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Nitric oxide enhances catechol estrogen-induced oxidative stress in LNCaP cells

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Abstract

Catechol estrogens (CEs), such as 4-hydroxyestradiol (4-OHE_2), undergo redox cycling during which reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$) and the chemically reactive estrogen semiquinone (CE-SQ) and quinone (CE-Q) intermediates are produced. The quinone's putative mutagenicity may be enhanced by ROS and/or reactive nitrogen species. High concentrations of nitric oxide (NO) present during inflammatory conditions may react with ($O_2^{\cdot -}$) to form peroxynitrite ($ONOO^-$), a potent oxidant implicated in many pathological conditions. In this study, the possible generation of peroxynitrite from the interaction of CEs and NO and its effect on plasmid DNA and intact cells were investigated. A combination of 4-OHE_2 and NO increased the level of single strand breaks (SSB) in plasmid DNA by more than 60% compared to vehicle controls in a metal-free buffer system. 4-OHE_2 alone or NO alone had no effect. Results obtained from use of different antioxidants and ROS scavengers suggested a role of peroxynitrite in oxidative stress. In cells, 4-OHE_2 or NO alone induced dose-dependent DNA damage as assessed by single cell gel electrophoresis. Co-treatment with 4-OHE_2 and NO had an additive effect at lower doses. Generation of intracellular ROS was measured by the oxidation of carboxy-2′,7′-dichlorofluorescein diacetate to the fluorescent compound carboxy-2′,7′-dichlorofluorescein. NO alone, in oxygenated media, generated little ROS whereas 4-OHE_2 produced approximately 70% increase in fluorescence. When added together 4-OHE_2 and NO, produced a 2-fold increase in ROS. The generation and involvement of peroxynitrite to this increase was implied since uric acid inhibited it. Generation of peroxynitrite was also observed by use of dihydrorhodamine 123. Therefore, we conclude that combined treatments with 4-OHE_2 and NO generated peroxynitrite seen from increased fluorescence and its inhibition by uric acid or combined SOD and catalase treatments. Results reported here suggest a role of peroxynitrite in causing damage to biomolecules when CEs and NO are present simultaneously. This may have biological relevance as high concentrations of NO formed during inflammatory conditions may exacerbate cancers due to estrogens.

Keywords: *Oxidative stress, DNA damage, catechol estrogens, nitric oxide, peroxynitrite*

Abbreviations: BSO, buthionine bulfoximine; CDCFDA, 5-(and 6)-carboxy-2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithio-*bis*-2-nitrobenzoic acid, Ellman's reagent; EDTA, Ethylenediamine-N,N,N′,N′-tetraacetic acid; GSSG, glutathione, oxidized form; KPB, potassium phoshate buffer; NOC5, 3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine; 4-OHE_2, 4-hydroxyestradiol; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dimutase; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl

Introduction

Estrogen is a known risk factor for breast cancer and possible mechanisms of carcinogenesis may also be similar for other types of cancers e.g. renal, brain, endometrial, ovarian and prostate cancers. Carcinogenesis by estrogens has mainly been focused on mitogenecity via estrogen receptor-mediated cellular events. However, estrogen receptor-independent

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events such as the genotoxic potential of catechol estrogens (CEs) are increasingly being recognized. 4-Hydroxyestradiol (4-OHE_2) undergoes redox cycling [1] during which free radicals such as superoxide ($O_2^{\cdot-}$) and the chemically reactive estrogen semiquinone (CE-SQ) and quinone (CE-Q) intermediates are produced. The CE-Q can react with DNA to form stable or depurinating adducts with the potential to cause mutations leading to tumorigenesis [2]. Another mechanism by which mutations may be formed is by the free radicals generated during the metabolic redox cycling of CEs [3]. The ($O_2^{\cdot-}$) formed during this process is not very reactive by itself, but is a source of more reactive species such as hydroxyl radicals and singlet oxygen.

Superoxide can also react with nitric oxide (NO) at a rate constant of $6.7 \times 10^9\,M^{-1}s^{-1}$ to produce peroxynitrite ($ONOO^-$), a powerful oxidant that has been shown to cause a variety of DNA lesions [4], modify and inactivate proteins and cause tissue damage. There is a possibility that, under certain conditions, e.g. reduced cellular SOD activity, increased estrogen metabolism or chronic inflammation, ($O_2^{\cdot-}$) generated from CEs may combine with NO to form peroxynitrite. The simultaneous presence of estrogens and NO may pose increased risks for major human cancers that are associated with chronic infections and inflammation. A synergistic interaction between NO and CEs was shown in acellular media by Yoshie and Ohshima [5], that implicates peroxynitrite in enhancing oxidative DNA damage. Indeed, Paquette et al. [6] confirmed the formation of $ONOO^-$ from CEs and NO in acellular media. However, it is not clear whether peroxynitrite may actually be formed from CEs and NO *in vivo* or in cell systems.

