it is well known that isolated NQO1 oxidizes NADH and NADPH virtually indiscriminately, the question of NQO1 electron donor specificity, if any, within intact cells and tissues has not been investigated [1,3]. The importance of identifying the donor relates to the capacity of the pulmonary endothelium to regenerate the required donor in oxidative stress or other pathophysiological conditions in which there may be a high competing demand for NAD(P)H and/or $NAD(P)^+$ to sustain both NQO1 activity and that of other protective mechanisms, e.g., NADPH for glutathione reductase or NAD^+ for poly-ADP ribose polymerase (PARP).

Therefore, the objective of the present study was to evaluate whether an electron donor preference for NQO1 activity could be observed in intact pulmonary arterial endothelial cells, and if so, whether NADH or NADPH could be identified as the donor. To address this question, we manipulated cellular pyridine nucleotide redox status of pulmonary arterial endothelial cells using metabolic inhibitors and treatment conditions directed at glycolysis and the pentose phosphate pathway. We also targeted pyridine nucleotide redox status using lactate, which

represents a physiological modulator of the cell redox environment. Intracellular NADH, NAD^+, NADPH and $NADP^+$ were quantified by KOH extraction of the cells followed by high performance liquid chromatography (HPLC), and intact cell NQO1 activity was measured using DQ as the electron acceptor, as we have previously described [4,24]. Parallel studies were carried out with the TPMET thiazine electron acceptor, toluidine blue O polymer (TBOP), to provide a basis of comparison with an established NADH dependent redox process, and to extend our previous observations of this pulmonary endothelial redox function [21,23,25,26].

Materials and Methods

Materials

Potassium hexacyanoferrate (III) (hereafter referred to as $K_3Fe(CN)_6^{3-}$ or ferricyanide), 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone, hereafter referred to as DQ), 2-deoxyglucose (2-DG), iodoacetic acid sodium salt (IOA), epiandrosterone (EPI), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), dicumarol, L-(+)-lactic acid sodium salt (lactate), ATP and other chemicals unless otherwise noted were purchased from Sigma Chemical (St. Louis, MO). Trypsin, penicillin-streptomycin and RPMI 1640 tissue culture medium and fetal calf serum were from Invitrogen (Grand Island, NY). Biosilon beads were from Nunc (Roskilde, Denmark). NAD^+, $NADP^+$, NADPH and NADH standards for HPLC were purchased from Boehringer Mannheim (Indianapolis, IN). The NQO1 inhibitor ES936 was the kind gift of Dr.'s David Siegel and David Roth (School of Pharmacy, University of Colorado Health Sciences Center, Denver, CO).

Endothelial cell culture

Bovine pulmonary arterial endothelial cells were isolated from segments of calf pulmonary artery obtained from a local slaughterhouse, and cells between passages 4 and 20 were cultured to confluence on Biosilon microcarrier beads (mean diameter 230 μm; surface area 255 cm^2/gm beads) in magnetic stirrer bottles (Techne Inc., Burlington, N.J.) containing RPMI 1640 medium supplemented with 20% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 30 mg/ml L-glutamine as previously described [26]. The cells were 99.5% positive for DiO-acetylated-low density lipoprotein (Biomedical Technologies) uptake as measured by fluorescence activated cell sorting and exhibited cobblestone morphology as observed by phase contrast microscopy.

Treatment Conditions

Approximately 0.3 - 0.2 ml packed volume of cell coated beads were aliquoted from the stirred culture flasks into spectrophotometric cuvettes or conical-bottomed centrifuge tubes. After the cell coated beads had settled, they were washed three consecutive times by resuspension in 3 ml of Hank's Balanced Salt Solution (HBSS) containing 10 mM HEPES, pH 7.4 (HBSS/HEPES), allowing the beads to settle between each wash. The cell coated beads were resuspended in 3 ml of HBSS/HEPES only (control) or HBSS/HEPES containing the inhibitor or lactate treatments. Except when specified the HBSS/HEPES contained 5.5 mM glucose. For the spectrophotometric studies, the treatment conditions were as follows: 5 mM lactate; 10 mM 2-DG in glucose-free buffer; 10 mM 2-DG and 5 mM lactate in glucose-free buffer; 0.4 mM IOA; 0.4 mM IOA and 5 mM lactate; 0.03 mM EPI; 0.03 mM EPI and 5 mM lactate; 10 μM dicumarol or 0.05 μM ES936. For the HPLC studies, the treatments were as follows: 5 mM lactate; 10 mM 2-DG in glucose-free buffer; 10 mM 2-DG and 5 mM lactate in glucose-free buffer; 0.4 mM IOA; 0.4 mM IOA and 5 mM lactate; 0.03 mM EPI; 0.03 mM EPI and 5 mM lactate; 0.05 mM DQ; 0.05 mM DQ and 0.03 mM EPI. The cells were incubated in the treatment media by mixing on a Nutator mixer at 37°C for 10 min before measurements of NQO1 and

TPMET activities or pyridine nucleotide and ATP concentrations. After the 10 min mixing period the buffer was removed from the cuvettes and saved for measurement of LDH activity.

Toluidine blue-O polymer

To prepare the toluidine blue-O polymer (TBOP), toluidine blue-O was incorporated in an acrylamide polymer by copolymerization of toluidine blue-O-methylacrylamide and acrylamide as previously described [23]. Polymer chains of less than 3.5KDa were removed by dialysis through a 3.5 KDa cutoff membrane. The amount of reducible TBO^+ per unit mass of the TBO polymer ($TBOP^+$) was approximately 17 nmoles/mg [25].

Protocol for measuring TPMET and NQO1 activity in intact cells

DQ or $TBOP^+$ mediated reduction of the cell membrane impermeant secondary electron acceptor, ferricyanide, were used as measures of NQO1 and TPMET activities, respectively, as previously described [4,23–25]. Following the 10-min treatment period described in *Treatment Conditions*, the cell coated beads were resuspended in 3 ml of fresh HBSS/HEPES containing 600 μM ferricyanide in the absence or presence of DQ (50 μM) or TBOP (0.2 mg/ml) with the same treatments to which the cells were exposed during the previous 10-min treatment incubations. The suspensions were mixed on a Nutator mixer at 37°C, and periodically the mixing was stopped, the cell coated beads allowed to settle at the bottom of the spectrophotometric cuvettes out of the spectrophotometer light path, and the absorbance of ferricyanide in the medium measured at 421 nm using a Beckman Model DU 7400 spectrophotometer.

