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Quinone and Nitrofurantoin Redox Cycling by Recombinant Cytochrome b₅ Reductase

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Abstract

NADH cytochrome b₅ reductase mediates electron transfer from NADH to cytochrome b₅ utilizing flavin adenine dinucleotide as a redox cofactor. Reduced cytochrome b₅ is an important cofactor in many metabolic reactions including cytochrome P450-mediated xenobiotic metabolism, steroid biosynthesis and fatty acid metabolism, hemoglobin reduction, and methionine and plasmalogen synthesis. Using recombinant human enzyme, we discovered that cytochrome b₅ reductase 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 was oxygen-dependent and preferentially utilized NADH as a co-substrate; NADH was 5–10 times more active than NADPH in supporting redox cycling. Redox cycling activity was greatest for 9,10-phenanthrenequinone and 1,2-naphthoquinone, followed by 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (menadione), nitrofurantoin and 2-hydroxyestradiol. Using menadione as the substrate, quinone redox cycling was found to inhibit reduction of cytochrome b₅ by cytochrome b₅ reductase, as measured by heme spectral changes in cytochrome b₅. Under anaerobic conditions where redox cycling is inhibited, menadione had no effect on the reduction of cytochrome b₅. Chemical redox cycling by cytochrome b₅ reductase may be important in generating cytotoxic reactive oxygen species in target tissues. This activity, together with the inhibition of cytochrome b₅ reduction by redox-active chemicals and consequent deficiencies in available cellular cytochrome b₅, are likely to contribute to tissue injury following exposure to quinones and related redox active chemicals.

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Keywords

cytochrome b₅; redox cycling; reactive oxygen species; free radicals

Introduction

Cytochrome b₅ reductase is an FAD-containing enzyme that mediates electron transfer from NADH to cytochrome b₅ (Elahian *et al.*, 2014; Strittmatter, 1965). Reduced cytochrome b₅ serves as a redox partner in an array of metabolic processes including cytochrome P450-mediated xenobiotic metabolism and steroid biosynthesis, fatty acid metabolism, hemoglobin reduction, and methionine and plasmalogen synthesis (Guengerich, 2005). Cytochrome b₅ reductase/cytochrome b₅ has also recently been reported to reduce cytoglobin, a heme containing protein thought to be important in protecting cells against hypoxia (Amdahl *et al.*, 2017). The importance of cytochrome b₅ reductase *in vivo* is illustrated by findings that endoplasmic reticulum-associated b₅ knockout mice display phenotypic abnormalities that include alterations in hepatic fatty acid metabolism and the development of diabetes and lipoatrophy (Xu *et al.*, 2011). Genetic deficiencies in cytochrome b₅ reductase in humans have also been linked to methemoglobinemia which is associated with accumulation of oxidized Fe⁺³ and a reduced oxygen carrying capacity of blood as well as neurodevelopmental disorders (Fermo *et al.*, 2008; Galeeva *et al.*, 2013; Hudspeth *et al.*, 2010; Nagai *et al.*, 1993).

Earlier studies demonstrated that cytochrome b₅ reductase can mediate the one electron reduction of quinones to their semiquinone form (Iyanagi *et al.*, 1984; Iyanagi and Yamazaki, 1969; Iyanagi and Yamazaki, 1970). Semiquinone intermediates are unstable and donate an electron to diatomic oxygen, forming superoxide anion and returning to the native quinone state (O'Brien, 1991; see Fig 1 for reaction scheme). The ability of a quinone to be reduced again by cytochrome b₅ reductase, cycling back and forth between redox states, a process referred to as chemical redox cycling or futile redox cycling, can result in tissue injury. The formation of superoxide anion through redox cycling generates cytotoxic oxidizing species including hydrogen peroxide (H₂O₂), hydroxyl radicals and, in the presence of nitric oxide, peroxynitrite; in cells this process can lead to oxidative and/or nitrosative stress (Wink *et al.*, 1997). Quinone redox cycling is an energetically favorable reaction which can inhibit the enzymes mediating this process. For example, quinone redox cycling is also known to be mediated by NADPH cytochrome P450 reductase, an enzyme that donates electrons to the cytochrome P450s (Szilagyi *et al.*, 2016). Redox cycling preferentially uses electrons from NADPH at the expense of their supply to the cytochrome P450s which in turn inhibits their monooxygenase activities (Jan *et al.*, 2015). Similarly, sepiapterin reductase, the rate limiting enzyme in tetrahydrobiopterin biosynthesis, is a mediator of quinone redox cycling, a process that readily inhibits activity of this enzyme (Yang *et al.*, 2013). In the present studies, we characterized chemical redox cycling using recombinant human cytochrome b₅ reductase and determined if this process interferes with electron flow from NADH to cytochrome b₅. The ability to generate reactive oxygen species as well as alter the reduction of cytochrome b₅ is likely to contribute to tissue injury induced by various redox active chemicals.

