Carcinogenesis vol.16 no.4 pp.891–895, 1995

Mitochondrial enzyme-catalyzed oxidation and reduction reactions of stilbene estrogen

Ronald D.Thomas and Deodutta Roy[1]

Environmental Toxicology Program, Department of Environmental Health Sciences, 720 South 20th Street, University of Alabama, Birmingham, AL 35294, USA

[1]To whom correspondence should be addressed

We have demonstrated for the first time that mitoplasts (i.e. mitochondria without outer membrane) were able to convert stilbene estrogen (diethylstilbestrol, DES) to reactive metabolites, which covalently bind to mitochondrial (mt)DNA. Depending on the cofactor used, mitochondrial enzymes catalyzed the oxidation and/or reduction of DES. DES was oxidized to DES quinone by peroxide-supported mitochondrial enzyme. A Lineweaver–Burk plot of rate of formation of DES quinone at various substrate concentrations yielded a K_m of 33 μM and V_{max} of 39 nmol/mg protein/min. The oxidation of DES to DES quinone by mitochondria was drastically decreased by known inhibitors of cytochromes P450. DES quinone was reduced to DES by mitoplasts in the presence of NADH. The K_m and V_{max} for the DES quinone reduction in the absence of mitoplasts and NADH were 3.2 μM and 5.6 nmol respectively. The reduction of DES quinone to DES by mitoplasts was significantly inhibited by inhibitors of cytochrome b5 reductase and diaphorase. DES quinone was also reduced to DES by pure diaphorase, a mitochondrial reducing enzyme, in the presence of NADH. The K_m and V_{max} for the DES quinone reduction by diaphorase were 9.0 μM and 4.3 nmol respectively. Under reaction conditions similar to oxidation of DES to DES quinone by mitoplasts, it was observed that mitochondrial metabolic products of DES were able to covalently bind to mtDNA. These data provide direct evidence of mitochondrial enzyme-catalyzed oxidation and reduction reactions of DES. In the cell, activation of DES in the mitochondria (the organelle in which mtDNA synthesis, mtDNA repair and transcription systems are localized) is of utmost importance, because an analogous *in vivo* mitochondrial metabolism of DES through covalent modifications in mitochondrial genome may produce instability in the mitochondrial genome of the cells. These modifications may in turn play a role in the development of DES-induced hepatocarcinogenicity.

Introduction

We have previously shown that estrogens are metabolized to reactive intermediates such as semiquinone and quinone (1,2). Diethylstilbestrol (DES*, a synthetic estrogen also known as stilbene estrogen) reactive metabolites have been shown to covalently bind to DNA (3) and nuclear proteins (4). It is believed that DES-reactive metabolites, mainly formed by microsomal cytochrome P4501A1 (2) in the endoplasmic reticulum, are transported to mitochondria and nuclei to bind covalently to macromolecules of subcellular components. While it is widely believed that metabolic activation of chemical carcinogens probably occurs in the endoplasmic reticulum, the concept that another organelle can play an important role in this process is gaining support (5,6). DES-reactive intermediates such as quinone and semiquinone are highy unstable (7). The inherent unstable nature of DES-reactive metabolites suggests that activation of DES most likely occurs *in situ* within the organelle (i.e. site of genotoxicity). It has been shown that the mitochondria contain cytochromes P450 which can activate benzo[*a*]pyrene and aflatoxin B_1 to their reactive metabolites (8–10). It is not known whether mitochondria are capable of participating in redox cycling of DES.

In the present study, oxidation of the stilbene estrogen, *E*-DES, to DES quinone and the subsequent reduction of DES quinone to *Z*-DES by mitoplasts have been investigated. This study provides direct evidence for mitochondrial enzyme-mediated oxidation and reduction reactions of DES. DES-reactive intermediates, generated during mitochondrial oxidation, are able to bind covalently to mitochondrial (mt)DNA. An analogous *in vivo* oxidation of DES to reactive metabolites and covalent modifications in DNA of mitochondria by reactive products may be factors in DES-induced toxicity/cell transformation/teratogenicity.

