Stilbene estrogen produces higher levels of mitochondrial DNA adducts than nuclear DNA adducts in the target organ of cancer (liver) of male Sprague Dawley rats

RONALD D. THOMAS[1] and DEODUTTA ROY[2]

[1]Environmental Toxicology Program, College of Pharmacy, Florida A & M University, Tallahassee, FL-32307-3800;
[2]Department of Environmental Health Sciences, University of Alabama at Birmingham, Birmingham, AL 35294-0022, USA

Received April 18, 2001; Accepted May 18, 2001

Abstract. We have previously demonstrated that diethylstilbestrol is metabolized to diethylstilbestrol reactive metabolites by mitochondrial enzymes *in vitro*. *In vitro*, these reactive intermediates bind to mitochondrial DNA. Here we have investigated the *in vivo* formation of diethylstilbestrol adducts with mitochondrial DNA and the nature of mitochondrial DNA-diethylstilbestrol adducts. Diethylstilbestrol exposure to male rats produced several adducts in mitochondrial DNA of both kidney and liver. The total relative adduct levels were 7-fold higher in mitochondrial DNA than in nuclear DNA in the target organ of cancer (liver) of Sprague Dawley rats. The chromatographic mobility of mitochondrial DNA adducts formed *in vivo* were similar to that of dGMP-DES quinone adducts formed *in vitro*. These findings suggest that mitochondrial DNA appears more susceptible to formation of diethylstilbestrol adducts than nuclear DNA, and the results suggest that obstruction of replication and/or transcription of the mitochondrial genes by covalent modifications of the mitochondrial DNA by diethylstilbestrol may produce mitochondrial genomic instability *in vivo* and may provide an explanation for the carcinogenic effects of DES.

Introduction

Stilbene estrogen (diethylstilbestrol, DES) is a hormonal chemical that possesses potent estrogenic activity. It is carcinogenic in both animals and humans (1). There are several case reports indicating that cancer patients developed liver cancer subsequent to DES treatment (2-4). DES causes hepatocarcinogenicity in Sprague Dawley rats (5) and in Armenian hamsters (6). The mechanism of DES-induced hepatocarcinogenesis is not clear. We have demonstrated the redox-cycling of DES using rat mitochondrial enzymes (7). During redox-cycling, DES is oxidized to DES semiquinone and further oxidized to DES quinone in the presence of cytochrome P450. The quinone is reduced back to hydroquinone (DES) by cytochrome P450 reductase. DES reactive metabolites, quinone and semiquinone are postulated to covalently attack macromolecules. Covalent modification in macromolecules, particularly DNA, may be involved in DES-induced liver neoplasm. It has been reported that a single dose of DES (100 mg/kg) produced nuclear (n)DNA adducts in the target organ of cancer (liver) in male Sprague Dawley rats four hours after administration. In the Syrian hamster we have shown that the total relative adduct levels were 5- to 6-fold higher in mitochondrial DNA than in nuclear DNA in the target organ of cancer (kidney) (8). The effects of DES on the mitochondrial genome in the liver of Sprague Dawley rats are not clear.

Therefore, in the present study using the highly sensitive ^{32}P-postlabeling method, we have investigated the covalent modification in mitochondrial (mt) DNA in the target organ of cancer (liver) and nontarget organ of cancer (kidney) of Sprague Dawley rats exposed to DES. We have selected Male Sprague Dawley rats as an experimental animal model to assess the mitochondrial genotoxic potential of DES for the reasons that DES is a hepatocarcinogen in this model. Findings of this study will help to elucidate the mechanism by which DES induces instability in the mitochondrial genome in the liver.

Materials and methods

Materials. Materials and chemicals needed for ^{32}P-postlabeling assays were obtained from the sources described previously (9,10). DES Q was prepared as described by Roy and Liehr (9).

Preparation of mitochondrial and nuclear DNA. Five male Sprague Dawley rats were treated with 150 mg/kg of DES i.p. After four hours, the animals were sacrificed. The mitochondria and nuclei were isolated by differential centrifugation. DNA was isolated from purified mitoplasts and nuclei (11,12).