There are numerous reports on effects of estrogens in breast cancer, but scanty reports on prostate cancer. There is accumulating evidence for the possible involvement of estrogens in initiation and/or progression of prostate tumorigenesis [7–13]. Furthermore, high and sustained concentrations of NO produced chiefly from induced NO synthase (iNOS) during chronic inflammation may increase mutations caused by estrogens. In the prostate, high iNOS expression has been associated with increased cellular proliferation, dedifferentiation and advanced stage cancer [14]. Wang et al. [15], also found increased expression of iNOS in neoplastic cultures and tissue sections compared to nonneoplastic ones.

In the present study, we explored further the involvement of peroxynitrite in reactions of CEs with NO. Generation of peroxynitrite was studied in cell-free systems and also in LNCaP.FGC cells, a prostate cancer cell line derived from a lymph node metastasis. Since estrogens may cause prostate cancer, this cell line was chosen as a model of estrogen-induced carcinogenesis in the prostate. The effects of CEs on DNA damage in the presence of NO were also studied.

Materials and methods

Chemicals

4-OHE_2, buthionine sulfoximine, dihydrorhodamine 123, rhodamine 123, and dimethyl sulfoxide were obtained from Sigma-Aldrich Chemicals Ltd. (St. Louis, USA). NOC5 (a NO donor) and SIN-1 (a peroxynitrite generator) were from Dojindo Laboratories (Kumamoto, Japan). Superoxide dismutase, catalase, and pUC18 DNA were from Wako Pure Chemicals Ltd. (Osaka, Japan). 5-(and 6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA) was obtained from Molecular Probes, Inc. (Eugene, OR, USA).

Induction of strand breaks in plasmid DNA

Agarose gel electrophoretic patterns of plasmid pUC18 DNA (2686 bp) were used to measure single strand breaks (SSB). The method is as described by Li and Trush [16], with some modifications. Briefly, 0.1 μg of DNA was incubated with various chemicals in KPB, pH 7.4 at 37°C for 1 h (final volume 10 μl). The reaction was terminated by the addition of 2 μl of electrophoresis loading buffer. Immediately after exposure, the DNA was loaded onto 1% agarose gel prepared in Tris Acetate/EDTA (TAE) buffer (40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, pH 7.0) and gel electrophoresis was carried out for 2 h at 8.5 V/cm in a horizontal gel electrophoresis apparatus. The gels, pre-stained with 0.5 μg/ml ethidium bromide, were exposed to UV light. Pictures were taken, scanned and the DNA strand breaks were measured by the conversion of supercoiled pUC18 double-stranded DNA (SC) to open circular (OC) and linear forms (L) by densitometry. The number of SSB was calculated as previously described [17].

Cell culture and treatment

LNCaP.FGC prostate cancer cells were cultured in Dulbecco's Modified Eagles's Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics at 37°C in 5% CO_2 in air.

To deplete cellular glutathione levels, cells were treated with 100 μM BSO, an inhibitor of glutathione synthesis, for 6, 12, 18 or 24 h. Total glutathione levels were analysed spectrophotometrically.

In DNA damage experiments using the comet assay, cells were seeded in 35-mm dishes, and treated with 100 μM BSO for 18 h. Media were replaced with DMEM containing 10% charcoal-stripped FBS. The NO donor, NOC5, and the CE,

$4OHE_2$ were typically administered for 90 min at 37°C. Stock solutions of $4OHE_2$ were dissolved in 99% DMSO and stored at −80°C. Further dilutions of the stock solution were with media just before cell treatment. Media containing the desired concentrations of $4OHE_2$ were added to the cells. The final concentration of DMSO in the media was <0.01%. A stock solution of 100 mM NOC5 in 10 mM NaOH was prepared and stored at −80°C. NOC5 was added directly to the cells. Cells were harvested by trypsinization, centrifuged and cell pellets were used either for the comet assay or for the cytotoxicity assay (trypan blue dye exclusion method).

Glutathione assay

Cells (70–80% confluent) were treated with 100 μM BSO for 0, 6, 12, 18 and 24 h. Cells were harvested using a rubber policeman, washed with PBS, and centrifuged at 200*g* for 10 min at 4°C. 5% SSA was added to the cell pellet and cells were lysed by 3 cycles of freezing and thawing and centrifuged at 8000*g* for 10 min. The supernatant was used for total glutathione determination by an enzymatic recycling method using glutathione reductase and DTNB [18]. Values were determined spectrophotometrically at 405 nm using a plate reader. Protein concentration from cell pellets was determined by the method of Lowry et al. [19]. Cell viability was determined by the trypan blue dye exclusion method.

