Environ Health Prev Med (2010) 15:319–326
DOI 10.1007/s12199-010-0146-1

REGULAR ARTICLE

DNA damage and estrogenic activity induced by the environmental pollutant 2-nitrotoluene and its metabolite

Chigusa Watanabe · Takashi Egami · Kaoru Midorikawa · Yusuke Hiraku · Shinji Oikawa · Shosuke Kawanishi · Mariko Murata

Received: 10 December 2009 / Accepted: 6 April 2010 / Published online: 28 April 2010

Abstract
Objectives The environmental pollutant 2-nitrotoluene (2-NO_2-T) is carcinogenic and reproductively toxic in animals. In this study, we elucidated the mechanisms of its carcinogenicity and reproductive toxicity.
Methods We examined DNA damage induced by 2-NO_2-T and its metabolite, 2-nitrosotoluene (2-NO-T), using ^{32}P-5′-end-labeled DNA. We measured 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage, in calf thymus DNA and cellular DNA in cultured human leukemia (HL-60) cells treated with 2-NO_2-T and 2-NO-T. 8-Oxoguanine DNA glycosylase (*OGG1*) gene expression in HL-60 cells was measured by real-time polymerase chain reaction (PCR). We examined estrogenic activity using an E-screen assay and a surface plasmon resonance (SPR) sensor.
Results In experiments with isolated DNA fragments, 2-NO-T induced oxidative DNA damage in the presence of Cu (II) and β-nicotinamide adenine dinucleotide disodium salt (reduced form) (NADH), while 2-NO_2-T did not. 2-NO-T significantly increased levels of 8-oxodG in HL-60 cells. Real-time polymerase chain reaction (PCR) analysis revealed upregulation of *OGG1* gene expression induced by 2-NO-T. An E-screen assay using the human breast cancer cell line MCF-7 revealed that 2-NO_2-T induced estrogen-dependent cell proliferation. In contrast, 2-NO-T decreased the cell number and suppressed 17β-estradiol-induced cell proliferation. The data obtained with the SPR sensor using estrogen receptor α and the estrogen response element supported the results of the E-screen assay.
Conclusions Oxidative DNA damage caused by 2-NO-T and estrogen-disrupting effects caused by 2-NO_2-T and 2-NO-T may play a role in the reproductive toxicity and carcinogenicity of these entities.

Keywords 2-Nitrotoluene · 2-Nitrosotoluene · Oxidative DNA damage · Reproductive toxicity · Estrogenic activity

C. Watanabe · T. Egami · K. Midorikawa · Y. Hiraku · S. Oikawa · S. Kawanishi · M. Murata (✉)
Department of Environmental and Molecular Medicine, Mie University School of Medicine, Tsu, Mie 514-8507, Japan
e-mail: mmurata@doc.medic.mie-u.ac.jp

S. Kawanishi
Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, Suzuka, Mie, Japan

Introduction

2-Nitrotoluene (2-NO_2-T) is used to synthesize agricultural and rubber chemicals; azo dyes; and dyes for cotton, wool, silk, leather, and paper [1]. Environmental surveys have demonstrated the presence of 2-NO_2-T in rivers and in drinking water [1]. Thus, humans are exposed to 2-NO_2-T in the workplace and in the environment.

A comparative study of *o*-, *m*-, and *p*- (2-, 3-, and 4-) nitrotoluene showed that all three chemicals caused toxicity in the kidney, spleen, liver, and/or reproductive system in rats, and the toxicity was most severe with the *ortho* isomer [2]. Testicular degeneration was observed in male rats treated with 2-NO_2-T, and was characterized by decreased numbers of germinal epithelial cells and the presence of syncytial giant cells (degenerative spermatids) in seminiferous tubules. Epididymal sperm density and testicular spermatid head counts were reduced, indicating male reproductive toxicity [3]. Furthermore, a National Toxicology Program (NTP) study demonstrated clear evidence of the carcinogenic activity of 2-NO_2-T. In both

male and female rats, incidences of subcutaneous skin neoplasms, hepatocellular adenoma or carcinoma, and mammary gland fibroadenomas were increased [2]. Epididymis mesothelioma was observed in rats treated with 2-NO_2-T [4]. The tumorigenicity in reproductive organs, such as the mammary gland fibroadenoma and epidermis mesothelioma, and the male reproductive toxicity suggest certain endocrine-disrupting effects. Some reports indicate that 2-NO_2-T may contribute to genetic lesions [2, 5, 6]. The carcinogenic and reproductively toxic mechanisms of 2-NO_2-T are not fully established. Therefore, it is important to clarify the mechanisms that contribute to the toxicity of 2-NO_2-T.

To elucidate the mechanism of genotoxicity, we examined DNA damage caused by 2-NO_2-T and its metabolite 2-nitrosotoluene (2-NO-T), using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* tumor suppressor gene. The amount of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage, was measured using an electrochemical detector coupled to an HPLC system (HPLC-ECD). An E-screen assay was performed to detect estrogenic activity, using the human breast cancer cell line MCF-7. Furthermore, we measured the binding of the 2-NO_2-T-liganded estrogen receptor (ER) to the estrogen response element (ERE), using a surface plasmon resonance (SPR) sensor in order to examine possible endocrine-disrupting effects.