The amount of the ferricyanide reduction product, ferrocyanide ($K_4Fe(CN)_6^{4-}$) in each sample was calculated from the decrease in ferricyanide absorbance at each time point (extinction coefficient = 1.0 $mM^{-1}\cdot cm^{-1}$). DQ or $TBOP^+$ mediated ferricyanide reduction rates were determined from linear regression fits of the individual ferricyanide versus time curves [4,23, 24]. The background rates of cell mediated ferricyanide reduction in the absence of DQ or TBOP were subtracted from the individual rates obtained in their presence, normalized to the cell protein, and then combined to obtain mean rates. The DQ or $TBOP^+$ reduction rates were calculated as one-half the zero order ferricyanide reduction rates in the presence of the electron acceptors. The underlying assumptions are that DQ and durohydroquinone (DQH_2) are freely permeable to intracellular sites of reduction and oxidation, respectively, whereas $TBOP^+$ is reduced at the cell surface via transplasma membrane electron transport (TPMET) [4,23,24]. In addition, ferricyanide reduction by DQH_2, and the reduced form of $TBOP^+$, TBOPH, is virtually instantaneous on the time course of the experiments, such that ferricyanide acts as a sink for the DQH_2 or TBOPH generated by the cells [4,23,24]. This approach is assumed to minimize the confounding contribution of cell mediated DQH_2 or TBOPH oxidation to the measurement, thereby allowing for an estimate of reduction rate.

HPLC measurements of intracellular pyridine nucleotides and ATP

Following the 10 min treatments described in *Treatment Conditions* above, the cell coated beads were allowed to settle and the experimental medium was removed. The details of the KOH extraction procedure and the HPLC separation method have been previously described [25]. The only difference for the present study was the HPLC equipment and software, which was composed of a binary pump (Beckman model 126 solvent module), autosampler (Beckman model 508), diode array detector (Beckman Coulochem II), a helium degasser for the mobile phase and Beckman 32 Karat chromatography software. Quantification was by peak area against standard curves generated by subjecting the standards to the same extraction treatment as the cells. The standard curves for all pyridine nucleotides and ATP were linear in the concentration range measured in the cell extracts. At the end of each experiment, the

microcarrier beads were dried and weighed to determine the cell culture surface area, providing a means for normalizing data between experiments.

NQO1 and glucose-6-phosphate dehydrogenase (G-6-PDH) activities in cytosol fractions

Cytosol-enriched fractions from untreated cells were obtained by sonication (3 pulses of 15 sec each with the power output set to 6 watts using a Microson Ultasonic Cell Disrupter, Farmington, NY) of ~ 0.2 ml of packed cell-coated beads on ice in 1 ml of 2 mM HEPES buffer, pH 7.4. The lysates were centrifuged for 30 min at 10,000 × g at 4°C to obtain a cytosol-enriched fraction.

NQO1 activity was determined by measuring the reduction rate of 2,6-dichlorophenolindophenol (DCPIP; 50 μM) spectrophotometrically at 600 nm (extinction coeffiecient 21.0 $mM^{-1}{\cdot}cm^{-1}$) following the addition of cell cytosol fraction (~10 μg protein) to a reaction mixture containing 25 mM Tris-HCl, 0.02% bovine serum albumin, 0.01% Tween 20, 5 μM flavin adenine nucleotide (FAD) and 200 μM NADPH, pH 7.4, as had been previously described [4]. The difference between the reaction rates in the absence and presence of 10 μM dicumarol was used to calculate the NQO1 activity. G-6-PDH activity was determined by measuring the rate of NADPH generation from $NADP^+$ spectrophotometrically at 340 nm (extinction coefficient 6.22 $mM^{-1}{\cdot}cm^{-1}$) at 25°C following the addition of cytosol-enriched fraction (~75 μg protein) to a reaction mixture containing 55mM Tris-HCl (pH 7.4), 3.3 mM $MgCl_2{\cdot}6H_2O$, 3.3 mM glucose-6-phosphate (G-6-P) or 3.3 mM 2-deoxyglucose-6-phosphate (2-DG-6-P) and 2.0 mM $NADP^+$ [27]. NQO1 and G-6-PDH activities in cytosol-enriched fractions were also determined in the presence of 0.1 mM and 0.03 mM EPI, respectively. The reaction rates were normalized to the protein content of the cytosol-enriched fractions as measured by the Bio-Rad protein assay.

Additional measurements

Lactate dehydrogenase (LDH) activity in the treatment medium and in the cells at the end of each experimental protocol for the spectrophotometric studies was determined as previously described [26]. The extracellular medium was removed from the cells, the cells were sonicated on ice, and LDH activity in the extracellular medium and the cell lysate was measured. The fraction of the total cell LDH released into the cell medium for all studies combined was 2.0 ± 0.1 and 2.5 ± 0.1% (mean ± SEM) during the 10-min treatment and the 30-min DQ- and $TBOP^+$-ferricyanide reaction periods, respectively. For all experiments for control and treated cells (n = 198), no significant differences in LDH release between control and the treatment conditions (Kruskal-Wallis one way ANOVA based on ranks, ($p > 0.05$) were detected.

To normalize the data for comparisons between studies, the protein content of the cells in each experiment was measured using the BioRad protein reagent as previously described [26]. NQO1 and TPMET activities are expressed as nmol of DQ or $TBOP^+$ reduction per min per mg protein ($nmol{\cdot}min^{-1}{\cdot}mg^{-1}$ protein). Pyridine nucleotide and ATP concentrations are expressed as pmol per cm^2 cell surface area. One cm^2 of cell surface area for endothelial cells cultured on the Biosilon microcarrier beads is equivalent to 29.4 ± 0.5 μg (mean ± SE) protein, ~1.7 mg of DNA and ~128,000 cells [25].

Statistical Analysis

Results are expressed as mean ± standard error (mean ± SEM). Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA). Differences between groups were evaluated using the unpaired *t*-test, one-way analysis of variance (one way ANOVA) followed by the Tukey post hoc test or when appropriate, the Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's post hoc test. Statistical significance was assumed at $p < 0.05$.

