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Diacetyl/L-Xylulose Reductase Mediates Chemical Redox Cycling in Lung Epithelial Cells

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Abstract

Reactive carbonyls such as diacetyl (2,3-butanedione) and 2,3-pentanedione in tobacco and many food and consumer products are known to cause severe respiratory diseases. Many of these chemicals are detoxified by carbonyl reductases in the lung, in particular, dicarbonyl/L-xylulose reductase (DCXR), a multifunctional enzyme important in glucose metabolism. DCXR is a member of the short-chain dehydrogenase/reductase (SDR) superfamily. Using recombinant human enzyme, we discovered that DCXR mediates redox cycling of a variety of quinones generating superoxide anion, hydrogen peroxide, and, in the presence of transition metals, hydroxyl radicals. Redox cycling activity preferentially utilized NADH as a cosubstrate and was greatest for 9,10-phenanthrenequinone and 1,2-naphthoquinone, followed by 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (menadione). Using 9,10-phenanthrenequinone as the substrate, quinone redox cycling was found to inhibit DCXR reduction of L-xylulose and diacetyl. Competitive inhibition of enzyme activity by the quinone was observed with respect to diacetyl ($K_i = 190 \mu\text{M}$) and L-xylulose ($K_i = 940 \mu\text{M}$). Abundant DCXR activity was identified in A549 lung epithelial cells when diacetyl was used as a substrate. Quinones inhibited reduction of this dicarbonyl, causing an accumulation of diacetyl in the cells and culture medium and a decrease in acetoin, the reduced product of diacetyl. The identification of DCXR as an enzyme activity mediating chemical redox cycling suggests that it may be important in generating cytotoxic reactive oxygen species in the lung. These activities, together with the inhibition of dicarbonyl/L-xylulose metabolism by redox-active chemicals, as well as consequent deficiencies in pentose metabolism, are likely to contribute to lung injury following exposure to dicarbonyls and quinones.

Graphical Abstract

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The authors declare no competing financial interest

Reactions of NAD(P)H DCXR

Introduction

Dicarbonyl/L-xylulose reductase (DCXR) is a multifunctional homotetrameric enzyme important in carbonyl detoxification and carbohydrate metabolism(1) (see Figure 1 for reactions catalyzed by DCXR). A member of the short-chain dehydrogenase/reductase (SDR) superfamily, it catalyzes the pyridine nucleotide-dependent reduction of various monosaccharides, including pentoses, tetroses, trioses, and ketones, as well as α -dicarbonyl compounds.(2, 3) DCXR is thought to play a key role in the glucuronic acid/uronate cycle of glucose metabolism, an alternative pathway mediating the oxidation of glucose-6-phosphate. (4–6) In this pathway, glucuronic acid is metabolized in a multistep process leading to the formation of the pentose, L-xylulose.(6) Subsequently, DCXR converts L-xylulose to xylitol, and additional reactions shuttle this metabolite into the pentose phosphate pathway, contributing to cellular energy metabolism.(6) In humans, mutations in DCXR result in pentosuria, a clinical condition characterized by high blood and urine levels of L-xylulose. (7) The enzyme is abundant in the lung, kidney, liver, and epididymis, and deficiencies in the enzyme are associated with diabetes, cancer, and male infertility.(1)

As an α -dicarbonyl reductase, DCXR mediates the reductive metabolism of reactive and potentially toxic carbonyl compounds.(3, 8, 9) These include carbonyls generated endogenously or exogenously derived carbonyls such as the water-soluble irritant diacetyl (butane-2,3-dione), methylglyoxal, 3,4-hexanedione, 2,3-heptanedione, and 1-phenyl-1,2-propanedione present in air pollution and tobacco products, and certain foods.(1) Evidence suggests that metabolism and detoxification of endogenous α -dicarbonyl compounds prevent the formation of advanced glycation end-products, reducing the development of pulmonary fibrosis, diabetes, cardiovascular disease, and stroke.(10–13)

It is well recognized that members of the SDR superfamily mediate chemical redox cycling. (14–17) Thus, in many tissues, including the lung, the enzymatic one electron reduction of

quinones, nitroaromatic compounds, and bipyridinium herbicides generates radical ions from these compounds. These ions react rapidly with molecular oxygen regenerating the parent compound and superoxide anion.(18, 19) The reaction of superoxide anion with nitric oxide can generate peroxynitrite, an oxidant and nitrating agent capable of damaging nucleic acids, proteins, and lipids.(20) Dismutation of superoxide anion either spontaneously or via the activity of superoxide dismutases also generates hydrogen peroxide (H₂O₂), an intermediate that can form highly toxic hydroxyl radicals in the presence of transition metals.(21) In the lung, these reactive oxygen and reactive nitrogen species cause injury and exacerbate diseases including asthma, chronic obstructive pulmonary disease, and fibrosis. (22)

The present studies demonstrate that DCXR mediates chemical redox cycling. Thus, purified recombinant human DCXR efficiently generated cytotoxic reactive oxygen species (ROS) by redox cycling a number of quinones. Moreover, the redox cycling process with quinones was associated with the inhibition of the α -dicarbonyl reductase activity of DCXR. Quinone redox cycling also inhibited the reduction of L-xylulose by DCXR. These data identify a novel activity of DCXR which may contribute to lung injury.

Materials and Methods

Chemicals and Reagents

Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). T4 DNA ligase, Amplex Red reagent, and Ni-NTA-agarose were from Thermo Fisher Scientific (Waltham, MA). The pGEM-T TA cloning kit was from Promega (Madison, WI). Pfu Easy A polymerase was from Agilent (Santa Clara, CA). Diacetyl, acetoin (3-hydroxy-2-butanone), horseradish peroxidase, menadione, NADH, NADPH, NADP(+), protease inhibitor cocktail, and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Protease inhibitor cocktail contained 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane), bestatin, leupeptin, and aprotinin.

Cell Culture

A549 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium supplemented with 110 mg/L sodium pyruvate, 4.5 g/L D-glucose, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 10% FBS. Cells were maintained at 37 °C with 5% CO₂ in a humidified incubator.

Cloning of the Human DCXR Gene into a pET28a Expression Vector

Total RNA was isolated from A549 cells using the TRIzol reagent (Thermo Fisher Scientific). cDNAs were synthesized using reverse transcription with Superscript II RNase H-RT according to the manufacturer's instructions (Thermo Fisher Scientific). The full length DCXR gene was amplified by PCR using Pfu Easy A polymerase (Agilent, Santa Clara, CA) with the primer pairs hDCXR-F ccgcgcgcgcagccataggagctgttcctc and hDCXR-R cgaattcggatccttatcagcagcccca. Forward primers (hDCXR-F) contain an NdeI restriction site and 15 nucleotides at the 5'-end of DCXR, and reverse primers (hDCXR-R) contain a

*Bam*HI restriction site and 15 nucleotides at the 3'-end of DCXR, according to the published human DCXR sequence (accession number: NM_016286.3, PubMed). The PCR products were purified using a PCR purification kit (Qiagen, Valencia, CA), cloned into the TA vector pGEM-T (Promega), and transformed into *Escherichia coli* DH10B cells. The pGEMT-hDCXR was isolated using a plasmid miniprep kit (Qiagen), digested with *Nde*I and *Bam*HI restriction enzymes, and then subcloned into the *Nde*I and *Bam*HI sites of pET28a, which carries a hexa-histidine tag coding sequence (Novagen, Madison, WI). Positive clones were selected and verified by sequence analysis (DNA Core facility, Rutgers University-Robert Wood Johnson Medical School, Piscataway, NJ).

Expression and Purification of Recombinant DCXR

The expression vector containing the DCXR gene was transformed into *E. coli* BL21 (DE3). Clones were selected and cultured in Terrific Broth medium containing 25 mg/L kanamycin at 37 °C. To induce recombinant human DCXR protein, cultures were incubated with 0.5 mM isopropyl D-thiogalactopyranoside for 4 h at 37 °C; under these conditions, the absorbance of the cultures reached 0.6 at 600 nm. Pellets from low speed centrifugation (6,000*g* for 20 min) were resuspended in buffer A (50 mM NaH₂PO₄/Na₂HPO₄, pH 7.0, 300 mM NaCl), sonicated on ice, and then centrifuged at 20,000*g* for 30 min. Recombinant proteins in supernatants were purified using a nickel affinity chromatography column (Thermo Fisher Scientific) under native conditions. Briefly, after loading the crude extracts, the Ni-NTA affinity column was equilibrated with buffer A and then washed with buffer B (buffer A containing 20 mM imidazole). The recombinant enzyme was eluted with buffer C (buffer A containing 150 mM imidazole and 10% glycerol). Eluted fractions were concentrated using a VivaSpin 6 column (VWR, West Chester, PA) and analyzed for protein using the DC Protein Assay Reagent (Bio-Rad) with bovine serum albumin as the standard. Recombinant enzymes were analyzed on 12% SDS–polyacrylamide gels using Coomassie Brilliant Blue R-250 staining.