Materials and methods

Materials

The restriction enzymes *Eco*RI and *Apa*I and proteinase K were purchased from Roche Molecular Biochemicals (Mannheim, Germany). The restriction enzyme *Hin*dIII and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA). [γ-^{32}P] ATP (222 TBq/mmol) was obtained from New England Nuclear (Boston, MA, USA). Superoxide dismutase (SOD; 3000 units/mg from bovine erythrocytes), catalase (45000 units/mg from bovine liver), and 2-NO_2-T were purchased from Sigma Chemical (St. Louis, MO, USA). RNase A and bacterial alkaline phosphatase were purchased from Sigma (Steinheim, Germany). β-Nicotinamide adenine dinucleotide disodium salt (reduced form) (NADH) was purchased from Kohjin (Tokyo, Japan). Diethylenetriamine-*N*, *N*, *N*′, *N*″, *N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were obtained from Dojin Chemicals (Kumamoto, Japan). Formamidopyrimidine-DNA glycosylase (Fpg; 20000 units/mg from *Escherichia coli*) was obtained from Trevigen (Gaithersburg, MD, USA). Dimethyl sulfoxide (DMSO) and nuclease P_1 were obtained from Wako Chemical (Osaka, Japan). 2-NO-T was obtained from Aldrich Chemical (Milwaukee, WI, USA). The lysis buffer for extracting DNA was obtained from Applied Biosystems (Foster City, CA, USA).

Preparation of ^{32}P-5′-end-labeled DNA fragments

Exon-containing DNA fragments obtained from the human *p53* tumor suppressor gene [7] were prepared, as described previously [8]. A 5′-end-labeled 650-bp fragment (*Hin*dIII*13972–*Eco*RI*14621) was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P] ATP and T_4 polynucleotide kinase (*, ^{32}P-label). The 650-bp fragment was further digested with *Apa*I to obtain a singly labeled 443-bp fragment (*Apa*I 14179–*Eco*RI*14621) and a 211-bp fragment (*Hin*dIII*13972–*Apa*I 14182).

Detection of DNA damage induced by 2-NO_2-T and its metabolite 2-NO-T in the presence of Cu (II) and NADH

The standard reaction mixture (in a 1.5-ml Eppendorf microtube) contained 2-NO_2-T or 2-NO-T, Cu (II), NADH, ^{32}P-5′-end-labeled DNA fragments, and calf thymus DNA (20 μM per base) in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 1 h, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min or treated with 10 units of Fpg protein in 10 μl of reaction buffer (10 mM hydroxyethylpiperazine ethane sulfonic acid [HEPES]–KOH [pH 7.4], 100 mM KCl, 10 mM ethylenediaminetetraacetate [EDTA], and 0.1 mg/ml bovine serum albumin [BSA]) at 37°C for 2 h as described previously [9]. The preferred cleavage sites were determined by direct comparison of the labeled, cleaved oligonucleotides with a standard 5′-end-labeled Maxam–Gilbert sequencing reaction [10] (LKB 2010 Macrophor; LKB Pharmacia Biotechnology Inc., Uppsala, Sweden). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer.

Analysis of 8-oxodG in calf thymus DNA

DNA fragments (100 μM per base) from calf thymus were incubated with 2-NO-T, Cu (II), and NADH at 37°C for 1 h, and 0.1 mM DTPA was added to stop the reaction. After ethanol precipitation, DNA was digested into nucleosides with nuclease P_1 and calf intestine phosphatase, and then analyzed by HPLC-ECD [7].

Measurement of 8-oxodG in DNA from cultured human leukemia cells treated with 2-NO_2-T and its metabolite 2-NO-T

Human leukemia HL-60 cells (1×10^6/ml) were treated with 2-NO_2-T or 2-NO-T at 37°C for 4 h in RPMI 1640 medium with 6% fetal bovine serum (FBS), and washed three times with cold phosphate-buffered saline (PBS). Under anaerobic conditions, DNA was extracted using lysis buffer, RNase A, and proteinase K. After ethanol precipitation, DNA was digested with nuclease P_1 and bacterial alkaline phosphatase, and then analyzed by HPLC-ECD [8].

Measurement of *OGG1* gene expression in cultured human leukemia cells treated with 2-NO_2-T and its metabolite 2-NO-T

Total RNA was isolated using an RNAqueous-4PCR kit (Ambion, Austin, TX, USA). The concentration and purity of RNA were confirmed using a UV-spectrometer (UVPC-1600; Shimadzu, Kyoto, Japan). The quality of RNA was checked by electrophoresis on a 1% agarose gel. cDNA was synthesized from total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription PCR was performed at 37°C for 60 min, and the reaction mixture was heated to 95°C for 5 min and held at 4°C. Gene expression was quantified by real-time PCR, using the ABI StepOne PCR system (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) as the primer and probe (glyceraldehyde-3-phosphate-dehydrogenase [GAPDH], Hs99999905_m1, OGG1, Hs00249902_m1) and TaqMan Gene Expression Master Mix (Applied Biosystems). OGG1 and GAPDH levels were determined from relative standard curves, which applied threshold cycle (Ct) values, and OGG1 was normalized to an endogenous reference (GAPDH).

Bioassay for measuring estrogenic activity (E-screen assay)

The E-screen assay was performed using a modified version [11] of the method established by Soto et al. [12]. Briefly, MCF-7 cells were trypsinized and plated into 12-well plates, at an initial concentration of 3×10^4 cells per well, with seeding medium (DMEM supplemented with 5% FBS and 100 ng/ml kanamycin). After the cells were allowed to attach for 24 h, the seeding medium was replaced with experimental medium (phenol red-free DMEM, supplemented with 5% FBS-charcoal dextran, 100 ng/ml kanamycin, and 4 mM L-glutamine), and then a test chemical was added. 2-NO_2-T, 2-NO-T, and 17β-estradiol (E2) as a positive control were dissolved in DMSO before being tested. The final concentration of solvent in the culture medium did not exceed 0.1%, as this concentration did not affect cell yields [11]. The control also contained 0.1% DMSO. Cells were incubated for 6 days after treatment with the test compounds, and were then trypsinized and harvested. Harvested cells were counted using a Coulter counter (Beckman Coulter, Fullerton, CA, USA).