Materials and Methods

Chemicals and reagents.

Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) was obtained from Invitrogen (Eugene, OR). Cytochrome b₅, acetylated cytochrome c, 9,10-phenanthrenequinone, 2-methyl-1,4-naphthoquinone (menadione), 1,4-naphthoquinone, nitrofurantoin (N-(5-nitro-2-furfurylidene)-1-aminohydantoin), 2-hydroxyestradiol, diethylenetriaminepentaacetic acid (DETAPAC) and all other chemicals were from SigmaAldrich (St. Louis, MO). cDNA expressing recombinant N-terminal histidine tagged human cytochrome b₅ reductase was a generous gift of Dr. Lauren Trepanier (University of Wisconsin-Madison, Madison, WI). The enzyme was expressed in *E. coli* as previously described (Kurian *et al.*, 2004) and purified using Ni-NTA chromatography (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Stock solutions of the enzyme ranged from 1–4 mg/ml and were >98% pure as determined by SDS-polyacrylamide gel electrophoresis followed by silver staining. The specific enzyme activity was assessed using the ferricyanide assay as previously published (Mihara and Sato, 1975) where 1 U of cytochrome b₅ reductase reduces 1 μ mole of ferricyanide per min. Enzyme protein was quantified using the DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Enzyme assays.

Unless otherwise stated, all assays were performed in 96-well flat bottom microwell plates at 37°C; changes in absorbance/fluorescence were measured using a SpectraMax M3 fluorescent microplate reader (Molecular Devices, Sunnyvale, CA). Data were collected using SoftMax Pro 6.3 software and analyzed using GraphPad Prism 5 (La Jolla, CA). Reactions were initiated using NADH or NADPH (200 μ M final concentration) as reducing cofactors. Enzyme concentrations in reaction mixes ranged from 0.02–12 U/mL, as specified in Fig. legends.

Measurements of reactive oxygen species in enzyme assays.

Changes in absorbance at 550 nm of acetylated cytochrome c were used to monitor production of superoxide anion (Azzi *et al.*, 1975). Briefly, reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.8), 50 μ M acetylated cytochrome c, 1 U/mL recombinant cytochrome b₅ reductase and 100 μ M menadione in a final volume of 0.1 mL. Unless otherwise stated, reactions were initiated by the addition of NAD(P)H (200 μ M). To demonstrate the specificity of the assay, superoxide dismutase (1000 U/mL) was used as a scavenger of superoxide anion. H₂O₂ generation was measured using the continuous Amplex red/horseradish peroxidase method as previously described (Mishin *et al.*, 2010). Briefly, reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.8), 0.2 U/mL recombinant cytochrome b₅ reductase, 100 μ M Amplex red, 1 U/mL horseradish peroxidase, and redox cycling compounds in a final volume of 0.1 mL. Reactions were initiated by the addition of NAD(P)H. To demonstrate the specificity of the assay, catalase (1000 U/mL) was used as a scavenger of H₂O₂. The fluorescence increase due to formation of resorufin (excitation 535 nm/emission 580 nm), was recorded, and the rates were calculated based on the linear phase of reactions after background subtraction. The terephthalate-based assay was used to measure the formation of hydroxyl radicals (Son *et*

