Aldehyde DNA lesions induced by catechol estrogens in calf thymus DNA

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The primary purpose of this research is to examine the hypothesis that reactive oxygen species generated by estrogen quinonoids are the main source for the formation of aldehydic DNA lesions (ADL) in genomic DNA. ADL induced by quinonoid metabolites of 17β-estradiol (E2), e.g. 4-hydroxyestradiol (4-OH-E2), 2-hydroxyestradiol (2-OH-E2), estrogen-3,4-quinones (E2-3,4-Q) and estrogen-2,3-quinone (E2-2,3-Q), were investigated in calf thymus DNA (CT-DNA) under physiological conditions. The abasic sites resulting from the spontaneous depurination–depyrimidination of the modified bases and the aldehydic base and sugar lesions resulting from the oxidative damage to deoxyribose moieties in the DNA molecules were measured by an aldehyde reactive probe and were estimated as the number of ADL per 10⁶ nucleotides. With the addition of NADPH (100 µM) and Cu(II) (20 µM), nanomolar levels (100 nM) of 4-OH-E2 and 2-OH-E2 induced ~10-fold increases in the number of ADL over control (P < 0.001). In parallel, increases in 8-oxoguanine were detected in DNA exposed to 4-OH-E2 and 2-OH-E2 (100 nM) plus Cu(II) and NADPH. Further investigation indicated that the ADL induced by estrogen catechols plus Cu(II) and NADPH were causally involved in the formation of hydroperoxide and Cu(I). Both E2-2,3-Q and E2-3,4-Q alone induced a 2-fold increase in the number of ADL over control (P < 0.05) in CT-DNA at high concentrations (1 mM). Neither neutral thermal hydrolysis nor lower ionic strength of the reaction medium induced further increases in the number of ADL in E2-3,4-Q-modified CT-DNA. Conversely, with the inclusion of Cu(II) and NADPH, both E2-3,4-Q and E2-2,3-Q (1 µM) induced parallel formation of DNA single strand breaks and ~20-fold increases in the number of ADL over control (P < 0.001). The data also demonstrated that the ADL induced by estrogen quinones with and without the presence of Cu(II) and NADPH contain 69 and 78% putrescine-excisable ADL in CT-DNA, respectively. Additionally, results of the ADL cleavage assay indicate that the ADL induced by estrogen quinones plus Cu(II) and NADPH in CT-DNA were predominantly T7 exonuclease-excisable (50%) and exonuclease III-excisable (20%) ADL, whereas the intact ADL, and other ADL accounted for 5 and 25%, respectively. These results suggest that the ADL induced by estrogen quinones in CT-DNA are derived from oxidative events rather than depurination/depyrimidination of labile estrogen quinone–DNA adducts. Overall, our results are at variance with the idea that depurination of estrogen quinone–DNA adducts is the major source for the formation of ADL in genomic DNA. We hypothesize that in addition to DNA adducts and oxidized bases, the ADL induced by estrogen quinonoid-mediated oxidative stress may play a role in estrogen-induced carcinogenicity.

Introduction

Carcinogenic properties of estrogen are conventionally attributed to the receptor-driven mitogenesis mechanism where elevated E2 forms a complex with the estrogen receptor (ER) in the nucleus, which subsequently interacts with estrogen responsive elements and leads to the subsequent expression of estrogen responsive genes and cell proliferation (1). Accumulating evidence suggests that the metabolic by-products, direct and oxidative DNA damage, play significant roles in estrogen carcinogenesis (2–7). Aromatic hydroxylation of endogenous estrogen, i.e. estrone (E1) and 17β-estradiol (E2), at C-2 and C-4 gives rise to biologically active catechol estrogens, i.e. 2-hydroxyestradiol (2-OH-E2) and 4-hydroxyestradiol (4-OH-E2), via hepatic and extrahepatic cytochrome P450 enzymes (8–11). 2-OH-E and 4-OH-E can be further oxidized to the corresponding estrogen-2,3-quinone (E2-2,3-Q) and estrogen-3,4-quinone (E2-3,4-Q) via enzymatic and non-enzymatic activation (12–14). It has been shown that 4-OH-E2 is carcinogenic in male Syrian golden hamsters but 2-OH-E2 is not (4). However, both 4-OH-E2 and 2-OH-E2 are capable of inducing uterine adenocarcinoma in female CD-1 neonatal mice (15).

