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Quantification of Oxidative DNA Lesions in Tissues of Long-Evans Cinnamon Rats by Capillary High-performance Liquid Chromatography-Tandem Mass Spectrometry Coupled with Stable Isotope-dilution Method

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Abstract

The purpose of our study was to develop suitable methods to quantify oxidative DNA lesions in the setting of transition metal-related diseases. Transition metal-driven Fenton reactions constitute an important endogenous source of reactive oxygen species (ROS). In genetic diseases with accumulation of transition metal ions, excessive ROS production causes pathophysiological changes, including DNA damage. Wilson's disease is an autosomal recessive disorder with copper toxicosis due to deficiency of ATP7B protein needed for excreting copper into bile. The Long-Evans Cinnamon (LEC) rat bears a deletion in Atp7b gene and serves as an excellent model for hepatic Wilson's disease. We used a sensitive capillary LC-ESI-MS/MS/MS method in conjunction with stable-isotope dilution technique to quantify several types of oxidative DNA lesions in liver and brain of LEC rats. These lesions included 5-formyl-2'-deoxyuridine, 5-hydroxymethyl-2'-deoxyuridine, and the 5'R and 5'S diastereomers of 8,5'-cyclo-2'-deoxyguanosine and 8,5'-cyclo-2'-deoxyadenosine. Moreover, the levels of these DNA lesions in the liver and brain increased with age and correlated with age-dependent regulation of the expression of DNA repair genes in LEC rats. These results provide significant new knowledge for better understanding the implications of oxidative DNA lesions in transition metal-induced diseases, such as Wilson's disease, as well as in ageing and ageing-related pathological conditions.

Introduction

Reactive oxygen species (ROS) can be induced by both endogenous and exogenous sources and maintaining cellular ROS homeostasis is essential for normal cellular function; excess generation of ROS can cause damage to lipids, proteins and DNA ¹. Transition metal ion-mediated Fenton reactions constitute an important endogenous source of ROS ². Understanding the role of these processes in DNA damage will be particularly important for defining mechanisms of genotoxicity as well as for developing potential therapeutic

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Supporting Information Available:

Instrument parameters for LC-MS/MS/MS analysis, LC-MS/MS/MS data, calibration curves, and histological grading for liver damage of rats used in this study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

interventions. This will be benefited by the availability of sensitive and effective diagnostic methods to characterize oxidative DNA lesions.

Among single-gene disorders of transition metal handling, Wilson's disease (WD) occupies a unique place due to defective excretion of copper into bile³, along with hepatic, neurological and renal abnormalities following copper toxicosis^{4, 5}. The WD gene encodes a copper-dependent P-type ATPase (ATP7B) and is highly expressed in the liver, kidney and placenta^{4, 5}. Long-Evans Cinnamon (LEC) rat, which was found in an inbred colony of healthy Long-Evans Agouti (LEA) rats⁶⁻⁸, bears a deletion in *Atp7b* gene⁹ and shares many attributes of WD^{8, 10}, including hepatic damage arising from copper accumulation, which worsens with age^{8,11, 12}. Thus, the LEC rat is an appropriate animal model for examining the pathophysiology of liver injury in Wilson's disease^{6, 8}, and the LEA rat constitutes an excellent healthy control.

The accumulation of copper in tissues of LEC rats may lead to the aberrant generation of ROS and elevated formation of oxidative DNA lesions in tissues of LEC rats. In this context, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) was found to be present at elevated levels in DNA of the liver, kidney and brain of LEC rats¹³. Nair et al.¹⁴ found by immunoaffinity/³²P-postlabeling assays that hepatic levels of two DNA lesions arising from byproducts of lipid peroxidation, 1,*N*⁶-etheno-2'-deoxyadenosine (ϵ dA) and 3,*N*⁴-etheno-2'-deoxycytidine (ϵ dC), increased with age in LEC rats, peaking at 8-12 weeks. This suggested that progressive accumulation of oxidatively damaged DNA may contribute to losses of hepatocytes in WD. Moreover, other oxidatively induced DNA lesions may be contributing to liver damage in WD. Along this line, a Fenton-like reagent, Cu(II)/H₂O₂/ascorbate, was found to induce a spectrum of oxidatively generated DNA lesions, including single-nucleobase lesions (8-oxodG, 5-FodU and 5-HmdU) and intrastrand cross-link lesions^{15, 16}. In alternative settings, quantification of 8-oxodG, 5-HmdU, 5-FodU, as well as cdG and cdA in cells after exposure to X-^{17, 18} or γ -rays¹⁹⁻²¹ or chemopreventive agents has been reported²². Similarly, cdG and cdA could be detected in mouse tissues²³⁻²⁵.

The oxidatively induced DNA lesions, if left unrepaired, may accumulate and confer deleterious biological consequences. Along this line, 5-HmdU could induce T $\rightarrow$ C transition in cellular DNA²⁶⁻²⁸, and 5-FodU could give rise to T $\rightarrow$ G and T $\rightarrow$ A transversions in mammalian cells²⁹. Host cell reactivation assay and *in-vitro* cleavage assay using mammalian cell nuclear extract revealed that cdA was a substrate for nucleotide excision repair (NER)^{30, 31}. Additionally, cdA is a strong blockade to transcription in mammalian cells³⁰ and it could induce transcription mutagenesis³². Furthermore, both diastereomers of cdA inhibit primer extension by T7 DNA polymerase as well as human DNA polymerases η and δ ^{31, 33}.

In the present study, we developed a sensitive capillary LC-ESI-MS³ method coupled with the stable isotope dilution technique and quantified the levels of 5-FodU, 5-HmdU, and the *R* and *S* diastereomers of cdG and cdA in DNA isolated from the liver and brain tissues of LEC and LEA rats. This provided information regarding the effect of *Atp7b* deficiency and ageing on the accumulation of endogenously induced DNA lesions in mammalian tissues.