Materials and methods

Chemicals

E-DES, NADPH, NADH, cumene hydroperoxide (CHP), α-naphthoflavone (ANF), β-naphthoflavone (BNF), diaphorase (EC 1.8.1.4), *p*-hydroxy mercuribenzoic acid, mercuric chloride, and α-naphthylisothiocyanate (ANIT) were purchased from Sigma Chemical Co., St Louis, MO. *Z*-DES and *Z,Z*-dienestrol (*Z,Z*-DIES) were a generous gift of Dr J.G.Liehr, UTMB, Galveston, TX. DES quinone was chemically synthesized as described previously (2), and the purity of DES quinone was determined by UV spectroscopy, HPLC and, after rearrangement to *Z,Z*-DIES, by GC–MS (2). Materials and chemicals needed for ^{32}P-postlabeling were purchased from sources described previously (3).

Mitochondrial preparation

Male Sprague–Dawley rats were treated with BNF (50 mg/kg i.p.) for 4 days. Liver mitochondria were isolated by differential centrifugation (11). To exclude the possibility of contamination with endoplasmic reticulum, the outer membrane of mitochondria was removed by treating with 1.6% digitonin. After 30 min of gentle stirring, samples were centrifuged and mitoplasts were collected (11).

mtDNA isolation

Pure mitoplasts were suspended in 50 mM Tris, 10 mM EDTA, pH 8.0, containing 1% SDS. After 5 min of gentle shaking, samples were treated with RNase A (150 μg/ml) and RNase T1 (20 U/ml) for 30 min, and then further incubated with proteinase K (500 μg/ml) for 1 h at 37°C. The mtDNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and three times with chloroform/isoamyl alcohol (24:1). Two volumes of ice-cold ethanol were added in aqueous extract, chilled at −70°C for 15 min and centrifuged at 11 000 r.p.m. The purity of mtDNA was checked by UV spectroscopy and agarose gel electrophoresis. The 260/280 ratio was 1.74.

*Abbreviations: DES, diethylstilbestrol; mtDNA, mitochondrial DNA; CHP, cumene hydroperoxide; ANF, α-naphthoflavone; BNF, β-naphthoflavone; ANIT, α-naphthylisothiocyanate; DIES Z,Z-dienestrol.

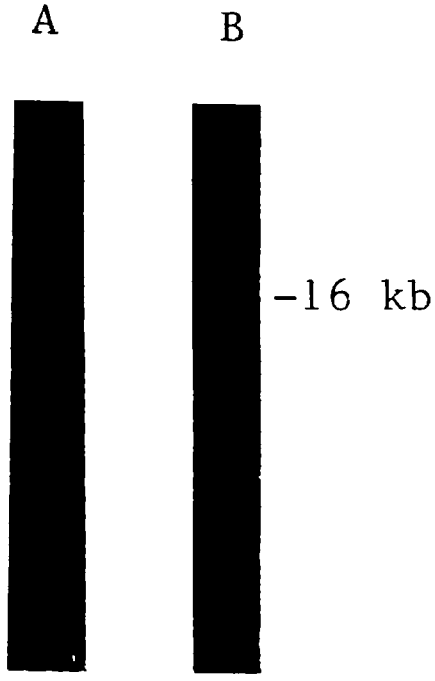

Fig. 1. Visualization of ethidium bromide staining of mtDNA separated on 1% agarose gel by UV. (**A**) DNA size standards ranging from 23 to 0.5 kb. (**B**) mtDNA (16 kb).

Determination of the purity of mitoplasts

The purity of mitoplasts was assessed by both morphological and biochemical analyses. Mitoplasts were stained with H&E. Phase-contrast microscopy did not reveal any cellular contamination. The determination of cytochrome c oxidase (12), an enzymatic marker of mitochondria, showed 110–120 μmol/mg protein/min specific activity. Microsomal contamination was assessed by measuring the activity of glucose 6-phosphatase, an enzymatic marker of endoplasmic reticulum (13). The activity of glucose 6-phosphatase in mitoplasts was <1% of that found in microsomes (4.0 pmol/mg protein/min in mitoplasts versus 636 pmol/mg protein/min in microsomes). This is in agreement with the report of Niranjan *et al.* (5,6). Visualization of ethidium bromide staining of DNA separated on 1% agarose gel by UV revealed a single band of 16 kb (Figure 1). Nuclear DNA or ribosomal RNA was not detected. These results suggested that mitoplast preparations were highly pure.