Chemical reactions between DES quinone and dGMP. One mg of dGMP (dissolved in 1.4 ml water) was reacted with one mg

Correspondence to: Dr Ronald D. Thomas, College of Pharmacy, Florida A & M University, Tallahassee, FL-32307, USA
E-mail: ronald.thomas@famu.edu

Key words: stilbene estrogen, mitochondrial DNA, DNA adducts

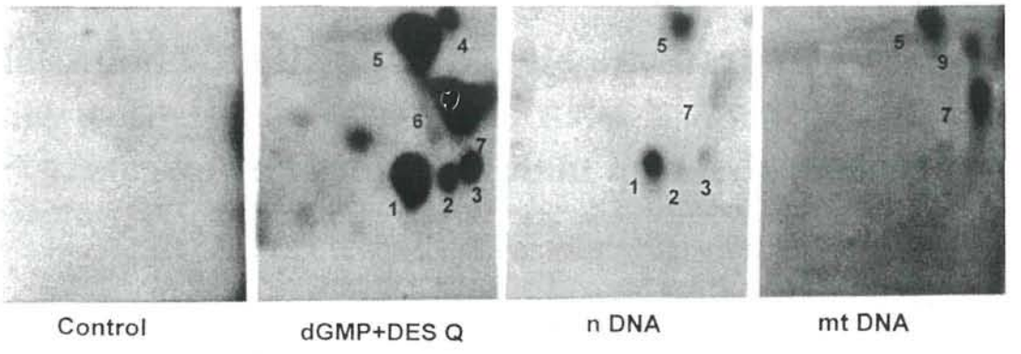

Figure 1. The two dimensional thin layer chromatography of mtDNA and nDNA adducts isolated from rat liver. Panel A, the two dimensional chromatograph of the dGMP-DES quinone adducts. Panel B, nDNA adducts isolated from rats treated with 150 mg/kg DES. Panel C, mtDNA adducts isolated from rats treated with 150 mg/kg DES.

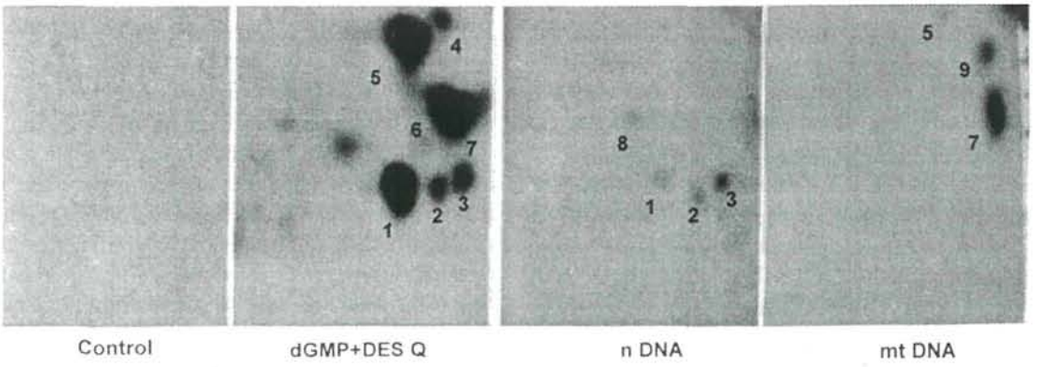

Figure 2. The two dimensional thin layer chromatography of mtDNA and nDNA adducts isolated from rat kidney. Panel A, the chromatographic profile of the DES quinone -dGMP adducts. Panel B, the chromatographic mobility of nDNA adducts isolated from rats treated with 150 mg/kg DES. Panel C, the chromatographic mobility of mtDNA adducts isolated from rats treated with 150 mg/kg DES.

DES quinone (dissolved in 0.6 ml ethanol) for four hours at 25°C. Reaction mixtures were extracted four times with chloroform and six times with water-saturated ethyl acetate. The concentration of dGMP remaining in the aqueous phase was estimated at 260 nm. The samples were stored at -70°C unless used immediately for adduct analysis.

DNA adducts analysis by ^{32}P-postlabeling. Analysis of covalent modification in DNA was carried out by the ^{32}P-postlabeling technique (10). DNA (10 μg) was digested with micrococcal nuclease, spleen phosphodiesterase, and nuclease P1 as described previously (10). The resolution of ^{32}P-postlabeled nucleotides was carried out by removing unmodified nucleotides on polyetheleneimine-cellulose TLC plates using the following solvent: D1, (2.3 M sodium phosphate, pH 5.7). A two dimensional adduct profile was generated by using D3, (4.2 M lithium formate and 6.8 M urea, pH 3.3) and D4 (0.8 M sodium phosphate, 0.5 M Tris and 8.5 M urea, pH 8.2). D5 (1.7 M phosphate, pH 6.0) was used to remove any unreacted radioactivity from the TLC sheet. The ^{32}P-labeled adduct spots were detected by autoradiography using Kodak X-Omat film. The spots containing ^{32}P-postlabeled adducts were excised from TLC plates. The levels of radioactivity in these adducts were determined by Cerenkov counting. The amount of adducts was calculated according to the procedures described previously (10).