Catechol estrogens form several DNA adducts via their quinone and semiquinone forms (16–19). Evidence indicates that E2-3,4-Q reacts with calf thymus DNA (CT-DNA) and gives rise to a labile 4-OH-E2-1(α,β)–N7-guanine adduct (4-OH-E2–N7G) (20). 4-OH-E2–N7G has been detected in female Sprague–Dawley rats treated with 4-OH-E2 and E2-3,4-Q. Conversely, E2-2,3-Q forms stable adducts with dG and dA that have miscoding potentials in mammalian cells (21). Recent findings also point to the induction of depurinating adducts derived from the reactions of E2-2,3-Q with deoxyguanosine and deoxyadenosine that give rise to the formation of N7 depurinating adducts, i.e. 2-OH-E2–6(α,β)–N7-guanine and 2-OH-E2–6(α,β)–N7-adenine (17). It has been suggested that depurination of estrogen quinone-derived DNA adducts generates abasic sites which play an important role in the initiation of estrogen carcinogenesis (2,20).

Abbreviations: ADL, aldehydic DNA lesions; AP, apurinic–apyrimidinic; ARP, aldehyde reactive probe; ASB, aldehyde slot blot; CT-DNA, calf thymus DNA; E2-2,3-Q, estrogen-2,3-quinone; E2-3,4-Q, estrogen-3,4-quinone; 2-OH-E2, 2-hydroxyestradiol; 4-OH-E2, 4-hydroxyestradiol; 8-oxoG, 8-oxoguanine; ROS, reactive oxygen radicals; SSβ, single strand breaks.
Reactions of CT-DNA with 4-OH-E2 and 2-OH-E2

Apurinic/apyrimidinic (AP) sites are formed in high numbers by the spontaneous hydrolysis of bases, generating the loss of up to 10,000 purines/dymanial cell (22). Evidence indicates that AP sites may be introduced by spontaneous depurination of labile oxidized bases and unmodified bases, and by DNA glycosylase-mediated cleavage of the N-glycosyl bond between the deoxiribose moiety and modified bases during the base excision repair processes (23). Endogenous reactive oxygen species (ROS) are also capable of inducing AP sites via hydrogen abstraction from the deoxyribose moiety of nucleic acid to generate AP sites (24,25). The AP sites resulting from the spontaneous depurination/depyrimidination of the modified bases and from the oxidative damage to the deoxyribose moiety of DNA molecules will lead to aldehydeic forms of DNA lesions (ADL). If not repaired, AP sites are promutagenic DNA lesions and are strong blockers of DNA synthesis leading to cell death. Quinonoid derivatives of estrogens are capable of inducing oxidative damage to genomic DNA by generating ROS via redox cycling (26–29) and are suggested to be responsible for the elevated oxidative DNA damage detected in target tissues of estrogen-treated hamsters (30,31). Information regarding the direct assay of the number of ADL induced by estrogen quinonoids has not been reported. The origin of the estrogen-induced ADL, as well as the pathways by which estrogen quinonoids generate the ADL, remain elusive. To examine the hypothesis that ROS generated by estrogen quinonoids are the main source for the formation of ADL in genomic DNA, we applied the aldehyde reactive probe (ARP)-Slot Blot (ASB) assay (32) to measure the number of ADL. The ring-opened forms of regular AP sites and other aldehydeic deoxyribose lesions, including aldehydeic bases, oxidized AP sites, were quantified in parallel with the measurements of DNA single strand breaks (DNA SSB) and 8-oxoguanine (8-oxoG). A mechanic link to the formation of ADL between estrogen quinonoid-mediated ROS versus depurination of estrogen quinonoid-DNA adducts is depicted.