DCXR Enzyme Assays

Kinetics of diacetyl reduction by recombinant DCXR was measured by recording initial rates of NADH or NADPH oxidation at 340 nm using flat bottomed transparent 96-well plates in a Spectramax M5 Fluorescence/Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). The reaction mixes in a final volume of 100 µL, contained 100 mM potassium phosphate buffer, pH 7.0, 5 mM diacetyl, NADH, or NADPH, and were initiated by the addition of enzyme protein. The rates of the reactions were calculated from calibration curves prepared using known NAD(P)H concentrations in the reaction mixes. In some experiments, DCXR activity was measured by monitoring the formation of acetoin in reaction mixes following diacetyl reduction using HPLC.(23) For these studies, cell lysates were prepared as previously described.(24) Standard reaction mixes in 100 µL contained 600 µM NADH, 2 mM diacetyl, and 1–2 µg purified DCXR or 100 µg of cell lysates. After increasing periods of time, reactions were stopped by the addition of 10 µL of 70% perchloric acid. Precipitated proteins were removed by centrifugation (20,000*g* for 10 min at room temperature) and clear supernatants mixed with 40 µL of 0.4% 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl, and reacted for 30 min with shaking. After centrifugation, supernatants were transferred to 1.5 mL Eppendorf tubes and DNPH

derivatives extracted using 250 μ L of chloroform with vigorous shaking for 5 min. After centrifugation at 10,000g for 4 min, 200 μ L of the chloroform phase was transferred into glass tubes (Thermo Fisher Scientific) and dried under nitrogen. Samples were then dissolved in 50 μ L of ethyl acetate, diluted with 50 μ L of 50% methanol, and 20 μ L aliquots analyzed by HPLC. Acetoin-DNPH derivatives generated were separated using a Jasco HPLC system (Easton, MD) fitted with an YMC Butyl (C4) column (150 $\times$ 4.6 mm, 200A, YMC America, INC, Allentown, PA). Ethanol (35%) in water (v/v) was used as the mobile phase and the flow rate set at 1.2 mL/min. Eluted peaks were monitored at 365 nm using a Jasco UV-FP-2075-plus detector. The chromatographic peaks were integrated using Jasco ChromNAV 1.11.02 version software. Acetoin concentrations were calculated using a calibration curve from which standard samples were treated under the same conditions.

DCXR activity was also assayed in intact A549 cells. Briefly, cells (8×10^5) grown in 3.5 cm culture dishes were treated with 2 mM diacetyl in 2 mL of growth medium. After increasing periods of time, 100 μ L of culture medium was removed and analyzed for diacetyl and acetoin content by the HPLC assay as described above. Western blotting using antibodies to DCXR (Thermo Fisher Scientific) were performed as previously described.(25)

Oxidation of xylitol to xylulose by DCXR was quantified as the reduction of NADP⁺ to NADPH in standard 0.1 mL reactions containing 100 mM glycine buffer, pH 9.5, supplemented with 3 mM MgCl₂, NADP⁺, and xylitol. Reactions were initiated by the addition of DCXR, and changes in absorbance at 340 nm were monitored using a microplate reader.

Chemical redox cycling by DCXR was quantified by measuring the formation of superoxide anion, H₂O₂, and hydroxyl radicals. Superoxide anion was quantified by monitoring the reduction of acetylated cytochrome *c* at 550 nm.(17) Reaction mixes (0.1 mL) contained 50 mM potassium phosphate buffer, pH 7.8, 50 μ M acetylated cytochrome *c*, 0.2 mM NADPH, 0.3 mM menadione, and varying amounts of DCXR. H₂O₂ production was assayed using the Amplex-Red/horseradish peroxidase method as previously described.(26) Briefly, standard 0.1 mL reaction mixes contained 50 mM phosphate buffer, pH 7.8, 25 μ M Amplex-Red, 0.1 U horseradish peroxidase, 0.2 mM NADPH (or 0.6 mM NADH), 2.6 μ g DCXR, and appropriate concentrations of redox active chemicals. Reactions were initiated by the addition of the enzyme and H₂O₂ quantified by the formation of fluorescent product, resorufin, which was recorded using the fluorescent microplate reader with excitation and emission wavelengths set at 530 and 587 nm, respectively. The concentrations of H₂O₂ formed were calculated from a calibration curve prepared using appropriate H₂O₂ standards. Hydroxyl radicals were measured by monitoring the formation of the 2-hydroxyterephthalate from terephthalate as described previously.(27) Briefly, standard 0.1 mL reaction mixes contained 50 mM phosphate buffer, pH 7.8, 0.2 mM NADPH (or 0.6 mM NADH), 100 μ M Fe²⁺, 110 μ M EDTA, 2 mM terephthalate, 3.0 μ g of DCXR, and 20 μ M 9,10-phenanthrenequinone. The kinetics of the formation of the fluorescent product, 2-hydroxyterephthalate, was detected using the fluorescent microplate reader with excitation and emission wavelengths set at 310 and 425 nm, respectively. In some experiments, the ability of hydroxyl radicals formed during redox cycling to nick closed circular plasmid DNA was assayed.(28) These reactions were run as described above in 20 μ L volumes and

contained 200 µg of pcDNA 5/TO (5.7 kb) (Thermo Fisher Scientific). After 1 h at room temperature, samples were mixed with EZ-Vision In-Gel Solution (Amresco, Solon, OH) and run on 0.8% agarose gels. DNA was detected by UV fluorescence using a FluorChem E imager (Protein Simple, San Jose, CA). Oxygen utilization in enzyme reactions during redox cycling was measured using a Clark-type oxygen electrode as described previously.(17) In these assays, 16 µg of recombinant DCXR/0.8 mL of reaction mix was used.

Molecular Modeling and Docking

The crystal structure of human DCXR was obtained from the Protein Data Bank (entry ID: 1WNT). For docking modeling, ligands and water were removed from the protein structure using Discovery Studio 2016 (Accelrys Inc., San Diego, CA). The addition of polar hydrogen to the DCXR protein and setting of rotatable bonds on docking molecules were performed using AutoDock Tools v1.5.6 (The Scripps Research Institute, La Jolla, CA) and then converted to pdbqt format for docking screening. Molecular docking was performed using AutoDock Vina v1.1.2 (The Scripps Research Institute). Each grid computation was performed with a grid box of $20 \times 40 \times 40$ Å with 1 Å spacing and a grid center at dimensions (x, y, and z) 38.448, 46.080 and 70.246, which covered all the active site residues and allowed for the flexible rotation of the ligand. All other parameters were set on default as described previously.(29) The best conformation with the lowest docked energy was extracted and visualized using PyMol molecular graphics system (Schrödinger, Cambridge, MA, v 1.8.2.2).