et al., 2015). In this assay, hydroxyl radicals are formed due to the reaction of H_2O_2 with Fe^{2+} and scavenged by the non-fluorescent substrate terephthalate, producing the fluorescent 2-hydroxyterephthalate (2-OH-TPT, excitation 315 nm/emission 425 nm). Reactions were run in the presence of 1 U/mL recombinant cytochrome b_5 reductase, 100 μM FeCl_3 , 110 μM EDTA and 1 mM terephthalate. To demonstrate the specificity of the assay, in some experiments DMSO (1%, ~125 mM) was used as a trap for hydroxyl radicals.

Analysis of NAD(P)H consumption and oxygen utilization in enzyme assays.

Oxygen uptake was measured using an Oxygraph Plus System fitted with a Clark-type oxygen electrode (Hansatech Instruments Ltd, UK) as previously described (Yang *et al.*, 2013). Decreases in absorbance at 340 nm were used to analyze the rates of NADH utilization initiated by cytochrome b_5 reductase (Bergmeyer, 1975). Briefly, reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.8), 1.0 mM sodium azide, 1.0 mM DETAPAC, and 600 μM NADH in a final volume of 0.1 ml. In these experiments the reactions were initiated by the addition of 1U/mL cytochrome b_5 reductase.

Spectral analysis of cytochrome b_5 heme.

The oxidation state of heme can be characterized using visible-wavelength spectral analysis (Lewis, 1954; North *et al.*, 1996). The enzymatic reduction of cytochrome b_5 heme by cytochrome b_5 reductase results in a red shift of its Soret peak from 412 to 425 nm (see Fig 4) (Aoyama *et al.*, 1990; Arinc and Cakir, 1999; Strittmatter and Velick, 1956). Heme-binding difference spectra were recorded on the microplate reader as previously described (Szilagyi *et al.*, 2016). Reaction mixtures for all spectral measurements contained 100 mM potassium phosphate buffer (pH 7.5), 0.1% Triton X-100, 12 U/mL cytochrome b_5 reductase and 12 ng/mL cytochrome b_5 . Prior to the addition of NADH, each sample served as its own reference cuvette. Reaction mixtures were incubated in the dark and, after 10 min at room temperature, 200 μM NADH was added and the mixture incubated for an additional 10 min. The optical absorbance from 300–500 nm was recorded before and after the addition of NADH. Difference spectra were generated by subtracting the absolute oxidized spectra from the absolute reduced spectra. Where applied, anaerobic conditions in the wells were generated by incubating reaction mixtures with 25 U/mL catalase, 50 mM glucose, and 12.5 U/mL glucose oxidase for 20 min prior to initiating spectral analysis (Penketh *et al.*, 2015).

Results

In initial studies, we characterized redox cycling by recombinant human cytochrome b_5 reductase. Redox cycling of quinones generates a semiquinone radical that reacts with molecular oxygen to regenerate the parent compound (O'Brien, 1991). Using menadione as the redox active quinone, the enzyme was found to readily consume oxygen in the presence of NADH (Fig. 2A). Both oxygen utilization and NADH consumption were time-dependent (Figs. 2A-B). The redox cycling process actively generated superoxide anion and H_2O_2 (Fig. 2C-D). Formation of reactive oxygen species was also dependent on time and the concentration of menadione. NADH was significantly more active in supplying reducing equivalents to cytochrome b_5 reductase for redox cycling when compared to NADPH (Fig. 2E); the rate of H_2O_2 formation in the presence of menadione (100 μM) per unit of

cytochrome b₅ reductase for NADH and NADPH was 595.8 ± 18.1 and 58.8 ± 7.8 pmole/min/U B₅R ($n = 3 \pm \text{SD}$), respectively. In the presence of Fe³⁺/EDTA complexes, redox cycling corresponded with generation of hydroxyl radicals (Fig. 2F). Accumulation of superoxide anion and H₂O₂ in redox cycling assays was abolished by superoxide dismutase and catalase, respectively, while hydroxyl radical production was inhibited by DMSO, a hydroxyl radical trap (Eberhardt and Colina, 1988). Cytochrome b₅ reductase did not produce any detectable reactive oxygen species in the absence of a redox cycling agent (data not shown).