Reactions of CT-DNA with 4-OH-E2 and 2-OH-E2

To determine the induction of ADL by estrogen-derived quinonoid metabolites, CT-DNA was incubated with estrogen catechols, i.e. 4-OH-E2 and 2-OH-E2, under physiological conditions. The incubation medium (final vol 0.4 ml) consisted of 150 mM phosphate-buffered saline (pH 7.4) and CT-DNA (250 μg). To these preparations, 4-OH-E2 or 2-OH-E2 (1–100 μM; dissolved in DMSO) was added, and the reaction was carried out at 37°C. After 2 h of incubation, the reaction was terminated by chilling the mixture in an ice bath. DNA was isolated by ethanol precipitation. For some experiments, metal ions, Cu(II) (20 μM; prepared from cupric chloride), NAPDH (100 μM), catalase (30 U), superoxide dismutase (30 U) and bathocuproine (50 μM) were added before the addition of catechol estrogens. In a separate experiment, methoxylamine-pretreated CT-DNA was incubated with low concentrations of 4-OH-E2 or 2-OH-E2 (10–100 mM; dissolved in DMSO) at 37°C for 2 h, and DNA was isolated and assayed for the presence of ADL by the ASB assay.

Reactions of CT-DNA with E2-3,4-Q and E2-2,3-Q

Synthesis of estrogen quinonoids followed the procedure of Abul-Hajj (34) and Dwivedy et al. (18). In brief, a solution of 4-OH-E2 or 2-OH-E2 (8.1 mg, 0.028 mmol) in acetonitrile was at −30°C under argon atmosphere with constant stirring. Activated MnO2 (5 mg, 0.055 mmol) was added to the stirring solution and the reaction was allowed to continue for an additional 3 min. The mixture was quickly filtered to remove MnO2. While being kept under −10°C, the filtrate was evaporated under a constant flow of argon to remove acetonitrile and the quinone residue was redissolved in DMSO. Immediately, estrogen quinones (final concentration 100–1000 μM) were reacted with CT-DNA (250 μg) in phosphate-buffered saline (150 mM, pH 7.4) at 37°C for 2 h. For some experiments, the reaction was carried out in the presence of Cu(II) (20 μM) and NAPDH (100 μM), while the quinone concentration was reduced to 0.1–10 μM. In a separate experiment, CT-DNA was incubated with E2-3,4-Q (1 mM) alone in different PBS (75 versus 150 mM of PBS) to determine whether differences in buffer concentration could modulate the formation of ADL by estrogen quinones.

Reactions of 4-OH-E2 and 2-OH-E2 with DNA isolated from freshly frozen calf thymus

In a separate experiment, the induction of oxidized bases by catechol estrogens plus Cu(II) (20 μM) and NAPDH (100 μM) was investigated in CT-DNA. The CT-DNA used in this experiment was extracted from a fresh thymus harvested from a newborn Holstein calf as described previously (33). The DNA isolation procedure was performed using TEMPO as an antioxidant as described previously by Nakamura et al. (22) and stored under −80°C. CT-DNA (200 μg) was incubated with 4-OH-E2 and 2-OH-E2 (10–100 μM) plus Cu(II) (20 μM) and NAPDH (100 μM) at 37°C for 2 h. DNA was isolated by ethanol precipitation and assayed for the presence of 8-oxoG.

Analysis of ADL by the aldehyde reactive probe (ARP)-slot-blot (ASB) assay

ADL were measured by the ASB assay as described by Nakamura et al. (32). The puricetine-cleavage assays were performed as described (22). DNA, 10 mM EDTA, and 100 mM putrescine were incubated in 10 mM Tris–HCl (pH 7.4) at 0°C for 30 min and immediately analyzed by the ASB assay.

The putrescine-cleavage assay is a modification of the ASB assay that further characterizes the ADLs.

Quantification of total ADLs. ARP-reacted DNA (275 ng) was resuspended in 10 μl 50 mM HEPES–KOH buffer (pH 7.5) containing 50 mM NaCl, 100 μg/ml BSA, 2 mM DTT and 5 mM MgCl2. After addition of 210 μl Tris-EDTA (TE) buffer, the samples were heat-denatured, and loaded on the nitrocellulose filter, followed by the ASB assay.

Exonuclease III (ExoIII)-excisable ADL. ARP-reacted DNA (275 ng) and 30 U ExoIII (New England BioLab, Beverly, MA) were incubated either in 10 μl 50 mM HEPES–KOH buffer (pH 7.5) for 10 min on ice or in 10 μl of 50 mM HEPES–KOH buffer (pH 7.5) with 8 mM CaCl2 instead of 5 mM MgCl2 at 37°C for 30 min. Immediately after the reaction, 210 μl TE buffer was added to the samples, followed by the ASB assay. The reduction of ADL from the number of total ADL corresponded to the number of ExoIII-excisable ADL.