Analysis of ER-ERE binding

The BIAcore-biosensor system (Biacore X; Pharmacia Biosensor, Uppsala, Sweden) permits the monitoring of macromolecular interaction in real time using an SPR sensor [13]. A single-stranded biotinylated oligonucleotide (35 mer, HPLC grade) with the sequence of the human *pS2* ERE [14] and the compensatory unbiotinylated oligonucleotide (35 mer, HPLC grade) were obtained from Invitrogen (Carlsbad, CA, USA). The biotinylated oligonucleotide's sequence is 5′-XGTCCAAAGTCAGGT CACGGTGGCCTGATCAAAGTT-3′ (X indicates biotin-labeled). The running buffer used for immobilization and the binding assay included 25 mM Tricine, 160 mM KCl, 5 mM $MgCl_2$, and 0.05% Tween 20 (pH 7.8). The biotinylation of the oligonucleotide was performed for immobilization to the streptavidin-treated sensor chip. The compensatory oligonucleotide was annealed to the immobilized ERE. Human estrogen receptor α (ERα 20 nM), obtained from Invitrogen, was liganded with 100 nM E2 or 10 µM 2-NO_2-T and its metabolite 2-NO-T by incubation at 37°C for 5 min. Then, the liganded ER was injected over surfaces coated with double-stranded ERE via a sample loop. Each binding cycle was performed with a constant flow of buffer of 20 µl/min at 25°C, as described previously [11]. The activity of the liganded ER to bind ERE was expressed as a percentage, with the response with 100 nM E2 taken as 100% and that without the chemical (DMSO 0.1%) as 0%. All samples contained 0.1% DMSO.

Results

Damage to ^{32}P-labeled DNA fragments induced by 2-NO_2-T and its metabolite 2-NO-T in the presence of Cu (II) and NADH

Figure 1a shows an autoradiogram of DNA fragments treated with 2-NO_2-T and its metabolite. Oligonucleotides were detected on the autoradiogram following DNA cleavage. 2-NO_2-T did not cause DNA damage even in the presence of Cu (II) and NADH under the condition used. 2-NO-T did not cause DNA damage in the presence of Cu

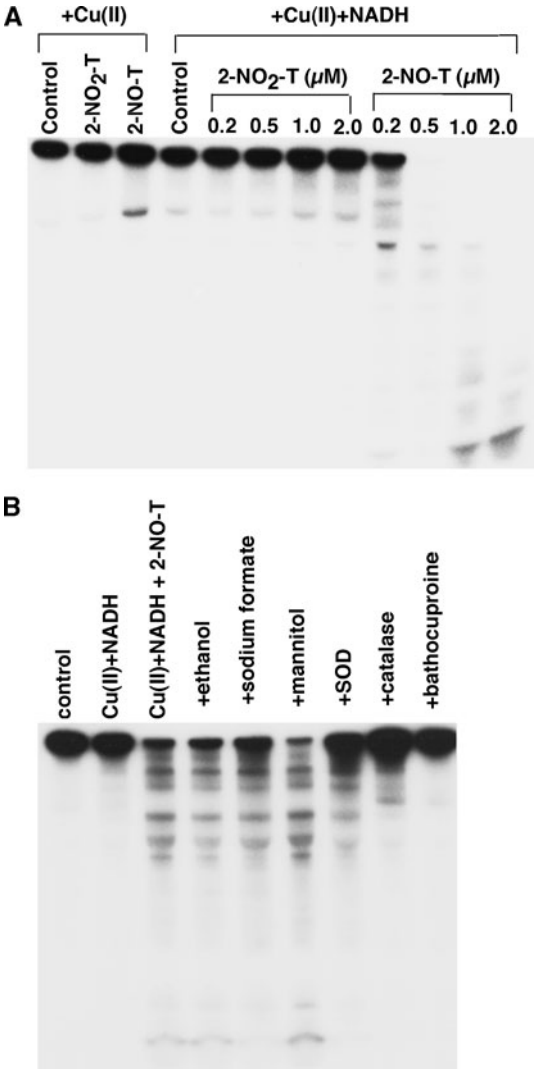

Fig. 1 Autoradiogram of ^{32}P-labeled DNA fragments incubated with 2-nitrotoluene (*2-NO_2-T*) and its metabolite -2-nitrosotoluene (*2-NO-T*) in the presence of Cu (II) and *β*-nicotinamide adenine dinucleotide disodium salt (reduced form) (NADH). **a** The reaction mixture contained the ^{32}P-5′-end-labeled 443-bp DNA fragment, 20 μM/base thymus DNA, the indicated concentrations of 2-NO_2-T or 2-NO-T, 20 μM $CuCl_2$, and 100 μM NADH in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM diethylenetriamine-*N*, *N*, *N*′, *N*″, *N*″-pentaacetic acid (DTPA). **b** The reaction mixture contained the ^{32}P-5′-end-labeled 443-bp DNA fragment, 20 μM/base thymus DNA, 0.5 μM 2-NO-T, 20 μM $CuCl_2$, 100 μM NADH, and a scavenger in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The scavenger or bathocuproine was added as follows: 5% ethanol, 0.1 M sodium formate, 0.1 M mannitol, 30 units/ml superoxide dismutase (SOD), 30 units/ml catalase, and 50 μM bathocuproine. The mixtures were incubated at 37°C for 1 h, and the DNA fragments were treated with 1 M piperidine at 90°C for 20 min, and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel

(II). 2-NO-T induced DNA damage in the presence of both Cu (II) and NADH. The intensity of damage increased with successive concentrations of 2-NO-T.