Results

Figure 1 illustrates the approach used to measure NQO1 and TPMET activities in the intact pulmonary arterial endothelial cells. The cell membrane impermeant secondary redox indicator, ferricyanide, was added to the medium surrounding the cells in the presence of DQ to measure NQO1 (Figure 1A) or $TBOP^+$ to measure TPMET (Figure 1C) activity. Also included were controls in which ferricyanide only was added to the medium surrounding the cells (Figures 1A and 1C). There was little detectable reduction of ferricyanide to its ferro form when ferricyanide only was present, indicating that the ferricyanide reduction observed in the presence of DQ or TBOP is secondary to cell mediated generation of durohydroquinone (DQH_2) or the reduced form of $TBOP^+$, TBOPH, respectively (Figures 1A and 1C), as we have previously described [4,23,24]. Under these reaction conditions, DQ and $TBOP^+$ are continually regenerated and ferricyanide reduction remains zero order until the ferricyanide is exhausted, as expected of a secondary electron acceptor that is essentially irreversibly reduced and does not influence the primary reduction rate.

Figures 1A and 1B also show that dicumarol, a competitive NQO1 inhibitor, and ES936, an irreversible NQO1 inhibitor, largely blocked DQ reduction, consistent with previous observations that DQ acts as predominately as an NQO1 electron acceptor in intact pulmonary arterial endothelial cells [4,19,24]. In contrast to its effects on DQ reduction, dicumarol had no appreciable effect on $TBOP^+$ reduction (Figures 1C and 1D). Together with the fact that, because of its size, TBOP is unable to enter cells in the oxidized or reduced form, these observations emphasize the utility of cell mediated DQ and $TBOP^+$ mediated ferricyanide reduction rates for non-destructive measurement of NQO1 and TPMET activities, respectively, in intact pulmonary endothelial cells [23].

Figures 2A and B show the effects of the treatment conditions on NQO1 and TPMET activities, respectively. 2-DG inhibited both NQO1 and TPMET activities whereas IOA preferentially inhibited TPMET, and EPI preferentially inhibited NQO1.

Figures 3A and B show the effects of the treatment conditions on the total KOH extractable cellular $NADPH/NADP^+$ and $NADH/NAD^+$ ratios. 2-DG decreased both the $NADPH/NADP^+$ and the $NADH/NAD^+$ ratios (Figures 3A and B) and IOA depressed the $NADH/NAD^+$ but not the $NADPH/NADP^+$ ratio (Figures 3A and B). EPI by itself did not affect the redox status of either pyridine nucleotide pair (Figures 3A and B), which was somewhat unexpected due to the marked effect of EPI on NQO1 activity (Figure 2A). However, the effect of DQ itself to depress the $NADPH/NADP^+$ ratio was more pronounced in the presence of EPI (DQ + EPI) (Figure 3A). Notably, the effects of DQ without or with EPI were specific to the $NADPH/NADP^+$ redox pair since there was no corresponding decrease in the $NADH/NAD^+$ ratio in either condition (Figure 3B).

The data in Figures 2 and 3 suggested that NQO1 activity was reflective of the cytosolic $NADPH/NADP^+$ redox status, whereas TPMET was more highly associated with the cytosolic $NADH/NAD^+$ ratios, the latter being consistent with previous studies [25]. If this were indeed the case, we would predict that treatment of the cells with lactate, which is reduced to NADH via lactate dehydrogenase, would overcome the effects of 2-DG and IOA on pulmonary endothelial $NADH/NAD^+$ redox status and TPMET activity, as observed in previous studies [25], but not the effects of 2-DG or EPI on $NADPH/NADP^+$ redox status or the NQO1 activity. As seen in Figures 4A and B, the addition of lactate to the 2-DG or IOA treatments overcame the inhibitor effects on both $NADH/NAD^+$ redox status and $TBOP^+$ reduction rate (i.e. TPMET activity). However, lactate could not overcome the effect of 2-DG on the DQ reduction rate (i.e., NQO1 activity), and the $NADPH/NADP^+$ ratio was not significantly different in the presence of 2-DG or 2-DG + lactate (Figures 5B and A, respectively).

The oxidized and reduced pyridine nucleotide concentrations used to calculate the NAD(P)H/$NAD(P)^+$ ratios in Figures 3–5, along with the ATP concentrations for each treatment condition are shown in Table 1.

Figure 6A and B show that EPI had the intended effect insofar as it inhibited G-6-PDH but not NQO1 activity as measured in cell cytosol fractions. Also shown in Figure 6B is that G-6-PDH activity in cell cytosol fractions was much lower when the substrate was 2-DG-6-P, the product of hexokinase catalyzed 2-DG phosphorylation, than with the physiological substrate, G-6-P (control). To emphasize this point, Figure 6B shows that 2-DG-6-P synergizes with EPI to inhibit G-6-PDH.

Discussion

The goal of the present study was to determine whether endogenous NQO1, which is predominately (>90%) located in the cell cytosol, exhibits a propensity for preferential oxidation of NADH or NADPH in intact pulmonary endothelial cells. The general strategy was to manipulate cytosolic redox status using treatment conditions directed at the glycolytic and pentose phosphate pathway, and lactate, and to evaluate the effects on pyridine nucleotide redox status and NQO1 activity. Although the treatments were anticipated to have secondary, unintended side-effects, the concept was that by using more than one treatment condition directed at each pyridine nucleotide redox pair, the combined data would reveal a preference of intact pulmonary endothelial cell NQO1 for endogenous NADH or NADPH.

The pyridine nucleotide measurements were of total cell KOH-extractable cellular pyridine nucleotides, with no distinction between protein-bound and free forms or information regarding intracellular compartmentation (e.g., cytosolic or mitochondrial). The supposition was that insofar as the treatment conditions were directed at glycolysis and the pentose phosphate pathway, or included lactate, the cytosolic compartment was a key contributor to the observed changes in total cell oxidized/reduced pyridine nucleotide ratios.