Results

Examination of adducts in untreated and DES-treated male rat kidney and liver mitochondrial DNA by ^{32}P-postlabeling revealed that DES treatment to Sprague Dawley rats produced several adducts in both kidney and liver mitochondrial DNA. No adducts were detected in DNA isolated from control animals (Figs. 1 and 2).

In the liver of rats treated with DES, we detected five distinct adducts in the nDNA and three distinct adducts in the mtDNA. The chromatographic mobility of some of the adducts (#5 and 7) was similar for both mt- and nDNA. There were some distinct fingerprints of adducts specific to nDNA (#1, 2 and 3) (Fig. 1). However, the mobilities of both mt- and nDNA sets of adducts were similar to the mobility of the chemically synthesized DES quinone-dGMP (Fig. 1). Two of the adducts in the nDNA of the liver (#5 and 7) cochromatographed with the mtDNA adducts (data not shown). Although there were more adducts in the nDNA than mitochondrial DNA, the levels of adducts were higher in the mtDNA compared to nDNA. The total adduct level was

Table I. Relative adduct levels (RALS) of the mtDNA and nDNA adducts in the liver from DES-treated male sprague Dawley rats.[a]

Adduct #	DGMP	nDNA	MtDNA
1	2.12×10^{-3}	7.69×10^{-9}	-
2	1.71×10^{-4}	1.11×10^{-9}	-
3	2.65×10^{-4}	2.33×10^{-9}	-
4	1.05×10^{-3}	-	-
5	9.27×10^{-4}	6.82×10^{-9}	9.00×10^{-8}
6	2.33×10^{-4}	-	-
7	2.90×10^{-4}	5.04×10^{-9}	4.19×10^{-8}
8	-	-	-
9	-	-	2.60×10^{-8}
Total adducts	-	2.29×10^{-8}	15.8×10^{-8}

[a]This table demonstrates the RALS for each of the adducts in Fig. 1. The spots in Fig. 1 were counted and the RALs were calculated (10).

Table II. Relative adduct levels (RALS) of the mtDNA and nDNA adducts in kidney from DES-treated male sprague Dawley rats.

Adduct #	DGMP	nDNA	mtDNA
1	2.12×10^{-3}	2.35×10^{-10}	-
2	1.71×10^{-4}	5.23×10^{-10}	-
3	2.65×10^{-4}	7.98×10^{-10}	-
4	1.05×10^{-3}	-	-
5	9.27×10^{-4}	-	2.20×10^{-9}
6	2.33×10^{-4}	-	-
7	2.90×10^{-4}	-	22.6×10^{-9}
8	-	1.62×10^{-10}	-
9	-	-	5.51×10^{-9}
Total adducts	-	1.72×10^{-9}	30.3×10^{-9}

[a]This table demonstrates the RALS for each of the adducts in Fig. 2. The spots in Fig. 2 were counted and the RALs were calculated (10).

2.29×10^{-8} and 15.8×10^{-8} in nDNA and mDNA, respectively (Table I). The ratio of mtDNA adducts to nDNA adducts was 7-fold greater in the target organ of cancer, liver (Table I). The total level of adducts in both the mtDNA and nDNA of the liver was 5- and 13-fold, respectively, higher than that of the nontarget organ of cancer, kidney (Tables I and II).

DES also formed DNA adducts in the mtDNA and nDNA of the kidney. Four adducts were detected in the nDNA whereas only three were detected in the mtDNA (Fig. 2). The chromatographic mobility of the mtDNA adducts was completely different from that of the nDNA, which was not the case in the liver. However, the mobilities of most of the mt- and nDNA adducts were similar to the mobilities of the chemically synthesized DES quinone-dGMP adducts (Fig. 2). The relative intensities of the adducts correlated with their concentrations (Table II). The major nDNA relative adduct level was 0.798×10^{-9} RAL. The total relative adduct level in the mtDNA was higher than the total relative adduct level in the nDNA. The total relative adduct level was 1.72×10^{-9} RAL and 30.3×10^{-9} RAL in the nDNA and mtDNA, respectively. The ratio of mtDNA adducts to nDNA adducts is greater than 17-fold in the kidney.

Discussion

The present work for the first time demonstrated that DES can covalently bind to liver and kidney mitochondrial DNA *in vivo*; mitochondrial DNA was more susceptible to the covalent attack by DES than nuclear DNA in both liver and kidney of Sprague Dawley rats. The total level of adducts in both the mtDNA and nDNA in the target organ of cancer (liver) was 5- and 13-fold higher, respectively, than that of the nontarget organ of cancer, kidney. The DNA adducts formed by DES were predominantly at guanine in nature.