Results

Characterization of Recombinant Human DCXR

In initial experiments, the human DCXR gene was cloned into a hexahistidine-tagged vector, expressed in *E. coli*, and purified by nickel affinity chromatography (Figure 2, panel A). The purified recombinant enzyme appeared as a single 28 kDa band on SDS–polyacrylamide gels, which is consistent with its reported molecular mass.(2) Using diacetyl and L-xylulose, DCXR enzyme activity was found to be dependent on a pyridine nucleotide cofactor (NADH or NADPH) for reducing equivalents, and both substrates were reduced by DCXR in a time- and concentration-dependent manner (Figure 2, panels B–E, and not shown). With respect to the reduction of diacetyl and L-xylulose, DCXR displayed greater activity with NADH, when compared to that of NADPH (Figure 2, panels C and D). Table 1 shows the kinetic constants for the reduction of diacetyl and L-xylulose by DCXR using NADPH as the cofactor ($V_{\max} = 39.7$ µM/min, $K_m = 0.170$ mM, $k_{\text{cat}} = 5.80$ s⁻¹, $k_{\text{cat}}/K_m = 33.90$ s⁻¹mM⁻¹, and $V_{\max} = 33.8$ µM/min, $K_m = 0.503$ mM, $k_{\text{cat}} = 1.09$ s⁻¹, $k_{\text{cat}}/K_m = 2.16$ s⁻¹ mM⁻¹, respectively) and NADPH at fixed concentrations of diacetyl and L-xylulose ($V_{\max} = 30.9$ µM/min, $K_m = 0.09$ mM, $k_{\text{cat}} = 1.6$ s⁻¹, $k_{\text{cat}}/K_m = 17.9$ s⁻¹mM⁻¹, and $V_{\max} = 36.2$ µM/min, $K_m = 0.024$ mM, $k_{\text{cat}} = 1.55$ s⁻¹, $k_{\text{cat}}/K_m = 64$ s⁻¹mM⁻¹, respectively). DCXR also mediated the oxidation of xylitol using NADP⁺ as the cofactor ($V_{\max} = 70.9$ µM/min, $K_m = 0.11$ mM, $k_{\text{cat}} = 1.7$ s⁻¹, and $k_{\text{cat}}/K_m = 15.211$ s⁻¹mM⁻¹) (Table 1).

Chemical Redox Cycling by Recombinant Human DCXR

Recombinant DCXR readily mediated redox cycling of menadione, as measured by the formation of superoxide anion and H_2O_2 (Figure 3, panels A and B). In the presence of Fe^{2+} , redox cycling generated hydroxyl radicals (Figure 3, panel C). The formation of superoxide anion and H_2O_2 was time- and concentration-dependent and suppressed by superoxide dismutase and catalase, respectively, while hydroxyl radical production was inhibited by DMSO, a hydroxyl radical trap (Figure 3 and not shown). DCXR displayed greater redox cycling activity with NADH, when compared to NADPH (Figure 2, panel E). Besides menadione, a number of quinones also redox cycled with DCXR including 9,10-phenanthrenequinone, 1,2-naphthoquinone, and 1,4-naphthoquinone (Figure 3, panel D). Of the redox cycling chemicals, 1,2-naphthoquinone and 9,10-phenanthrenequinone were the most active (EC_{50} 's = 1.5 μM and 1.7 μM , respectively), followed 1,4-naphthoquinone (EC_{50} = 5.4 μM) and menadione (EC_{50} = 228 μM) (Figure 3, panel D and Table 2). Hydroxyl radicals are known to damage DNA;(30) we next determined if DNA was damaged by hydroxyl radicals formed during redox cycling. Using a plasmid DNA unwinding assay,(28) we found that plasmid DNA was nicked during redox cycling (inset, Figure 3, panel C). In the presence of Fe^{2+} /EDTA complexes, hydroxyl radicals are formed from H_2O_2 during redox cycling; catalase was found to readily inhibit DNA damage.

Oxygen is required for the generation of ROS during chemical redox cycling.(17) Consistent with this, DCXR-mediated redox cycling of 9,10-phenanthrenequinone was found to utilize molecular oxygen (Figure 4). By comparison, oxygen utilization was not evident when DCXR enzyme activity was assayed with diacetyl or L-xylulose as substrates in the absence of a redox cycling quinone (not shown).

Redox cycling quinones were found to inhibit DCXR activity, as measured by the reduction of diacetyl to acetoin (Figure 5, panels A and B). It is possible that the quinones, because of their ability to form reactive semiquinones during redox cycling, were site-directed irreversible inhibitors of DCXR. However, this is unlikely because the quinones did not induce a time-dependent inhibition of DCXR substrate reduction (data not shown); moreover, diacetyl was found to readily reverse enzyme inhibition resulting from preincubation with menadione (Figure 6, panel A). Each of the quinones was found to be a competitive inhibitor of the DCXR substrate reduction reaction (Figure 5, panel C and Table 3). 1,2-Naphthoquinone (IC_{50} = 2.9 μM , K_i = 1.4 μM) was the most potent, followed by 9,10-phenanthrenequinone (IC_{50} = 9.3 μM , K_i = 4.3 μM), 1,4-naphthoquinone (IC_{50} = 1.8 μM , K_i = 5.4 μM), and menadione (IC_{50} = 52.8 μM , K_i = 24.3 μM) (Figure 5, panel B). Diacetyl and L-xylulose also inhibited quinone redox cycling by DCXR, blocking the formation of ROS (Figure 6). Figure 6 (panel B) shows that diacetyl readily inhibits superoxide anion formation by menadione redox cycling. In redox cycling assays using 9,10-phenanthrenequinone as the substrate, diacetyl and L-xylulose were competitive inhibitors (Figure 6, panels C and D); the K_i for these inhibitors was 190 μM and 940 μM , respectively (Table 3). Consistent with their ability to inhibit redox cycling, diacetyl and L-xylulose also blocked oxygen utilization in enzyme redox cycling assays (Figure 4 and not shown).

DCXR has been reported to be inhibited by short chain fatty acids including butyric acid, pentanoic acid, hexanoic acid, and heptanoic acid.(2) Each of these organic acids was found to inhibit the reduction of diacetyl by DCXR (not shown), and butyric acid was the most active ($IC_{50} = 0.95$ mM) followed by pentanoic acid ($IC_{50} = 4.4$ mM), hexanoic acid ($IC_{50} = 6.1$ mM), and heptanoic acid ($IC_{50} = 19.9$ mM). Redox cycling was also inhibited by these short chain fatty acids; butyric acid ($IC_{50} = 6.8$ mM) was the most active followed by heptanoic acid ($IC_{50} = 25.1$ mM), pentanoic acid ($IC_{50} = 28.4$ mM), and hexanoic acid ($IC_{50} = 36.0$ mM). In agreement with these data, the organic acids also inhibited oxygen utilization during redox cycling (Figure 4, panels C and D).

Effects of Menadione on DCXR Activity in Lung Epithelial Cells

Human airway epithelium has been shown to express DCXR.(2) In primary cultures, these cells are highly sensitive to diacetyl and readily metabolize diacetyl to acetoin.(31) Similarly, we found that in human A549 lung epithelial cells containing DCXR as revealed by Western blotting (Figure 7), DCXR readily reduced diacetyl to acetoin, which is released into the culture medium (Figure 7). Acetoin production was time- and concentration-dependent (Figure 7, inset and not shown). Treatment of A549 cells with 300 μ M menadione inhibited DCXR activity, as measured by the reduced formation of acetoin following diacetyl treatment of the cells (Figure 8). After 3 h, menadione inhibited the formation of acetoin by approximately 65% (Figure 8, inset).

Discussion

The present studies demonstrate that DCXR, a carbonyl reductase important in diacetyl and xylulose metabolism, can mediate chemical redox cycling. This involves the one electron reduction of redox-active quinones including 9,10-phenanthrenequinone, 1,2-naphthoquinone, 1,4-naphthoquinone, and menadione, at the expense of a reduced nicotinamide adenine dinucleotide cofactor. The reaction of semiquinone radicals formed from these quinones with molecular oxygen regenerates the parent compound and cytotoxic ROS. The quinones were found to vary in their DCXR redox cycling activity with 1,2-naphthoquinone and 9,10-phenanthrenequinone identified as the most active, followed by 1,4-naphthoquinone and menadione. In general, the catalytic activity of the quinones directly correlated with their redox potentials. Of note, under our assay conditions, NADH was significantly more active than NADPH in supplying electrons to DCXR, not only for redox cycling but also for the reduction of diacetyl and L-xylulose. Other enzymes that mediate redox cycling including cytochrome *b5* reductase and ubiquinone oxidoreductase also preferentially utilize NADH for substrate reduction.(32, 33) Our findings that ROS formed during redox cycling by DCXR are in accord with earlier studies showing that purified human xylulose reductase generates superoxide anion by redox cycling with 9,10-phenanthrenequinone.(16) With respect to the reduction of diacetyl and L-xylulose, the observation that there was greater activity with NADH is consistent with reports that DCXR enzyme activity in yeast and in hamster sperm is NADH-dependent.(34, 35) Mammalian DCXR and various structurally related carbonyl reductases have been reported to largely utilize NADPH.(1, 2) Differences between these studies and ours may be due to differences in the conditions used for assessing enzyme activity. In mice, lung carbonyl reductase, which

is closely related to DCXR, Lys17, and Arg39 in the active site of the enzyme are thought to be responsible for binding NADPH, as they form a positively charged environment.(36, 37) Substitutions/mutations in the active of the enzyme have been reported to change its specificity from NADPH to NADH.(37) Further studies are required to more precisely define the structural features in human DCXR responsible for its selectivity toward NADH.