Evaluation of intracellular reactive oxygen species (ROS)

Intracellular production of ROS was measured by using CDCFDA. This nonpolar compound passively diffuses into cells, where it is converted to the membrane-impermeant polar derivative CDCF by esterases [20]. CDCF is nonfluorescent but is rapidly oxidized to the highly fluorescent CDCF by intracellular H_2O_2 and other peroxides. Stocks of CDCFDA (5 mM) were made in DMSO and stored at −80°C. Cells were pre-treated with BSO for 18 h prior to addition of CDCFDA at a final concentration of 5 μM for 30 min. Cells were washed extensively with PBS and treated with 4-OHE2 and/or NOC5 or ROS scavengers. After treatment they were harvested by trypsinization and resuspended in PBS. Fluorescence was recorded (500 nm excitation, 536 nm emission) using a JASCO FP-777 spectrofluorometer.

The production of peroxynitrite from the interaction of NO and $4\text{-}OHE_2$ was determined by the oxidation of dihydrorhodamine 123 (DHR) to the fluorescent product rhodamine. LNCaP cells were seeded in six-well 35-mm tissue culture dishes at 1×10^5 cells/dish and allowed to attach overnight. Cells were pre-treated with BSO for 18 h after which they were loaded with fresh medium containing 5μM DHR and incubated for 2 h at 37°C to allow the dye to enter the cells and then washed two times with PBS to remove extracellular DHR. Following indicated treatments, cells were trypsinized, collected, washed and resuspended in 10 mM Tris-HCl/25 mM sucrose, pH 7.4, sonicated for 20 s and centrifuged at 10,000*g* for 15 min at 4°C. Supernatant fluorescence was measured using a fluorescence spectrophotometer with excitation and emission wavelengths of 500 and 536 nm, respectively, at room temperature [21]. Authentic rhodamine 123 standards, 0 to 400 nM, were used to generate a standard curve.

Comet assay

The comet assay was performed according to the method of Singh et al. [22] with some modifications [23]. Briefly, slides were coated with a first layer of 1% normal agarose. Approximately 20,000 cells were suspended in 50 μl 0.5% low melting point agarose and layered onto the slides, which were then immediately covered with cover slips. After agarose solidification at 4°C for 5 min, cover slips were removed and slides were immersed for 1 h at 4°C in fresh lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, pH 10) containing 1% Triton X-100. The slides were equilibrated in alkaline solution (1 mM Na_2EDTA, 300 mM NaOH, pH > 13) for 40 min. Electrophoresis was carried out for 30 min at 1 V/cm. Afterwards, slides were neutralized by washing them three times with 0.4 M Tris buffer (pH 7.5) every 5 min. Slides were stained with ethidium bromide (2 μg/ml). Images were scored using a fluorescent microscope (Olympus B × 50 equipped with a 520–550 nm excitation filter). Based on the extent of strand breakage, cells were classified according to their tail length in five categories, ranging from 0 (no visible tail) to 4 (still a detectable head of the comet but most of the DNA in the tail). The following formula [24] was used to calculate scores in which N is the number of cells in each category (e.g. N_4 is the number of cells in category 4).

$$\text{Score} = (N_0 + N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4) \times 100/(N_0 + N_1 + N_2 + N_3 + N_4)$$

Experiments were done in duplicate and repeated at least twice.

Statistical analysis

Differences in means were assessed by analysis of variance (ANOVA), followed by Fisher's protected least significant difference test. Statistical significance was considered at $P < 0.05$.

Results

Induction of strand breaks in plasmid DNA

Incubation of pUC18 plasmid DNA with 4-OHE_2 and NOC5 produced SSB (Figure 1A). 4-OHE_2 or NOC5 alone had no effect on the level of strand breaks. The extent of DNA damage increased with increasing concentrations of NOC5 and reached a peak at 250 μM. This result is similar to that of Yoshie and Ohshima [5]. The level of single strands produced was moderate as no linear or fragmented DNA was observed after agarose gel electrophoresis. In order to