The common effects of the treatment conditions on the $NADPH/NADP^+$ ratios and the reduction rates of the NQO1 electron acceptor, DQ, suggest NADPH as the endogenous electron donor for NQO1. The evidence consists of the observations that the pentose phosphate pathway inhibitor, EPI, decreased NQO1 activity and that when DQ was also present, the $NADPH/NADP^+$ but not the $NADH/NAD^+$ ratios were depressed. The absence of an effect of the glycolytic inhibitor, IOA, on NQO1 activity and the $NADPH/NADP^+$ ratios provided indirect support for NADPH as the endogenous NQO1 electron donor, as did the observation that lactate did not overcome the inhibitory effect of 2-DG on NQO1 activity.

The inhibitor sensitivity profile for TPMET was markedly distinct from that of NQO1. The insensitivity of TPMET to pentose phosphate pathway blockade, the sensitivity to glycolytic inhibitors, the responsiveness to lactate, and the overall relationship between $NADH/NAD^+$ redox status and TPMET activity were in sharp contrast with NQO1. These observations strongly suggested NADH rather than NADPH as the cytosolic TPMET electron donor, confirming our previous observations [25].

The 2-DG treatment was carried out in the absence of glucose, a combination that might be expected to decrease glycolytic flux and pentose phosphate pathway activity due to G-6-P depletion and also inhibit glycolysis via 2-DG inhibition of phosphohexose isomerase and hexokinase [28]. An additional mechanism to depress pentose phosphate activity is suggested by the Figure 5 study showing that 2-DG-6-P is not as good a substrate as the physiological G-6-P (Control) in a cytosol fraction G-6-PDH assay, consistent with observations in other studies [29,30]. Whereas distinguishing the relative contributions of these mechanisms to the observed effects was outside the scope of the present study, the key point is that 2-DG inhibition

of both NQO1 and TPMET activities is consistent with the decreased capacity to regenerate both NADH via glycolysis and NADPH via the pentose phosphate pathway.

IOA was used to target glycolysis via inhibition of glyceraldehyde-3-phosphate dehydrogenase [31]. Accordingly, there was a dramatic decrease in the NADH/NAD^+ ratio and TPMET activity, both of which were overcome with lactate, consistent with previous observations [25]. However, IOA had no detectable effect on NQO1 activity, suggesting that a decrease in the glycolytic supply of NADH was not a limiting factor for DQ reduction via NQO1.

Both 2-DG and IOA decreased the total NAD^+ + NADH and $NADP^+$ + NADPH concentrations in the cells, revealing a compromised ability to maintain normal pyridine nucleotide levels. One explanation is depletion of the total pyridine nucleotide pool via activation of protective mechanisms including, e.g., NAD^+ dependent poly(ADP-ribose) polymerase-1 (PARP-1), sirtuins or other pathways [32,33]. Regardless of the mechanisms involved, there was no detectable effect on cell viability, total cell protein or total cell LDH activity (see Methods Section) over the experimental time course.

EPI is a G-6-PDH inhibitor that has been used to evaluate the contribution of pentose phosphate pathway derived NADPH to various processes, including hypoxic pulmonary vasoconstrictor responses [34,35]. The observation that EPI inhibited NQO1 activity is thus consistent with a role of pentose phosphate derived NADPH in NQO1 mediated DQ reduction in the cells. However, an impact of EPI on the NADPH/$NADP^+$ ratio was only revealed when DQ was also present. This might be explained by noting again that the pyridine nucleotide measurements were of total KOH extractable pyridine nucleotides, and therefore do not give information regarding distribution in intracellular compartments [36–38]. Thus, the apparent maintenance of a normal NADPH/$NADP^+$ ratio in the presence of EPI may reflect domination of the measured total cell NADPH/$NADP^+$ ratio by a cellular compartment that depends on, e.g., $NADP^+$-isocitrate dehydrogenase, malate dehydrogenase and/or nicotinamide nucleotide transhydrogenase for regeneration of NADPH. Alternatively, it may be that under resting conditions, there is little drain on the NADPH pool, and that blockade of the pentose phosphate pathway has no appreciable impact on resting NADPH/$NADP^+$ because there is no need to replenish NADPH when NQO1 is not activated by DQ. This would be analogous to observations in bovine coronary arteries in which glucose deprivation alone does not alter cytosolic NADPH levels, but a hypoxia-induced depression in cytosolic NADPH was exacerbated in glucose-free medium [39].

An alternative or additional explanation for the DQ or DQ + EPI induced decline in the NADPH/$NADP^+$ ratio is NADPH depletion via one-electron DQ reduction and subsequent redox cycling. However, our previous studies suggest that redox cycling is not a predominant pathway of DQ metabolism in pulmonary endothelial cells under these or very similar reaction conditions we have studied. First, as compared to the classical redox cycling quinone, menadione, there was little detectable DQ mediated superoxide generation from these cells, measured as a BMPO-radical adduct signal by electron paramagnetic resonance spectroscopy [30]. This was consistent with observations of other investigators that DQ did not elicit appreciable reactive oxygen species formation from rat hepatocytes or PC12 cells in a series of other, more reactive benzoquinones [40]. Secondly, at the same DQ concentrations used in the present study (50 μM) nearly all the DQ reduction mediated by the pulmonary endothelial cells was blocked with NQO1 inhibitors and, in the absence of NQO1 inhibitors, all the DQ added to the cells could be accounted for as either DQ or DQH_2 at the end of a 30 minute incubation [30]. Furthermore, DQH_2 is a relatively stable hydroquinone with a low propensity to autooxidize over the experimental time frame [30].

The results of the present study taken together with the aforementioned observations strongly suggest that the vast majority of DQ metabolism in pulmonary endothelial cells is attributable to NQO1 driven reduction to DQH_2, and that NQO1 utilization of NADPH provides a reasonable explanation for DQ mediated NADPH depletion. One implication is that the capacity to supply NADPH at a sufficient rate to maintain optimal NQO1 activity under oxidative stress or other conditions constituting an additional drain on NAD(P)H may limit the therapeutic utility of NQO1 as a target for activation of blood borne chemotherapeutic and antioxidant compounds [16,41–43].