Of interest were our findings that chemical redox cycling was associated with inhibition of the α -dicarbonyl and L-xylulose reductase activity of DCXR. Using 9,10-phenanthrenequinone as a substrate, inhibition of DCXR was found to be competitive with respect to diacetyl and L-xylulose, suggesting that the reaction occurs at the active site of the enzyme. These data are in accord with our findings that diacetyl also inhibited redox cycling. Fatty acids have been shown to inhibit short chain dehydrogenases/reductases including carbonyl reductase and DCXR.(2) Similarly, we found that butyric acid, pentanoic acid, hexanoic acid, and heptanoic acid inhibited diacetyl reduction by DCXR and that these short chain fatty acids also inhibited ROS formation during redox cycling, as well as oxygen utilization. Our findings that butyric acid was the most active inhibitor are consistent with earlier studies using recombinant DCXR's from other species including humans.(2) Conversely, in our studies, higher concentrations of short chain fatty acids were required to inhibit DCXR activity. This is most likely due to differences in enzyme assay conditions.

The present studies also demonstrate that A549 lung epithelial cells contain DCXR activity; the reaction product generated from diacetyl is the α -hydroxyketone, acetoin, which is rapidly transported out of the cells into the culture medium. These data are in accord with reports that DCXR is expressed in the mouse, rat, hamster, guinea pig, and human lung.(2) In a number of rodent models, diacetyl has been reported to induce extensive lung injury including degeneration of the airway epithelium.(38–40) These effects are thought to be due to diacetyl-induced protein modifications followed by protein turnover and autophagy.(41) The observation that DCXR knockout mice exhibit increased susceptibility to diacetyl-induced pulmonary injury provides support for the notion that DCXR is important in the pathogenic response.(42) We also found that quinone redox cycling inhibits diacetyl metabolism in A549 cells. These data are consistent with our observation that quinones inhibit diacetyl reduction by recombinant DCXR. It should be noted that A549 cells contain additional aldo-keto reductases which may also metabolize diacetyl. Further studies are required to determine the extent to which these enzymes redox cycle quinones and if the redox cycling inhibits diacetyl metabolism.

Crystallographic studies of human DCXR (xylulose reductase) complexed with NADPH have shown that the enzyme is a homotetramer consisting of 26 kDa subunits(42–44) that shares a cofactor binding sequence (GxxxGxxG(A)) with other members of the short chain dehydrogenase family. In DCXR, Lys17, Thr38, and Arg39 form hydrogen bonds with the phosphate group in NADPH.(36, 45) A conserved sequence motif in the catalytic center of the human enzyme contains a critical triad pocket composed of Ser136, Tyr149, and Lys153. This triad is presumably important in maintaining substrate/cofactor proximity, stabilizing enzyme structure, and in facilitating proton transfer.(42, 46) To better understand the structure of substrates bound to DCXR and their effects on quinone redox cycling, we performed molecular docking analysis of diacetyl and L-xylulose in the DCXR active site.

Both diacetyl and L-xylulose were found to be positioned in proximity to NADP⁺, within the hydrophobic pocket of the catalytic center of DCXR (Figure 9). L-Xylulose appeared to anchor by forming hydrogen bonds with the side chain of Tyr149 (2.2 Å) in the triad pocket, the oxygen atom on the nicotinamide moiety of NADP⁺ (2.1 Å), the imidazole ring of His146 (2.1 and 2.5 Å), and the indole ring of Trp191 (2.2 and 2.9 Å) in the hydrophobic cleft, extending from the catalytic center of the enzyme. Diacetyl was found to complex with DCXR via hydrophobic interactions with amino acids Tyr149, Leu89, Val181, and NADP⁺, as well as by forming hydrogen bonds with His146 (2.1 Å) and Trp191 (2.0 Å). These findings are in accord with site-directed mutagenesis studies of amino acids in the catalytic triad or its surrounding hydrophobic residues (Gln137, Val143, His146, Ala190, and Trp191) in rat DCXR which demonstrated a complete loss or moderate change in the rate of substrate reduction of L-xylulose and diacetyl.(46)

Molecular docking analysis with 1,2-naphthoquinone, 1,4-naphthoquinone, menadione, and 9,10-phenanthrenequinone, revealed that all of these redox cyclers fit within the hydrophobic cleft of the active site of DCXR, forming hydrophobic interactions with Met186, Val181, Trp191, His146, Leu89, and Tyr149 (Figure 9 and not shown). For example, 1,4-naphthoquinone resides in close proximity (~2.9 Å) to His146, forming hydrogen bonds with the imidazole ring of histidine and the phenyl ring of Tyr149. The quinone is also in close proximity to the nicotinamide moiety of the NADP⁺ cofactor (~3.0 Å) forming π -stacking interactions which are likely important in the one electron reduction of 1,4-naphthoquinone to its semiquinone and, in the presence of molecular oxygen, the formation of ROS, and the regeneration of the parent quinone. The fact that the quinone moiety superimposes in the binding sites for L-xylulose and diacetyl in DCXR implies that these compounds act as mutual competitive inhibitors. This is supported by our findings that redox cyclers are capable of inhibiting substrate reduction by DCXR and that the substrates of DCXR act as inhibitors for chemical redox cycling.

Chemical redox cycling and consequent generation of ROS is an important mechanism contributing to tissue injury.(22) Products derived from superoxide anion and H₂O₂ including hydroxyl radicals can modify many cellular components including proteins, nucleic acids, and membrane lipids;(47) reactions of hydroxyl radicals with membranes initiate lipid peroxidation, a process that generates cytotoxic lipid peroxidation end products. (48) A growing number of enzymes have been identified as mediators of chemical redox cycling including flavin and nonflavin containing enzymes such as NADPH-cytochrome P450 reductase, various isoforms of nitric oxide synthase, NADH-cytochrome *b5* reductase, thioredoxin reductase, several aldo-keto reductases including sepiapterin reductase and, as shown in the present studies, DCXR.(17, 49–54) All of these enzymes require NADPH and/or NADH as the source of reducing equivalents, and the role of several of these enzyme in oxygen activation by quinones has been described.(55–57) Enzymes such as the NADPH-dependent nitric oxide synthases and NADPH-cytochrome P450 reductase contain FMN and FAD as cofactors; these flavins are thought to mediate the redox cycling process by accepting single electrons from NADPH. NADH-cytochrome *b5* reductase and thioredoxin reductase contain FAD, and redox cycling may be due to the ability of this flavin to form a semiquinone intermediate.(58) In contrast, DCXR, aldo-keto reductases, and sepiapterin reductase do not contain cofactors in the form of prosthetic groups.(2, 17, 59) Redox cycling

by these enzymes may be due to the close proximity of the redox active quinones and electron donor groups in the active site of the enzyme. Our findings that quinones are competitive DCXR inhibitors with respect to diacetyl and L-xylulose provide support for this notion. Given that there are many enzymes in tissues capable of chemical redox cycling and generating ROS, a question arises as to which are responsible for mediating toxicity. In general, this depends on localized concentrations of the enzymes and their relative ability to generate ROS with a redox active chemical in a tissue such as the lung. It should be noted, however, that some of these enzymes (e.g., aldo-keto reductases) can also detoxify redox active quinones, which may be important in limiting ROS formation and tissue injury.(58)

The toxicity of α -dicarbonyls is thought to be due, in part, to their ability to produce advanced glycation end-products, a complex mixture of chemicals generated via nonenzymatic reactions of carbohydrates and proteins.(60) In the lung, the formation of advanced glycation end products has been associated with fibrosis, acute lung injury, acute respiratory distress syndrome, and cancer.(61, 62) Thus, detoxification of α -dicarbonyls in the lung by DCXR is key to preventing toxicity.(8) The present studies demonstrate that DCXR can be inhibited by redox cycling chemicals present in air pollution and cigarette smoke; this suggests a novel mechanism of quinone toxicity. Thus, DCXR-mediated redox cycling not only generates cytotoxic ROS but also reduces localized concentrations of intracellular oxygen and reducing equivalents from NADH/NADPH, which can contribute to tissue injury. Moreover, inhibition of metabolism by DCXR can increase tissue concentrations of cytotoxic α -dicarbonyls or deleterious advanced glycation end products. Similarly, inhibition of DCXR can result in deficiencies in pentose metabolism, and this, either alone or in combination with other metabolic effects of α -dicarbonyls and/or redox cycling quinones, may also adversely affect tissue functioning. Further studies are needed to better understand the role of DCXR in detoxifying reactive intermediates, as well as interactions with redox cycling chemicals that contribute to pulmonary injury.