Oxidation of DES to DES quinone by mitoplasts

The reaction condition for the conversion of DES to DES quinone as described by Roy and Liehr (2) was used. Cofactor was either NADPH or CHP. The mitochondrial activation system contained mitoplasts (0.29 mg equivalent mitochondrial protein), 2 mM NADPH, or 120 or 255 μM CHP, and various concentrations of DES (0–100 μM) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. Some of the reactions were carried out in the presence of inhibitor of cytochromes P450 (20 μM ANF or 10 μM ANIT). The conversion of DES to DES quinone was monitored as a gradual increase in UV absorption in the range of 300–500 nm. The oxidation product formation was also analyzed by HPLC (2). DES metabolites were extracted with ether. The extraction recovery of synthetic DES quinone was 95 ± 5%. The identity of DES quinone was confirmed by matching with the peak of synthetic DES quinone and GC–MS analyses.

Reduction of DES quinone

The reaction condition for the reduction of quinone as described by Roy and Liehr (2) was used. The reduction reaction mixture contained mitoplasts (0.29 mg equivalent mitochondrial protein) or 0.07 mg pure enzyme diaphorase, 0.05 mM NADH and DES quinone (0–0.02 mM) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. Some of the reactions were carried out in the presence of inhibitor (50 μm *p*-hydroxy mercuribenzoic acid or 50 μM mercuric chloride). The reduction of quinone was monitored by its gradual disappearance in UV scans. The reduction product, Z-DES, was also analyzed by HPLC (2).

Covalent binding of DES to mtDNA

The reaction condition consisted of 0.145 mg equivalent mitoplast protein, 250 μM DES and 255 μM CHP or 2 mM NADPH in a final volume of 1 ml 10 mM phosphate buffer, pH 7.4. After 2 h reaction, the mtDNA was isolated. mtDNA–DES adducts were analyzed by nuclease P1 ^{32}P-postlabeling (3). The solvent system consisted of D1 = 2.3 M $NaPO_4$, pH 5.7; D3 = 4.2 M lithium formate plus 7.5 M urea, pH 3.3; D4 = 0.8 M $NaPO_4$ plus 8.5 M urea plus 0.5 M Tris, pH 8.2; and D5 = 1.7 M $NaPO_4$, pH 6.0.

Table I. Oxidation of DES to DES quinone by mitoplasts

Conditions	DES quinone (pmol/mg protein/min)
−Cumene hydroperoxide	<5
−Mitoplasts	<5
Complete system	196 ± 38 (100)
+ANF (20 μM)	28 ± 12 (14)
+ANIT (10 μM)	37 ± 2 (20)

The reaction system consisted of 290 μg/ml equivalent mitochondrial protein, 120 μM cumene hydroperoxide, 100 μM DES in a final volume of 1 ml 10 mM potassium phosphate buffer, pH 7.5 (complete system). Some of the reactions were carried out in the presence of cytochromes P450 inhibitor, α-naphthoflavone (ANF), or α-naphthylisothiocyanate (ANIT). The rearrangement product of DES quinone, Z,Z-dienestrol, was analyzed by HPLC (2). Each value is a mean of three or four experiments ± SD. The values in parentheses represent the percentage of total oxidation activity.

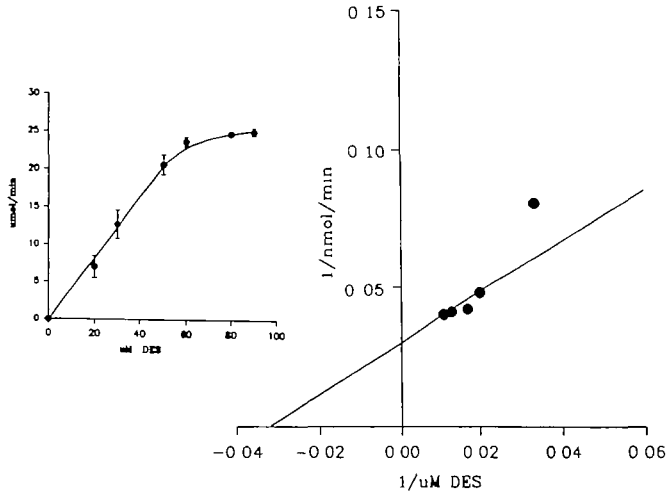

Fig. 2. Influence of various substrate concentrations on the rate of oxidation of DES to DES quinone by mitoplasts The reaction mixture consisted of purified mitoplasts from β-naphthoflavone-treated male Sprague–Dawley rat liver (0.29 mg equivalent protein), 120 μM cumene hydroperoxide, and various concentrations of DES (0–100 μM) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. The oxidation was monitored by both UV spectroscopy and HPLC as described in Materials and methods. The data are presented as a double reciprocal plot and as substrate-dependent product formation (inset). Values represent the means of three or four experiments.