Experimental Section

Materials

Nuclease P1 and phosphodiesterases 1 and 2 were purchased from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase and proteinase K were obtained from New England Biolabs (Ipswich, WA), and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) hydrochloride was from Tocris Bioscience (Ellisville, MO).

Synthesis and Characterization of Compounds

The structures of the isotope-labeled DNA lesions used in the present study are shown in Scheme 1. The stable isotope-labeled 5-FodU and 5-HmdU were synthesized previously¹⁶.

[1,3,7,9-¹⁵N₄-(2-amino-¹⁵N)]-8,5'-cyclo-2'-deoxyguanosine

The title compound was synthesized at a sub-milligram scale following published procedures for the preparation of un-labeled 8-bromo-2'-deoxyguanosine (8-BrdG) and cdG with some modifications^{34, 35}. [1,3,7,9-¹⁵N₄-(2-amino-¹⁵N)]-2'-deoxyguanosine (1 mg) was suspended in a mixture of acetonitrile (2.1 mL) and water (0.9 mL), to which was added *N*-bromosuccinimide (1.3 mg) solution in three portions. The mixture was stirred at room temperature for 1.5 h. After the solvent was removed, ethyl ether (1 mL) was added to the mixture. The solution was subsequently stirred at room temperature for 2 h and incubated at -20°C overnight. The mixture was then centrifuged and the supernatant discarded. The precipitate was washed twice with cold acetone and dried to give the uniformly ¹⁵N-labeled 8-bromo-2'-deoxyguanosine.

A 1-mM solution of the above-prepared 8-bromo-2'-deoxyguanosine, dissolved in acetonitrile under argon atmosphere, was irradiated with 254-nm UV light from a TLC lamp (UVGL-58, UVP Inc., Upland, CA) at room temperature for 3 h. The resulting 5'*R* and 5'*S* diastereomers of [1,3,7,9-¹⁵N₄-(2-amino-¹⁵N)]-8,5'-cyclo-2'-deoxyguanosine were purified from the reaction mixture by HPLC (Figure S1). Exact mass measurements gave *m/z* 271.0746 and *m/z* 271.0752 for the [M + H]⁺ ions of the uniformly ¹⁵N-labeled 5'*R*- and 5'*S*-cdG, respectively, which are in keeping with the calculated *m/z* of 271.0741. The high-resolution mass spectra (HRMS) were acquired on an Agilent 6510 Q-TOF LC/MS instrument equipped with an Agilent HPLC-Chip Cube MS interface as described previously³⁶. The isotopic purity was found to be better than 99.9% based on LC-MS/MS analysis.

[1,3,7,9-¹⁵N-(2-amino-¹⁵N)]-8,5'-cyclo-2'-deoxyadenosine

The isotope-labeled cdA was synthesized from the uniformly ¹⁵N-labeled 2'-deoxyadenosine using the above-described procedures for the preparation of the two diastereomers of uniformly ¹⁵N-labeled cdG (Figure S2). Exact mass measurements yielded *m/z* 255.0801 and *m/z* 255.0796 for the [M + H]⁺ ions of the uniformly ¹⁵N-labeled 5'*R*- and 5'*S*-cdA, respectively, which are again consistent with the calculated *m/z* of 255.0792. The isotopic purity was found to be better than 99.1% based on LC-MS/MS analysis.

Animals

Breeding of rats was approved by the Animal Care and Use Committee at Albert Einstein College of Medicine. Rats were housed in the Institute for Animal Studies at Einstein under 14 h light/10 h dark cycles with unrestricted access to tap water and normal rodent chow containing 11.8 mg copper/kg (Ralston Purina, St. Louis, MO). LEA rats were periodically backcrossed with LEC-/- rats to maintain their inbred status. LEA and LEC-/- rats were crossed to obtain heterozygous LEC+/- rats. The genotype of animals was verified by polymerase chain reaction (PCR) of genomic DNA from tail as described previously³⁷. Animals were housed without any manipulations for various time periods and sacrificed for collecting tissues in liquid nitrogen, which were stored under -80°C. Grading of tissue changes was performed by histological examination of hematoxylin and eosin-stained tissue sections; the following changes in hepatocytes were graded: macro- and microvesicular steatosis, polyploidy, apoptosis, and mitosis³⁸. The grades were summed with a maximal possible score of 13 (see results and Supporting Information).

Extraction of Nuclear DNA

Genomic DNA was isolated using a high-salt method³⁹. Briefly, rat tissue was ground under liquid nitrogen into fine powders in a mortar. The powders were subsequently transferred to a 50-mL centrifuge tube, to which was added a 5-mL lysis buffer containing 20 mM Tris (pH 8.1), 20 mM EDTA, 400 mM NaCl and 1% SDS (w/v) as well as 25 μ L of proteinase K (20 mg/mL). The tube was incubated in a water bath at 55°C overnight. Next day, 0.5 volume of saturated NaCl solution was added to the digestion mixture, which was vortexed for 1 min, and then incubated at 55°C for 15 min. The mixture was centrifuged at ~10,000 rpm for 30 min and the supernatant was collected and centrifuged again under the same conditions. The nucleic acids in the supernatant were precipitated from ethanol. To the nucleic acid mixture was added 2.5 μ L solution of RNase A (10 mg/mL) and 1 μ L solution of RNase T1 (25 units/ μ L), and the solution was incubated at 37°C for 1 h followed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) twice. The DNA was then precipitated from the aqueous layer by ethanol. The mixture was subsequently centrifuged at 7,000 rpm for 15 min. The resulting DNA pellet was washed twice with 70% cold ethanol and allowed to air-dry at room temperature for overnight. The pellet was then dissolved in TE buffer and the amount of nuclear DNA was quantified by using UV spectrophotometry.

