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Oxidative DNA damage induced by nitrotyrosine, a biomarker of inflammation☆

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Abstract

Inflammation has been postulated as a risk factor for several cancers. 3-Nitrotyrosine is a biochemical marker for inflammation. We investigated the ability of nitrotyrosine and nitrotyrosine-containing peptides (nitroY-peptide) to induce DNA damage by the experiments using ^{32}P-labeled DNA fragments obtained from the human *p53* tumor suppressor gene and an HPLC-electrochemical detector. Nitrotyrosine and nitroY-peptide caused Cu(II)-dependent DNA damage in the presence of P450 reductase, which is considered to yield nitroreduction. Catalase inhibited DNA damage, suggesting the involvement of H_2O_2. Nitrotyrosine and nitroY-peptide increased 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation, an indicator of oxidative DNA damage. Nitrotyrosine-containing peptides of histone induced 8-oxodG formation more efficiently than free nitrotyrosine. We propose the possibility that nitrotyrosine-induced H_2O_2 formation and DNA damage contribute to inflammation-associated carcinogenesis.

Keywords: Nitrotyrosine; Oxidative DNA damage; P450 reductase; Copper; Hydrogen peroxide; Inflammation; Carcinogenesis

Inflammation has been postulated as a risk factor for several cancers [1–5]. Inflammation and infection activate a variety of inflammatory cells, which produce nitric oxide (NO) and superoxide ($O_2^{\cdot-}$), yielding peroxynitrite ($ONOO^-$), and other types of reactive nitrogen species (RNS) [6,7]. The reaction of RNS with protein-bound tyrosine residues causes nitrotyrosine formation in inflammatory and infected tissues. Many studies revealed the presence of 3-nitrotyrosine in human tissues and fluids due to inflammation and infectious diseases [8–11]. Nitrotyrosine serves as a biochemical marker for inflammation. Increases of protein tyrosine nitration were observed in cancer sites [12,13]. Interestingly, a recent study has suggested that histones are the most prominent nitrated proteins in the Mutatec tumor tissue exposed to NO [14]. Irie et al. [15] have demonstrated that histone is a substrate for "denitrase" that removes the nitro group of nitrotyrosine in proteins. The existence of a repair mechanism for nitrated tyrosine in histone has led us to an idea that nitrotyrosine may have deleterious effects on biological system. There arises a possibility that nitrotyrosine can be involved in DNA damage, which may participate in inflammation-associated carcinogenesis.

We investigated the ability of nitrotyrosine and nitrotyrosine-containing peptides of histone to induce DNA damage using ^{32}P-labeled DNA fragments obtained from the human *p53* and *p16* tumor suppressor genes. We also analyzed 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation in calf thymus DNA with an electrochemical detector coupled to an HPLC (HPLC-ECD), as an indicator of oxidative DNA damage.

Materials and methods

Materials. Restriction enzymes (*Eco*RI, *Mro*I, and *Apa*I) and calf intestine phosphatase were purchased from Boehringer–Mannheim (Germany). Restriction enzymes (*Hin*dIII and *Ava*I) and T_4 polynucleotide

☆ *Abbreviations:* nitroY-peptide, nitrotyrosine-containing peptide; RNS, reactive nitrogen species; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine (and also known as 8-hydroxy-2′-deoxyguanosine); DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; HPLC-ECD, high performance liquid chromatography coupled with an electrochemical detector; NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); P450 reductase, NADPH-cytochrome P450 reductase; SOD, superoxide dismutase.

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kinase were purchased from New England Biolabs. [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear. Superoxide dismutase (SOD, 3000 U/mg from bovine erythrocytes) and catalase (45,000 U/mg from bovine liver) were from Sigma Chemical. Nitrotyrosine-containing peptides (nitroY-peptide) were supplied by Sawady Technology (Tokyo, Japan; nitroY1-peptide) and Nihon Sigma Genosys Biotechnologies (Hokkaido, Japan; nitroY3-peptide). The amino acid sequences of nitrated tyrosine residue on histone were from reference [14] that identified them in tumor tissue by mass spectrometry as follows; nitroY1-peptide; nitroY-R-P-G-T-V-A-L-R and nitroY3-peptide; E-S-nitroY-S-V-nitroY-V-nitroY-K. NADPH, $NADP^+$, acrylamide, bisacrylamide, and piperidine were obtained from Wako Pure Chemical Industries (Osaka, Japan). NADPH-cytochrome P450 reductase (P450 reductase) from rat microsome was a kind gift from Dr. Y. Kumagai (Tsukuba University). Ethanol and $CuCl_2$ were from Nakalai Tesque (Kyoto, Japan). Nuclease P_1 was from Yamasa Shoyu (Chiba, Japan). Bathocuproinedisulfonic acid was from Dojin Chemicals (Kumamoto, Japan).