It is perhaps not surprising that NADPH would be the endogenous electron donor for NQO1 even though isolated NQO1 has little intrinsic preference for it over NADH. The majority of the reducing power for phase II and antioxidant enzymes is attributable to NADPH, and total cell NADPH levels in the pulmonary endothelial cells, as well as in liver, are 2–3 times higher than total NADH [36,38,44]. This discrepancy is even greater when considering the cytosolic compartment, where NADPH concentrations are estimated to be orders of magnitude greater than NADH, and where protein binding further depletes the NADH pool [36,38,44–46]. Finally, although studies of the isolated enzyme show that the V_{max} for NQO1 is about the same regardless of whether NADH or NADPH is the electron donor, the K_m for NADPH is about one-half that of NADH [1]. Taken together, the weight of the evidence suggests that NADH would be unlikely to be present in a high enough concentration in the cytosol to compete with NADPH for NQO1. However, a key point of this study is that despite the very low relative abundance of NADH as compared to NADPH in the cytosolic compartment, the capacity to supply NADH is sufficient to maintain TPMET activity at a rate comparable to NADPH supported NQO1 mediated DQ reduction.

In their initial studies of NQO1, Ernster et. al proposed it as the predominant NADPH oxidizing enzyme in the liver cell cytosol, and hypothesized a role in regulating oxidation of cytosolic reduced pyridine nucleotides [3]. At that time, it was considered that NQO1 might participate in providing reducing equivalents to the mitochondrial electron transport chain [47]. This concept formed the basis for the therapeutic utility of quinones in treatment of defects in mitochondrial respiration, and recent observations in NQO1 knockout mice are consistent with the prediction that NQO1 participates in maintenance of pyridine nucleotide redox status [14,43].

In conclusion, the present study demonstrates that cytoplasmic redox status, as reflected in the $NADH/NAD^+$ and $NADPH/NADP^+$ ratios, affects TPMET reduction of thiazine compounds and NQO1 mediated quinone reduction. The results also provide confirmation of previous observations that TPMET activity reflects the $NADH/NAD^+$ ratio, and suggest, that in contrast to TPMET, NQO1 activity is more closely tied to the $NADPH/NADP^+$ ratio. In addition, future studies may be warranted to evaluate $TBOP^+$ and DQ reduction as nondestructive indices of changes in cell redox status.

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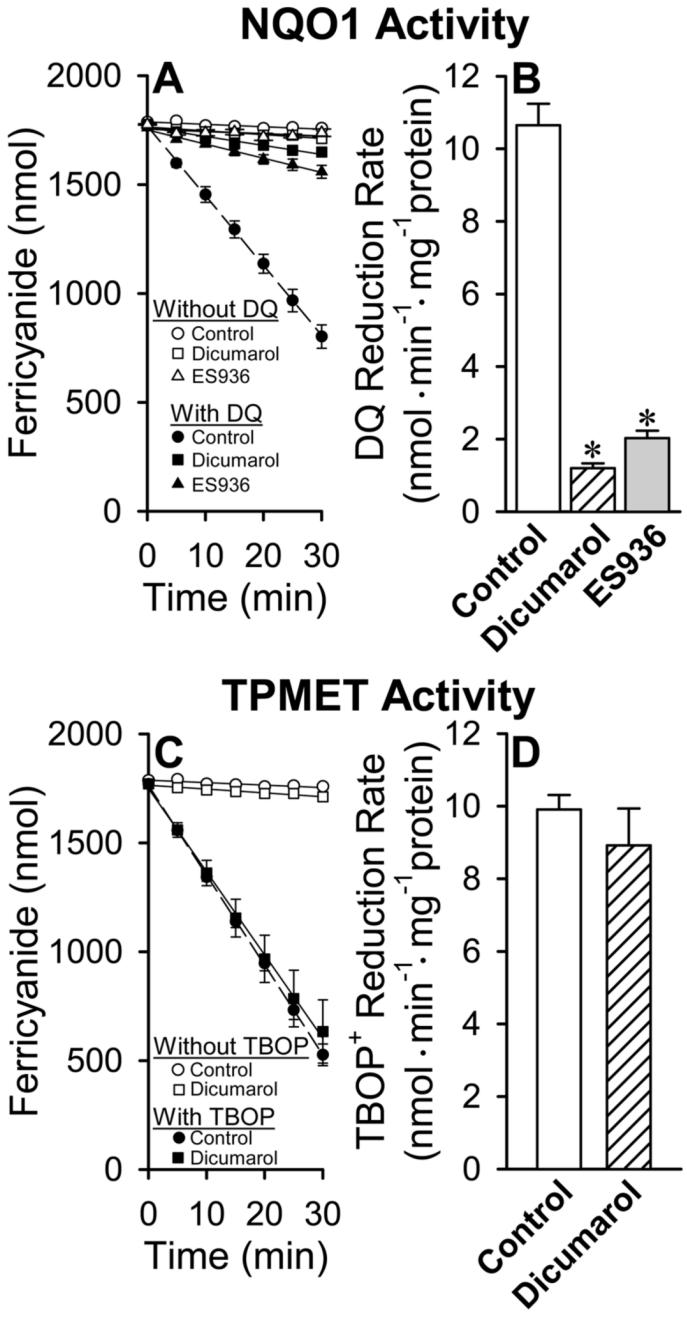

Figure 1. Experimental approach for measuring intact cell (A) NQO1 and (C) TPMET activity

The ferricyanide concentrations in the extracellular medium vs time for example experiments. (A) For the Without DQ conditions (open symbols), the extracellular medium contained ferricyanide alone (control) or ferricyanide and dicumarol or ES936. For the With DQ conditions (closed symbols), the extracellular medium contained ferricyanide with DQ (control) or with DQ and dicumarol or ES936. (B) For the Without TBOP conditions (open symbols) the extracellular medium contained ferricyanide alone (control) or ferricyanide with dicumarol. For the With TBOP conditions (closed symbols) the extracellular medium contained ferricyanide with TBOP (control) or with TBOP with dicumarol. The number of experiments (n) carried out for each condition were as follows: (A) without DQ, control (3),

dicumarol (3), ES936 (3); with DQ, control (26), dicumarol (8), ES936 (4); (B) without TBOP, control (3), dicumarol (3); with TBOP, control (21), dicumarol (6). The cell protein (mean ± SEM) in (A) was 1.57 ± 0.06 mg per sample and in (B) was 1.68 ± 0.07 mg per sample, and there were no significant differences between the protein concentrations within the (A) studies or within the (C) studies. *Significantly different from control ($p<0.05$), Kruskal-Wallis one-way ANOVA on ranks and Dunn's test.