Results

Oxidation of DES

DES quinone was detected by UV spectroscopy and HPLC as an oxidation product of DES in CHP-dependent mitochondrial reactions (Table I). The rate of DES quinone formation in the presence of mitoplasts and CHP was dependent on the concentration of DES (Figure 2), and a Lineweaver–Burk plot of rate of formation of DES quinone at various DES concentrations yielded a K_m of 33 μM and a V_{max} of 39 nmol/mg protein/min. DES quinone formation was below detectable level in control reactions, i.e. in the absence of cofactor (CHP) or mitoplasts (Table I).

Two well-characterized inhibitors of cytochromes P450—

Table II. Reduction of DES quinone by mitoplasts

Conditions	Reduction products (pmol Z-DES formed/mg protein/min)
−NADH	<5
−Mitoplasts	<5
Complete system	502 ± 40 (100)
+PHMB (50 μM)	283 ± 24 (47)

The reaction system consisted of 290 μg/ml equivalent mitoplast proteins, 50 μM NADH, 18 μM DES quinone in a final volume of 1 ml 10 mM potassium phosphate buffer, pH 7.5 (complete system). Some of the reactions were carried out in the presence of *p*-hydroxy mercuribenzoic acid (PHMB). The reduction product (Z-DES) was analyzed by HPLC (2). Each value is a mean of three to four experiments ± SD. The values in parentheses represent the percentage of total reduction activity.

Table III. Reduction of DES quinone by diaphorase

Conditions	Reduction products (pmol Z-DES pmol/mg protein/min)
−NADH	<5
−Diaphorase	<5
Complete system	806 ± 122
+PHMB (50 μM)	<5
+$HgCl_2$ (50 μM)	<5

The reaction system consisted of 70 μg/ml pure diaphorase, 50 μM NADH, 18 μM DES quinone in a final volume of 1 ml 10 mM potassium phosphate buffer, pH 7.5 (complete system). In some reactions, *p*-hydroxy mercuribenzoic acid (PHMB) or $HgCl_2$ was added. The reduction product (Z-DES) was analyzed by HPLC (2). Each value is a mean of three or four experiments ± SD.

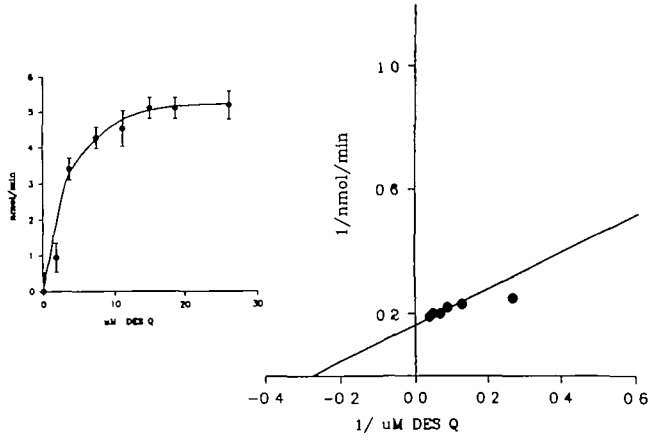

Fig. 3. Influence of various substrate concentrations on the reduction of DES quinone catalyzed by mitoplasts in the presence of NADH. The reduction reaction mixture contained purified mitoplasts from β-naphthoflavone-treated male Sprague−Dawley rat liver (0.29 mg equivalent protein), 50 μM NADH, and various concentrations of DES quinone (0–30 μM) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. The reduction was monitored by both UV spectroscopy and HPLC as described in Materials and methods. The data are presented as double reciprocal plot and as substrate-dependent product formation (inset). Values represent the means of three or four experiments.