T7 exonuclease (Exo) treatment. DNA (275 ng) pre-reacted with ARP and 25 U T7 exonuclease (United States Biochemical Corp., Cleveland, OH) was incubated in 10 μl of 50 mM HEPES–KOH buffer (pH 7.5) at 0°C for 30 s, followed by addition of 210 μl TE buffer and measurement by the ASB assay. The reduction of ADL from the number of total ADL corresponded to the number of T7 Exo-excisable ADL.

ExoIII and T7 Exo treatment. DNA (275 ng) reacted with ARP and 30 U ExoIII in 10 μl of 50 mM HEPES–KOH buffer (pH 7.5) was incubated at 0°C for 10 min. The samples and 25 U T7 Exo were further incubated at 0°C for 30 s. Immediately after the reaction, 210 μl TE buffer was added to the sample, followed by the ASB assay. Quantification of intact ADL was performed by treating the ARP-tagged DNA with ExoIII and T7 Exo followed by ASB assay. The number of intact ADL was determined by the number of total ADL minus the total number of T7 Exo-excisable, ExoIII-excisable ADL and the residual estimated by this assay.

Induced depurination/depyrimidination of CT-DNA exposed to E2-3,4-Q was performed by incubating the quinone-modified DNA with methoxylamine to reduce the presence of ADL followed by ethanol precipitation. The recovered DNA was subject to neutral thermal depurination by heating at 70°C in PBS buffer (pH 7.4) for 2 h to induce depurination/depyrimidination, and immediately followed by the ASB assay for regular ADL.

Analysis of 8-oxoguanine by HPLC–electrochemical detection

Quantification of 8-oxoG was based on a HPLC–electrochemical detection method as described previously by Beckman et al. (35). In brief, DNA (50 μg in 200 μl) was dissolved in reaction buffer (50 mM phosphate, pH 7.5, 10 mM NaCl and 0.5 mM EDTA) and was treated with E.coli repair enzyme.
formamidopyrimidine glycosylase (2 μg), at 37°C for 1 h to release the 8-oxoG. The digest was centrifuged to separate 8-oxoG from DNA fibre using Microcon-10 (Amicon). The filtrate containing 8-oxoG was analyzed by reversed phase HPLC using an electrochemical array detector (ESA, Chelmsford, MA). In a serial array of electrodes at increasing potential (100, 150, 250, 300, 350, 400, 450, 500 mV), 8-oxoG was present in DNA digests, with maximum oxidation occurring at 250 mV. The remaining DNA fibre was digested with 350, 400, 450, 500 mV, 8-oxoG was present in DNA digests, with maximum oxidation occurring at 250 mV. The remaining DNA fibre was digested with the addition of 0.1 N HCl and incubated at 70°C for 1 h. The digests containing guanine were analyzed by HPLC-UV (λ254 nm). The quantification of 8-oxoG and guanine was based upon peak area relative to the calibration curves of the corresponding authentic standards (r² > 0.999). The limit of detection was defined as 5 × 10⁻⁹ 8-oxoG per g when 50 μg of DNA was used.

DNA single-strand break (DNA SSB) assay
DNA SSBs were assayed using agarose gel electrophoresis after glyoxal denaturation of DNA as described previously (36). Briefly, 20 μg of CT-DNA (dissolved in deionized water) was denatured by reacting with glyoxal at 50°C for 1 h. The denatured DNA was electrophoresed on a 0.7% agarose gel in ten mM sodium phosphate buffer and soaked in ddH2O at 4°C overnight. The DNA was visualized under UV irradiation (312 nm) and was photographed with a Polaroid camera.

Statistical analysis
All data are expressed as mean ± standard deviation. The significance of differences in the results was evaluated with one-way ANOVA, followed by Dunnett’s multiple comparison test.