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Abbreviations

DCXR	dicarbonyl/L-xylulose reductase
SDR	short-chain dehydrogenase/reductase
ROS	reactive oxygen species
DNPH	2,4-dinitrophenylhydrazine

References

1. Ebert B, Kisiela M, Maser E. Human DCXR - another 'moonlighting protein' involved in sugar metabolism, carbonyl detoxification, cell adhesion and male fertility? *Biol Rev Camb Philos Soc*. 2015; 90:254–278. [PubMed: 24720935]

2. Nakagawa J, Ishikura S, Asami J, Isaji T, Usami N, Hara A, Sakurai T, Tsuritani K, Oda K, Takahashi M, Yoshimoto M, Otsuka N, Kitamura K. Molecular characterization of mammalian dicarbonyl/l-xylulose reductase and its localization in kidney. *J Biol Chem*. 2002; 277:17883–17891. [PubMed: 11882650]
3. Asami J, Odani H, Ishii A, Oide K, Sudo T, Nakamura A, Miyata N, Otsuka N, Maeda K, Nakagawa J. Suppression of AGE precursor formation following unilateral ureteral obstruction in mouse kidneys by transgenic expression of alpha-dicarbonyl/l-xylulose reductase. *Biosci Biotechnol, Biochem*. 2006; 70:2899–2905. [PubMed: 17151462]
4. Touster O, Hutcheson RM, Rice L. The influence of D-glucuronolactone on the excretion of l-xylulose by humans and guinea pigs. *Biochim Biophys Acta*. 1955; 215:677–684.
5. Winegrad AI, Burden CL. l-xylulose metabolism in diabetes mellitus. *N Engl J Med*. 1966; 274:298–305. [PubMed: 5903211]
6. Sochor M, Baquer NZ, McLean P. Glucose overutilization in diabetes: evidence from studies on the changes in hexokinase, the pentose phosphate pathway and glucuronate-xylulose pathway in rat kidney cortex in diabetes. *Biochem Biophys Res Commun*. 1979; 86:32–39. [PubMed: 435303]
7. Wang YM, Van Eys J. The enzymatic defect in essential pentosuria. *N Engl J Med*. 1970; 282:892–896. [PubMed: 4392213]
8. Sudo T, Ishii A, Asami J, Uematsu Y, Saitoh M, Nakamura A, Tada N, Ohnuki T, Komurasaki T, Nakagawa J. Transgenic mice over-expressing dicarbonyl/l-xylulose reductase gene crossed with KK-Ay diabetic model mice: an animal model for the metabolism of renal carbonyl compounds. *Exp Anim*. 2005; 54:385–394. [PubMed: 16365515]
9. Odani H, Asami J, Ishii A, Oide K, Sudo T, Nakamura A, Miyata N, Otsuka N, Maeda K, Nakagawa J. Suppression of renal alpha-dicarbonyl compounds generated following ureteral obstruction by kidney-specific alpha-dicarbonyl/l-xylulose reductase. *Ann N Y Acad Sci*. 2008; 1126:320–324. [PubMed: 18079483]
10. Chen L, Wang T, Wang X, Sun BB, Li JQ, Liu DS, Zhang SF, Liu L, Xu D, Chen YJ, Wen FQ. Blockade of advanced glycation end product formation attenuates bleomycin-induced pulmonary fibrosis in rats. *Respir Res*. 2009; 10:55. [PubMed: 19552800]
11. Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M. Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proc Natl Acad Sci U S A*. 1991; 88:11555–11558. [PubMed: 1763069]
12. Faria A, Persaud SJ. Cardiac oxidative stress in diabetes: Mechanisms and therapeutic potential. *Pharmacol Ther*. 2017; 172:50. [PubMed: 27916650]
13. Kizer JR, Benkeser D, Arnold AM, Ix JH, Mukamal KJ, Djousse L, Tracy RP, Siscovick DS, Psaty BM, Zieman SJ. Advanced glycation/glycoxidation endproduct carboxymethyl-lysine and incidence of coronary heart disease and stroke in older adults. *Atherosclerosis*. 2014; 235:116–121. [PubMed: 24825341]
14. Jarabak J. Polycyclic aromatic hydrocarbon quinone-mediated oxidation reduction cycling catalyzed by a human placental NADPH-linked carbonyl reductase. *Arch Biochem Biophys*. 1991; 291:334–338. [PubMed: 1659323]
15. Usami N, Kitahara K, Ishikura S, Nagano M, Sakai S, Hara A. Characterization of a major form of human isatin reductase and the reduced metabolite. *Eur J Biochem*. 2001; 268:5755–5763. [PubMed: 11722560]
16. Matsunaga T, Kamiya T, Sumi D, Kumagai Y, Kalyanaraman B, Hara A. L-Xylulose reductase is involved in 9,10-phenanthrenequinone-induced apoptosis in human T lymphoma cells. *Free Radical Biol Med*. 2008; 44:1191–1202. [PubMed: 18206670]
17. Yang S, Jan YH, Gray JP, Mishin V, Heck DE, Laskin DL, Laskin JD. Sepiapterin reductase mediates chemical redox cycling in lung epithelial cells. *J Biol Chem*. 2013; 288:19221–19237. [PubMed: 23640889]
18. Rashba-Step J, Cederbaum AI. Generation of reactive oxygen intermediates by human liver microsomes in the presence of NADPH or NADH. *Mol Pharmacol*. 1994; 45:150–157. [PubMed: 8302274]
19. Bonneh-Barkay D, Reaney SH, Langston WJ, Di Monte DA. Redox cycling of the herbicide paraquat in microglial cultures. *Mol Brain Res*. 2005; 134:52–56. [PubMed: 15790529]

20. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*. 2007; 87:315–424. [PubMed: 17237348]
21. Winterbourn CC. Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol Lett*. 1995; 82–83:969–974.
22. Kimbrough RD, Gaines TB. Toxicity of paraquat to rats and its effect on rat lungs. *Toxicol Appl Pharmacol*. 1970; 17:679–690. [PubMed: 5495991]
23. Baggetto LG, Lehninger AL. Formation and utilization of acetoin, an unusual product of pyruvate metabolism by Ehrlich and AS30-D tumor mitochondria. *J Biol Chem*. 1987; 262:9535–9541. [PubMed: 3597423]
24. Yang S, Jan YH, Mishin V, Richardson JR, Hossain MM, Heindel ND, Heck DE, Laskin DL, Laskin JD. Sulfa drugs inhibit sepiapterin reduction and chemical redox cycling by sepiapterin reductase. *J Pharmacol Exp Ther*. 2015; 352:529–540. [PubMed: 25550200]
25. Jan YH, Heck DE, Casillas RP, Laskin DL, Laskin JD. Thioredoxin Cross-Linking by Nitrogen Mustard in Lung Epithelial Cells: Formation of Multimeric Thioredoxin/Thioredoxin Reductase Complexes and Inhibition of Disulfide Reduction. *Chem Res Toxicol*. 2015; 28:2091–2103. [PubMed: 26451472]
26. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem*. 1997; 253:162–168. [PubMed: 9367498]
27. Son Y, Mishin V, Welsh W, Lu SE, Laskin JD, Kipen H, Meng Q. A novel high-throughput approach to measure hydroxyl radicals induced by airborne particulate matter. *Int J Environ Res Public Health*. 2015; 12:13678–13695. [PubMed: 26516887]
28. Mariano TM, Vetrano AM, Gentile SL, Heck DE, Whittemore MS, Guillon CD, Jabin I, Rapp RD, Heindel ND, Laskin JD. Cell-impermeant pyridinium derivatives of psoralens as inhibitors of keratinocyte growth. *Biochem Pharmacol*. 2002; 63:31–39. [PubMed: 11754871]
29. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem*. 2010; 31:455–461. [PubMed: 19499576]
30. Kukiela E, Cederbaum AI. DNA strand cleavage as a sensitive assay for the production of hydroxyl radicals by microsomes: role of cytochrome P4502E1 in the increased activity after ethanol treatment. *Biochem J*. 1994; 302(3):773–779. [PubMed: 7945202]
31. Zacccone EJ, Goldsmith WT, Shimko MJ, Wells JR, Schwegler-Berry D, Willard PA, Case SL, Thompson JA, Fedan JS. Diacetyl and 2,3-pentanedione exposure of human cultured airway epithelial cells: Ion transport effects and metabolism of butter flavoring agents. *Toxicol Appl Pharmacol*. 2015; 289:542–549. [PubMed: 26454031]
32. Yubisui T, Takeshita M. Characterization of the purified NADH-cytochrome b5 reductase of human erythrocytes as a FAD-containing enzyme. *J Biol Chem*. 1980; 255:2454–2456. [PubMed: 7358682]
33. King MS, Sharpley MS, Hirst J. Reduction of hydrophilic ubiquinones by the flavin in mitochondrial NADH:ubiquinone oxidoreductase (Complex I) and production of reactive oxygen species. *Biochemistry*. 2009; 48:2053–2062. [PubMed: 19220002]
34. Verho R, Putkonen M, Londesborough J, Penttila M, Richard P. A novel NADH-linked l-xylulose reductase in the l-arabinose catabolic pathway of yeast. *J Biol Chem*. 2004; 279:14746–14751. [PubMed: 14736891]
35. Ishikura S, Usami N, Kitahara K, Isaji T, Oda K, Nakagawa J, Hara A. Enzymatic characteristics and subcellular distribution of a short-chain dehydrogenase/reductase family protein, P26h, in hamster testis and epididymis. *Biochemistry*. 2001; 40:214–224. [PubMed: 11141073]
36. Nakanishi M, Kakumoto M, Matsuura K, Deyashiki Y, Tanaka N, Nonaka T, Mitsui Y, Hara A. Involvement of two basic residues (Lys-17 and Arg-39) of mouse lung carbonyl reductase in NADP(H)-binding and fatty acid activation: site-directed mutagenesis and kinetic analyses. *J Biochem*. 1996; 120:257–263. [PubMed: 8889808]
37. Scrutton NS, Berry A, Perham RN. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. *Nature*. 1990; 343:38–43. [PubMed: 2296288]