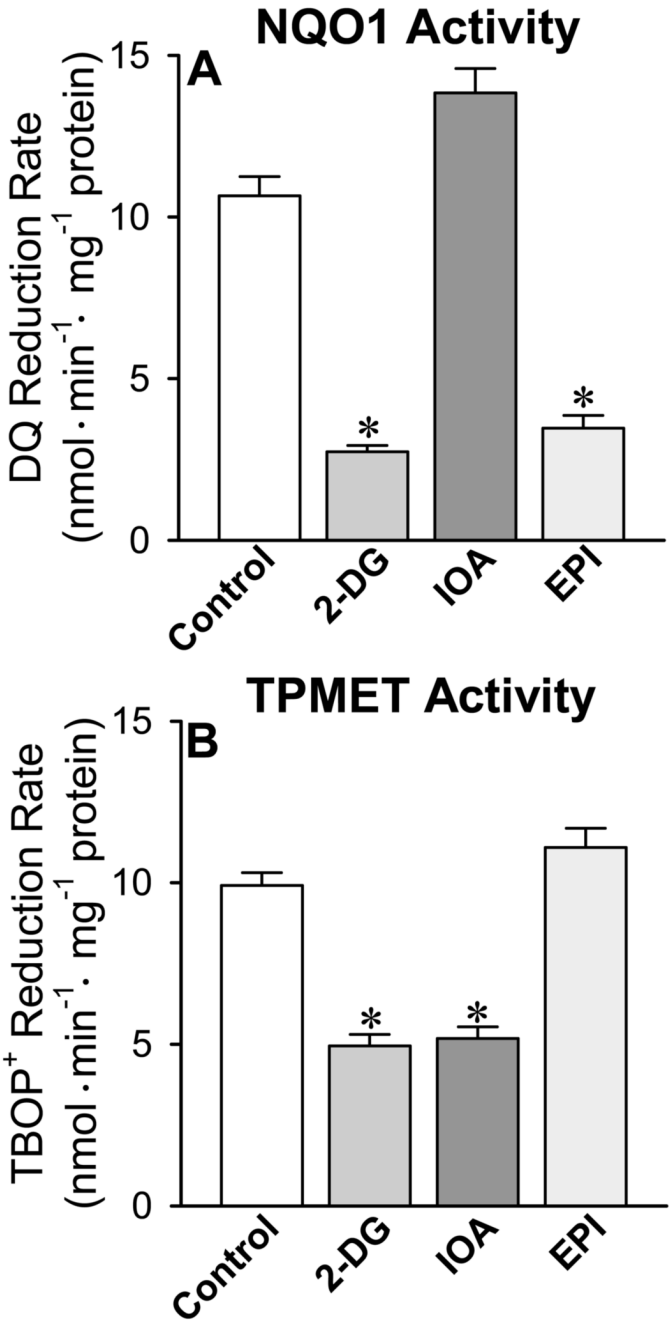

Figure 2. Effects of inhibitors of glycolysis and pentose phosphate pathway on NQO1 (A) and TPMET (B) activities

The activities were calculated from the ferricyanide concentration versus time curves (exemplified in Figure 1) in the presence of DQ to measure NQO1 activity (A) or in the presence of $TBOP^+$ to measure TPMET activity (B), in the absence (control) and presence of the inhibitors. The number of experiments (n) carried out for each condition were as follows: (A) control (26), 2-DG (10), IOA (17), EPI (10); (B) control (21), 2-DG (10), IOA (11), EPI (11). *Significantly different from control ($p<0.05$), Kruskal-Wallis one-way ANOVA on ranks and Dunn's test.

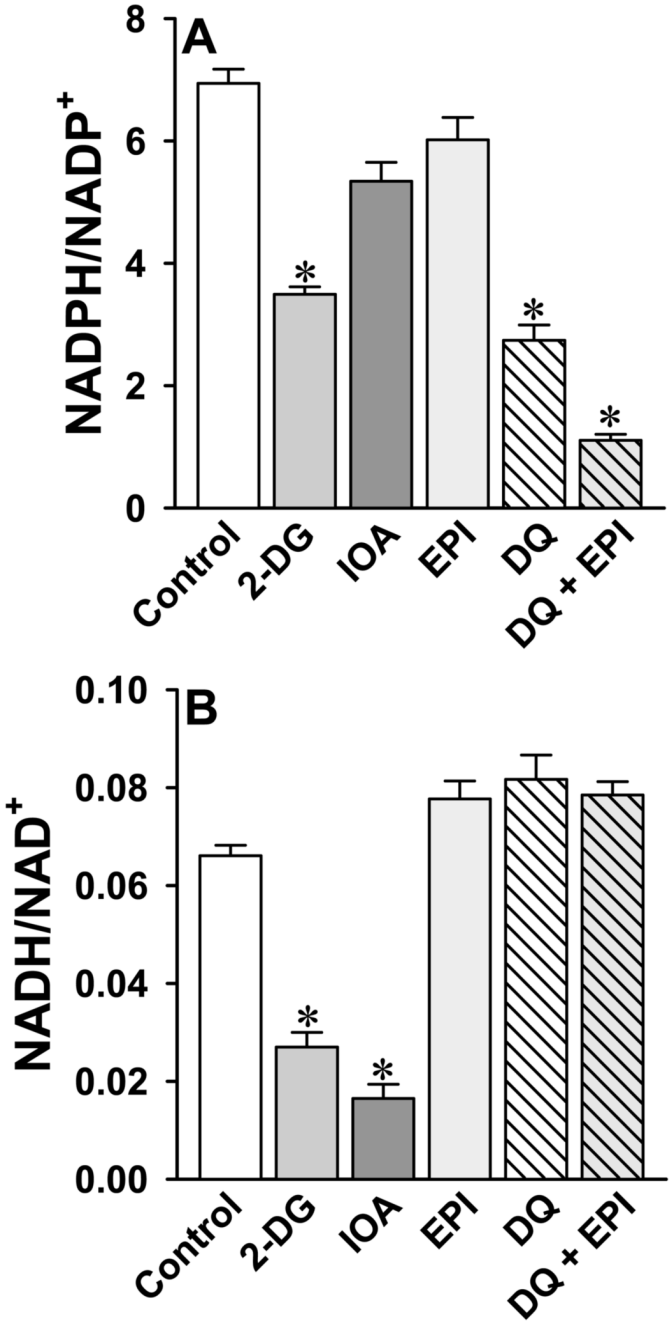

Figure 3.