Enzymatic Digestion of Nuclear DNA

Eight units of nuclease P1, 0.01 unit of phosphodiesterase 2, 20 nmol of EHNA and a 20- μ L solution containing 300 mM sodium acetate (pH 5.6) and 10 mM zinc chloride were added to 80 μ g of DNA. In this context, some enzymes used for DNA digestion were contaminated with adenine deaminase, which can induce the deamination of dA to 2'-deoxyinosine (dI). Because dI shared a very similar retention time as *S*-cdG during HPLC enrichment (Figure S3), the presence of considerable amount of dI in the digestion mixture diminished substantially the sensitivity for *S*-cdG measurement. Therefore, we added EHNA prior to the enzymatic digestion⁴⁰, which reduced the deamination of dA to less than 2%, thereby rendering *S*-cdG to be detected with adequate sensitivity. The above digestion was continued at 37°C for 48 h. To the digestion mixture were then added 8 units of alkaline phosphatase, 0.02 unit of phosphodiesterase 1 and 40 μ L of 0.5 M Tris-HCl buffer (pH 8.9). The digestion mixture was incubated at 37°C for 2 h and subsequently neutralized by addition of formic acid. To the mixture were then added isotopically labeled standard lesions, which included 2.5 pmol of 5-FodU, 1.5 pmol of 5-HmdU, 80 fmol of *R*-cdG, 40 fmol of *S*-cdG, 60 fmol of *R*-cdA and 20 fmol of *S*-cdA (Scheme 1). The enzymes in the digestion mixture were subsequently removed by chloroform extraction. The resulting aqueous layer was dried, reconstituted in doubly distilled water and subjected to off-line HPLC separation for the enrichment of the lesions under study.

HPLC

A 4.6 $\times$ 250 mm Alltima HP C18 column (5 μ m in particle size, Grace Davison, Deerfield, IL) was used for the enrichment of oxidatively induced DNA lesions from the enzymatic digestion products of DNA. A solution of 10 mM ammonium formate (solution A) and methanol (solution B) were used as mobile phases, and the flow rate was 1 mL/min. A gradient of 42 min 0% B, 1 min 0-5% B, 32 min 5% B, 5 min 5-20% B, and 10 min 20% B was employed. A typical HPLC trace is depicted in Figure S3. A minor RNA contamination (less than 5% for most DNA samples) was often observed for the DNA samples based on the chromatograms obtained during the HPLC enrichment. Correction for RNA contamination was made for each DNA sample according to the peak areas of adenosine and guanosine in the HPLC traces relative to those of 2'-deoxyadenosine (dA) and 2'-deoxyguanosine (dG). The HPLC fractions eluting at 8.5-10.0, 17.0-20.0, 21.0-24.0, 29.5-32.5, 32.5-36.0 and 56.0-63.0 min were pooled for *R*-cdG, 5-HmdU, *R*-cdA, *S*-cdG, 5-

FodU and S-cdA, respectively. The collected fractions were dried in the Speed-vac, redissolved in H₂O, and injected for LC-MS/MS/MS analysis.

LC-MS/MS/MS Analysis

A 0.5×250 mm Zorbax SB-C18 column (particle size, 5 μm, Agilent) was used for the separation of the above-enriched lesion fractions, and the flow rate was 8.0 μL/min, which was delivered by using an Agilent 1100 capillary HPLC pump (Agilent Technologies). A solution of 0.1% (v/v) formic acid in water (solution A) and a solution of 0.1% (v/v) formic acid in methanol (solution B) were used as mobile phases for the analyses of all the ROS-induced DNA lesions after HPLC enrichment, and a gradient of 5 min 0-20% B, 21 min 20-35% B, and 9 min 35-80% B was employed for the separation.

The effluent from the LC column was directed to an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific), which was set up for monitoring the fragmentation of the labeled and unlabeled R-cdG, S-cdG, R-cdA and S-cdA in the positive-ion mode or the labeled and unlabeled 5-FodU and 5-HmdU in the negative-ion mode. The temperature for the ion transport tube was maintained at 275°C, the sheath gas flow rate was 15 arbitrary units, and no auxiliary gas was used. In the positive-ion mode, the electrospray, capillary and tube lens voltages were 4.5 kV, 5 V and 20 V, respectively; each MS³ scan was composed of three microscans and the maximum time for each microscan was 250 ms. In the negative-ion mode, the electrospray, capillary and tube lens voltages were 4 kV, -10 V and -100 V, respectively; each scan had one microscan and the maximum time for each microscan was 100 ms. Prior to analyzing the lesions isolated from tissue DNA samples, the sensitivities for detecting all these lesions were optimized by varying two working parameters of the LTQ mass spectrometer, i.e., normalized collision energy and activation Q (Table S1). The detection limits (LOD and LOQ) were determined based on three independent LC-MS³ measurements performed in three separate days (Table S1). The LOD and LOQ referred to the amounts of a lesion that gave rise to signal-to-noise ratios (S/N) of 3 and 10, respectively, in the selected-ion chromatogram for the transitions employed for the lesion quantification. A single technical replicate analysis was performed for each tissue DNA sample.

Real-time Quantitative Reverse Transcription-Polymerase Chain Reaction (Real-time qRT-PCR)

mRNA was isolated from the liver and brain tissues of LEC^{-/-} and LEC^{+/-} rats with RNeasy mini kit (Qiagen, Valencia, CA) following the vendor's recommended procedures. One μg of total RNA was subsequently reverse-transcribed with 1 μL iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, CA) and 4 μL 5×iScript reaction mixture in a 20 μL-volume reaction. The reaction was carried out at 25°C for 5 min and at 42°C for 30 min, and the reverse transcriptase was then deactivated by heating at 85°C for 5 min.