Several additional redox active chemicals generated reactive oxygen species by redox cycling with cytochrome b₅ reductase including 9,10-phenanthrenequinone, 1,4-naphthoquinone, and the catechol estrogen, 2-hydroxyestradiol and nitrofurantoin (Fig. 3 and Table 1). Of these chemicals, 9,10-phenanthrenequinone was most active ($K_m = 5.7 \mu\text{M}$, $k_{\text{cat}} = 5.8 \text{ min}^{-1}$, $k_{\text{cat}}/K_m = 1.0 \text{ min}^{-1}\mu\text{M}^{-1}$). Less activity was noted for 1,4-naphthoquinone ($K_m = 9.8 \mu\text{M}$, $k_{\text{cat}} = 0.99 \text{ min}^{-1}$, $k_{\text{cat}}/K_m = 0.10 \text{ min}^{-1}\mu\text{M}^{-1}$), 2-hydroxyestradiol ($K_m = 2.6 \mu\text{M}$, $k_{\text{cat}} = 0.17 \text{ min}^{-1}$, $k_{\text{cat}}/K_m = 0.065 \text{ min}^{-1}\mu\text{M}^{-1}$), menadione ($K_m = 77 \mu\text{M}$, $k_{\text{cat}} = 3.5 \text{ min}^{-1}$, $k_{\text{cat}}/K_m = 0.046 \text{ min}^{-1}\mu\text{M}^{-1}$) and nitrofurantoin ($K_m = 270 \mu\text{M}$, $k_{\text{cat}} = 0.99 \text{ min}^{-1}$, $k_{\text{cat}}/K_m = 0.0037 \text{ min}^{-1}\mu\text{M}^{-1}$). Cytochrome b₅ reductase-mediated redox cycling was also observed with other catechol estrogens, including 4-hydroxyestradiol, 4-hydroxyestrone and 2-hydroxyestriol. The parent estrogens, estradiol, estrone, and estriol, and the methoxy-estrogen metabolite 2-methoxyestradiol were not able to redox cycle with the enzyme (data not shown).

Cytochrome b₅ is an endogenous ligand for cytochrome b₅ reductase (Strittmatter, 1965). As a heme-containing protein, reduction of cytochrome b₅ by cytochrome b₅ reductase can be monitored by difference spectroscopy (Aoyama, et al., 1990; Strittmatter and Velick, 1956). In this assay, reduction of cytochrome b₅ by cytochrome b₅ reductase results in a red shift of its Soret peak from 410 nm to 425 nm (Fig 4)(Aoyama, et al., 1990). We found that by redox cycling with cytochrome b₅ reductase, both menadione (10 μM and 100 μM) and 2-hydroxyestradiol (100 μM) blocked reduction of cytochrome b₅ (Fig. 5A-B). As redox cycling is oxygen-dependent, we next analyzed the effects of depleting oxygen on cytochrome b₅-mediated reduction of cytochrome b₅ in reaction mixtures. In the presence of NADH, cytochrome b₅ reductase effectively supplied electrons to cytochrome b₅ under both aerobic and anaerobic conditions, as measured by distinctive changes in the cytochrome b₅ difference spectra. However, under anaerobic conditions, menadione was unable to inhibit the reduction of cytochrome b₅ (Fig. 5C).