Results

Induction of ADL in CT-DNA by 4-OH-E2 and 2-OH-E2
To test the hypothesis that the quinonoid derivatives of estrogen induce the formation of ADL in genomic DNA via the formation of ROS, we investigated the induction of ADL in CT-DNA by reacting CT-DNA with 4-OH-E2 and 2-OH-E2. We demonstrated that the number of ADL was increased in CT-DNA treated with 4-OH-E2 (3 μM) with the addition of Cu(II) (20 μM) under physiological conditions (P < 0.001) (Figure 1A). Similar patterns of the induction of ADL were observed in CT-DNA treated with 2-OH-E2 (10 μM) plus Cu(II) (20 μM), where the number of ADL in catechol estrogen-treated DNA increased 13-fold over control (P < 0.001) (Figure 1B). 4-OH-E2 (100 μM) and 2-OH-E2 (100 μM) alone did not induce significant increases in the number of ADL compared with control (Figure 1A and B). When NADPH (100 μM) and Cu(II) (20 μM) were included in the incubates, 4-OH-E2 (100 nM) and 2-OH-E2 (100 nM) induced 8–13-fold increases in the number of ADL over the corresponding control (P < 0.001) (Figure 2A).

Further investigation into the types of ROS species that mediated the induction of ADL indicated that when catalase (C) and the Cu(I) chelator, bathocuproine, were included in the reaction, the number of ADL in CT-DNA treated with catechol estrogens and Cu(II) was reduced to levels comparable with that of control (Figure 3A and B), suggesting the involvement of hydrogen peroxide and Cu(I). In contrast, the addition of SOD did not prevent the formation of ADL in CT-DNA exposed to 4-OH-E2 or 2-OH-E2 plus Cu(II). This result suggests that superoxide anions are not directly involved in the formation of ADL.

Induction of 8-oxoG in isolated CT-DNA by estrogen quinonoids
To confirm whether the origin of catechol estrogen-induced ADL was mediated by oxidative stress, we use formamidopyrimidine glycosylase cleavage assay to measure the catechol estrogen-modified CT-DNA for increases in 8-oxoG as an indicator of oxidative stress. Results indicate that in the presence of NADPH (100 μM) and Cu(II) (20 μM), increases of 8-oxoG were detected in CT-DNA treated with 100 nM 4-OH-E2 or 2-OH-E2 (35.5 ± 0.675 and 29.8 ± 3.28 8-oxoG/10⁶ G) versus 5.28 ± 0.827 8-oxoG/10⁶ G for the NADPH plus Cu(II)-treated control (P < 0.001) (Figure 2B). The amount of 8-oxoG present in the DMSO-treated control CT-DNA was undetectable when the limit of detection was defined as 5 × 10⁻¹⁷ 8-oxoG/G when 50 μg of DNA was used in the assay. The increases in the concentration of 8-oxoG in CT-DNA treated with catechol estrogens plus Cu(II) and NADPH were in parallel with the increases in the number of ADL, suggesting that the ADL resulted from oxidative stress (Figure 2A and B).

Induction of ADL in CT-DNA by E2-3,4-Q and E2-2,3-Q with and without Cu(II) plus NADPH
Evidence indicates that estrogen quinones are capable of reacting with DNA to form unstable quinone–DNA adducts, which subsequently undergo depurination/depyrimidination to form AP sites (17–20). Redox cycling of quinonoid derivatives in the presence of reducing equivalents (i.e. NADPH) and metal ions [e.g. Cu(II)] is known to generate ROS, which are sources of oxidant-mediated formation of ADL (37). To determine whether estrogen quinones are capable of inducing the formation of ADL in genomic DNA, we incubated CT-DNA with E2-3,4-Q or E2-2,3-Q (1–100 μM) with or without the presence of NADPH (100 μM) and Cu(II) (20 μM). Results demonstrated that at 100 μM, E2-3,4-Q or E2-2,3-Q alone did not induce increases in the number of ADL compared with
controls (Figure 4A and B). At high doses (1 mM), both E2-2,3-Q and E2-3,4-Q alone induced a 2-fold increase in the number of ADL over control (P < 0.05) in CT-DNA (Figure 5).

In contrast, in the presence of NADPH and Cu(II), low doses of E2-3,4-Q or E2-2,3-Q (1 mM) induced 16±23-fold increases in the number of ADL over control (P < 0.001) (Figure 4A and B). This finding suggests that the presence of reducing equivalents and metal ions enhanced redox cycling of E2-3,4-Q and E2-2,3-Q to form ADL. These findings were in good agreement with our previous investigation in pentachlorophenol-derived quinones that induced ADL in CT-DNA and HeLa S3 cells (36,38).