Analyses of the transcripts for rat NTHL1, NEIL1, XPA, ERCC1, CSA and CSB were performed by real-time qRT-PCR using iQTM SYBR Green Supermix kit (Bio-Rad) according to the manufacturer's recommendations on a Bio-Rad iCycler system (Bio-Rad). The real-time PCR was performed by running at 95°C for 3 min and 45 cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 45 s (The primer sequences are listed in Table S3). The comparative cycle threshold (*C_t*) method was used for the relative quantification of gene expression⁴¹. The GAPDH gene was used as the internal control, which was included on each plate to correct for sample variations. The mRNA level of each gene in each sample was normalized to that of the internal control. Relative mRNA level in LEC^{-/-} rats with respect to 1-month old animal tissue was represented as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t_{\text{gene of interest}}} - C_{t_{\text{internal control}}})_{3 \text{ or } 6 \text{ month}} - (C_{t_{\text{gene of interest}}} - C_{t_{\text{internal control}}})_{1 \text{ month}}$.

Relative mRNA levels in 3- and 12-month old LEC+/- rats were calculated in a similar fashion. All data shown were the mean $\pm$ S.D. of results from three separate experiments.

Results

1. LC-MS³ method for quantifying ROS-induced DNA lesions in rat tissues

To quantify low levels of DNA lesions in isolated and cellular DNA, we enriched lesions from the enzymatic digestion mixture by off-line HPLC with an analytical column and then subjected lesion-containing fractions to LC-MS analysis on an LTQ linear ion-trap mass spectrometer^{16, 42-44}. To achieve unambiguous identification and quantification of low levels of ROS-induced bulky lesions in complicated sample matrices^{16, 42, 43}, we employed MS³ mode of detection using parameters detailed in Table S1. We also assessed analytical performance of LC-MS³ monitored in the positive- or negative-ion mode (See Experimental Section) for all lesions (Table S1).

Here we describe the detection of *S*-cdG to illustrate the application of LC-MS³ for quantifying this lesion in rat tissues. The selected-ion chromatograms (SICs) and MS³ spectra for unlabeled and ¹⁵N-labeled *S*-cdG are displayed in Figure 1. We monitored the m/z 266 $\rightarrow$ 180 $\rightarrow$ 163 and m/z 271 $\rightarrow$ 185 $\rightarrow$ 167 transitions for *S*-cdG and its uniformly ¹⁵N-labeled counterpart, respectively. The identity of the component eluting at 13.6 min in the SIC depicted in Figure 1A was determined to be *S*-cdG based on its co-elution with ¹⁵N-labeled *S*-cdG and similar fragment ions found in MS³ of the analyte and its uniformly ¹⁵N-labeled standard (Figure 1). As shown in Scheme 2, the ions of m/z 180 and 185 arise from cleavages of both the *N*-glycosidic linkage and the bond between the 5'-C and 4'-C of the 2-deoxyribose moiety of *S*-cdG and ¹⁵N-labeled *S*-cdG, respectively⁴⁵. Further fragmentation of the ion at m/z 180 yielded three major fragment ions of m/z 163, 152 and 135, which form from the neutral losses of NH₃, CO and [NH₃ + CO], respectively (The proposed structures for these fragment ions are depicted in Scheme 2). The corresponding fragment ions were found in the MS³ of the internal standard. In addition, the relative abundances of these three major fragment ions emanating from the cleavage of the ions of m/z 180 and 185 were very similar, thereby confirming the identity of the component eluting at 13.6 min to be *S*-cdG. Other purine cyclonucleosides were assessed by LC-MS/MS/MS in a similar fashion (Figures S4-S6). We also constructed calibration curves for the quantification of all these lesions from results of three independent sets of experiments performed in three separate days (Tables S5-S10 and Figures S7-S12); the relatively small variations in measurement results highlighted the excellent reproducibilities of the LC-MS/MS/MS method.

2. The levels of ROS-induced DNA lesions in liver and brain tissues of LEA and LEC rats

With the above LC-MS³ method, we quantified all lesions in DNA isolated from rat liver and brain tissues. The data are summarized in Table S2 and plotted in Figures 2-3, where the lesion levels are reported as number of lesions per 10⁶ nucleosides. These values were calculated from amounts (in moles) of lesions measured by LC-MS³ (based on peak areas found in the corresponding selected-ion chromatograms for the internal standards and analytes, the calibration curves and the amounts of internal standards added) and the amount of nucleosides (in moles, measured by UV absorbance measurement at 260 nm) in the genomic DNA employed for the digestion.

To manage the number of samples, we studied 3-month old LEA rats since liver copper remains normal in these animals without age-dependent changes. Similarly, no alteration in liver histology was observed in LEA rats over age (Table S4). In addition, we established that biliary copper excretion is normally maintained in heterozygous LEC+/- rats and as a result liver copper content also remains normal. This was confirmed by normal liver

histology in heterozygous LEC+/- rats, which was similar to LEA rats (Table S4). Nevertheless, we included heterozygous LEC+/- rats of 3 and 12 month ages in our studies of DNA lesions. By contrast, LEC rats begin to exhibit significant changes in hepatic copper content at early ages, and extensive copper-induced liver damage becomes apparent within several weeks of age (Table S4).