Effects of scavengers and bathocuproine on DNA damage induced by 2-NO-T

Figure 1b shows the effects of scavengers and bathocuproine, a Cu (I)-specific chelator, on DNA damage induced by 2-NO-T in the presence of Cu (II) and NADH. Hydroxyl radical (OH) scavengers, such as ethanol, sodium formate, and mannitol, did not have an inhibitory effect. SOD reduced the amount of DNA damage. Catalase and bathocuproine inhibited the DNA damage, suggesting the involvement of hydrogen peroxide (H_2O_2) and Cu (I). These results suggest that the main reactive species is not ·OH but Cu (I) OOH.

Site specificity of DNA cleavage by 2-NO-T

To examine site specificity, an autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensity of DNA cleavage in the human *p53* tumor suppressor gene. 2-NO-T/Cu (II)/NADH generated piperidine-labile sites predominantly at thymine and cytosine residues (Fig. 2a), and Fpg-sensitive sites at guanine and cytosine residues (Fig. 2b) of the *p53* gene.

Formation of 8-oxodG in calf thymus DNA induced by 2-NO-T in the presence of Cu (II) and NADH

We examined the Cu (II)-mediated formation of 8-oxodG in calf thymus DNA treated with 2-NO-T in the presence and absence of NADH, using HPLC-ECD (Fig. 3). 2-NO-T did not cause oxidative DNA damage in the presence of Cu (II), but the addition of NADH significantly increased the amount of 8-oxodG. These results, as well as the above-mentioned results, suggest that 2-NO-T requires both NADH and Cu (II) to induce DNA damage, in a cell-free system.

Formation of 8-oxodG and *OGG1* gene expression in human cultured cells treated with 2-NO_2-T and its metabolite 2-NO-T

Figure 4 shows 8-oxodG levels and *OGG1* expression in HL-60 cells. 2-NO-T significantly increased the amount of 8-oxodG, whereas there was no significant increase in 8-oxodG in the cells treated with 2-NO_2-T (Fig. 4a). The amount of 8-oxodG increased with successive concentrations of 2-NO-T (Fig. 4b). 2-NO-T significantly increased *OGG1* mRNA levels compared with the control, but 2-NO_2-T did not (Fig. 4c). OGG1 is a DNA repair enzyme that incises DNA at 8-oxo-guanine residues. These results suggest that 2-NO-T induced oxidative damage to cellular DNA.

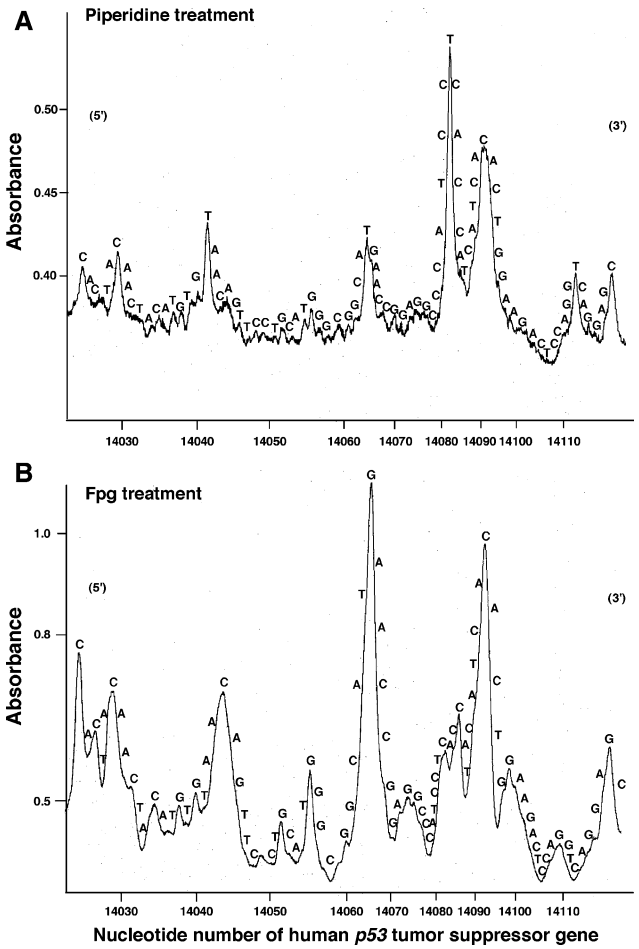

Fig. 2 Site specificity of DNA damage induced by 2-NO-T in the presence of Cu (II) and NADH. The reaction mixture contained the ^{32}P-5′-end labeled 211-bp (*Hin*dIII*13972–*Apa*I 14182) DNA fragment, 20 μM/base calf thymus DNA, 0.2 μM or 0.5 μM 2-NO-T, 20 μM $CuCl_2$, and 100 μM NADH in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation for 1 h at 37°C, the DNA fragments were treated with piperidine (**a**) or formamidopyrimidine-DNA glycosylase (*Fpg*) protein (**b**) and electrophoresed by the method described in "Materials and methods". The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer