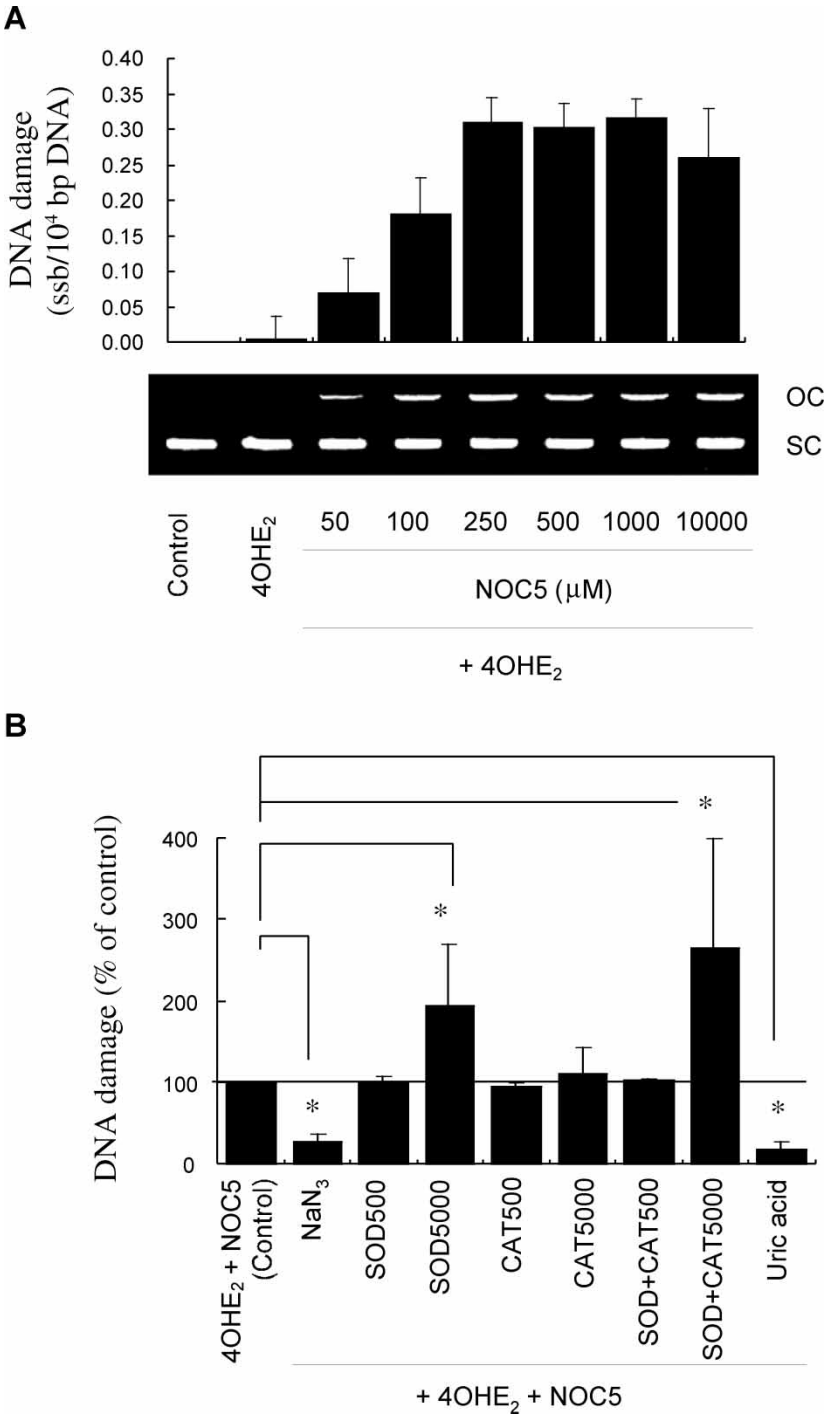

Figure 1. Induction of single strand breaks (SSB) in pUC18 plasmid DNA. (A) DNA was incubated with 10 μM 4-Hydroxyestradiol and different concentrations of NOC5 for 1 h at 37°C. DNA damage was expressed as SSB per 10^4 base pairs (bp) DNA (upper panel); Representative agarose gel electrophoresis pattern of the DNA after treatment (lower panel). (B) Effect of antioxidants and free radical scavengers on SSB in plasmid DNA. DNA was incubated with 10 μM 4-Hydroxyestradiol and 500 μM NOC5 for 1 h at 37°C in the presence of either 1 mM sodium azide (NaN_3), 500 or 5000 U/ml superoxide dismutase (SOD500, SOD5000) or catalase (CAT500, CAT5000) or both, or 1 mM uric acid. Following treatments, SSB were analysed as explained in "Materials and methods".

assess the nature ROS or RNS involved in inducing the strand breaks, selected scavengers were used (Figure 1B). Sodium azide (NaN_3) reduced strand breaks caused by 4-OHE_2 and NOC5. This implies the involvement of singlet oxygen ($^1O_2$) as NaN_3 is known to scavenge this reactive radical. Superoxide dismutase, however increased the level of DNA damage in the presence of 4-OHE_2 and NO. This may be due to formation of the nitroxyl radical (NO^-). Heat inactivated SOD, catalase or SOD and catalase combinations had no effect on DNA damage in the presence of 4-OHE_2 and NO. Furthermore, SOD alone or catalase alone did not induce strand breaks in DNA (data not shown). Use of combined SOD and catalase at the lower concentration showed no effect. The higher concentration revealed increased strand breaks. Catalase at 5000 U/ml was not able to protect against SSB suggesting H_2O_2 is not important in this system. Uric acid almost completely inhibited strand breaks caused by 4-OHE_2 and NO. This implies the involvement of peroxynitrite. Uric acid may scavenge peroxynitrite by forming a nitrated uric acid product, which was detected by Skinner et al. [25].

Depletion of cellular glutathione levels

Cells were treated with 100 μM BSO for the indicated duration (in hours) (Table I). Following 24 h of treatment, total glutathione levels were reduced to about 7% of control (untreated) cells. Total glutathione measured in control cells was 17.82 nmol/mg protein. BSO did not cause cell death at any of the time points tested.