Discussion

The present studies demonstrate that a variety of redox active chemicals, including quinones and nitrofurantoin, redox cycle with purified recombinant cytochrome b₅ reductase. These data are consistent with earlier studies using electron spin resonance, which demonstrated that cytochrome b₅ reductase catalyzes the one electron reduction of quinones including menadione (Iyanagi and Yamazaki, 1969; 1970). The generation of reactive oxygen species during chemical redox cycling is an important mechanism contributing to tissue injury (Hochstein, 1983). Hydroxyl radicals derived from superoxide anion and H₂O₂ are highly

reactive; modifications on cellular macromolecules can disrupt cellular function. Reactive oxygen species can also initiate lipid peroxidation, a process that can disrupt lipid membranes and generate cytotoxic lipid peroxidation end products. In addition to cytochrome b₅ reductase, a number of other enzymes have been reported to mediate redox cycling, including various aldo-keto reductases, nitric oxide synthases and thioredoxin reductases, cytochrome P450 reductase, sepiapterin reductase and dicarbonyl/L-xylulose reductase (Garner *et al.*, 1999; Gray *et al.*, 2007; Shultz *et al.*, 2011; Szilagyi, et al., 2016; Yang, et al., 2013; Yang *et al.*, 2017). At the present time, the individual contributions of the enzymes to tissue injury in response to different quinones and other redox active chemicals as a consequence of redox cycling, is unknown. It is likely that toxicity depends, not only on the relative ability of individual enzymes to generate reactive oxygen species during redox cycling, but also on enzyme concentrations and their subcellular distribution in different cell types. In this regard, the ability of microsomal forms of cytochrome b₅ reductase and cytochrome P450 reductase to generate reactive oxygen species during redox cycling may compromise microsomal functioning including xenobiotic metabolism. However, redox cycling enzymes such as various aldo-keto reductases and cytochrome P450s can also metabolize various redox active chemicals. Together with various cellular antioxidants, these detoxifying enzymes are important in limiting tissue damage resulting from exposure to redox active chemicals.

It is well recognized that estrogens are an important risk factor for the development of breast cancer (Santen *et al.*, 2015). Estrogens can be metabolized to catechols, as well as reactive quinones and semiquinones (Parl *et al.*, 2009). Catechol estrogens are known to redox cycle, and their ability to generate cytotoxic reactive oxygen species may contribute to the carcinogenic process (Parl, et al., 2009). Both breast cancer epithelial cells and purified NADPH cytochrome P450 reductase mediate catechol estrogen redox cycling and generate reactive oxygen species (Fussell *et al.*, 2011). The present studies demonstrate that cytochrome b₅ reductase mediates redox cycling of certain catechol estrogens. Depending on the localized concentrations of catechol estrogens and enzymes that mediate redox cycling such as cytochrome b₅ reductase, this may be an important mechanism contributing to the development of breast cancer.

Nitrofurantoin is a commonly used nitroaromatic redox-active antibiotic; it has also been used as an antitumor agent (Kamat and Lamm, 2004; Oscier *et al.*, 2002). Adverse reactions to the drug, which include liver injury (Paiva *et al.*, 1992), pulmonary fibrosis (Witten, 1989), hematological disorders (Gait, 1990), and peripheral polyneuropathy (Jacknowitz *et al.*, 1977), are thought to be due to the generation of reactive oxygen species during redox cycling (Martin, 1983; Rossi *et al.*, 1988). Our data showing that cytochrome b₅ reductase mediates nitrofurantoin redox cycling indicates that this enzyme may be an off-target for the drug. Redox cycling by cytochrome b₅ reductase, as well as other enzymes reported to redox cycle nitroaromatic compounds, including nitric oxide synthase (Boelsterli *et al.*, 2006), thioredoxin reductase (Cenas *et al.*, 2006), ferredoxin:NADP⁺ reductase (Miskiniene *et al.*, 1997) and cytochrome P450 reductase (Wang *et al.*, 2008) may contribute to cytotoxicity of nitrofurantoin. Inhibition of the production of reactive oxygen species by these enzymes during redox cycling might translate into improved medical use of nitrofurantoin and related drugs.