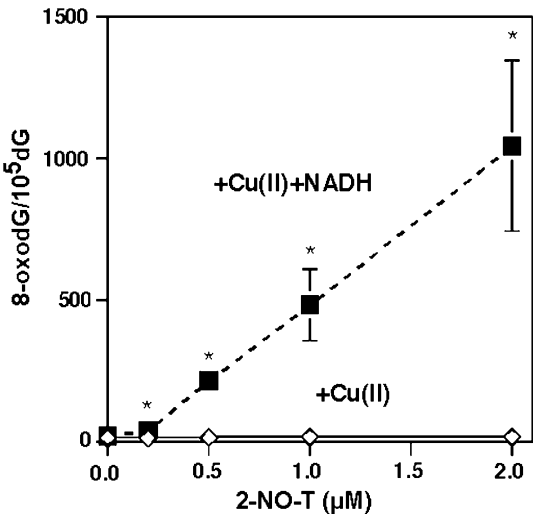

Fig. 3 Formation of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (*8-oxodG*) in calf thymus DNA treated with 2-NO-T in the presence of Cu (II) and NADH. The reaction mixture contained 100 μM/base calf thymus DNA, the indicated concentrations of 2-NO-T, 20 μM $CuCl_2$, and no or 100 μM NADH in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 1 h, DNA was precipitated in ethanol and enzymatically digested into nucleosides. Then, the amounts of 8-oxodG were measured by an electrochemical detector coupled to an HPLC system (HPLC-ECD). Results are expressed as the means and SEM for four independent experiments. *Significant difference compared with the control by Student's *t* test with Bonferroni correction (the level of significance was less than 0.05)

Cell proliferative activity of 2-NO_2-T and 2-NO-T in estrogen-sensitive MCF-7 cells

The effects of 2-NO_2-T and 2-NO-T on cell proliferation were measured with an E-screen assay (Fig. 5a). 2-NO_2-T induced proliferation in MCF-7 cells, indicating an estrogenic effect. On the other hand, 2-NO-T decreased the cell numbers of MCF-7 cells. E2, as a positive control, significantly induced cell proliferation compared with the negative control (DMSO 0.1%), and the addition of 2-NO-T significantly suppressed cell proliferation compared with the effect of E2 alone. The suppression of the E2-induced cell proliferation by 2-NO-T was similar to that shown by the E2 antagonist 4-hydroxytamoxifen (data not shown), suggesting an anti-estrogenic effect of 2-NO-T. 2-NO_2-T induced cell proliferation and 2-NO-T suppressed it in a dose-dependent manner (Fig. 5b).

Binding of liganded ERα to ERE

The binding activity of liganded ERα and ERE was measured by SPR sensor (Fig. 5c). The reactivity of the E2-liganded ER to ERE was detected as the peak height of the sensorgram, expressed as 100%, and the reactivity without the chemical (DMSO 0.1%) was expressed as 0%. 2-NO_2-T-liganded ERα had about 30% E2-binding activity. On the contrary, 2-NO-T decreased the binding to ERE, of which the sensorgram was below the negative control (DMSO 0.1%) level. This was a similar pattern to that shown by 4-hydroxytamoxifen (data not shown), suggesting that 2-NO-T has anti-estrogenic activity.

Discussion

The present study has shown that 2-NO-T can cause oxidative DNA damage, including the formation of 8-oxodG, in the presence of Cu (II) and NADH. Possible mechanisms of oxidative DNA damage caused by 2-NO-T are shown in

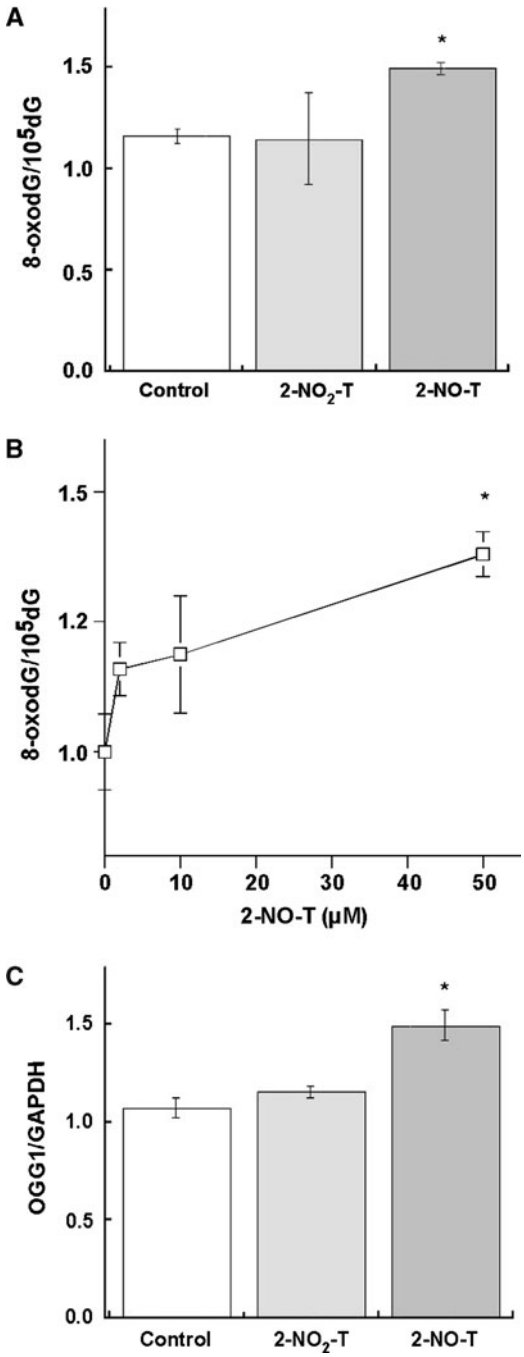

Fig. 4 Intracellular 8-oxodG formation and 8-oxoguanine DNA glycosylase (*OGG1*) gene expression induced by 2-NO_2-T and its metabolite 2-NO-T in HL-60 cells. HL-60 cells were treated with 50 μM 2-NO_2-T and 2-NO-T (**a**, **c**) or the indicated concentrations of 2-NO-T (**b**) in the experimental medium at 37°C for 4 h. **a**, **b** DNA was extracted and treated as described in "Materials and methods". Amounts of 8-oxodG were determined by HPLC-ECD. **c** RNA was extracted and reverse-transcribed to cDNA. *OGG1* gene expression was determined by real-time polymerase chain reaction (PCR) as described in "Materials and methods". Results are expressed as the means ± SEM for four independent experiments. *Significant difference compared with the control by Student's *t* test with Bonferroni correction (the level of significance was less than 0.05). *GAPDH* glyceraldehyde-3-phosphate-dehydrogenase