Considering the unstable nature of DES reactive intermediates, the presence of an ample quantity of nucleophilic molecules in the primary organelle of metabolic activation (endoplasmic reticulum) and the ample quantity of nucleophilic molecules in the cytoplasm capable of scavenging reactive intermediates; it seems unlikely that highly reactive and short-lived DES genotoxic metabolites will traverse the distance from endoplasmic reticulum to mitochondria. It has been previously shown that mitochondria have cytochromes P450 and have the metabolic activation system to metabolize polycyclic aromatic hydrocarbons (7). Also, our recent study revealed that mitochondria are capable of catalyzing oxidation and reduction reactions of stilbene estrogen, and stilbene estrogen reactive metabolites are able to covalently bind to mtDNA. These data provide support to the concept that the cellular metabolic activation of stilbene estrogens to genotoxic metabolites can also occur in mitochondria (9). The fact that cellular activation of DES occurs in the mitochondria is important because certain DES metabolites may be 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 *in vivo*.

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 (13). The adduct level generated in the mtDNA was 40 times higher than that of the nuclear DNA (13,14). The mtDNA adducts persisted for longer time than that of nuclear DNA adducts (14). The mitochondria of tumor cells are frequently structurally and functionally different from those isolated from normal cells (15-17). 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 (5) and in Armenian hamsters (6). The mechanism of DES-induced

hepatocarcino-genesis is not clear. DES reactive metabolites bind covalently to mtDNA as shown in this *in vivo* study. This study revealed that DES covalently bind *in vivo* to hepatic mtDNA of Sprague Dawley rats, and the adduct level generated in the mtDNA is seven times higher than that of the nuclear DNA. DES-mtDNA adducts may produce instability in mitochondrial genome presumably by affecting transcription and replication and may provide one possible explanation for the carcinogenic effects of DES.

Thus, the study presented here shows that mtDNA appear more susceptible to formation of DES adducts than nuclear DNA *in vivo*. Studies are in progress to determine the structure of the DES-mt DNA adducts.

Acknowledgments

This research work was supported by the NIH grant CA52584.

References

1. IARC: Monographs on the evaluation of hormonal contraception and postmenopausal hormonal therapy. IARC 72: 399-530, 1999.
2. Rosinus V and Maurer R: Diethylstilbestrol-induced liver cancer. Schweiz Med Wochenschr 111: 1139-1142, 1981.
3. Ham JM, Pirola RC and Crouch RL: Hemangioendothelial sarcoma of the liver associated with long-term estrogen therapy in a man. Dig Dis Sci 11: 879-883, 1980.
4. Hoch-Ligeti C: Angiosarcoma of the liver associated with diethylstilbestrol. JAMA 29: 1510-1511, 1978.
5. Williams GM, Iatropoulos M, Cheung R, Radi L and Wang CX: Diethylstilbestrol liver carcinogenicity and modification of DNA in rats. Cancer Lett 68: 193-198, 1993.
6. Coe JE, Ishak KG and Ross MJ: Estrogen induction of hepatocellular carcinoma in Armenian hamsters. Hepatology 11: 570-577, 1990.
7. Thomas RD and Roy D: Mitochondrial enzyme-catalyzed oxidation and reduction reactions of stilbene. Carcinogenesis 16: 891-895, 1995.
8. Thomas R and Roy D: *In vivo* mitochondrial genotoxicity of diethyl-stilbestrol. Proc Am Assoc Cancer Res 35: 260, 1994.
9. Roy D and Liehr JG: Catalysis of the oxidation of steroid and stilbene estrogens to estrogen quinone metabolites by the naphthoflavone-inducible cytochrome P450I family. Arch Biochem Biophys 296: 450-456, 1992.
10. Reddy M and Randerath K: Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. Carcinogenesis 7: 1543-1551, 1986.
11. Greenwalt JW: The isolation of outer and inner mitochondrial membranes. Meth Enzymol 31: 310-332, 1974.
12. Wharton D and Tzagoloff A: Cytochrome oxidase from beef heart. Meth Enzymol 10: 245-250, 1967.
13. Allen J and Coombs ML: Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA. Nature 287: 244-245, 1980.
14. Bagged L: Role of mitochondria in carcinogenesis. Eur J Cancer 29A: 156-159, 1993.
15. Torroni A, Stepien G, Hodge J and Wallace D: Neoplastic transformation is associated with coordinate induction of nuclear and cytoplasmic oxidation phosphorylation genes. J Biol Chem 265: 20589-20593, 1990.
16. Wilkie D, Egilsson V and Evans I: Mitochondria in oncogenesis. Lancet 198: 697-698, 1975.
17. Pedersen P: Tumor mitochondria and the bioenergetics of cancer cells. Progr Exp Tumor Res 22: 190-274, 1978.