Evaluation of intracellular ROS

Cells were pre-treated with BSO prior to addition of 4OHE_2, NOC5 or any ROS scavengers. Figure 2A shows the detection of intracellular ROS with the fluorescent probe CDCFDA. Fluorescence intensity was increased by 12.7% with 0.5 mM NOC5 alone relative to the control (untreated cells). 4-OHE_2 alone at 10 μM increased ROS production by approximately 1.7-fold, whereas a combination of 4-OHE_2 and NOC5 at the same concentrations produced a 2.2-fold increase. A measure of 10 μM SIN-1, a peroxynitrite generator, increased fluorescence by about 3.8-fold. Uric acid, but not TEMPO, inhibited ROS production by 4-OHE_2 and NOC5 (Figure 2B). TEMPO is a metal-independent, membrane-permeable superoxide dismutase-mimetic that scavenges O_2^- to hydrogen peroxide (H_2O_2) and oxygen. The concentration of 1 mM used is based on effective concentrations previously used by other researchers [26,27]. 4-OHE_2 and NOC5 dose-dependently increased dihydrorhodamine oxidation in cells (Figure 2C), with the lower concentration (10 μM 4OHE_2/500 μM NOC5) generating approximately 51.9 nM rhodamine and the higher concentration (20 μM 4OHE_2/1000 μM NOC5) generating 318.1 nM. Uric acid and, to a lesser extent, SOD and catalase combined inhibited DHR oxidation (Figure 2D). These results suggest that peroxynitrite can be produced intracellularly in the presence of the CE, 4-OHE_2, and NO.

Table I. Total gluthione levels in LNCaP cells.

Time (h)	TGSH (nmol/mg protein)	TGSH (% of control)	Viability (%)
0 (control)	17.82 ± 0.19	100.0	83.3 ± 0.9
6	4.85 ± 0.47	27.2	82.6 ± 3.9
12	4.11 ± 0.27	23.0	84.1 ± 3.1
18	2.74 ± 0.07	15.4	84.5 ± 4.4
24	1.29 ± 0.60	7.2	81.5 ± 2.8

Data represent mean ± S.D., $n = 3$.
TGSH = Total glutathione (GSH + GSSG).

Comet assay

All cells used in the comet assay were pre-treated with BSO in order to reduce the cytoprotective role of glutathione against ROS. In preliminary experiments, 4OHE_2 increased DNA damage in cells in a dose-dependent fashion up to 50 μM after which there was no further increase (data not shown). NO also increased the level of damage in cells dose-dependently up to 1 mM. A higher concentration, 2 mM, was not included in the assay as it compromised cell viability (data not shown). Increasing concentrations of 4OHE_2 and NOC5 in equivalent ratios also had a similar effect on DNA damage (Figure 3). In comparison to corresponding concentrations of either 4OHE_2 alone or NOC5 alone, 4OHE_2 and NOC5 combined only caused additive effects at the lower concentrations (2.5 μM 4OHE_2/0.125 mM NOC5 and 5 μM 4OHE_2/0.25 mM NOC5).

Discussion

Results presented in this study demonstrate the generation of peroxynitrite in situations where both CEs and NO are present. This has important biological implications as peroxynitrite or its conjugate, peroxynitrous acid (HOONO), have been linked to various pathological processes, including cancer, and a large body of evidence now links estrogens to tumorigenesis.

4-OHE_2 in the presence of NO causes SSB in plasmid DNA (Figure 1). This result is in agreement with that of Yoshie and Ohshima [5]. The level of strand breaks was dose-dependent but reached a peak after which no further increase was observed. CEs generate strong strand breakage in the presence of metal ions, such as copper [28]. DNA damage in these systems has been attributed to the hydroxyl radical