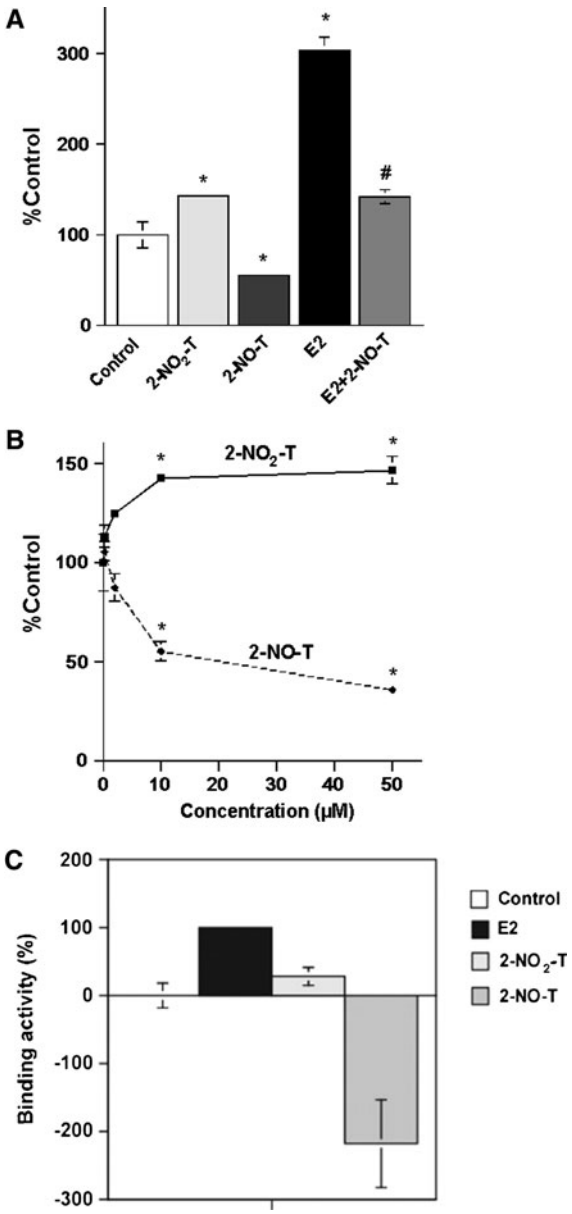

Fig. 5 Estrogenic activities of 2-NO_2-T and 2-NO-T. **a** An E-screen assay was performed as described in "Materials and methods". MCF-7 cells were incubated with 10 μM 2-NO_2-T, 100 pM 17β-estradiol (*E2*), and 10 μM 2-NO-T + 100 pM E2 at 37°C for 6 days. **b** MCF-7 cells were incubated with the indicated concentrations of 2-NO_2-T and 2-NO-T at 37°C for 6 days. Then the cells were trypsinized, harvested, and counted. Results are expressed as the means ± SEM for four independent experiments. *Significant difference compared with the control by Student's *t* test with Bonferroni correction (the level of significance was less than 0.05). #Significant difference between E2 and 2-NO-T + E2 by Student's *t* test. **c** Estrogen receptor α and estrogen response element (ER–ERE) binding was assessed by surface plasmon resonance (SPR). Human ERα (20 nM) was liganded with 100 nM E2, 10 μM 2-NO_2-T, or 2-NO-T by incubation at 37°C for 5 min. Then, the liganded ER was introduced by a 40 μl-injection over a sensor chip immobilized with double-stranded human *pS2* ERE. The binding of liganded ER to ERE was expressed as a percentage; that is, with the response to 100 nM E2 taken as 100% and that to no ligand (DMSO, 0.1%) as 0%. Results are expressed as the means for two independent experiments

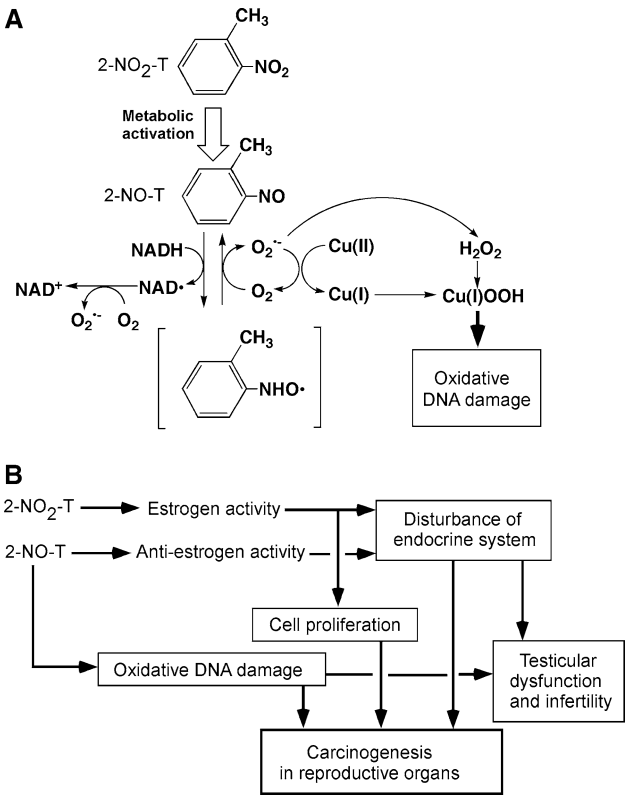

Fig. 6 Possible mechanisms of **a** DNA damage and **b** estrogen-disrupting effects caused by 2-NO_2-T and 2-NO-T

