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Original Contribution

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 uses 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, iodoacetate, or epiandrosterone in the absence or presence of lactate, NQO1 activity was measured in intact cells using duroquinone as the electron acceptor, and pyridine nucleotide redox status was measured in total cell KOH extracts by high-performance liquid chromatography. 2-Deoxyglucose decreased 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. Iodoacetate decreased NADH/NAD^+ but had no detectable effect on NADPH/$NADP^+$ or NQO1 activity. Epiandrosterone decreased NQO1 activity by 67%, and although epiandrosterone alone did not alter the NADPH/$NADP^+$ or NADH/NAD^+ ratio, when the NQO1 electron acceptor duroquinone was also present, NADPH/$NADP^+$ decreased by 84% with no impact on NADH/NAD^+. Duroquinone alone also decreased NADPH/$NADP^+$ but not NADH/NAD^+. The results suggest that NQO1 activity is more tightly coupled to 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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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 bloodborne 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 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 the extracellular medium of pulmonary arterial endothelial cells in culture, durohydroquinone (DQH_2) is generated in the perfusate and extracellular medium, respectively,

Abbreviations: NQO1, NAD(P)H:quinone oxidoreductase; DQ, duroquinone; DQH_2, durohydroquinone; PARP, poly-ADP ribose polymerase; HPLC, high-performance liquid chromatography; TPMET, transplasma membrane electron transport; TPOP, toluidine blue O polymer; 2-DG, 2-deoxyglucose; IOA, iodoacetic acid sodium salt; EPI, epiandrosterone; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HBSS, Hanks' balanced salt solution; LDH, lactate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase.

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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, for example, 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 for 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, USA). Trypsin, penicillin–streptomycin, RPMI 1640 tissue culture medium and fetal calf serum were from Invitrogen (Grand Island, NY, USA). Biosilon beads were from Nunc (Roskilde, Denmark). NAD^+, $NADP^+$, NADPH and NADH standards for HPLC were purchased from Boehringer Mannheim (Indianapolis, IN, USA). The NQO1 inhibitor ES936 was the kind gift of Dr. David Siegel and Dr. David Roth (School of Pharmacy, University of Colorado Health Sciences Center, Denver, CO, USA).

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/g beads) in magnetic stirrer bottles (Techne Inc., Burlington, NJ, USA) 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 di-O-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

An approximately 0.2-ml packed volume of cell-coated beads was 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 Hanks' 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, 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; 50 μM DQ; 50 μM DQ and 0.03 mM EPI. The cells were incubated in the treatment medium by mixing on a Nutator mixer at 37 °C for 10 min before measurements of NQO1 and TPMET activities or pyridine nucleotides and ATP. After the 10-min mixing period, the buffer was removed from the cuvettes and saved for measurement of lactate dehydrogenase activity.

Toluidine blue O polymer

To prepare the TBOP, toluidine blue O was incorporated into an acrylamide polymer by copolymerization of toluidine blue O–methylacrylamide and acrylamide as previously described [23]. Polymer chains less than 3.5 kDa 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 nmol/mg [25].

Protocol for measuring TPMET and NQO1 activities in intact cells

DQ- and $TBOP^+$-mediated reduction of the cell membrane-impermeant secondary electron acceptor ferricyanide was used as a measure of NQO1 and TPMET activities, respectively, as previously described [4,23–25]. Following the 10-min treatment period described under 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 were allowed to settle at the bottom of the spectrophotometric cuvettes out of the spectrophotometer light path, and the absorbance of ferricyanide in the medium was 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} cm^{-1}). DQ- or $TBOP^+$-mediated ferricyanide reduction rates