38. Morgan DL, Flake GP, Kirby PJ, Palmer SM. Respiratory toxicity of diacetyl in C57BL/6 mice. *Toxicol Sci.* 2008; 103:169–180. [PubMed: 18227102]
39. Palmer SM, Flake GP, Kelly FL, Zhang HL, Nugent JL, Kirby PJ, Foley JF, Gwinn WM, Morgan DL. Severe airway epithelial injury, aberrant repair and bronchiolitis obliterans develops after diacetyl instillation in rats. *PLoS One.* 2011; 6:e17644. [PubMed: 21464978]
40. Hubbs AF, Cumpston AM, Goldsmith WT, Battelli LA, Kashon ML, Jackson MC, Frazer DG, Fedan JS, Goravanahally MP, Castranova V, Kreiss K, Willard PA, Friend S, Schwegler-Berry D, Fluharty KL, Sriram K. Respiratory and olfactory cytotoxicity of inhaled 2,3-pentanedione in Sprague-Dawley rats. *Am J Pathol.* 2012; 181:829–844. [PubMed: 22894831]
41. Hubbs AF, Fluharty KL, Edwards RJ, Barnabei JL, Grantham JT, Palmer SM, Kelly F, Sargent LM, Reynolds SH, Mercer RR, Goravanahally MP, Kashon ML, Honaker JC, Jackson MC, Cumpston AM, Goldsmith WT, McKinney W, Fedan JS, Battelli LA, Munro T, Bucklew-Moyers W, McKinstry K, Schwegler-Berry D, Friend S, Knepp AK, Smith SL, Sriram K. Accumulation of ubiquitin and sequestosome-1 implicate protein damage in diacetyl-Induced cytotoxicity. *Am J Pathol.* 2016; 186:2887–2908. [PubMed: 27643531]
42. El-Kabbani O, Ishikura S, Darmanin C, Carbone V, Chung RP, Usami N, Hara A. Crystal structure of human l-xylulose reductase holoenzyme: probing the role of Asn107 with site-directed mutagenesis. *Proteins: Struct Funct, Genet.* 2004; 55:724–732. [PubMed: 15103634]
43. El-Kabbani O, Carbone V, Darmanin C, Ishikura S, Hara A. Structure of the tetrameric form of human L-Xylulose reductase: probing the inhibitor-binding site with molecular modeling and site-directed mutagenesis. *Proteins: Struct Funct, Genet.* 2005; 60:424–432. [PubMed: 15906319]
44. Zhao HT, Endo S, Ishikura S, Chung R, Hogg PJ, Hara A, El-Kabbani O. Structure/function analysis of a critical disulfide bond in the active site of l-xylulose reductase. *Cell Mol Life Sci.* 2009; 66:1570–1579. [PubMed: 19337691]
45. Nakanishi M, Matsuura K, Kaibe H, Tanaka N, Nonaka T, Mitsui Y, Hara A. Switch of coenzyme specificity of mouse lung carbonyl reductase by substitution of threonine 38 with aspartic acid. *J Biol Chem.* 1997; 272:2218–2222. [PubMed: 8999926]
46. Ishikura S, Isaji T, Usami N, Nakagawa J, El-Kabbani O, Hara A. Identification of amino acid residues involved in substrate recognition of l-xylulose reductase by site-directed mutagenesis. *Chem-Biol Interact.* 2003; 143–144:543–550.
47. Radak Z, Zhao Z, Goto S, Koltai E. Age-associated neurodegeneration and oxidative damage to lipids, proteins and DNA. *Mol Aspects Med.* 2011; 32:305–315. [PubMed: 22020115]
48. Negre-Salvayre A, Coatrieux C, Ingueneau C, Salvayre R. Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. *Br J Pharmacol.* 2008; 153:6–20. [PubMed: 17643134]
49. Wang Y, Gray JP, Mishin V, Heck DE, Laskin DL, Laskin JD. Distinct roles of cytochrome P450 reductase in mitomycin C redox cycling and cytotoxicity. *Mol Cancer Ther.* 2010; 9:1852–1863. [PubMed: 20501808]
50. Day BJ, Patel M, Calavetta L, Chang LY, Stamler JS. A mechanism of paraquat toxicity involving nitric oxide synthase. *Proc Natl Acad Sci U S A.* 1999; 96:12760–12765. [PubMed: 10535996]
51. Marin A, Lopez de Cerain A, Hamilton E, Lewis AD, Martinez-Penuela JM, Idoate MA, Bello J. DT-diaphorase and cytochrome B5 reductase in human lung and breast tumours. *Br J Cancer.* 1997; 76:923–929. [PubMed: 9328153]
52. Gray JP, Heck DE, Mishin V, Smith PJ, Hong JY, Thiruchelvam M, Cory-Slechta DA, Laskin DL, Laskin JD. Paraquat increases cyanide-insensitive respiration in murine lung epithelial cells by activating an NAD(P)H:paraquat oxidoreductase: identification of the enzyme as thioredoxin reductase. *J Biol Chem.* 2007; 282:7939–7949. [PubMed: 17229725]
53. Matsunaga T, Arakaki M, Kamiya T, Endo S, El-Kabbani O, Hara A. Involvement of an aldo-keto reductase (AKR1C3) in redox cycling of 9,10-phenanthrenequinone leading to apoptosis in human endothelial cells. *Chem-Biol Interact.* 2009; 181:52–60. [PubMed: 19442656]
54. Matsunaga T, Shinoda Y, Inoue Y, Shimizu Y, Haga M, Endo S, El-Kabbani O, Hara A. Aldo-keto reductase 1C15 as a quinone reductase in rat endothelial cell: its involvement in redox cycling of 9,10-phenanthrenequinone. *Free Radical Res.* 2011; 45:848–857. [PubMed: 21623689]