Fig. 6a. The nitroso metabolite of 2-NO_2-T, 2-NO-T, can be reduced to hydronitroxide radicals by an endogenous reductant, NADH. When the radicals autoxidize to 2-NO-T, the generation of $O_2^{\cdot-}$ would then occur from molecular oxygen. Thereafter, $O_2^{\cdot-}$ is dismutated to generate H_2O_2 together with the reduction of Cu (II) to Cu (I). The inhibitory effects of catalase and bathocuproine on the DNA damage induced by the metabolite suggest that H_2O_2 and Cu (I) are involved. NADH reduces the nitroso form to hydronitroxide radicals, and the radicals autoxidize again to the nitroso form, resulting in the generation of reactive oxygen species and DNA damage through the redox cycle. NADH itself is oxidized to NAD·, and then NAD^+ with the formation of $O_2^{\cdot-}$. Several studies indicate that NADH may react nonenzymatically with some xenobiotics and mediate their reduction [15, 16]. The cycling of redox reactions would cause DNA damage with the excessive generation of reactive oxygen species at low concentrations of 2-NO-T.

The E-screen assay in the present study revealed that 2-NO_2-T induced cell proliferation in estrogen-dependent MCF-7 cells. The data obtained with the SPR sensor showed that 2-NO_2-T-liganded ERα had little ability to bind ERE. These data indicate that 2-NO_2-T has estrogenic activity. On the other hand, 2-NO-T decreased cell proliferation, and suppressed E2-induced cell proliferation. 2-NO-T-liganded ER had lower reactivity to ERE compared with the control (DMSO 0.1%). These results shown by 2-NO-T were similar to those shown by the E2 antagonist, 4-hydroxytamoxifen. Several studies have suggested that the three-dimensional folding of the hormone-binding domain induced by ligand exhibits a difference in the ionic charge at the surface of the ligand-receptor complexes [17]. The estrogen antagonist such as 4-hydroxytamoxifen induced conformational modifications of the estrogen receptor that do not preclude binding to the ERE but fail to promote events needed for gene transcription [18]. In the present study, 2-NO-T exhibited an SPR sensorgram similar to that of 4-hydroxytamoxifen, and the results of SPR may indicate a change in the ionic charge at the surface of the estrogen receptor (ER) induced by the binding of 2-NO-T, which contributes to the alteration in binding ability to ERE. The method we used here is one of the established methods employed to determine the sequence-dependent receptors' binding capacity by monitoring the binding of ER to ERE sequences immobilized on a sensor surface [19, 20]. In the assessment by SPR, 2-NO-T decreased the binding activity dramatically (Fig. 5b). However, in the E-screen assay, only moderate inhibition was observed (Fig. 5a). SPR detects simply the reactivity of liganded ER to ERE. Because cells have additional determinants of receptor function, including cofactors, there may be some discrepancies between the two assay systems. However, SPR is a useful method with which to detect specific protein–DNA interactions, which play an initial role in transcription and further biological processes. Experiments with the E-screen assay and SPR sensor indicate that 2-NO_2-T and its metabolite may have endocrine-disrupting effects.

It is known that estrogen inhibits the production of testosterone in Leydig cells [21], disturbing spermatogenesis. The reproductive toxicity of 2-NO_2-T in males may be related to alterations in endocrine status. Alternatively, 2-NO_2-T may have direct effects on the reproductive organs. Several reproductive toxicants have DNA-damaging effects in male germ cells [22]. In addition, the male reproductive organs are highly susceptible to oxidative damage [23]. That is, oxidative DNA damage caused by 2-NO-T may contribute to male reproductive toxicity, such as a decreased sperm count. On the other hand, cancers in reproductive organs such as the testes and mammary glands are assumed to develop as follows. According to the hypothesis of estrogen-induced carcinogenesis [24, 25], metabolites of estrogen play a role in tumor initiation through DNA damage, whereas estrogen itself induces tumor promotion and/or progression by enhancing cell proliferation. Several studies indicate that materials with endocrine-disrupting effects contribute to breast or testicular cancer and a reduction in sperm counts [26–28].

Although the mechanisms are not completely understood, the binding of ERE and ER liganded with some interaction of 2-NO_2-T or 2-NO-T may be responsible for such endocrine-disrupting effects. A possible mechanism of endocrine disruption induced by 2-NO_2-T and 2-NO-T is shown in Fig. 6b. The present results and previous findings may be interpreted as indicating that oxidative DNA damage caused by 2-NO-T, and endocrine disruption caused by 2-NO_2-T and 2-NO-T, play a role in carcinogenicity and reproductive toxicity; for example, in mammary gland fibroadenoma, epididymis mesothelioma, and testicular degeneration.