Preparation of ^{32}P-5′-end-labeled DNA fragments. DNA fragments obtained from the human *p53* tumor suppressor gene [16] containing exons were prepared, as described previously [17]. The 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-labeled). 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). DNA fragment was also obtained from the human *p16* tumor suppressor gene [18]. The 5′ end-labeled 490-bp fragment (*Eco*RI*5841–*Eco*RI*6330) containing exon 1 of the human *p16* tumor suppressor gene was obtained by pGEM-T Easy Vector (Promega). The 490-bp fragment was further digested with *Mro*I to obtain a singly labeled 328-bp fragment (*Eco*RI*5841–*Mro*I 6168) and a 158-bp fragment (*Mro*I 6173–*Eco*RI*6330) as described previously [19].

Detection of DNA damage by nitrotyrosine. The standard reaction mixtures (in a microtube; 1.5 mL; Eppendorf) containing nitrotyrosine, 100 μM NADPH, and P450 reductase in 20 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 25 °C for 30 min. After pre-incubation, ^{32}P-5′-end-labeled DNA fragments, calf thymus DNA (20 μM/base), and 20 μM $CuCl_2$ were added to the mixtures (total 200 μL), followed by the incubation at 37 °C for 1 h. Then, the DNA fragments were treated in 10% (v/v) piperidine at 90 °C for 20 min, or treated with 6 U Fpg protein in 21 μL of reaction buffer (10 mM Hepes–KOH (pH 7.4), 100 mM KCl, 10 mM EDTA, and 0.1 mg/ml BSA) at 37 °C for 2 h. The treated DNA was electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing X-ray film to the gel [19].

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure [20] using a DNA-sequencing system (LKB 2010 Macrophor). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltroScan XL).

Analysis of 8-oxodG formation in calf thymus DNA by nitrotyrosine. The standard reaction mixture (in a microtube; 1.5 mL; Eppendorf) containing nitrotyrosine, 100 μM NADPH, and P450 reductase in 20 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 25 °C for 30 min. After pre-incubation, calf thymus DNA (100 μM/base) and 20 μM $CuCl_2$ were added, and then incubated at 37 °C for 1 h. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase, and analyzed by an HPLC-ECD, as described previously [21].

Measurement of $NADP^+$ amount. The standard reaction mixtures (in a microtube; 1.5 mL; Eppendorf) containing nitrotyrosine, 100 μM NADPH and P450 reductase in 20 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 25 °C for 30 min, followed by incubation at 37 °C for 1 h. $NADP^+$ amount was analyzed by HPLC with a Shimadzu photodiode array UV detector (SPD-M10A, Kyoto, Japan) at 260 nm with Wako Pure Chemical ODS (46 mm × 150 mm) in mobile phase containing 2% methanol and 100 mM potassium phosphate buffer (pH 6) at flow rate 1 mL/min.

Results

Damage to ^{32}P-labeled DNA fragments by nitrotyrosine in the presence of P450 reductase, NADPH, and Cu(II)

Free nitrotyrosine and nitrotyrosine-peptides of histone caused Cu(II)-mediated DNA damage when