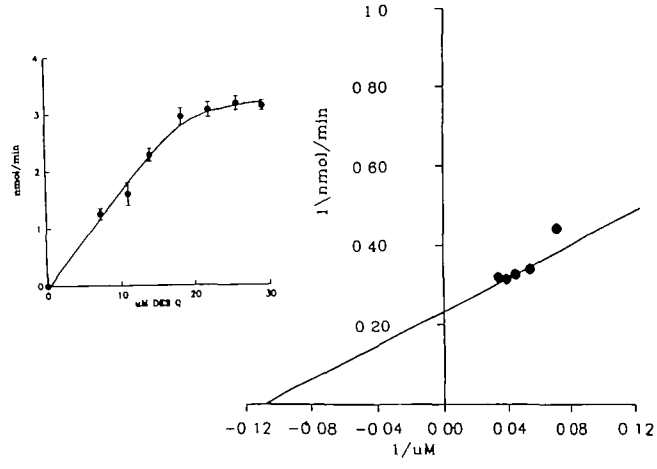

Fig. 4. Reduction of DES quinone by a mitochondrial reducing enzyme, diaphorase. The reduction reaction mixture contained purified diaphorase (0.07 mg equivalent protein), 50 μM NADH, and various concentrations of DES quinone (0–30 μM) in a final volume of 1 ml 10 mM phosphate buffer, pH 7.5. The reduction of DES quinone was monitored as described above. The data are presented as a double reciprocal plot and as substrate-dependent product formation (inset). Values represent the means of three or four experiments.

ANF and ANIT—inhibited peroxidase-dependent mitochondrial oxidation of DES by 81–83% (Table I). The marked inhibition by cytochromes P450 inhibitors suggests that mitochondrial cytochromes P450 were presumably involved in conversion of DES into DES quinone.

Reduction of DES quinone

Both UV spectroscopy and HPLC analyses demonstrated that DES quinone was rapidy reduced to Z-DES by mitoplasts in the presence of NADH (Table II). The rate of DES quinone reduction in the presence of mitoplasts and NADH was dependent on the concentration of DES quinone (Figure 3, left panel), and a Lineweaver−Burk plot of rate of reduction of DES quinone at various substrate concentrations yielded K_m = 3.2 μM and V_{max} = 5.6 nmol/mg protein/min (Figure 3, right panel). *p*-Hydroxy mercuribenzoic acid inhibited the reduction of DES quinone to Z-DES by 43% (Table II). In control reactions, i.e. in the absence of NADH or mitoplasts, reduction of DES quinone to Z-DES was not detected.

The reduction of DES quinone was also accomplished by using a mitochondrial enzyme, diaphorase, in the presence of NADH (Table III). Using the rate of reduction of DES quinone at various substrate concentrations, kinetic constants for the reduction reaction catalyzed by diaphorase were determined (Figure 4). The V_{max} was 4.3 nmol and the K_m was 9 μM. The addition of *p*-hydroxy mercuribenzoic acid or $HgCl_2$ inhibited the reduction of DES quinone by 95% (Table III). In control reaction, i.e. in the absence of the enzyme, the reduction of DES quinone was minimal (Table III).

DES−mtDNA adducts

Incubation of DES with mitoplasts in the presence of CHP showed one major adduct (spot 1) and several minor adducts (spots 8, 10–15) (Figure 5B). In control reactions, i.e. in the absence of CHP or mitoplasts, formation of adducts was below detectable level by autoradiography (Figure 5A). The level of adduct formed in DNA in the presence of mitoplasts and cofactor CHP (1951 amol/μg mtDNA) was drastically lowered by ANIT, an inhibitor of cytochromes P450 (221 amol/μg mtDNA) (Figure 5C). Incubation of mtDNA with DES in the presence of mitoplasts and NADPH also formed a major (spot 1) and a minor adduct (spot 10) (Figure 5D). The level of binding of DES to mtDNA in the presence of CHP (1951 amol/