Acknowledgments This work was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. IARC working group. Printing processes and printing inks, carbon black and some nitro compounds. In: IARC monographs on the evaluation of the carcinogenic risks to humans, vol. 65. Lyon: IARC publisher; 1996.
2. National Toxicology Program, National Institutes of Health, US Department of Health and Human Services. Toxicology and carcinogenesis studies of *o*-nitrotoluene sulfone (CAS no. 88-72-2) in F344/N rats and B6C3F(1) mice (feed studies), Natl Toxicol Program Tech Rep Ser. 2002;504:1–357.
3. Dunnick JK, Elwell MR, Bucher JR. Comparative toxicities of *o*-, *m*-, and *p*-nitrotoluene in 13-week feed studies in F344 rats and B6C3F1 mice. Fundam Appl Toxicol. 1994;22:411–21.
4. National Toxicology Program, National Institutes of Health, US Department of Health and Human Services. Comparative toxicity and carcinogenicity studies of *o*-nitrotoluene and *o*-toluidine hydrochloride (CAS Nos. 88-72-2 and 636-21-5) administered in feed to male F344/N rats, Toxic Rep Ser. 2000;44:1–C8.
5. Hong HL, Ton TV, Devereux TR, Moomaw C, Clayton N, Chan P, Dunnick JK, Sills RC. Chemical-specific alterations in ras, p53, and beta-catenin genes in hemangiosarcomas from B6C3F1 mice exposed to o-nitrotoluene or riddelliine for 2 years. Toxicol Appl Pharmacol. 2003;191:227–34.
6. Sabbioni G, Jones CR, Sepai O, Hirvonen A, Norppa H, Jarventaus H, Glatt H, Pomplun D, Yan H, Brooks LR, Warren SH, Demarini DM, Liu YY. Biomarkers of exposure, effect, and susceptibility in workers exposed to nitrotoluenes. Cancer Epidemiol Biomarkers Prev. 2006;15:559–66.
7. Murata M, Moriya K, Inoue S, Kawanishi S. Oxidative damage to cellular and isolated DNA by metabolites of a fungicide orthophenylphenol. Carcinogenesis. 1999;20:851–7.
8. Murata M, Kawanishi S. Oxidative DNA damage by vitamin A and its derivative via superoxide generation. J Biol Chem. 2000;275:2003–8.
9. Iwamoto T, Hiraku Y, Oikawa S, Mizutani H, Kojima M, Kawanishi S. Oxidative DNA damage induced by photodegradation products of 3(′)-azido-3(′)-deoxythymidine. Arch Biochem Biophys. 2003;416:155–63.
10. Maxam AM, Gilbert W. A new method for sequencing DNA. Proc Natl Acad Sci USA. 1977;74:560–4.
11. Murata M, Midorikawa K, Koh M, Umezawa K, Kawanishi S. Genistein and daidzein induce cell proliferation and their metabolites cause oxidative DNA damage in relation to isoflavone-induced cancer of estrogen-sensitive organs. Biochemistry. 2004;43:2569–77.
12. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect. 1995;103(Suppl 7):113–22.
13. Rich RL, Hoth LR, Geoghegan KF, Brown TA, LeMotte PK, Simons SP, Hensley P, Myszka DG. Kinetic analysis of estrogen receptor/ligand interactions. Proc Natl Acad Sci USA. 2002;99:8562–7.
14. Lu D, Kiriyama Y, Lee KY, Giguere V. Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. Cancer Res. 2001;61:6755–61.
15. Fann YC, Metosh-Dickey CA, Winston GW, Sygula A, Rao DN, Kadiiska MB, Mason RP. Enzymatic and nonenzymatic production of free radicals from the carcinogens 4-nitroquinoline N-oxide and 4-hydroxylaminoquinoline N-oxide. Chem Res Toxicol. 1999;12:450–8.
16. Murata M, Kobayashi M, Kawanishi S. Nonenzymatic reduction of nitro derivative of a heterocyclic amine IQ by NADH and Cu(II) leads to oxidative DNA damage. Biochemistry. 1999;38:7624–9.
17. Sabbah M, Gouilleux F, Sola B, Redeuilh G, Baulieu EE. Structural differences between the hormone and antihormone estrogen receptor complexes bound to the hormone response element. Proc Natl Acad Sci USA. 1991;88:390–4.
18. McGee TD, Edwards J, Roitberg AE. Preliminary molecular dynamic simulations of the estrogen receptor alpha ligand binding domain from antagonist to apo. Int J Environ Res Public Health. 2008;5:111–4.
19. Cheskis BJ, Karathanasis S, Lyttle CR. Estrogen receptor ligands modulate its interaction with DNA. J Biol Chem. 1997;272:11384–91.
20. Teh HF, Peh WY, Su X, Thomsen JS. Characterization of protein–DNA interactions using surface plasmon resonance spectroscopy with various assay schemes. Biochemistry. 2007;46:2127–35.
21. Murono EP, Derk RC. The reported active metabolite of methoxychlor, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane, inhibits testosterone formation by cultured Leydig cells from neonatal rats. Reprod Toxicol. 2005;20:503–13.
22. Shin JH, Mori C, Shiota K. Involvement of germ cell apoptosis in the induction of testicular toxicity following hydroxyurea treatment. Toxicol Appl Pharmacol. 1999;155:139–49.
23. Wu X, Faqi AS, Yang J, Pang BP, Ding X, Jiang X, Chahoud I. 2-Bromopropane induces DNA damage, impairs functional antioxidant cellular defenses, and enhances the lipid peroxidation process in primary cultures of rat Leydig cells. Reprod Toxicol. 2002;16:379–84.
24. Hiraku Y, Yamashita N, Nishiguchi M, Kawanishi S. Catechol estrogens induce oxidative DNA damage and estradiol enhances cell proliferation. Int J Cancer. 2001;92:333–7.
25. Lavigne JA, Goodman JE, Fonong T, Odwin S, He P, Roberts DW, Yager JD. The effects of catechol-O-methyltransferase inhibition on estrogen metabolite and oxidative DNA damage levels in estradiol-treated MCF-7 cells. Cancer Res. 2001;61:7488–94.
26. Hardell L, Bavel B, Lindstrom G, Eriksson M, Carlberg M. In utero exposure to persistent organic pollutants in relation to testicular cancer risk. Int J Androl. 2006;29:228–34.
27. Pflieger-Bruss S, Schuppe HC, Schill WB. The male reproductive system and its susceptibility to endocrine disrupting chemicals. Andrologia. 2004;36:337–45.
28. Safe S. Clinical correlates of environmental endocrine disruptors. Trends Endocrinol Metab. 2005;16:139–44.