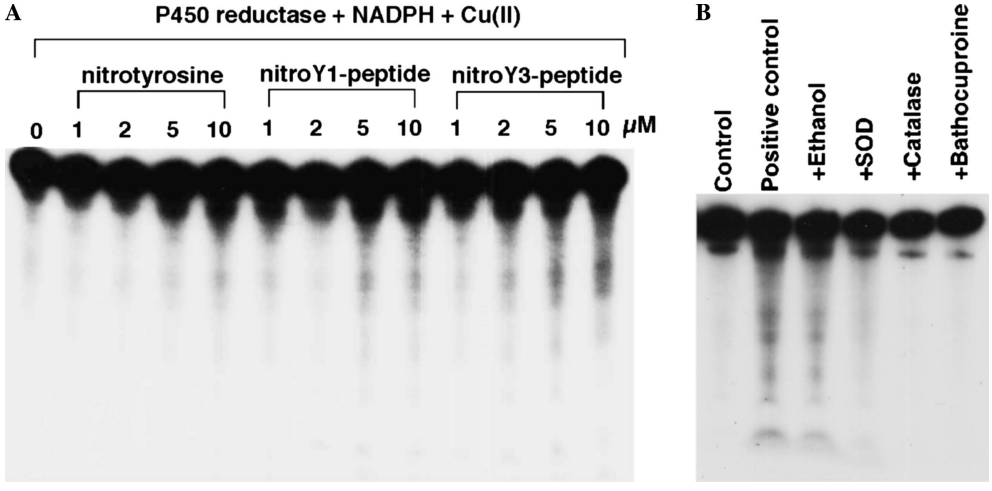

Fig. 1. Autoradiograms of ^{32}P-labeled DNA fragments incubated with free nitrotyrosine and nitroY-peptides in the presence of P450 reductase, NADPH, and Cu(II). (A) The reaction mixtures containing indicated concentrations of nitrotyrosine or nitroY-peptide, 100 μM NADPH, and 2.1 μg/mL P450 reductase in 20 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 25 °C for 30 min. After pre-incubation, ^{32}P-5′-end-labeled 158-bp DNA fragments, calf thymus DNA (20 μM/base), and 20 μM $CuCl_2$ were added to the mixtures (total 200 μL), followed by the incubation at 37 °C for 1 h. (B) Scavengers were added after pre-incubation of 10 μM nitroY3-peptide as follows: 5% (v/v) ethanol; 30 U SOD; 30 U catalase; 50 μM bathocuproine. After the incubation, the DNA fragments were treated with hot piperidine and electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing X-ray film to the gel.

they were treated with P450 reductase (Fig. 1A). Without P450 reductase, free nitrotyrosine and nitroY-peptides caused no DNA damage even in the presence of Cu(II) (data not shown). In the absence of Cu(II), DNA damage was not observed. Free nitrotyrosine induced slight DNA damage. NitroY-peptides damaged DNA more efficiently than free nitrotyrosine. The peptide containing three nitrotyrosine residues (nitroY3-peptide) induced DNA damage stronger than that containing one nitrotyrosine (nitroY1-peptide). The amount of oligonucleotides was increased by piperidine treatment, suggesting the involvement of base modification/liberation (data not shown).

Effects of scavengers and bathocuproine on DNA damage induced by nitrotyrosine

Fig. 1B shows the effects of scavengers and bathocuproine, a Cu(I)-specific chelator, on DNA damage induced by P450 reductase-treated nitroY3-peptide in the presence of Cu(II). Catalase and bathocuproine inhibited DNA damage, suggesting the involvement of hydrogen peroxide (H_2O_2) and Cu(I). Ethanol, a typical free hydroxyl radical (·OH) scavenger, did not attenuate DNA damage. SOD partly inhibited DNA damage. Similar results were obtained with nitroY1-peptide (data not shown).