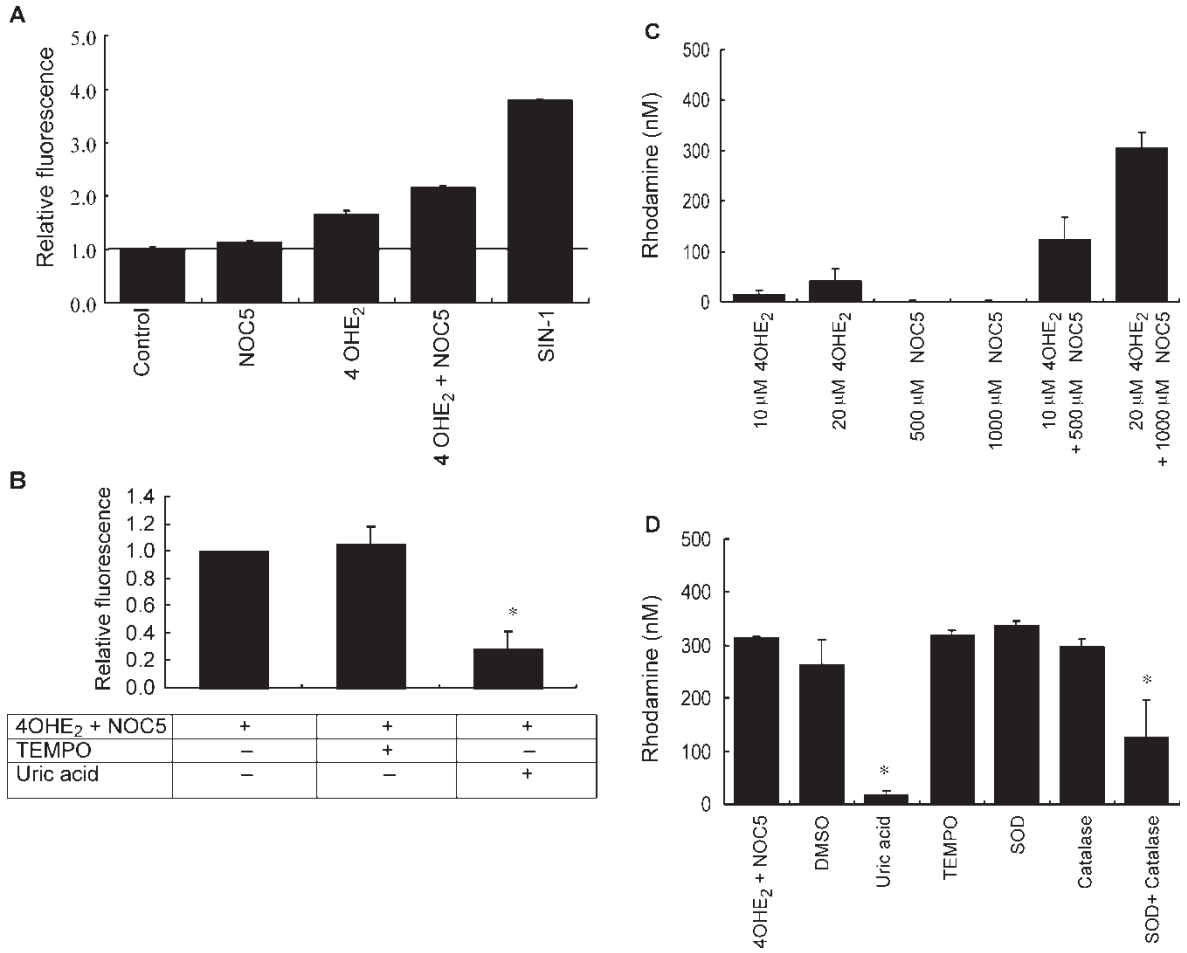

Figure 2. Intracellular generation of ROS by 4-OHE_2 and/or NOC5 in LNCaP cells. (A) Detection of ROS with CDCFA in cells treated with 10 μM 4OHE_2, 0.5 mM NOC5 or both for 90 min. A measure of 50 μM SIN-1 was administered to another group of cells. Data represent results from three independent experiments. (B) Effect of ROS scavengers on CDCFA fluorescence in cells treated with 10 μM 4-OHE_2 + 0.5 mM NOC5 for 90 min. TEMPO (1 mM) or Uric acid (1 mM) was administered to cells prior to 4-OHE_2 + NOC5 treatment. Data represent results from three independent experiments. *Significantly different from 4-OHE_2 + NOC5, $P < 0.05$. (C) Intracellular oxidation of DHR 123 by 10 or 20 μM 4-OHE_2 + 0.5 or 1 mM NOC5, respectively. Data are means ± SD of five experiments done in duplicate. (D) Effect of ROS scavengers on 4-OHE_2/NOC5 oxidation of DHR. Scavengers used were DMSO (1%), uric acid (1 mM), TEMPO (1 mM), SOD (500 U/ml), and catalase (500 U/ml). Data are means ± SD of two experiments done in triplicate. *Significantly different from 4-OHE_2 + NOC5, $P < 0.05$.

produced from H_2O_2 in the Fenton reaction. Hydrogen peroxide can be produced in the diffusion-controlled reaction of $O_2^{\cdot-}$ with Cu^+, as well as by the slower non-catalytic dismutation of $O_2^{\cdot-}$ [29]. In the present experiment, strand breaks were presumably caused by peroxynitrite possibly formed by the reaction of NO with $O_2^{\cdot-}$, which is produced during autoxidation of CE-SQ to CE-Q by dioxygen [1,28,30]. In contrast to Yoshie and Ohshima [5], this DNA damage was not inhibitable by SOD and was, in fact, increased with high SOD concentrations. SOD is expected to increase the concentration of H_2O_2 as it dismutates the $O_2^{\cdot-}$ anions, with subsequent formation of hydroxyl radicals. However, this result is not a consequence of hydroxyl radicals since the classical Fenton reaction may not occur in our system due to the elimination of metal ions with DTPA. The increased strand breakage may be due to formation of the nitroxyl anion (NO^-). SOD catalyzes the conversion of NO to nitroxyl [31,32], which is known to cause SSB [33]. In this case, H_2O_2 may not be formed if all the Cu(I), Zn-SOD reacts with NO instead of superoxide ($O_2^{\cdot-}$). This may explain the lack of effect of catalase when combined with SOD. Catalase alone was also ineffective suggesting H_2O_2 was not involved in the DNA damage. Conversion of CE to CE-SQ in the presence of NO may also generate NO^- using the quinone/hydroquinone redox system [34]. Hydrogen peroxide increases DNA damage in the presence of NO^- [32]. The singlet oxygen scavenger, sodium azide, inhibited strand breakage. In this system, the source of $^1O_2$ may be the spontaneous dismutation of $O_2^{\cdot-}$. There is also the possibility that H_2O_2 may be formed from $O_2^{\cdot-}$ generated from CE and NO, and this reacts with $ONOO^-$ to form singlet oxygen [35,36].