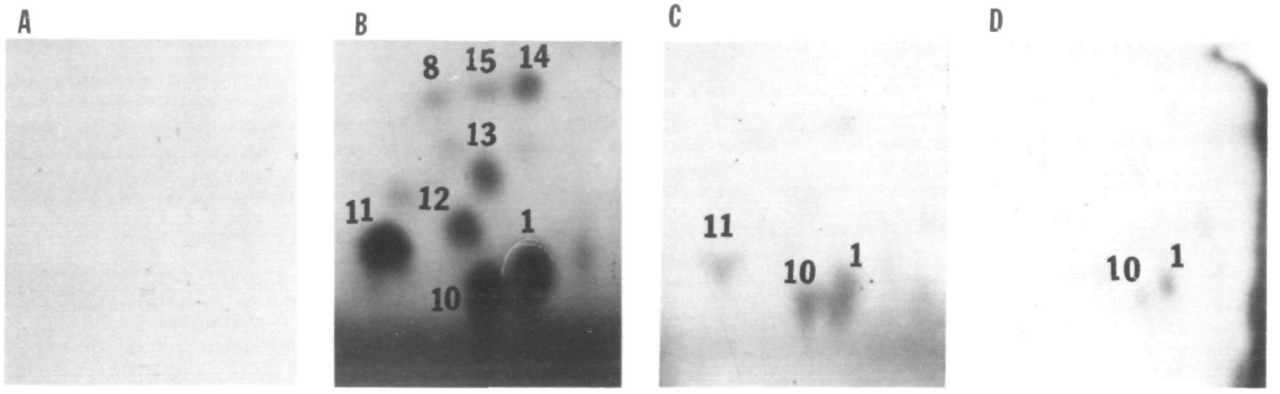

Fig. 5. Formation of adducts in mtDNA reacted with DES in the presence of mitoplasts and cofactor cumene hydroperoxide. The reaction condition was the same as described for the oxidation of DES to DES quinone. mtDNA was isolated and formation of mtDNA−DES adducts was analyzed by ^{32}P-postlabeling (3). Labeled adducts were located by autoradiography for 16 h at −70°C. (**A**) mtDNA from unreacted mitoplasts; (**B**) 250 μM DES + 0.145 mg equivalent protein from mitoplasts + 255 μM cumene hydroperoxide; (**C**) 500 μM α-naphthylisothiocyanate + 0.145 mg equivalent protein from mitoplasts + 250 μM DES + 255 μM cumene hydroperoxide; and (**D**) 250 μM DES + 0.145 mg equivalent protein from mitoplasts + 2 mM NADPH.

μg mtDNA) was higher than that observed in the presence of NADPH (584 amol/μg mtDNA). Higher binding of DES to mtDNA during CHP-dependent reaction was possible because only oxidation of DES to DES quinone occurs (2). Reduction of DES quinone does not occur, since CHP is not a cofactor of reductase (2). However, during the NADPH-dependent mitochondrial activation of DES, both oxidation of DES to DES quinone and reduction of DES quinone to DES occurs, which may account for the lower binding of DES to mtDNA.

Discussion

Direct evidence for mitochondrial enzyme-catalyzed oxidation and reduction reactions of stilbene estrogen is established. It is shown here for the first time that stilbene estrogen, DES, can be oxidized to DES quinone by CHP-supported mitochondrial cytochromes P450 oxidase. NADPH-dependent mitochondrial incubation did not exhibit a detectable level of estrogen quinone. Previous studies of NADPH-dependent microsomal oxidation of estrogen or xenobiotics (1,2,14) also failed to provide direct evidence of quinone formation. Rapid reduction of quinone to hydroquinone may be the likely reason for the undetectable level of quinone. Like other xenobiotic quinones (14), estrogen quinone is rapidly reduced to hydroquinone in the presence of cytochrome P450 reductase or microsomes and NADPH (1,2). Indirect evidence in support of NADPH-dependent mitochondrial oxidation of DES to DES-reactive metabolites is apparent from covalent mtDNA−DES adduct formation, because DES in the absence of cofactor did not form detectable amounts of mtDNA−DES adducts. Moreover, in CHP-supported mitochondrial conversion of DES to quinone, in which only the oxidation reaction is possible due to the absence of cofactor for reducing enzyme (2), mtDNA−DES adduct formation was observed. In analogy to previous work with microsomal redox cycling of estrogens (1,2), it is postulated that semiquinone is formed during oxidation of DES and reduction of DES quinone by mitochondrial enzymes (Figure 6). The resulting semiquinone may react with O_2 to form O_2^- and quinone. Thus, this study provides evidence for mitochondrial enzyme-mediated oxidation and reduction reactions of DES.