Of interest were our findings that chemical redox cycling was associated with inhibition of the cytochrome b₅ reduction by cytochrome b₅ reductase. This was oxygen-dependent, a finding consistent with the fact that oxygen is required for quinone redox cycling by cytochrome b₅ reductase. Cytochrome b₅ reductase catalyzes electron transfer from NADH, a two electron carrier, to cytochrome b₅, a one electron carrier, via FAD binding domains (Strittmatter, 1965). Transfer of electrons from NADH generates a fully reduced form of FAD while transfer of single electrons to cytochrome b₅ generates FAD semiquinone intermediates. Redox cycling presumably occurs via single electron transfer between FAD, semiquinones and the redox active chemical. Inhibition of cytochrome b₅ reduction of cytochrome b₅ as a result of redox cycling is likely because this is an energetically favorable process (Iyanagi, et al., 1984). Thus, electrons preferentially transfer to redox active chemicals at the expense of the reduction of cytochrome b₅. Inhibition of cytochrome b₅ reduction may result in deficiencies in processes dependent on this cofactor including fatty acid and cholesterol synthesis, methemoglobin and cytoglobin reduction, and xenobiotic oxidation (Amdahl, et al., 2017; Galeeva, et al., 2013; Guengerich, 2005; Hudspeth, et al., 2010; Nagai, et al., 1993; Xu, et al., 2011). Interestingly, high doses of primaquine, a quinone used to treat malaria, have been shown to induce methemoglobinemia in humans (Carmona-Fonseca *et al.*, 2009; Kantor, 1992; Sin and Shafran, 1996), and this may be due to redox cycling. However, the extent to which redox cycling affects different metabolic pathways mediated by cytochrome b₅ reductase/cytochrome b₅ remains to be determined.

In summary, the present studies show that cytochrome b₅ reductase-mediated redox cycling, not only generates cytotoxic reactive oxygen species, but also has the capacity to reduce localized concentrations of intracellular oxygen and reducing equivalents from pyridine nucleotide cofactors, which can contribute to tissue damage. These activities, together with the inhibition of cytochrome b₅ reduction, are likely to contribute to cytotoxicity following exposure to quinones and other redox active chemicals. However, further studies are needed to determine how redox active chemicals bind to cytochrome b₅ reductase and the precise mechanism of electron transfer during redox cycling reactions.

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- Cytochrome b₅ reductase (cypb5) mediates chemical redox cycling
- Chemical redox cycling by cypb5 generates reactive oxygen species
- Redox cycling by cypb5 is NADH- and oxygen-dependent
- Redox cycling inhibits electron transfer from cypb5 to cytochrome b₅.

Fig 1. Reactions mediated by cytochrome b5 reductase.

Upper panel: Reduced cytochrome b5 (Cytb5-Fe³⁺) serves as a redox partner for a variety of enzymes including those mediating reduction of hemoglobin, xenobiotics and fatty acid/lipid metabolism and steroid, plasmalogen and methionine biosynthesis. Cytochrome b₅ is maintained in the reduced state by cytochrome b₅ reductase at the expense of reducing equivalents supplied by NADH. *Lower panel:* Cytochrome b₅ reductase also mediates chemical redox cycling. In this reaction, the enzyme forms semiquinone radicals from redox active quinones such as menadione (2-methyl-1,4-naphthoquinone) from electrons donated by NADH. Reaction of the semiquinone with molecular oxygen regenerates the parent compound and in the process, generates superoxide anion. Spontaneous and enzyme supported dismutation of superoxide anion generates H₂O₂ and, in the presence of a transition metal, hydroxyl radicals.

Fig 2. Characterization of redox cycling by cytochrome b₅ reductase.