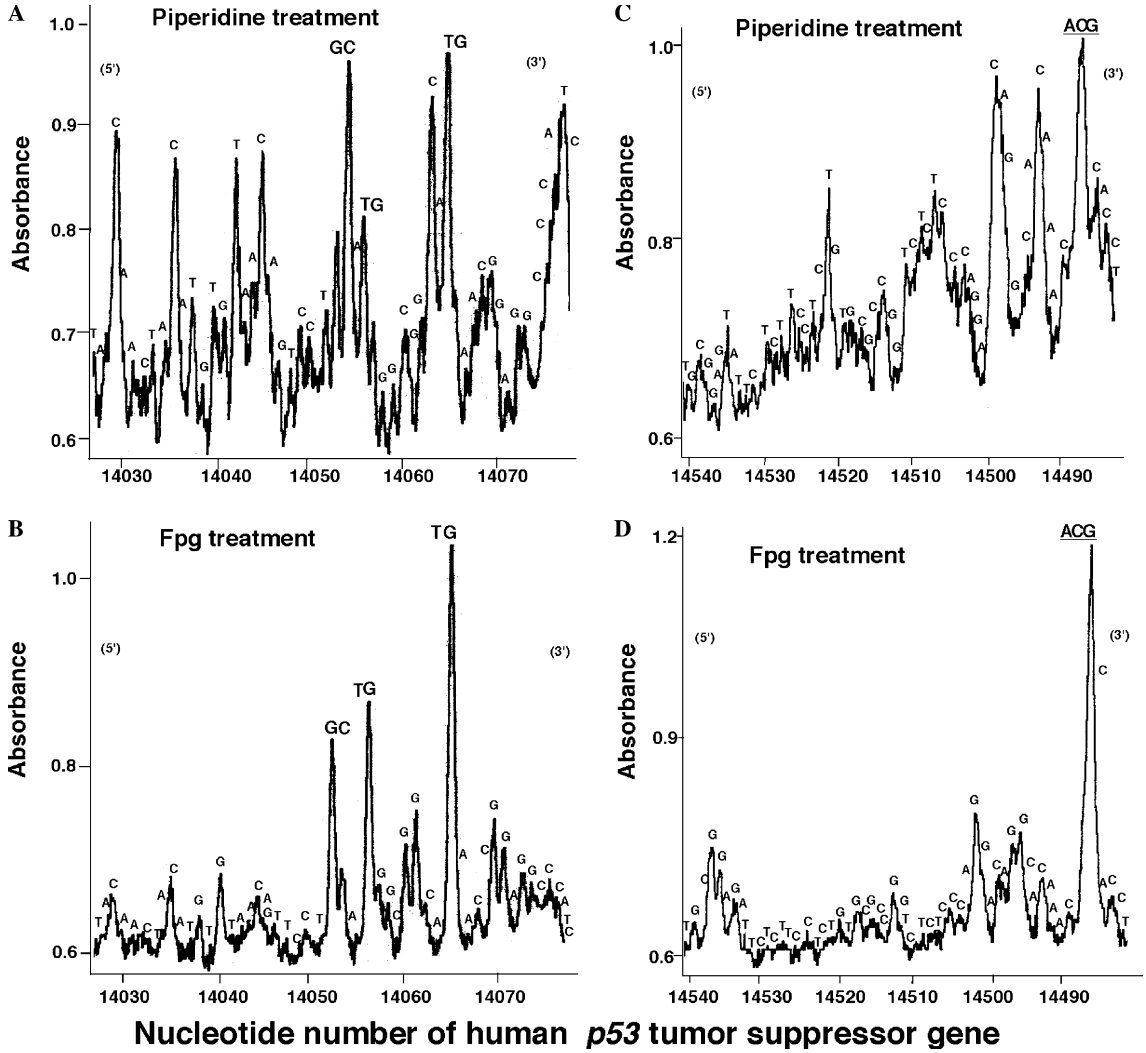

Fig. 2. Site specificity of Cu(II)-mediated DNA cleavage induced by nitroY3-peptide in the presence of P450 reductase. The reaction mixtures containing 10 μM nitroY3-peptide, 100 μM NADPH, and 2.1 μg/mL P450 reductase in 20 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 25 °C for 30 min. After pre-incubation, ^{32}P-5′-end-labeled 211-bp (A,B) or 443-bp (C,D) DNA fragments, calf thymus DNA (20 μM/base), and 20 μM $CuCl_2$ were added to the mixtures. Reaction mixtures were incubated at 37 °C for 1 h, followed by hot piperidine (A,C) and Fpg treatment (B,D). DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel using a DNA-sequencing system and the autoradiogram was obtained by exposing X-ray film to the gel. The relative amounts of oligonucleotide were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene and underscoring shows the complementary sequence to codon 273 (nucleotide numbers 14486–14488).

Site specificity of DNA cleavage by nitrotyrosine

An autoradiogram was obtained and scanned with a laser densitometer to measure relative intensity of DNA cleavage in the human *p53* tumor suppressor gene (Fig. 2). P450 reductase-treated nitroY-peptide induced piperidine-labile sites relatively at thymine and cytosine residues in the presence of Cu(II) (Figs. 2A and C). With Fpg treatment, DNA cleavage occurred mainly at guanine residues (Figs. 2B and D). Collectively, damage at neighboring guanine and pyrimidine residues such as 5′-TG-3′ and 5′-GC-3′ sites was observed (Figs. 2A and B), suggesting that double-base lesion occurred. NitroY-peptides caused piperidine-labile and Fpg-sensitive lesions at CG in the 5′-ACG-3′ sequence, a well-known hotspot of the *p53* gene [22] (Figs. 2C and D).