Considering the unstable nature of reactive intermediates and the presence of an ample quantity of nucleophilic molecules in the main organelle of metabolic activation (endoplasmic reticulum) and the cytoplasm capable of scavenging reactive

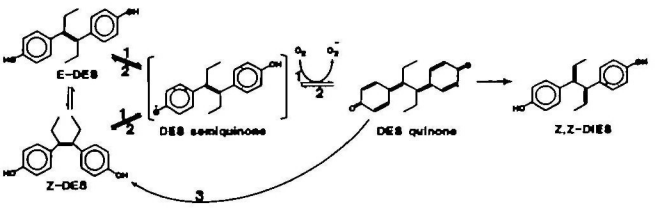

Fig. 6. Scheme of redox cycling of stilbene estrogen (DES) by mitochondrial enzymes. (**1**) Both *E*- and *Z*-DES are oxidized by mitochondrial cytochrome P450 oxidase. The intermediacy of a DES semiquinone in this process is postulated but not demonstrated. (**2**) The DES quinone is reduced via DES semiquinone to *Z*-DES by cytochrome b5 reductase (1), diaphorase (as shown in this study) or ubiquinone reductase (remains to be shown). (**3**) The DES quinone is directly reduced to DES by quinone reductase (1). The DES semiquinone and quinone generated during mitochondrial redox cycling of DES are postulated to bind covalently to mtDNA.

intermediates, it seems unlikely that highly reactive and short-lived DES genotoxic metabolites will traverse the distance from endoplasmic reticulum to mitoplasts. It has been previously shown that mitochondria have cytochromes P450 (5,6) and have the metabolic activation system to metabolize polycyclic aromatic hydrocarbons (5,6,8–10). Stilbene estrogen and catecholestrogens, the major metabolites of estrogens, have been shown to be primarily metabolized to reactive metabolites by cytochrome P4501A1 (2) in the endoplasmic reticulum of the cell. Estrogen-reactive metabolites are able to bind covalently to macromolecules of nuclear components (4). DES-reactive intermediates such as quinone and semiquinone are highly unstable (1,7). The findings of this study that mitochondria are capable of catalyzing oxidation and reduction reactions of DES, and DES-reactive metabolites are able to bind covalently to mtDNA, provide support to the concept that in the cell the metabolic activation of stilbene estrogens to genotoxic metabolites can also occur in mitochondria. The fact that in the cell the activation of DES occurs in the mitoplasts may be very important because certain DES metabolites may have been prevented from reacting with mtDNA by selective trapping or transport out of the cell. This may also explain the possible mechanism by which DES metabolites covalently bind to mtDNA.

Recently it has been shown that mtDNA is the primary site of attack by reactive metabolites of procarcinogens such as benzo[*a*]pyrene, aflatoxin B_1, 7,12-dimethyl[*a*]anthracene and

3-methylcholanthrene (8–10). The adduct level generated in the mtDNA was 40 times higher than that of the nuclear DNA (6). The mtDNA adducts persisted for a longer time than that of nuclear DNA adducts (8–10). The mitochondria of tumor cells are frequently structurally and functionally different from those isolated from normal cells (15,16). These findings support the hypothesis that insults to the mitochondria may play a role in chemically induced carcinogenesis. DES causes hepatocarcinogenicity in Sprague–Dawley rats (17) and in Armenian hamsters (18). The mechanism of DES-induced hepatocarcinogenesis is not clear. DES-reactive metabolites bind covalently to mtDNA as shown in this *in vitro* study. DES has been shown to bind *in vivo* to hepatic mtDNA of Sprague–Dawley rats, and the adduct level generated in the mtDNA was 15 times higher than that of the nuclear DNA (19). Demonstration of the ability of mitochondria to oxidize DES to DES-reactive metabolites, which covalently attack mitochondrial DNA, suggests that DES-reactive metabolites may produce genetic instability in mitochondria by producing mutational changes in mitochondrial genome. It is postulated that an analogous *in vivo* damage to mtDNA by DES-reactive metabolites may play a role in DES-induced hepatocarcinogenicity (17).

In summary, formation of DES quinone has been directly detected as an oxidation product of mitochondrial enzyme in CHP-supported reactions. DES quinone was rapidly reduced by mitochondrial reducing enzymes. DES metabolites generated by mitochondrial enzymes were able to covalently modify mtDNA. Whether these modifications are a factor in the development of DES-induced toxicity/cancer/teratogenicity, however, remains to be established.