Induction of DNA SSB in CT-DNA by E2-3,4-Q or E2-2,3-Q with and without Cu(II) plus NADPH

To examine whether estrogen quinones induce parallel formation of DNA SSB and ADL, the estrogen quinone-treated DNA was assayed by agarose gel electrophoresis after glyoxal denaturation of DNA to avoid artifacts that may contribute from alkaline-labile oxidized bases and ADL. The results indicate that increased DNA SSB were detected in DNA exposed to E2-3,4-Q or E2-2,3-Q in the presence of Cu(II) and NADPH (Figure 6A and B, lanes 9–12) whereas E2-3,4-Q or E2-2,3-Q (100–1000 μM) alone did not induce significant increases in DNA SSB over control (Figure 6A and B, lanes 5–8). The corresponding increases in the number of ADL and DNA SSB in CT-DNA treated with estrogen quinones in the presence of Cu(II) and NADPH suggest that the origins of these ADL could have been cleaved either at 5’ or 3’ of the ADL.

Modulation of the induction of ADL in estrogen quinone-modified CT-DNA

To test whether heat-labile bases are introduced by estrogen quinones, the number of AP sites in CT-DNA exposed to E2-3,4-Q was quantified as the number of ADL per total nucleotides after neutral thermal hydrolysis to induce depurination of modified DNA bases. The E2-3,4-Q-modified DNA was pre-treated with methoxylamine (100 mM) to reduce the number of ADL. The number of AP sites in DNA exposed to E2-3,4-Q was quantified after neutral thermal hydrolysis at 70°C for 2 h. Results indicated that the number of AP sites in DNA treated with E2-3,4-Q after heat incubation was equivalent to that in control (P > 0.05) (Figure 7A). This result suggested that E2-3,4-Q did not introduce a significant amount of heat-labile base lesions and that they are unlikely to generate significant amounts of ADL. Similar results were observed in DNA exposed to E2-3,4-Q plus Cu(II) and NADPH (P > 0.05). Overall, this evidence suggests that depurination–depyrimidination of direct and oxidized DNA bases in DNA exposed to E2-3,4-Q is unlikely to contribute significant amounts of ADL under physiological conditions.

To determine whether differences in buffer concentration may modulate the formation of ADL by estrogen quinones, we incubated CT-DNA with E2-3,4-Q (1 mM) alone in different concentrations of PBS. Results demonstrated that at high doses (1 mM), E2-3,4-Q alone induced increases in the number of ADL under physiological conditions (150 mM PBS) compared with controls (P < 0.05) (Figure 7B). Similar
observations were detected in DNA treated with E2-3,4-Q (1 mM) under non-physiological conditions with PBS concentrations at 75 mM (P < 0.05). This result suggests that E2-3,4-Q (1 mM) alone induced increases in the number of ADL at high doses and that differences in the reaction medium (150 versus 75 mM of PBS) did not significantly modulate the formation of ADL (P > 0.05).

Putrescine-excisable ADL in CT-DNA induced by estrogen quinonoids

To examine the origin of the ADL induced by estrogen quinones, the DNA exposed to E2-3,4-Q (1 mM) at 37°C for 2 h with or without the presence of Cu(II) (20 µM) and NADPH (100 µM). Lane M, DNA markers; lanes 1 and 2, DMSO; lanes 3 and 4, DMSO plus Cu(II) and NADPH; lanes 5 and 6, estrogen quinone (1 mM); lanes 7 and 8, estrogen quinone (1 µM) plus Cu(II) and NADPH; lanes 9 and 10, estrogen quinone (10 µM) plus Cu(II) and NADPH.
To characterize the ADL induced by estrogen quinones, the DNA exposed to E2-3,4-Q in the presence of Cu(II) and NADPH was tagged with ARP, incubated with either ExoIII (3' → 5' exonuclease) and/or T7 Exo (5' → 3' exonuclease), and followed by ASB assay for the number of ADL. The summarized fractions of ExoIII-excisable, T7 Exo-excisable, and residual ADL are shown in Figure 9. Results of the ADL cleavage assay indicate that the ADL induced by estrogen quinones plus Cu(II) and NADPH were predominantly (50%) T7 Exo-excisable ADL in CT-DNA, whereas the endogenous ExoIII-excisable ADL, intact ADL, and other ADL accounted for 20, 5 and 25%, respectively.