We next describe the levels of oxidatively induced DNA lesions in livers of LEA, LEC+/- and LEC-/- rats. First, we did not observe significant differences in the levels of oxidatively induced DNA lesions in 3-month old LEA and LEC+/- rats (Figures 2-3), which was in agreement with normal liver copper content and histological appearances. On the other hand, appreciably higher levels of 5-FodU, 5-HmdU, and both diastereomers of cdA were observed in the liver of 3-month old LEC-/- rats (Figures 2-3). The two diastereomers of cdG were present at slightly higher levels in liver of LEC-/- rats than age-matched LEA and LEC+/- rats, but the difference was statistically insignificant. Additionally, we observed that the liver of 12-month old LEC+/- rats contained higher levels of ROS-induced lesions than the corresponding 3-month old LEA or LEC +/- rats. We found greatest levels of 5-FodU and S-cdA in LEC-/- rats at 3-months of age compared with those at 1- or 6-months of age, which may reflect changes associated with acute-to-chronic liver injury, which is a known feature of hepatitis in LEC-/- rats. However, no statistically significant difference was observed for other lesions among LEC-/- rats at various ages, except for S-cdG, which was present at higher levels in 6- than 1-month old rats.

While LEC rats exhibited extensive liver damage due to copper toxicosis, no neurological abnormalities or brain damage have been reported in these animals, which differs from WD in humans ⁴⁶ and is attributable to the lack of copper accumulation in the brain of LEC rats. However, examination of oxidative DNA lesions in the brain of these rats revealed some similarities and differences with those in the liver. In this respect, we again observed similar levels of all quantified oxidative DNA lesions in 3-month old LEA and LEC+/- rats. Likewise, we found age-dependent accumulation of all quantified oxidative DNA lesions, except for S-cdG and S-cdA in brain of LEC+/- rats. In contrast to liver tissues, we observed a slightly higher level of 5-FodU in the brain of LEC-/- rats compared with LEA or LEC+/- rats. Furthermore, we found an age-dependent increase in the levels of all the quantified ROS-induced DNA lesions in brain of LEC-/- rats.

3. Real-time qRT-PCR for monitoring expression levels of genes involved in DNA repair

Previous studies revealed a decreased expression of some DNA repair genes in liver tissues of LEC rats in the acute hepatitis stage ⁴⁷. We reasoned that the altered expression of DNA repair genes may also have contributed to age-related levels of oxidative DNA lesions in the brain and liver tissues of LEC rats. Therefore, we studied six DNA repair genes, including two BER genes, NTHL1 and NEIL1, two NER genes, XPA and ERCC1, and two genes involved in transcription-coupled repair, CSA and CSB ⁴⁸.

Expression of NTHL1, NEIL1, XPA, and CSB was markedly lower in the liver of 3-month old LEC-/- rats compared with 1-month old LEC-/- rats, while the expression of these genes was restored, fully or in part, in 6-month old LEC-/- rats (Figure 4). The transcript levels of CSA were not substantially different in liver of 1- and 3-month old LEC-/- rats, whereas the liver of 6-month old animals exhibited significantly higher expression of these two genes. On the other hand, expression of NTHL1 and CSA was slightly decreased in the brain of 3-month old LEC-/- rats. Furthermore, we found expression of NEIL1 increased with age, whereas expression of XPA, ERCC1 and CSB exhibited an age-dependent decrease in the brain of LEC-/- rats. Together, the brain and liver of LEC-/- rats displayed different age-dependent expression of DNA repair genes, which may have been responsible, at least in part, for the differences in the profiles of DNA lesions in these tissues.

We also assessed, by employing real-time PCR analysis, the levels of expression of the above six DNA repair genes in the liver and brain tissues of 3- and 12-month old LEC+/- rats. Compared to what we observed for the LEC-/- rats, relatively small differences were observed between the 3- and 12-month old LEC+/- rats, particularly for the brain tissues (Figure S13). Thus, the higher levels of the oxidatively induced DNA lesions observed for the brain and liver tissues of 12-month old LEC+/- rats might reflect the age-associated accumulation of these lesions.

Discussion

In this study, we found MS³ detection mode offered more reliable quantification of ROS-induced DNA lesions than the MS² mode that was employed to analyze these types of lesions in most previous studies²³⁻²⁵. For instance, Figure S5 shows the SICs and MS³ results for monitoring *S*-cdA in a rat DNA sample, where two pairs of major fragment ions at *m/z* 136 and 119, and at *m/z* 141 and 123 were observed for the unlabeled and labeled *S*-cdA, respectively. Apart from these fragment ions, other ions emanating from isobaric interferences (e.g. the ions of *m/z* 151 and 152 in Figure S5B) were also detected. Similar interferences were observed for some other lesions quantified in the present study. Thus, MS³ afforded more reliable detection of these lesions in cellular DNA.

We found higher levels of 5-FodU, 5-HmdU, and two diastereomers of cdA in the liver of 3-month old LEC-/- than age-matched LEC+/- or LEA rats. This was in keeping with previous observation of greater 8-oxodG levels in LEC rats, especially at the acute hepatitis stage¹³. In contrast, LEC-/- rats deficient in *Atp7b* did not show greater levels of all the quantified ROS-induced lesions, except for 5-FodU in the brain, which was in agreement with the absence of gross neurological defects in these rats⁴⁹.