Formation of 8-oxodG in calf thymus DNA by nitrotyrosine

Using an HPLC-ECD, we measured 8-oxodG content in calf thymus DNA treated with nitrotyrosine after P450 reductase treatment (Fig. 3). NitroY3-peptide increased the amount of 8-oxodG up to 2 μM and then decreased gradually. Significant increases ($p < 0.01$) were observed in all conditions treated with 1 μM and higher concentrations of nitroY3-peptide. NitroY1-peptide induced the increase of 8-oxodG formation with increasing its concentration (1 μM; $p < 0.05$, 2 μM and higher; $p < 0.01$). Free nitrotyrosine induced the increase of 8-oxodG formation significantly at 10 μM ($p < 0.05$). Nitrotyrosine residue-containing histone-peptides induced 8-oxodG formation much more efficiently than free nitrotyrosine.

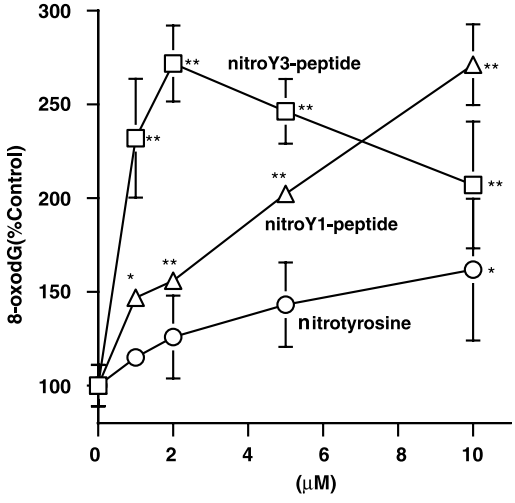

Fig. 3. Cu(II)-mediated formation of 8-oxodG in calf thymus DNA by nitrotyrosine and nitroY-peptides in the presence of P450 reductase. The reaction mixtures containing nitrotyrosine or nitroY-peptides, 100 μM NADPH, and 2.1 μg/mL P450 reductase in 20 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 25 °C for 30 min. After pre-incubation, DNA fragments (100 μM/base) from calf thymus and 20 μM $CuCl_2$ were added and then incubated at 37 °C for 1 h. After ethanol precipitation, DNA was enzymatically digested to the nucleosides and analyzed by an HPLC-ECD. Results are expressed as means (control; 100%) and SEM of values obtained from three independent experiments. Asterisks indicate significant difference compared with control by Student's *t* test (*$p < 0.05$, **$p < 0.01$).

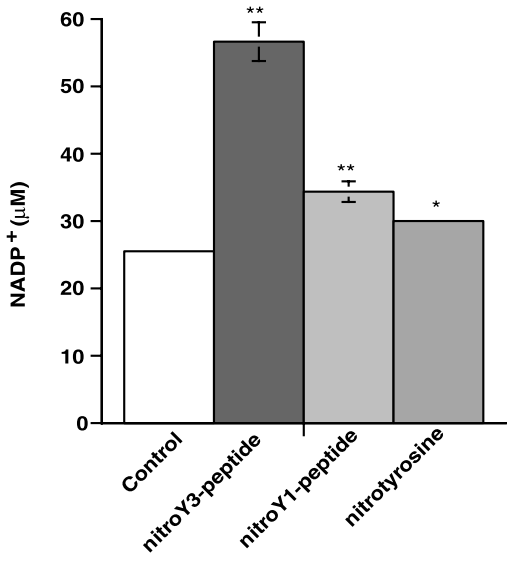

Fig. 4. Production of $NADP^+$ through reaction of nitrotyrosines and P450 reductase. The reaction mixtures containing 10 μM nitrotyrosine or nitroY-peptides, 100 μM NADPH, and 2.1 μg/mL P450 reductase in 20 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 25 °C for 30 min, followed by incubation at 37 °C for 1 h. $NADP^+$ amount was analyzed by HPLC with photodiode array UV detector (260 nm). Results are expressed as means and SEM of values obtained from three independent experiments. Asterisks indicate significant difference compared with control (*$p < 0.05$, **$p < 0.01$) by Student's *t* test.