**Discussion**

Accumulating evidence indicates that estrogen quinones are reactive electrophiles capable of alkylating genomic DNA, as well as being able to undergo redox cycling to generate ROS and the formation of oxidative DNA damage (20,26,27). Our previous investigation on pentachlorophenol-derived quinones has shown that ROS are sources for the formation of putrescine-excisable ADL in CT-DNA in the presence of Cu(II) and NADPH. In an effort to understand the formation of ADL induced by estrogen quinones and to characterize the identity of these ADL in genomic DNA, we treated CT-DNA with E2-3,4-Q (1 mM) plus Cu(II) and NADPH at 37°C for 2 h. Modified DNA was treated with (+Put) or without (−Put) putrescine (Put), which induces mild β-elimination and cleaved DNA 3'-side to ADL, followed by ASB assay. (B) Fractions (%) of putrescine-excisable ADL induced by E2-3,4-Q (1 mM) or E2-3,4-Q (1 mM) in the presence of Cu(II) (20 mM) and NADPH (100 μM).
with individual estrogen quinonoids under physiological conditions and measured the ADL by the ASB assay.

When CT-DNA was treated with catechol estrogen alone, no increase in ADL was detected (Figure 1). As transition metals, i.e. iron and copper, are known to enhance estrogen-induced oxidative DNA damage, we further demonstrated that with the inclusion of Cu(II), both 4-OH-E2 and 2-OH-E2 induce corresponding increases in the number of ADL (Figure 1). When NADPH (100 μM) and Cu(II) were added in the incubates, both 4-OH-E2 and 2-OH-E2 were capable of producing parallel formation of ADL and 8-oxoG at nanomolar concentrations in CT-DNA (Figure 2A and B), suggesting ROS as the main source for the formation of ADL mediated by catechol estrogens. This result is in good agreement with that reported by Thibodeau and Paquette (39) and Hiraku et al. (27), where NADH enhanced copper-dependent oxidative DNA damage was induced by catechol estrogens. Subsequent investigations revealed that these aldehydic deoxyribose lesions induced by catechol estrogens plus Cu(II) were abolished with the inclusion of catalase and Cu(I) chelator, but not by SOD. This finding supports the notion that formation of Cu(I) and H2O2 via redox cycling of estrogen quinonoids contributes to the subsequent induction of oxidant-mediated ADL. As copper is an essential transition metal in living cells, the ADL induced by estrogen quinonoids at nanomolar concentrations is a physiologically relevant pathway leading to oxidative damage to genomic DNA. In addition, results from the measurements of ADL and 8-oxoG suggest that both 4-OH-E2 and 2-OH-E2 induce oxidative DNA damage, despite the fact that only 4-OH-E2 is known to induce kidney tumors in Syrian golden hamster (4,40) and that the carcinogenic potency of 4-OH-E2 is greater than that of 2-OH-E2 in female CD-1 neonatal mice (15). Overall, it is likely that the parallel formation of ADL and 8-oxoG induced by estrogen quinonoid plus Cu(II) in CT-DNA is mediated by the reactive intermediate, Cu(I)-peroxide [Cu(I)OOH] complex (41,42). Figure 10 depicts the proposed pathway leading to the formation of ADL, 8-oxoG and DNA SSB in genomic DNA induced by redox cycling of estrogen quinonoids and Cu(II) and the subsequent formation of Cu(I) and H2O2.