Formation of reactive oxygen species by cytochrome b₅ reductase during redox cycling was initiated in the presence of menadione. *Panel A*, Oxygen consumption activated by menadione redox cycling was measured using a Clark oxygen electrode. Reaction mixtures contained 0.2 U/mL cytochrome b₅ reductase, 100 μM menadione, and 200 μM NADH. Note that the addition of menadione to the reaction mix, as indicated by the arrow, initiated oxygen consumption. Oxygen calibration was performed with the addition of several grains of sodium dithionite, as indicated by the arrow. *Panel B*, Consumption of NADH by cytochrome b₅ reductase (1 U/mL) in the presence and absence of 100 μM menadione. Then the reactions were initiated by adding NADH to a final concentration of 600 μM. Reactions were initiated by adding NADH to a final concentration of 600 μM. *Panel C*, NADH is a more effective electron donor in cytochrome b₅ reductase-mediated redox cycling. Reactions contained 0.2 U/ml cytochrome b₅ reductase, 200 μM NADH or NADPH, and increasing concentrations of menadione. Rates of H₂O₂ formation are presented as the mean ± SD (n = 3). *Panels D and E*, Time-dependent formation of superoxide anion and H₂O₂ during menadione redox cycling. Reaction mixtures contained 100 μM menadione, 200 μM NADH and 1 U/mL cytochrome b₅ reductase to measure formation of superoxide anion or 0.2 U/mL cytochrome b₅ reductase to measure formation of H₂O₂ formation. Superoxide anion was assayed using acetylated cytochrome c as described in the Materials and Methods. As indicated in some experiments, superoxide dismutase (10³ U/mL) or catalase (10³ U/ml) was added to the reaction mixtures to demonstrate the specificity of the assays. *Panel F*, Production of hydroxyl radicals in the presence of Fe²⁺ during menadione redox cycling. Reactions contained 100 μM menadione, 200 μM NADH, 1 U/mL cytochrome b₅ reductase, 100 μM FeCl₃, and 110 μM EDTA. In some reactions, DMSO (1%) was added as a hydroxyl radical trap.

Fig. 3. Comparison of chemical redox cycling by cytochrome b_5 reductase using different redox active quinones and nitrofurantoin.

Reaction mixtures contained 0.2 U/mL cytochrome b_5 reductase and were initiated by the addition of NADH to a final concentration of 200 μM . *Left panel*, H_2O_2 concentrations at various times were measuring in reaction mixtures contained increasing concentrations of menadione. Data are the mean $\pm$ SD ($n = 3$). *Right panel*, Reactions were run in the presence of increasing concentrations of 2-hydroxyestradiol (2-OH E2), 9,10-phenanthrenequinone (PHEN), 1,4-napthoquinone (1,4 NQ), menadione (MD), and nitrofurantoin (NFT). H_2O_2 concentrations were measured after 5 min.

Fig 4. Spectral properties of oxidized and reduced cytochrome b_5 .

Absolute spectra were recorded as described in Materials and Methods. Reaction mixtures contained 12 U/mL cytochrome b_5 reductase and 12 ng/mL cytochrome b_5 . Oxidized and reduced spectra were recorded in the presence and absence of NADH (200 μ M final concentration), respectively. Reduction of cytochrome b_5 is characterized by a shift in its Soret peak from 414 to 425 nm (Mihara and Sato, 1975; Strittmatter, 1965).

Fig 5. Effects of redox cycling agents on the reduction of cytochrome b_5 by cytochrome b_5 reductase.

Difference spectra were recorded as described in Materials and Methods. Reaction mixtures contained 12 U/mL cytochrome b_5 reductase, 12 ng/mL cytochrome b_5 , and menadione or 2OHE2 (*Left panel*, 10 μ M; *Center panel*, 100 μ M). The spectrum of each sample was recorded before and after the addition of NADH to a final concentration of 200 μ M. Spectra in the absence of NADH for each sample served as the reference. *Right panel*, Reduction of cytochrome b_5 by cytochrome b_5 reductase in the presence and absence of menadione under aerobic and anaerobic conditions. Reaction mixtures contained 12 U/mL cytochrome b_5 reductase, 12 ng/mL cytochrome b_5 , and 100 μ M menadione. Anaerobic conditions were generated *in situ* using the glucose/glucose oxidase method as described in the Materials and Methods.

Table 1

Kinetic constants for NADH-cytochrome b5 reductase-mediated H₂O₂ generation by redox cycling agents.

Compound	K _M	K _{cat}	K _{cat} /K _M (min ⁻¹ μM ⁻¹)
Menadione	77	3.5	0.046
9,10-Phenanthrenequinone	5.7	5.8	1.0
1,4-Napthoquinone	9.8	0.99	0.10
Nitrofurantoin	270	0.99	0.0037
2OHE2	2.6	0.17	0.065