We observed age-dependent increases in ROS-induced DNA lesions in liver and brain of LEC+/- rats and brain of LEC-/- rats (Figures 2-3). Randerath et al.⁵⁰ demonstrated by ³²P-postlabeling assays that bulky DNA lesions increase with age in animals. Some of these indigenous lesions ("I-compounds") were identical to those induced in isolated DNA exposed to Fenton-type reagents and the levels of these lesions were elevated in kidneys of rodents treated with prooxidant carcinogens⁵¹. Thus, these so-called "Type II-I compounds" were attributed to be induced by endogenous ROS⁵¹. Later, some of the Type-II I compounds were identified to be *S*-cdA with different flanking 5' nucleosides⁵². The covalent linkage between the C8 carbon of adenine and the C5' carbon of 2-deoxyribose in the lesion inhibits cleavage of the flanking 5' phosphodiester bond by nucleases in the ³²P-post-labeling assay⁵². However, the structures of other bulky DNA lesions demonstrated by the ³²P-post-labeling assay are yet to be established. Our studies showed that all four purine cyclonucleosides could be detected in animal tissues and all of them exhibited age-dependent increases in brain of LEC-/- rats and brain and liver of LEC+/- rats. Thus, aside from previously identified *S*-cdA, other bulky lesions, including *R*-cdA, *S*-cdG and *R*-cdG, should constitute new members of the Type II I-compound family. In this context, it is worth noting that we assessed the lesion levels in tissues of animals from a relatively small number of age groups. In the future, it will be important to expand the study to more age groups of rats and to other mammalian species.

Among our noteworthy findings were that we did not observe age-dependent increases in ROS-induced DNA lesions in the liver of LEC-/- rats. Indeed, the liver of 3-month old LEC-/- rats exhibited greater levels of 5-FodU and *S*-cdA than 1- and 6-month old LEC-/- rats. Similar age-associated prevalences of DNA lesions were previously observed for ϵ dA and ϵ dC in liver of LEC rats, where the levels of these two lesions peaked at 8-12 weeks¹⁴. Additionally, the age-dependent accumulation of ϵ dA and ϵ dC paralleled the liver copper

content of these rats¹⁴. Furthermore, accumulation of hepatic copper in LEC rats was shown to compromise the capacity for DNA repair. In the latter respect, the enzymatic activities and expression levels of two major DNA glycosylases, endonuclease III (NTHL1) and 8-oxoguanine DNA glycosylase (OGG1), were greatly diminished in LEC rat liver extracts during acute hepatitis at 16-18 weeks of age⁴⁷. No substantial differences in DNA repair activity or expression level of DNA repair genes were observed for LEA rats with ages ranging from 8 to 40 weeks⁴⁷. In keeping with these previous findings, the transcript levels of NTHL1, NEIL1, XPA and CSB genes in 3-month old LEC^{-/-} rats were lower than those in 1-month old LEC^{-/-} rats. The products of the former two genes are important for repairing thymine methyl group oxidation products^{53, 54}, and NEIL1 is important for repairing cdA⁵⁵. Additionally, cdA is a substrate for mammalian NER pathway^{30, 31}. On the other hand, CSA and CSB are important in transcription-coupled repair; CSB plays a key role as a coupling factor to recruit NER proteins to lesion-stalled RNA polymerase II, whereas CSA is dispensable for this process⁵⁶. Along this line, the CSB^{-/-} mouse embryonic fibroblast (MEF) is 2-fold more sensitive to γ rays than wild-type MEF, whereas the CSA^{-/-} MEF exhibited similar sensitivity as the wild-type counterpart⁵⁷. In particular, CSB was also shown to be important in repairing S-cdA⁵⁸. Therefore, diminished hepatic expression of NTHL1, NEIL1, XPA, and CSB in 3-month old LEC rats may have contributed to the elevated levels of 5-FodU and S-cdA in the liver of these rats.

It is worth discussing the relative levels of the ROS-induced purine cyclonucleosides with respect to 5-HmdU and 5-FodU. In this regard, our results (Figures 2-3) revealed that cdA and cdG levels were approximately 2 orders of magnitude lower than those of 5-FodU and 5-HmdU in the liver and brain of LEA and LEC rats. The relative levels of these lesions reflect a dynamic interplay between the rates of their formation and repair in animal tissues.

Taken together, the age- and genotype-dependent accumulation of oxidative DNA lesions, as revealed in the present study, should help in understanding the implications of oxidative DNA damage in the pathophysiology of WD, as well as in ageing and ageing-associated pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-FodU	5-formyl-2'-deoxyuridine
5-HmdU	5-hydroxymethyl-2'-deoxyuridine
cdG	8,5'-cyclo-2'-deoxyguanosine
cdA	8,5'-cyclo-2'-deoxyadenosine
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
dI	2'-deoxyinosine
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
LEC	Long-Evans Cinnamon

LEA	Long-Evans Agouti
ROS	reactive oxygen species
SIC	selected-ion chromatogram
LOD	limit of detection
LOQ	limit of quantification
BER	base excision repair
NER	nucleotide excision repair
OGG1	8-oxoguanine DNA-glycosylase
WD	Wilson's disease
ESI	electrospray ionization
MS	mass spectrometry.