Fig. 10. Diagram of the proposed pathway leading to the formation of various types of DNA damage by redox cycling of estrogen quinonoids.
To examine the formation of AP sites induced by estrogen quinones, E2-3,4-Q or E2-2,3-Q (0.1–1 mM, in DMSO 1% v/v) was incubated with CT-DNA in PBS buffer (150 mM sodium-potassium phosphate, pH 7.4) at 37°C under continuous stirring for 2 h. At 100 mM, neither of E2-3,4-Q nor E2-2,3-Q induced statistically significant increases in the number of ADL compared with vehicle control (Figure 4A and B). However, at 1 mM, both E2-2,3-Q and E2-3,4-Q did induce a 2-fold increase in the number of ADL (Figure 5). Cavalieri et al. (20) reported that the depurinating adduct, 4-OHE1(E2)-1-N7G, was detected in CT-DNA treated with E2-3,4-Q (1 mM) with the levels of depurinated adducts estimated to be 59–213 μmol/dNA-phosphate (equivalent to 59–213 ADL/10⁸ nucleotides) which is ~5 times higher than the number of ADL detected by ASB assay. We noticed that differences in buffer concentration (150 versus 67 mM PBS) may modify stability of alkylated DNA adducts (23). Further investigation of the differences in ionic strength of the reaction buffer did not identify significant changes in the formation of ADL induced by E2-3,4-Q (Figure 7) (P=0.05). Additionally, our current investigation demonstrated that the excisability of E2-3,4-Q-induced ADL by putrescine was 78 and 69% in the presence and absence of Cu(II) and NADPH, respectively (Figure 8B). Since regular AP sites at 3′-termini generated by spontaneous depurination/depyrimidation and β-elimination or by DNA glycosylase/AP-lyase are not excisable by putrescine (33), the ADL induced by estrogen quinones in CT-DNA under physiological conditions are likely to derive from oxidation processes more than depurination–depyrimidination of labile estrogen quinone–DNA adducts. The origin of the treatment-related increases in ADL in estrogen quinone-modified DNA may involve depurination of estrone quinone–DNA adducts, as well as hydride transfer from carbon to oxygen, due to the high redox potential of estrogen quinones. It is likely that high concentrations of estrogen quinones alone can serve as an oxidizing agent to mediate direct hydrogen abstraction on the C4′-position of deoxyribose to produce ADL (37,43). However, these increases in the formation of ADL in genomic DNA induced by high concentrations of estrogen quinones (1 mM) are unlikely to be of biological relevance. Additionally, 4-OH-E2(E2)-1-N7G was detected in mammmary gland tissue in female Sprague–Dawley rats exposed to a single dose of 4-OH-E2 and E2-3,4-Q (200 nmol/animal) (20), and the levels of 4-OH-E2(E2)-1-N7G were estimated to be ~2 μmol/mol DNA-phosphate which is equivalent to 2 ADL/10⁸ nucleotides. However, endogenous ADL in living cells have been estimated to be 5–10 ADL/10⁸ nucleotides (22). The intact ADL induced by depurination of E2-3,4-Q adducts in estrogen carcinogenesis is unlikely to be of biological significance.

Evidence indicates that estrogen-mediated ROS can induce sugar lesions on DNA double-helix by hydrogen abstraction from deoxyribose resulting in DNA strand breaks (44). The parallel formation of DNA SSB and ADL strongly suggests that the ADL induced by estrogen quinones plus Cu(II) and NADPH are predominantly cleaved ADL (Figures 4 and 6). Further investigation in DNA exposed to E2-3,4-Q plus Cu(II) and NADPH indicated that the predominant types of ADL are excisable by ExoIII and T7 Exo (Figure 9). This finding suggests that ~70% of the ADL induced by E2-3,4-Q plus Cu(II) and NADPH are aldehyde oxidized deoxyribose lesions at 3′-termini and 5′-termini. It is likely that estrogen quinone induces the formation of ROS which subsequently proceed to mediate the hydrogen abstraction on the C-5′ position of deoxyribose leading to the parallel formation of DNA SSB and ADL. The intact ADL (~5% of the total number of ADL) induced by estrogen quinones/Cu(II)/NADPH are likely to be derived from the spontaneous depurination of labile oxidized bases, direct quinone–DNA adducts, and C-4 oxidized AP sites. Recently, the endogenous ADL in genomic DNA have been characterized predominantly as being ExoIII-excisable and putrescine-excisable ADL. This evidence suggests that endogenous ADL are 3′-cleaved oxidized ADL (unpublished data). Estrogen quinonoid/Cu(II) may contribute to the accumulation of 3′-cleaved oxidized ADL in living cells. However, the impact of estrogen quinonoid-induced ADL in living cells is not known thus far and warrants further investigation.

In summary, these results provide evidence that in addition to oxidized bases, the ADL induced by estrogen quinoid-mediated ROS in genomic DNA represent a major type of oxidative damage to the DNA backbone. Overall, our results are in agreement with the hypothesis that depurination of estrogen quinone–DNA adducts is the major source of the formation of AP sites in genomic DNA under physiological condition and that the ADL induced by estrogen quinoid-mediated ROS may serve as a potential causative factor in estrogen carcinogenesis.

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