NADPH oxidation by nitrotyrosine in the presence of P450 reductase

NADPH oxidation to $NADP^+$ was analyzed by HPLC with photodiode array (Fig. 4). Free nitrotyrosine and nitroY-peptides significantly induced $NADP^+$ formation compared with control ($p < 0.05$ and $p < 0.01$, respectively). The amounts of $NADP^+$ by nitroY3-peptide and nitroY1-peptide were significantly higher than that of free nitrotyrosine ($p < 0.01$).

Discussion

The present study has demonstrated that nitrotyrosine and nitroY-peptides of histone have an ability to cause Cu(II)-mediated DNA damage via the activation with P450 reductase. Inhibitory effects of catalase and bathocuproine suggested that H_2O_2 and Cu(I) were required for DNA damage. A possible mechanism of

oxidative DNA damage induced by enzymatically activated nitrotyrosine can be speculated as accounting for most of the observations and references as follows. P450 reductase converts nitrotyrosine to corresponding nitro radical anion in the presence of NADPH via one-electron reduction [23,24]. The nitro radical anion reacts with O_2, followed by production of $O_2^{\cdot-}$ [25] and the consequent oxidation to the parent nitrotyrosine. Alternatively, there is a possibility that further nitroreduction by P450 reductase contributes to the generation of nitroso and/or *N*-hydroxy forms [26]. Any of reduced derivatives such as nitro radical anion, nitroso, and *N*-hydroxy forms may be oxidized again to yield redox cycle with generation of $O_2^{\cdot-}$. Subsequently, the generation of H_2O_2 by $O_2^{\cdot-}$ dismutation and reduction of Cu(II) to Cu(I) concomitantly occur. H_2O_2 reacts with Cu(I) to form a metal–oxygen complex such as Cu(I)-hydroperoxide causing DNA damage. This idea is supported by the observations that a typical free ·OH scavenger, ethanol, did not offer DNA protection. The complex DNA–Cu(I)–hydroperoxide may be considered to be a bound hydroxyl radical, which can release ·OH causing DNA damage. The ·OH released from the bound hydroxyl radical may immediately attack an adjacent constituent of DNA before it is scavenged by free ·OH scavengers [27]. NitroY-peptides damaged DNA more efficiently than free nitrotyrosine did. Relevantly, Krainev et al. [24] showed that nitrotyrosine residue within leucine enkephalin pentapeptide (Tyr–Gly–Gly–Phe–Leu) had a higher affinity for enzymatic reduction with higher production of $O_2^{\cdot-}$ than does free nitrotyrosine. We assessed the efficacy of redox cycling reaction by measuring $NADP^+$ as NADPH oxidation. The result also supported the idea that nitroY-peptides are more easily reduced by NADPH-P450 reductase than free nitrotyrosine. This can reasonably account for different DNA damaging potentials of free nitrotyrosine and nitrotyrosine-containing peptides.

It is considered that tyrosine plays a role in interactions of DNA-binding proteins and histones with DNA [28], probably through the close proximity of thymine and tyrosine in chromatin. Relevantly, Altman et al. [29] provided evidence for the formation of DNA–protein crosslinks between thymine and tyrosine in chromatin when cultured mannalian cells were treated with metal ions. When the tyrosine residues in histone are nitrated, reactive species derived from nitrotyrosine residue will immediately attack DNA. $ONOO^-$ is a potent DNA oxidizing agent [30], but it is very short-lived. On the other hand, nitrotyrosine has a long half-life in vivo. Nitrated proteins were detected for at least 24 h in rat skin singly exposed to $ONOO^-$ [31]. We have shown that enzymatically activated nitrotyrosine in histone peptide induced oxidative DNA damage in the presence of Cu(II). Abundant RNS are generated in inflammatory and infected tissues, and histone proteins are nitrated especially at tyrosine residues [14]. Most of nitrotyrosine in histone will be repaired by "denitrase" [15]. If nitrotyrosine residues still remain in histone, they may cause DNA damage in the presence of P450 reductase. This assumption is supported by the observation that P450 reductase exists in the nuclear fraction [32] although the amount of P450 reductase in nuclear fraction is smaller than that in cytosolic fraction. On the other hand, nitrotyrosine may be more easily catalyzed to reactive species by P450 reductase in cytosolic fraction. Therefore, both nitrotyrosines in cytosol and in nuclear histone may participate in DNA damage. In addition to the fact that nitrotyrosine is a marker for inflammation, we propose the possibility that nitrotyrosine-induced H_2O_2 formation and subsequent DNA damage contribute to inflammation-associated carcinogenesis.

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