55. Nohl H, Jordan W, Youngman RJ. Quinones in biology: functions in electron transfer and oxygen activation. *Adv Free Radical Biol Med*. 1986; 2:211–279.
56. Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ. Role of quinones in toxicology. *Chem Res Toxicol*. 2000; 13:135–160. [PubMed: 10725110]
57. Monks TJ, Jones DC. The metabolism and toxicity of quinones, quinonimines, quinone methides, and quinone-thioethers. *Curr Drug Metab*. 2002; 3:425–438. [PubMed: 12093358]
58. Tegoni M, Janot JM, Labeyrie F. Regulation of dehydrogenases/one-electron transferases by modification of flavin redox potentials. Effect of product binding on semiquinone stabilization in yeast flavocytochrome b2. *Eur J Biochem*. 1986; 155:491–503. [PubMed: 3007132]
59. Jin Y, Penning TM. Aldo-keto reductases and bioactivation/detoxication. *Annu Rev Pharmacol Toxicol*. 2007; 47:263–292. [PubMed: 16970545]
60. Vlassara H, Fuh H, Makita Z, Krungkrai S, Cerami A, Bucala R. Exogenous advanced glycosylation end products induce complex vascular dysfunction in normal animals: a model for diabetic and aging complications. *Proc Natl Acad Sci U S A*. 1992; 89:12043–12047. [PubMed: 1465438]
61. Guo WA, Knight PR, Raghavendran K. The receptor for advanced glycation end products and acute lung injury/acute respiratory distress syndrome. *Intensive Care Med*. 2012; 38:1588–1598. [PubMed: 22777515]
62. Buckley ST, Ehrhardt C. The receptor for advanced glycation end products (RAGE) and the lung. *J Biomed Biotechnol*. 2010
63. Butler J, Hoey BM. The one-electron reduction potential of several substrates can be related to their reduction rates by cytochrome P-450 reductase. *Biochim Biophys Acta, Protein Struct Mol Enzymol*. 1993; 1161:73–78.
64. Roginsky VA, Barsukova TK, Stegmann HB. Kinetics of redox interaction between substituted quinones and ascorbate under aerobic conditions. *Chem-Biol Interact*. 1999; 121:177–197. [PubMed: 10418963]
65. Ham SW, Choe JI, Wang MF, Peyregne V, Carr BI. Fluorinated quinoid inhibitor: possible “pure” arylator predicted by the simple theoretical calculation. *Bioorg Med Chem Lett*. 2004; 14:4103–4105. [PubMed: 15225735]

Figure 1.

Enzymatic reactions of DCXR. DCXR can mediate the two electron NAD(P)H-dependent reduction of typical substrates including diacetyl and L-xylulose. In the absence of these substrates, DCXR can also mediate the NAD(P)H-dependent one electron reduction of quinones forming semiquinones. In this reaction, NAD(P)H generates two molecules of the semiquinone. Reaction of the semiquinones with molecular oxygen regenerates the parent compound forming a superoxide anion in the process. It should be noted that it is possible that DCXR also mediates the NAD(P)H-dependent two electron reduction of quinones to the hydroquinone. Autooxidation of the hydroquinone can result in the one electron reduced quinone, which can then react with oxygen in the redox cycling process.

Figure 2.

Purification and activity of recombinant human DCXR. (Panel A) SDS-PAGE analysis of recombinant human DCXR expressed in *E. coli*; lane M, protein standard. Lane 1, crude extract of *E. coli* containing DCXR induced with 0.5 mM isopropyl-D-thiogalactopyranoside; lane 2, flow-through fraction from the Ni-NTA column; lane 3, imidazole eluted fraction from nickel-affinity column. The arrow indicates the purified enzyme. (Panel B) Effects of increasing concentrations of DCXR on reduction of 5 mM diacetyl as measured by NADH oxidation. Reactions contained 600 μM NADH. (Inset) Effects of increasing DCXR protein in reaction mixes on rates of diacetyl reduction. (Panels C and D) Comparison of NADH and NADPH in supporting diacetyl or L-xylulose reduction by DCXR. Reaction mixes contained 200 μM NADH or NADPH and enzyme activity measured by changes in absorbance at 340 nm. (Panel E) Comparison of H_2O_2 generation by DCXR in reaction containing NADH or NADPH in the presence of 20 μM 9,10-

phenanthrenequinone. Data are presented as the mean $\pm$ SE ($n = 4$), significantly more DCXR activity was evident in all reactions containing NADH when compared to NADPH ($p < 0.001$, from t test analysis using Sigmaplot).

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Figure 3.

Generation of reactive oxygen species by human recombinant DCXR. (Panel A) Effects of menadione on superoxide anion production by DCXR in the presence of 600 μM NADH. Menadione (300 μM) was added to reaction mixes as indicated by the arrow to stimulate superoxide anion formation. Reaction mixes in 100 μL contained 2.56 μg DCXR. Superoxide dismutase (SOD) (400 U/mL) was added to the reactions after 3.5 min as indicated by the arrowhead. Control reactions did not contain DCXR. (Panel B) Effects of menadione (300 μM) on H₂O₂ production by DCXR. Reactions were run in the presence and absence of catalase (2 kU/mL). Menadione was added at time zero. Reaction mixes in 100 μL contained 1.93 μg of DCXR. (Panel C) Effects of 9,10-phenanthrenequinone (9,10-PQ) on the production of hydroxyl radicals by DCXR. Reactions were run in the presence and absence of DMSO (15 mM). 9,10-Phenanthrenequinone (20 μM) was added at time zero. Reaction mixes in 100 μL contained 1.6 μg of DCXR and 2 mM terephthalate. (Inset) Ability of hydroxyl radicals generated by DCXR redox cycling to nick closed circular plasmid DNA. All samples in 20 μL reaction mixes contained 200 ng of plasmid DNA and 20 μM 9,10-phenanthrenequinone. Reactions were run in the absence (lane 1) and presence of DCXR (lanes 2–5). Samples in lanes 3 and 5 contained 100 μM Fe²⁺ and 110 μM EDTA; samples in lanes 4 and 5 contained 100 U of catalase. Reaction mixes were incubated for 1 h and then analyzed on a 0.8% agarose gel. Closed circular plasmid DNA and nicked plasmid DNA bands were visualized under ultraviolet light and are indicated by arrowhead and

arrow, respectively. (Panel D) Ability of different quinones to redox cycling with DCXR. H_2O_2 formation was measured in the presence of 200 μM NADPH and increasing concentrations of redox cycling chemicals. The maximum values of H_2O_2 production by DCXR for each quinones are listed in Table 2.

Figure 4.

Oxygen consumption by human recombinant DCXR during chemical redox cycling. The reaction was initially run in the presence of NADH (600 μM) and 9,10-phenanthrenequinone (20 μM) to establish a stable baseline. DCXR was then added to start the reaction. (Panel A) Inhibition of oxygen consumption by diacetyl (5 mM) which was added after DCXR (arrow). (Panel B) Pretreatment with diacetyl inhibited oxygen consumption during redox cycling by DCXR. Diacetyl was added to the reaction mix at time zero; 9,10-phenanthrenequinone (9,10-PQ) was used as the redox cycling chemical. (Panels C and D) Inhibition of oxygen consumption by hexanoic acid (50 mM) and butyric acid (10 mM) during redox cycling by DCXR. The short chain fatty acids were added at the time points indicated.

Figure 5.

Redox cyclers inhibit acetoin formation during diacetyl reduction by recombinant human DCXR. (Panel A) Effects of menadione on diacetyl reduction by DCXR. Reaction mixes contained 2 mM diacetyl, 600 μ M NADH, and 10 μ g/mL DCXR. At the indicated times, aliquots were removed and analyzed by HPLC for diacetyl and acetoin as described in the Materials and Methods. The top HPLC tracing shows the acetoin standard (10 nmol). The second and third HPLC tracings show diacetyl metabolism in reaction mixes after 0 and 10 min, respectively. The lower HPLC tracing shows the effects of menadione (300 μ M) on the reduction of diacetyl. (Panel A, inset) In the presence of 300 μ M menadione (MD), > 90% of the DCXR mediated reduction of diacetyl was inhibited. (Panel B) Effects of redox cyclers on diacetyl reduction by recombinant DCXR. Enzyme activity was assayed by the formation of acetoin in reaction mixes by HPLC. IC_{50} values for 1,2-naphthoquinone (1,2-NQ), 9,10-phenanthrenequinone (9,10-PQ), 1,4-naphthoquinone (1,4-NQ), and menadione (MD) are 2.9, 9.3, 11.8, and 52.8 μ M, respectively. (Panel C) Lineweaver-Burk plot showing a competitive type of inhibition of diacetyl reduction by menadione.

Figure 6.