References

- (1). Finkel T, Holbrook NJ. *Nature* 2000;408:239–247. [PubMed: 11089981]
- (2). Cadet J, Delatour T, Douki T, Gasparutto D, Pouget JP, Ravanat JL, Sauvaigo S. *Mutat. Res* 1999;424:9–21. [PubMed: 10064846]
- (3). Danks, DM. Disorders of copper transport. McGraw-Hill; New York: 1989.
- (4). Ala A, Walker AP, Ashkan K, Dooley JS, Schilsky ML. *Lancet* 2007;369:397–408. [PubMed: 17276780]
- (5). Ferenci P. *Clin. Gastroenterol. Hepatol* 2005;3:726–733. [PubMed: 16233999]
- (6). Mori M, Hattori A, Sawaki M, Tsuzuki N, Sawada N, Oyamada M, Sugawara N, Enomoto K. *Am. J. Pathol* 1994;144:200–204. [PubMed: 8291609]
- (7). Sasaki M, Yoshida MC, Kagami K, Takeichi N, Kobayashi H, Dempo K, Mori M. *Rat News Lett* 1985;14:4–6.
- (8). Yoshida MC, Masuda R, Sasaki M, Takeichi N, Kobayashi H, Dempo K, Mori M. *J. Hered* 1987;78:361–365. [PubMed: 3429843]
- (9). Wu J, Forbes JR, Chen HS, Cox DW. *Nat. Genet* 1994;7:541–545. [PubMed: 7951327]
- (10). Li Y, Togashi Y, Sato S, Emoto T, Kang JH, Takeichi N, Kobayashi H, Kojima Y, Une Y, Uchino J. *J. Clin. Invest* 1991;87:1858–1861. [PubMed: 2022751]
- (11). Masuda R, Yoshida MC, Sasaki M, Dempo K, Mori M. *Jpn. J. Cancer Res* 1988;79:828–835. [PubMed: 2459092]
- (12). Sawaki M, Enomoto K, Takahashi H, Nakajima Y, Mori M. *Carcinogenesis* 1990;11:1857–1861. [PubMed: 1698569]
- (13). Yamamoto F, Kasai H, Togashi Y, Takeichi N, Hori T, Nishimura S. *Jpn. J. Cancer Res* 1993;84:508–511. [PubMed: 8320167]
- (14). Nair J, Strand S, Frank N, Knauff J, Wesch H, Galle PR, Bartsch H. *Carcinogenesis* 2005;26:1307–1315. [PubMed: 15790590]
- (15). Cao H, Wang Y. *Nucleic Acids Res* 2007;35:4833–4844. [PubMed: 17626047]
- (16). Hong H, Cao H, Wang Y. *Chem. Res. Toxicol* 2006;19:614–621. [PubMed: 16696563]
- (17). D'Errico M, Parlanti E, Teson M, de Jesus BM, Degan P, Calcagnile A, Jaruga P, Bjoras M, Crescenzi M, Pedrini AM, Egly JM, Zambruno G, Stefanini M, Dizdaroglu M, Dogliotti E. *EMBO J* 2006;25:4305–4315. [PubMed: 16957781]
- (18). D'Errico M, Parlanti E, Teson M, Degan P, Lemma T, Calcagnile A, Iavarone I, Jaruga P, Ropolo M, Pedrini AM, Orioli D, Frosina G, Zambruno G, Dizdaroglu M, Stefanini M, Dogliotti E. *Oncogene* 2007;26:4336–4343. [PubMed: 17297471]

- (19). Dizdaroglu M, Dirksen ML, Jiang HX, Robbins JH. *Biochem. J* 1987;241:929–932. [PubMed: 3496079]
- (20). Pouget JP, Frelon S, Ravanat JL, Testard I, Odin F, Cadet J. *Radiat. Res* 2002;157:589–595. [PubMed: 11966325]
- (21). Hong H, Wang Y. *Anal. Chem* 2007;79:322–326. [PubMed: 17194156]
- (22). Bhimani RS, Troll W, Grunberger D, Frenkel K. *Cancer Res* 1993;53:4528–4533. [PubMed: 7691399]
- (23). Jaruga P, Xiao Y, Nelson BC, Dizdaroglu M. *Biochem. Biophys. Res. Commun* 2009;386:656–660. [PubMed: 19559005]
- (24). Kirkali G, de Souza-Pinto NC, Jaruga P, Bohr VA, Dizdaroglu M. *DNA Repair (Amst)* 2009;8:274–278. [PubMed: 18992371]
- (25). Egler RA, Fernandes E, Rothermund K, Sereika S, de Souza-Pinto N, Jaruga P, Dizdaroglu M, Prochownik EV. *Oncogene* 2005;24:8038–8050. [PubMed: 16170382]
- (26). Boorstein RJ, Teebor GW. *Cancer Res* 1988;48:5466–5470. [PubMed: 3416303]
- (27). Bilimoria MH, Gupta SV. *Mutat. Res* 1986;169:123–127. [PubMed: 3512995]
- (28). Shirname-More L, Rossman TG, Troll W, Teebor GW, Frenkel K. *Mutat. Res* 1987;178:177–186. [PubMed: 2953970]
- (29). Kamiya H, Murata-Kamiya N, Karino N, Ueno Y, Matsuda A, Kasai H. *Mutat. Res* 2002;513:213–222. [PubMed: 11719107]
- (30). Brooks PJ, Wise DS, Berry DA, Kosmoski JV, Smerdon MJ, Somers RL, Mackie H, Spoonde AY, Ackerman EJ, Coleman K, Tarone RE, Robbins JH. *J. Biol. Chem* 2000;275:22355–22362. [PubMed: 10801836]
- (31). Kuraoka I, Bender C, Romieu A, Cadet J, Wood RD, Lindahl T. *Proc. Natl. Acad. Sci. U. S. A* 2000;97:3832–3837. [PubMed: 10759556]
- (32). Marietta C, Brooks PJ. *EMBO Rep* 2007;8:388–393. [PubMed: 17363972]
- (33). Kuraoka I, Robins P, Masutani C, Hanaoka F, Gasparutto D, Cadet J, Wood RD, Lindahl T. *J. Biol. Chem* 2001;276:49283–49288. [PubMed: 11677235]
- (34). Gillet LCJ, Scharer OD. *Org. Lett* 2002;4:4205–4208. [PubMed: 12443059]
- (35). Jimenez LB, Encinas S, Miranda MA, Navacchia ML, Chatgililoglu C. *Photochem. Photobiol. Sci* 2004;3:1042–1046. [PubMed: 15570393]
- (36). Xiong L, Wang Y. *J. Proteome Res* 2010;9:1129–1137. [PubMed: 20050688]
- (37). Ahmed S, Deng J, Borjigin J. *Brain Res. Mol. Brain Res* 2005;137:63–69. [PubMed: 15950762]
- (38). Malhi H, Bhargava KK, Afriyie MO, Volenberg I, Schilsky ML, Palestro CJ, Gupta S. *J. Nucl. Med* 2002;43:246–252. [PubMed: 11850492]
- (39). Miller SA, Dykes DD, Polesky HF. *Nucleic Acids Res* 1988;16:1215. [PubMed: 3344216]
- (40). Lim KS, Jenner A, Halliwell B. *Nat. Protoc* 2006;1:1995–2002. [PubMed: 17487188]
- (41). Livak KJ, Schmittgen TD. *Methods* 2001;25:402–408. [PubMed: 11846609]
- (42). Hong H, Cao H, Wang Y. *Nucleic Acids Res* 2007;35:7118–7127. [PubMed: 17942427]
- (43). Jiang Y, Hong H, Cao H, Wang Y. *Biochemistry* 2007;46:12757–12763. [PubMed: 17929946]
- (44). Goodenough AK, Schut HA, Turesky RJ. *Chem. Res. Toxicol* 2007;20:263–276. [PubMed: 17305409]
- (45). Jaruga P, Birincioglu M, Rodriguez H, Dizdaroglu M. *Biochemistry* 2002;41:3703–3711. [PubMed: 11888287]
- (46). Lorincz MT. *Ann. N. Y. Acad. Sci* 2010;1184:173–187. [PubMed: 20146697]
- (47). Choudhury S, Zhang R, Frenkel K, Kawamori T, Chung FL, Roy R. *Cancer Res* 2003;63:7704–7707. [PubMed: 14633694]
- (48). Friedberg, EC.; Walker, GC.; Siede, W.; Wood, RD.; Schultz, RA.; Ellenberger, T. *DNA Repair and Mutagenesis*. ASM Press; Washington, D.C.: 2006.
- (49). Ahn TB, Cho SS, Kim DW, Jeon BS. *Neurol. Res* 2005;27:409–411. [PubMed: 15949239]
- (50). Randerath K, Reddy MV, Disher RM. *Carcinogenesis* 1986;7:1615–1617. [PubMed: 3742734]
- (51). Randerath K, Randerath E, Zhou GD, Li DH. *Mutat. Res* 1999;424:183–194. [PubMed: 10064860]