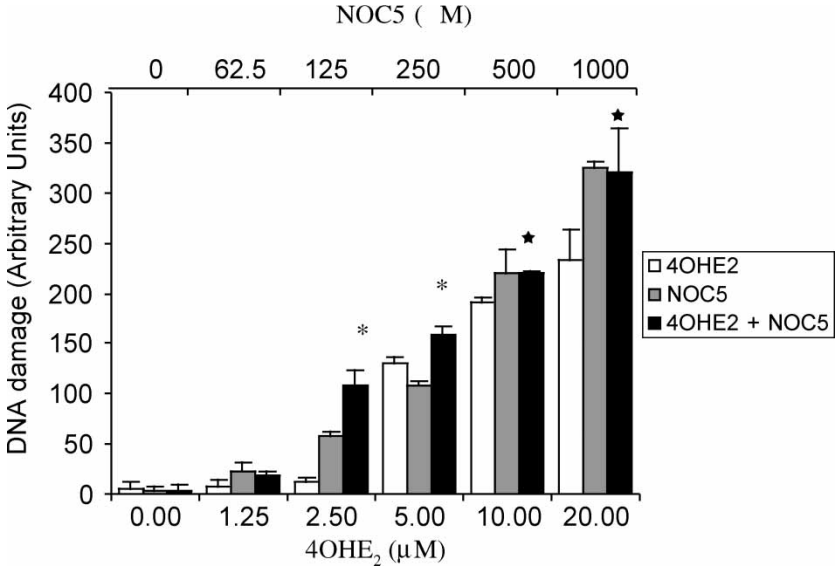

Figure 3. Induction of DNA damage in LNCaP cells treated with 1.25–20 μM 4-OHE_2 (white bar), 62.5–1000 μM NOC5 (grey bar), or 4OHE_2 + NOC5 (black bar) at the indicated concentrations. Cells were processed for the comet assay as described in "Materials and methods". *4-OHE_2 + NOC5 significantly different from either 4-OHE_2 alone or NOC5 alone, $P < 0.05$.★ 4-OHE_2 + NOC5 significantly different from 4-OHE_2 alone, $P < 0.05$. Data are expressed as mean ± SD, $n = 3$.

Furthermore, H_2O_2 may also react with NO to form singlet oxygen [37]. However, experiments performed on plasmid DNA in 0.1 M KPB, pH 7.4/0.1 mM DTPA with the direct addition of H_2O_2 and NOC5 failed to produce any DNA damage (data not shown). Uric acid efficiently abrogated strand breakage suggesting peroxynitrite was the major responsible species for the DNA strand breakage. In separate experiments, uric acid dose-dependently (0.5 to 2 mM) inhibited strand breaks elicited by 100 μM SIN-1 in plasmid DNA (data not shown). Peroxynitrite causes strand breakage in plasmid DNA as well as in eukaryotic cell DNA. The major reactions of peroxynitrite are oxidative in nature with generation of products such as 8-hydroxyguanine, 8-hydroxyadenine and oxazolone [4]. However, peroxynitrite can also mediate nitration of deoxyguanosine to form 8-nitroguanine.