Effects of inhibitors of glycolysis and pentose phosphate pathway on intracellular NADPH/$NADP^+$ (A) and NADH/NAD^+ (B). The number of experiments (n) carried out were as follows: control (61), 2-DG (11), IOA (12), EPI (23), DQ (9), DQ + EPI (7). *Significantly different from control ($p<0.05$), Kruskal-Wallis one-way ANOVA on ranks and Dunn's test.

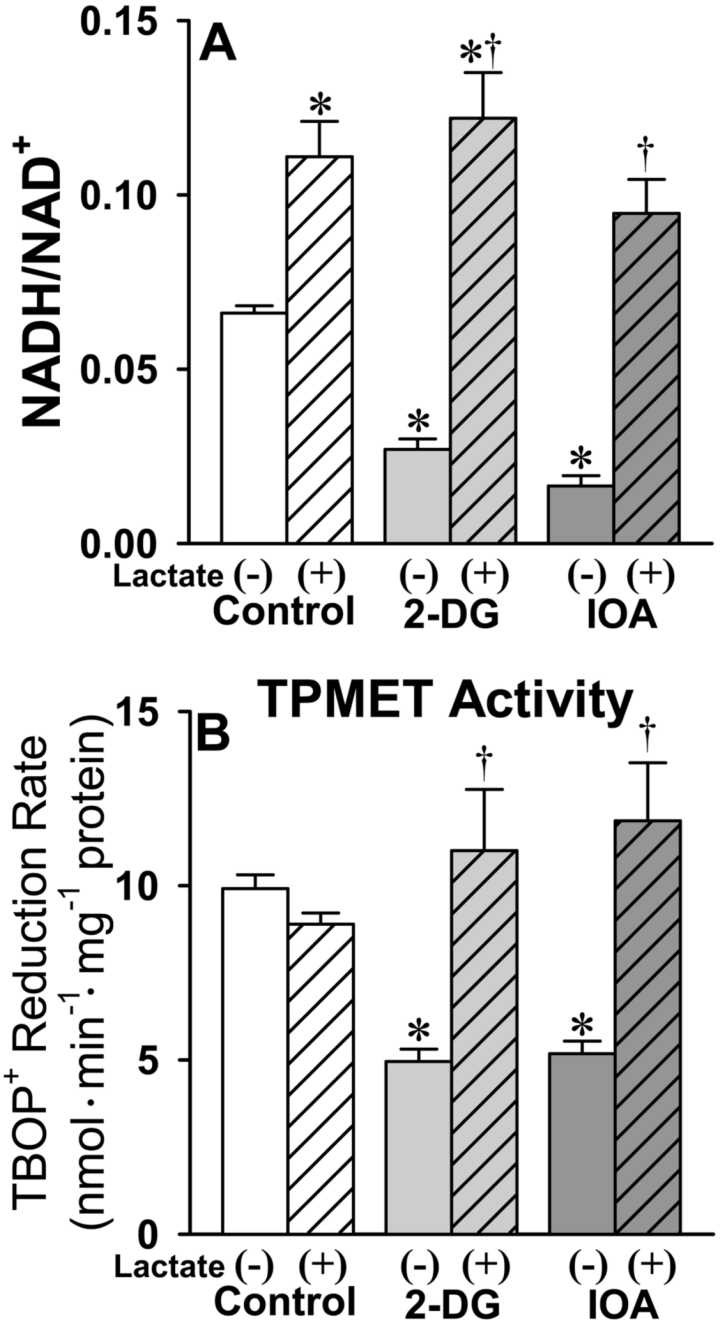

Figure 4. Lactate restores NADH/NAD$^+$ ratios (A) and TPMET activity (B) in the presence of 2-DG or IOA

(A) The NADH/NAD$^+$ ratios were calculated from the values shown in Table 1. (B) The number of experiments (n) carried were as follows: control, − lactate (21), + lactate (6); 2-DG, − lactate (10), + lactate (4); IOA, − lactate (11), + lactate (5). *Significantly different from control ($p<0.05$); † significantly different from paired treatment without lactate (− lactate), ($p<0.05$), Kruskal-Wallis one-way ANOVA on ranks and Dunn's test.