- (52). Randerath K, Zhou GD, Somers RL, Robbins JH, Brooks PJ. *J. Biol. Chem* 2001;276:36051–36057. [PubMed: 11454870]
- (53). Zhang QM, Yonekura S, Takao M, Yasui A, Sugiyama H, Yonei S. *DNA Repair* 2005;4:71–79. [PubMed: 15533839]
- (54). Miyabe I, Zhang QM, Kino K, Sugiyama H, Takao M, Yasui A, Yonei S. *Nucleic Acids Res* 2002;30:3443–3448. [PubMed: 12140329]
- (55). Jaruga P, Xiao Y, Vartanian V, Lloyd RS, Dizdaroglu M. *Biochemistry* 2010;49:1053–1055. [PubMed: 20067321]
- (56). Fousteri M, Vermeulen W, van Zeeland AA, Mullenders LH. *Mol. Cell* 2006;23:471–482. [PubMed: 16916636]
- (57). de Waard H, de Wit J, Andressoo JO, van Oostrom CT, Riis B, Weimann A, Poulsen HE, van Steeg H, Hoeijmakers JH, van der Horst GT. *Mol. Cell. Biol* 2004;24:7941–7948. [PubMed: 15340056]
- (58). Kirkali G, de Souza-Pinto NC, Jaruga P, Bohr VA, Dizdaroglu M. *DNA Repair* 2009;8:274–278. [PubMed: 18992371]

Figure 1. Selected-ion chromatograms (SICs) for monitoring the m/z 266→180→163 [A, for unlabeled *S*-cdG] and m/z 271→185→167 [B, for labeled *S*-cdG] transitions of the digestion mixture of genomic DNA from the liver tissue of a 6-month old LEC^{-/-} rat. Shown in the insets are the positive-ion MS³ spectra for the unlabeled and labeled *S*-cdG.

Figure 2.

Levels of 5-FodU and 5-HmdU in genomic DNA isolated from the liver (L) and brain (B) tissues of LEA (3m), LEC+/- (3m or 12m) and LEC-/- (1m, 3m or 6m) rats. The values represent the mean $\pm$ S.D. of results obtained from tissues of three rats. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The P values were calculated by using paired t -test.

Figure 3.

Levels of *R*-cdG, *S*-cdG, *R*-cdA and *S*-cdA in genomic DNA isolated from the liver (L) and brain (B) tissues of LEA (3m), LEC+/- (3m or 12m) and LEC-/- (1m, 3m or 6m) rats. The values represent the mean $\pm$ S.D. of results obtained for tissue samples from three different rats. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The P values were calculated by using paired t -test.

Figure 4.

The mRNA levels of NTHL1, NEIL1, XPA, ERCC1, CSA and CSB in the liver (A) and brain (B) tissues of LEC^{-/-} (1m, 3m or 6m) rats. The results were obtained by real-time qRT-PCR with the use of GAPDH as standard. The data represent the mean $\pm$ S.D. of three separate experiments.

Scheme 1.

Chemical structures of oxidative DNA lesions measured in this study. The “N” in bold italic font represents ^{15}N , and the site of deuterium atom incorporation is indicated with a “D”.

Scheme 2.

Proposed major fragmentation pathways for the $[M+H]^+$ ion of unlabeled *S*-cdG observed in MS/MS and MS/MS/MS.