Acknowledgements

This research was supported by NIH grant CA52584.

References

1. Liehr,J.G. and Roy,D. (1990) Free radical generation by redox cycling of estrogens. *Free Radical Biol. Med.*, **8**, 415–423.
2. Roy,D. and Liehr,J.G. (1992) Catalysis of the oxidation of steroid and stilbene estrogens to estrogen quinone metabolites by the β-naphthoflavone-inducible cytochrome P450IA family. *Arch. Biochem. Biophys.*, **296**, 450–456.
3. Liehr,J.G., Roy,D. and Gladek,A. (1989) Mechanism of inhibition of estrogen induced renal carcinogenesis in male Syrian hamsters by vitamin C. *Carcinogenesis*, **10**, 1983–1988.
4. Roy,D. and Pathak,D.N. (1993) Modifications in the low mobility group nuclear proteins by reactive metabolites of diethylstilbestrol. *Biochem. Mol. Biol. Int.*, **31**, 923–934.
5. Niranjan,B.G. and Avadhani,N.G. (1980) Activation of aflatoxin B_1 by a mono-oxygenase system localized in rat liver mitochondria. *J. Biol. Chem.*, **255**, 6575–6578.
6. Niranjan,B.G., Avadhani,N.G. and DiGiovanni,J. (1985) Formation of benzo(*a*)pyrene metabolites and DNA adducts catalyzed by a rat liver mitochondrial monooxygenase system. *Biochem. Biophys. Res. Commun.*, **131**, 935–942.
7. Roy,D., Kalyanraman,B. and Liehr,J.G. (1991) Xanthine oxidase-catalyzed reduction of estrogen quinones to semiquinones and hydroquinones. *Biochem. Pharmacol.*, **42**, 1627–1631.
8. Allen,J.A. (1980) Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA. *Nature*, **287**, 244–245.
9. Backer,J.M. and Weinstein,I.B. (1980) Mitochondrial DNA is a major target for a dihydrodiol-epoxide derivative of benzo(*a*)pyrene. *Science*, **209**, 297–299.
10. Niranjan,B.G., Bhat,N.K. and Avadhani,N.G. (1982) Preferential attack of mitochondrial DNA by aflatoxin B_1 during hepatocarcinogenesis. *Science*, **215**, 73–75.
11. Greenawalt,J.W. (1974) The isolation of outer and inner mitochondrial membranes. *Methods Enzymol.*, **31**, 310–332.
12. Wharton,D. and Tzagoloff,A. (1967) Cytochrome oxidase from beef heart. *Methods Enzymol.*, **10**, 245–250.
13. Baginski,E.S., Foa,P.P. and Zak,B. (1974) Glucose-6-phosphatase. In Bergmeyer,H.U. (ed.), *Methods in Enzymatic Analysis*. Academic Press, New York, Vol. 2, pp. 876–880.
14. Fluck,D.S., Rappaport,S.M., Eastmond,D.A. and Smith,M.T. (1984) Conversion of 1-naphthol to naphthoquinone metabolites by rat liver microsomes: demonstration by high performance liquid chromatography with reductive electrochemical detection. *Arch. Biochem. Biophys.*, **235**, 351–358.
15. Pedersen,P.L. (1978) Tumor mitochondria and the bioenergetics of cancer cells. *Prog. Exp. Tumor Res.*, **22**, 190–274.
16. Wilkie,D., Egilsson,V. and Evans,I.H. (1975) Mitochondria in oncogenesis. *Lancet*, 697–698.
17. Williams,G.W., Iatropoulos,M., Cheung,R., Radi,L. and Wang,C.X. (1993) Diethylstilbestrol liver carcinogenicity and modifications of DNA in rats. *Cancer Lett.*, **68**, 193–198.
18. Coe,J.E., Ishak,K.G. and Ross,M.J. (1990) Estrogen-induction of hepatocellular carcinomas in Armenian hamsters. *Hepatology*, **11**, 570–577.
19. Thomas,R.D. and Roy,D. (1994) Mitochondrial DNA is a major target of attack by diethylstilbestrol metabolites *in vivo*. *Proc. Am. Assoc. Cancer Res.*, **35**, 260.

Received on August 18, 1994; revised on November 29, 1994; accepted on December 9, 1994