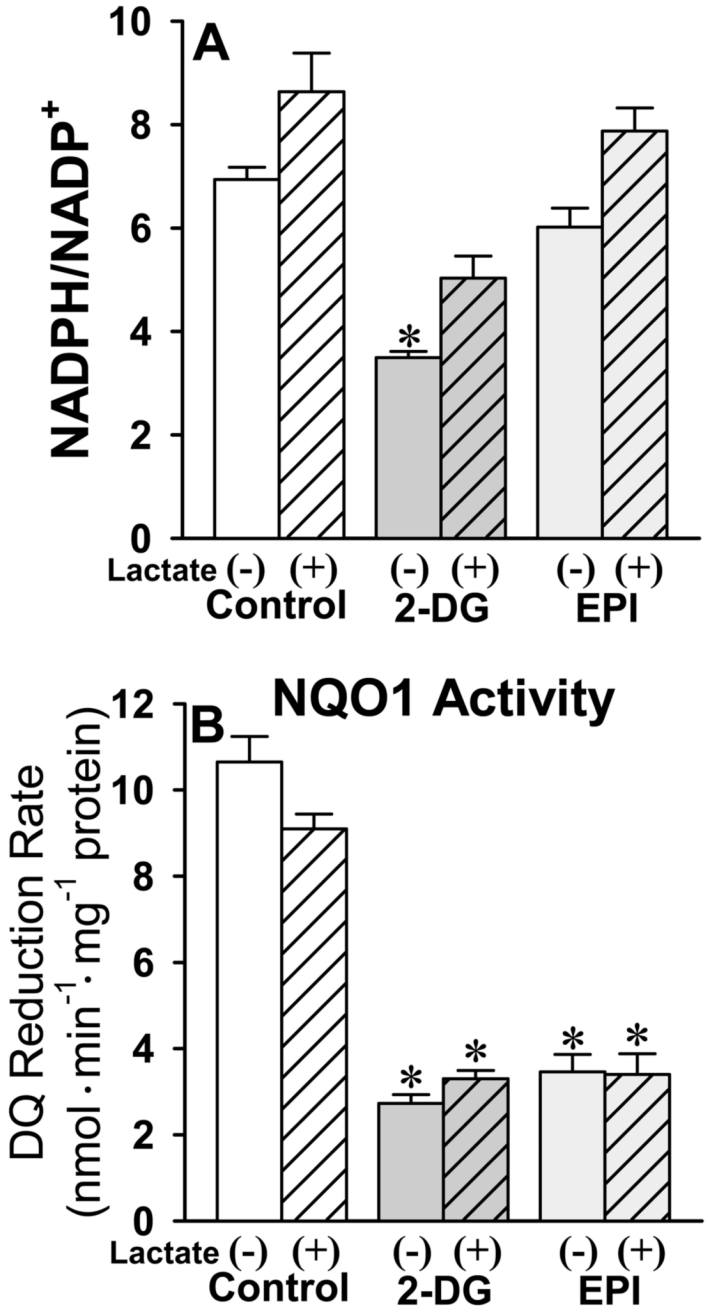

Figure 5. Lactate does not affect NADPH/NADP$^+$ ratios (A) or NQO1 activity (B) in the presence of 2-DG or EPI

(A) The NADPH/NADP$^+$ ratios were calculated from the values shown in Table 1. (B) The number of experiments (n) carried out were as follows: control, − lactate (26), + lactate (11); 2-DG, − lactate (10), + lactate (4); EPI, − lactate (10), + lactate (4). *Significantly different from controls ($p<0.05$), Kruskal-Wallis one-way ANOVA on ranks and Dunn's test.

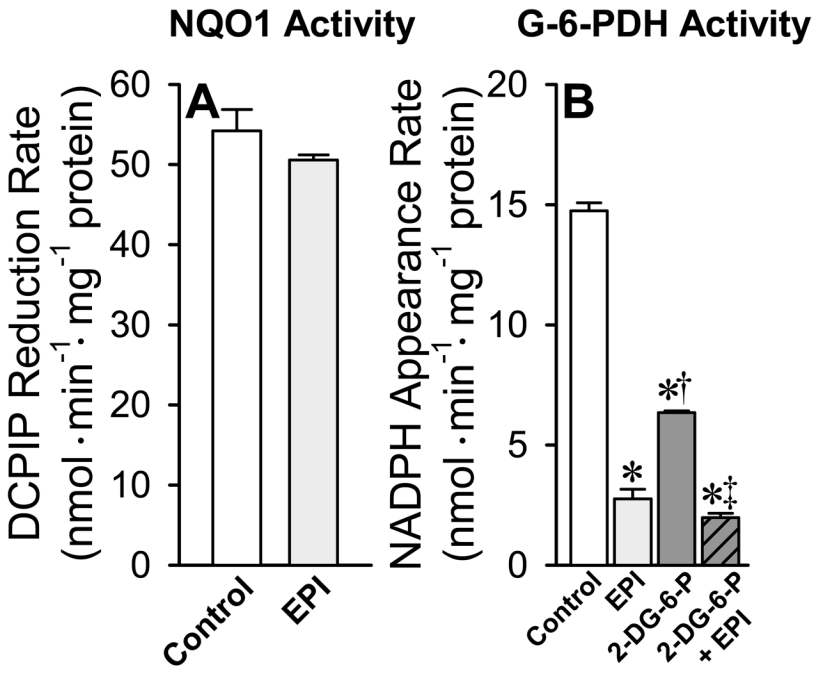

Figure 6. EPI does not inhibit NQO1 activity directly, but does inhibit G-6-PDH activity in cell cytosol fractions

The numbers of experiments (n) carried out in were as follows: (A) control (4), EPI (4); (B) control (10), EPI (6), 2-DG-6-P (6), 2-DG-6-P + EPI (5). *Significantly different from control; †significantly different from EPI; ‡ significantly different from 2-DG-6P, $p<0.05$, one way ANOVA and Tukey test.

Table 1

Pyridine nucleotide and ATP concentrations in cells

Treatment	n	NADH	NAD^+	$NADH+NAD^+$	NADPH	$NADP^+$	$NADPH+NADP^+$	ATP
		pmol per cm^2 cell surface area						
Control	61	5.1±0.2	78.9±2.3	84.0±2.4	10.7±0.3	1.6±0.1	12.4±0.3	696±22
Lactate	10	7.2±0.3	69.0±6.2	76.2±6.3	10.5±0.4	1.3±0.1	11.8±0.5	649±48
2-DG	11	1.5±0.2*	54.9±3.3*	56.4±3.5*	6.5±0.4*	1.9±0.1	8.4±0.4*	363±28*
2-DG + Lactate	8	7.6±0.8	63.0±3.7	70.6±4.0	8.3±0.5	1.7±0.1	10.0±0.6	494±26
IOA	12	1.2±0.2*	68.5±4.9	69.7±5.0	8.5±0.8	1.6±0.1	10.1±0.9	434±50*
IOA + Lactate	9	7.2±0.6	77.8±4.4	85.0±4.4	11.1±0.5	1.7±0.1	12.8±0.5	725±43
EPI	23	5.0±0.2	66.1±2.8	71.2±2.9	8.7±0.3	1.5±0.1	10.2±0.3	585±22
EPI + Lactate	7	7.4±0.5	56.2±1.9	63.6±1.6	9.1±0.4	1.2±0.1	10.3±0.6	547±25
DQ	9	4.9±0.4	60.1±4.0	64.9±4.2	7.5±0.6*	2.9±0.3	10.4±0.9	569±41
DQ + EPI	7	3.5±0.2	44.6±2.2*	48.1±2.4*	3.9±0.1*	3.7±0.3*	7.7±0.3*	511±12

Pyridine nucleotide and ATP concentrations expressed as pmol/cm^2 cell culture surface area in control cells and cells exposed to metabolic inhibitors in the absence or presence of lactate or to DQ in the absence or presence of EPI, measured by HPLC. Values are the means ± SEM, and n is the number of experiments carried out.

*Significantly different from control values, $p<0.05$, Kruskal-Wallis one way ANOVA on ranks and Dunn's test.