Effects of diacetyl and quinones on DCXR. (Panel A) Diacetyl restores DCXR substrate reduction inhibited by menadione (MD). DCXR reactions were run in the absence (gray bars) and presence (black bars) of 300 μM menadione, 200 μM NADPH, and 5 mM diacetyl. After 0, 5, or 10 min, substrates and cofactors were removed using Histag-agarose beads. Imidazole (200 mM) was used to elute DCXR from the beads, and then 10 μL of eluent fraction was added to 90 μL of enzyme reaction mixes which contained 200 μM NADPH and 5 mM diacetyl. Imidazole (20 mM) in the final reaction mix did not affect enzyme activity. Lane a is a positive control. In lane b, menadione was added back to the reaction mixtures where indicated. In lanes c–h, no menadione was added back to the reaction mixtures. DCXR activity was then measured over an additional 10 min incubation time. Reduction of diacetyl to acetoin was measured by HPLC as described in the Materials and Methods. These data demonstrate that DCXR-mediated inhibition of diacetyl reduction by menadione was reversible. (Panel B) Diacetyl (5 mM) inhibits superoxide anion formation by DCXR in the presence of 300 μM menadione. Reaction mixes in 100 μL contained 1.6 μg of DCXR and 600 μM NADH. (Inset) In the presence of diacetyl, menadione-stimulated superoxide anion formation by DCXR was inhibited by approximately 75%. Data are presented as the mean ± SE ($n = 3$). (Panels C and D) Lineweaver–Burk plots showing competitive type inhibition of chemical redox cycling by diacetyl and L-xylulose, respectively, of cytochrome *c* reduction induced by 9,10-phenanthrenequinone. Reaction mixes in 100 μL contained either 1.6 μg or 1.2 μg of DCXR in panels B and C, respectively.

Figure 7. Metabolism of diacetyl by lung epithelial cells. A549 cells were treated with diacetyl (2 mM) as described in Materials and Methods. After 0, 2, and 3 h, the culture medium was assayed for diacetyl and acetoin. The upper HPLC tracing shows an acetoin standard. The lower HPLC tracings show acetoin accumulation in the culture medium after 0, 2, and 3 h. An additional peak at about 20 min was generated in the reaction. This peak likely responds to another metabolite of diacetyl. (Inset) Western blot showing the expression of DCXR in A549 cells from two independent samples and quantification of acetoin accumulation in the culture medium of A549 cells after the addition of diacetyl. In the Western blot, β -actin was used as a protein loading control.

Figure 8.

Effects of menadione on the metabolism of diacetyl in lung epithelial cells. A549 cells were treated with diacetyl (2 mM) in the absence and presence of menadione (MD, 300 μM). The upper HPLC tracing shows an acetoin standard. The center two HPLC tracings show acetoin accumulation in the culture medium at 0 and 3 h after the addition of diacetyl. The lower HPLC tracing shows that acetoin formation in the cells is inhibited in the presence of menadione. (Inset) After 3 h, menadione inhibits diacetyl metabolism by approximately 65%.

Figure 9.

Molecular docking of L-xylulose, diacetyl, and redox cycling quinones to human DCXR. The docking model of substrates or redox cyclers bound to human DCXR (PDB ID: 1WNT) was performed using AutoDock Vina; the best-ranked docking poses were analyzed using PyMOL. (A,B) Docking of L-xylulose to human DCXR. The crystal structure of DCXR is shown as a ribbon diagram in green with critical amino acids (including catalytic triad S136, Y149, and K153) depicted as sticks with green backbones (oxygen, red; nitrogen, blue; and phosphor, orange). NADP is shown as sticks with magenta backbones. L-Xylulose is shown in spherical form (A) or in stick form (B) with cyan backbones. Residues within 4 Å of L-xylulose are indicated. Hydrogen bonds are presented in red broken lines, and the corresponding distances (Å) are indicated. The best-ranked docking pose of L-xylulose is situated in proximity to the nicotinamide moiety of NADP and the catalytic residues (S136 and Y149) of DCXR. L-Xylulose forms H bond contacts with Y149, H146, W191, and the oxygen atom on the nicotinamide moiety of NADP. (C) Interactions of diacetyl with key amino acid residues in DCXR. Diacetyl, shown in sticks with gray backbones, is located

within van der Waals contacts with Y149, L89, and V181 and forms H bond interactions with H146 and W191. (D) Interactions of 1,4-naphthoquinone with key amino acid residues in DCXR. 1,4-Naphthoquinone, shown as sticks with yellow backbones, fits in the hydrophobic cleft in the active site of DCXR forming hydrophobic interactions with M186, V181, W191, H146, L89, and Y149. (E) Superposition of L-xylulose, diacetyl, and 1,4-naphthoquinone in human DCXR. Note that the docked positions of L-xylulose, diacetyl, and 1,4-naphthoquinone are highly similar in the active site of DCXR, suggesting that these compounds may act as competitive substrates/inhibitors of the enzyme. (F) Superposition of diacetyl and 9,10-phenanthrenequinone in human DCXR. 9,10-Phenanthrenequinone is shown as sticks with orange backbones.

Table 1

Kinetic constants of recombinant human DCXR

		DCXR		
Reduction		<i>K_m</i> (mM)	<i>k_{cat}</i> (S⁻¹)	<i>k_{cat}/K_m</i> (S⁻¹ mM⁻¹)
Substrate I	Substrate II (fixed)			
Diacetyl	200 μM NADPH	0.170	5.80	33.90
NADPH	2 mM diacetyl	0.090	1.60	17.90
Xylulose	0.6 mM NADPH	0.503	1.09	2.16
NADPH	6 mM xylulose	0.024	1.55	64.00
Dehydrogenation (reverse reaction)		<i>K_m</i> (mM)	<i>k_{cat}</i> (S⁻¹)	<i>k_{cat}/K_m</i> (S⁻¹ mM⁻¹)
Substrate I	Substrate II (fixed)			
NADP(+)	200 mM xylitol	0.11	1.7	15.211
Redox cycling		<i>K_m</i> (μM)	<i>k_{cat}</i> (min⁻¹)	<i>k_{cat}/K_m</i> (min⁻¹ μM⁻¹)
Substrate I	Substrate II (fixed)			
9,10-PQ	200 μM NADPH	0.73	8.92	12.210
1,4-NQ	200 μM NADPH	10.75	1.70	0.158
Menadione	200 μM NADPH	365.80	1.32	0.004
NADPH	20 μM 9,10-PQ	31.00	6.91	0.223

The diacetyl and xylulose reduction was detected in 100 mM phosphate buffer, pH 7.0. Xylitol dehydrogenation was assayed in 100 mM glycine buffer with 3 mM MgCl₂. Redox cycling activities were assayed either following superoxide anion formation or production of H₂O₂. The details were described in the Materials and Methods.

Table 2

Redox cycling activity of quinones by DCXR

Chemicals	Structures	EC50 (μM) ^a	Redox potential (E, μV) ^b
1,2-naphthoquinone		1.52	-89
9,10-phenanthrenequinone		1.73	-124
1,4-naphthoquinone		5.37	-140
menadione		228.00	-206

^aEC50 is the half maximal concentration of quinones activating H₂O₂ production by redox cycling with DCXR. Assays were performed using 5 $\mu\text{g/ml}$ recombinant DCXR and 200 μM NADH. Maximal H₂O₂ production in enzyme assays with 1,2 - naphthoquinone was 93 pmol/min/ μg DCXR, 9,10- phenanthrenequinone was 252 pmol/min/ μg DCXR, 1,4 - naphthoquinone was 40 pmol/min/ μg DCXR and menadione was 142 pmol/min/ μg DCXR.

^bData are from Refs 63–65.

Table 3

Effects of inhibitors on DCXR activity

Inhibitor	DCXR activity ^I	Type of inhibition	Ki (μM)
Diacetyl	redox cycling	competitive	190.00
Xylulose	redox cycling	competitive	940.00
1, 2-naphthoquinone	diacetyl reduction	competitive	1.35
9,10-phenanthrenequinone	diacetyl reduction	competitive	4.27
1, 4-naphthoquinone	diacetyl reduction	competitive	5.40
menadione	diacetyl reduction	competitive	24.30

^IDiacetyl and xylulose reactions and redox cycling by DCXR were measured as described in the Materials and Methods. For redox cycling with diacetyl and xylulose as inhibitors, 20 μM 9,10-phenanthrenequinone was used.