Intracellular formation of peroxynitrite was detected when NOC5 and 4-OHE_2 were administered to cells (Figure 2). Using the carboxy-dichlorofluorescein probe, 4-OHE_2 alone increased fluorescence about 1.7-fold above that of the control (Figure 2A). Endogenous CEs can be oxidized by virtually any oxidative enzyme and/or metal ion to give CE-Qs [38]. Hence, superoxide generated during redox cycling may be responsible for this increased fluorescence as it is a source of H_2O_2. In the presence of cellular peroxidases H_2O_2 is capable of oxidizing dichlorofluorescein [39]. The slight increase in fluorescence by NOC5 alone may be due to the formation of nitrogen dioxide ($^{\cdot}NO_2$) in the presence of oxygen, as was demonstrated by Crow [40]. NO on its own has little or no effect on dichlorofluorescein oxidation [41–43]. When 4-OHE_2 and NOC5 were administered together, there was a greater increase in fluorescence (2.2-fold higher than control) and this was attributed to the formation of $ONOO^-$, which is a potent oxidant of dichlorofluorescein. Uric acid reduced fluorescence to 10.7% of control values (Figure 2B). In comparison, peroxynitrite generated from SIN-1 used in the positive control increased fluorescence by about 3.8-fold. No inhibition of fluorescence in the presence of TEMPO was observed. TEMPO may have limited peroxynitrite formation by dismutating the $O_2^{\cdot-}$, but the decrease in fluorescence may have been offset by the H_2O_2-induced fluorescence. In addition, the excess NO spared by TEMPO, may form NO_2 in the presence of oxygen. The fluorophore, DHR, produced similar results with 4-OHE_2 treatment leading to increased fluorescence, presumably from the formation of hydrogen peroxide and hydroxyl radicals. 4-OHE_2 and NOC5 combined at two different concentrations dose-dependently increased fluorescence (Figure 2C). However, no oxidation of DHR was seen with NOC5 alone. This is because neither NO nor nitrogen dioxide can oxidize DHR [40]. Even though, hydroxyl radicals were not involved in DHR oxidation in the cell-free system, they may still be partially responsible for oxidation in the cells since DMSO slightly reduced oxidation (Figure 2D). However, this finding did not reach any statistical significance. Fluorescence in the presence of SOD (or TEMPO) was inhibited by catalase (Figure 2D) indicating that H_2O_2 is responsible for maintaining fluorescence when the $O_2^{\cdot-}$ ion is scavenged. Exogenous SOD has been shown to influence intracellular oxidation of fluorescent

probes [44], but the precise mechanism by which it does this is obscure as both SOD and the superoxide anion have limited membrane permeability in live cells. Superoxide anions formed intracellularly may escape into the extracellular space via anion channels [45]. In this case, SOD may have access to $O_2^{\cdot-}$, in the same manner that catalase detoxifies the freely diffusable H_2O_2. In addition, exogenous SOD may also reduce extracellular formation of peroxynitrite, which would otherwise contribute to intracellular oxidation of indicator probes. Peroxynitrite can cross cell membranes by either passive diffusion when in its protonated form or via anion channels when in its anionic form [46].

Cells are normally resistant to the deleterious effects of electrophiles and ROS due to the high concentration of glutathione. Glutathione also serves as an antioxidant and a substrate for a number of enzymes, such as peroxidases and transferases. In studies involving cells, intracellular glutathione levels were depleted with BSO, an inhibitor of the synthetic enzyme, γ-glutamylcysteine synthetase. This was so as to make cells more sensitive to the effects of CEs and ROS as was done by Mobley and Brueggemeier [47], using MCF-7 breast cancer cells. Conjugation of CE quinones with GSH results in a decrease in DNA damage [47–49]. Total glutathione was depleted to only about 7% of control levels after 24 h of incubation with BSO (Table I). In this study, cells were routinely incubated with BSO for 18 h (approximately 15% reduction in intracellular glutathione). In preliminary experiments, depletion of glutathione in LNCaP cells made them susceptible to the damaging effects of 4-OHE_2 or NOC5 without compromising their survival (data not shown). Treatment with 4-OHE_2 alone or NOC5 alone caused DNA damage in a concentration-dependent manner (Figure 3). To our knowledge, this is the first study using single cell gel electrophoresis demonstrating induction of DNA strand breaks in the LNCaP.FGC cell line. NO can cause DNA damage in cells at concentrations found under inflammatory conditions [50]. Co-treatment of cells with both 4-OHE_2 and NOC5 had a similar pattern of results (Figure 3). Since both 4-OHE_2 and NOC5 cause DNA damage when given singly, it was interesting to see their combined effects. Additive effects were observed at the lower concentrations of 4-OHE_2 and NOC5. As it is clear that peroxynitrite is produced in the cells (Figure 2) from co-treatment with 4-OHE_2 and NOC5, it may be partially responsible for increased DNA damage at the lower concentrations. At higher concentrations no additive effect was observed probably due to the higher concentration of peroxynitrite formed activating DNA repair mechanisms, such as those mediated by poly(ADP-ribose) polymerase (PARP-1). Increased peroxynitrite formation can lead to SSB (or double strand breaks) that activate the nuclear enzyme PARP-1 [51]. PARP-1 is involved in DNA repair and thus may restrict any further damage to DNA, even though excessive strand breakage may also promote apoptotic or necrotic cell death due to depletion of cellular energy levels [51,52].

In summary, the present study provides further support for the formation of peroxynitrite from CEs and NO. Evidence reported here points to a role of peroxynitrite, at least in part, in causing damage to biomolecules when CEs and NO are present simultaneously. The contributory role of other ROS and/or reactive nitrogen species that may be formed cannot be excluded. These interactions may have biological relevance as high concentrations of NO formed during inflammatory conditions may exacerbate cancers due to estrogens.

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