Free Radical Biology & Medicine 50 (2011) 1536–1545

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Site-specific radical formation in DNA induced by Cu(II)–H_2O_2 oxidizing system, using ESR, immuno-spin trapping, LC-MS, and MS/MS

Suchandra Bhattacharjee [a,*], Leesa J. Deterding [b], Saurabh Chatterjee [a], JinJie Jiang [a], Marilyn Ehrenshaft [a], Olivier Lardinois [a], Dario C. Ramirez [c], Kenneth B. Tomer [b], Ronald P. Mason [a]

[a] Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA
[b] Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA
[c] Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA

ARTICLE INFO

Article history:
Received 28 January 2011
Revised 23 February 2011
Accepted 25 February 2011
Available online 5 March 2011

Keywords:
Spin trapping
DNA radicals
Copper
Nucleosides
Cells
ESR
IST
LC-MS/MS
Detect
Identify
Free radicals

ABSTRACT

Oxidative stress-related damage to the DNA macromolecule produces a multitude of lesions that are implicated in mutagenesis, carcinogenesis, reproductive cell death, and aging. Many of these lesions have been studied and characterized by various techniques. Of the techniques that are available, the comet assay, HPLC-EC, GC-MS, HPLC-MS, and especially HPLC-MS/MS remain the most widely used and have provided invaluable information on these lesions. However, accurate measurement of DNA damage has been a matter of debate. In particular, there have been reports of artifactual oxidation leading to erroneously high damage estimates. Further, most of these techniques measure the end product of a sequence of events and thus provide only limited information on the initial radical mechanism. We report here a qualitative measurement of DNA damage induced by a Cu(II)–H_2O_2 oxidizing system using immuno-spin trapping (IST) with electron paramagnetic resonance (EPR), MS, and MS/MS. The radical generated is trapped by DMPO immediately upon formation. The DMPO adduct formed is initially EPR active but subsequently is oxidized to the stable nitrone, which can then be detected by IST and further characterized by MS and MS/MS.

Published by Elsevier Inc.

DNA is continuously exposed to exogenous and endogenous mutagens including reactive oxygen and nitrogen species that could alter its integrity [1–3]. Oxidatively generated DNA damage is an inevitable consequence of cellular metabolism. Injury to this macromolecule can have severe biological consequences including mutation, cell death, carcinogenesis, and aging [4]. DNA lesions include strand breaks, DNA–protein or DNA–DNA crosslinks, abasic sites, and modified bases. Despite the abundance of oxidatively generated DNA damage, the products exist in a much larger background (10^5–10^6) of unaltered nucleosides, which themselves may be prone to oxidation during sample preparation and analysis [5].

Major efforts have been devoted in the past several years to the development of accurate assays aimed at measuring oxidative base damage [6–11]. Several analytical methods have been developed to quantify modified bases in a research field that remains very challenging. Two different types of approaches have been developed for monitoring different damage to DNA. First, indirect approaches such as the alkaline comet assay [12] or the alkaline elution technique [13] are very sensitive methods for measuring DNA strand breaks [9,14]. The alkaline single cell gel electrophoresis assay, particularly in its basal version, is not able to measure defined types of DNA damage because strand breaks thus measured may consist of frank nicks together with abasic sites and several alkali-labile oxidized bases. However, the use of repair enzymes before the comet assay analysis allows the detection of classes of damage including oxidized pyrimidine bases and purine base modifications [15]. Despite all of the above, these approaches still do not detect radicals, but only products thought to be formed from radicals. These methods are very sensitive, but they are inherently not specific [16] to free radical chemistry in that the free radical nature of the strand break is only inferred, as in the case of radiation damage. A second approach, which requires the extraction of DNA, is very useful for studying oxidative base lesions. It uses gas chromatography–mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC)-EC to measure 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), one of the most widely studied DNA-damage adducts. However, with GC-MS, the artifactual generation of oxidized bases during the derivatization steps, as well as the lack of stability of several oxidized

* Corresponding author. Fax: +1 919 541 1043.
E-mail address: bhattac1@niehs.nih.gov (S. Bhattacharjee).

0891-5849/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2011.02.034

base modifications, prevents accurate, valid measurements. These methods cannot detect the primary radical species and can also cause artifactual oxidation during workup, thus leading to erroneously high damage estimates. The levels of 8-oxodGuo reported in the literature are highly variable [17]. HPLC-EC is currently a more popular method for the detection of 8-oxodGuo, but this method has also been criticized on the grounds that the variability of the assay is unacceptable [5,18–20]. Its application in the oxidative detection mode is restricted to only a few electroactive DNA lesions having a low oxidation potential, such as 8-oxodGuo. In addition to the lack of versatility, the assay suffers from insufficient sensitivity [9], which hinders the accurate measurement of low levels of DNA lesions.

A significant improvement in the measurement of oxidatively damaged DNA has been obtained by the use of HPLC coupled to MS and MS/MS. This assay combines the efficiency of HPLC separation with the sensitivity of mass spectrometry [9–11]. Consequently, this method has been gaining prominence and has the potential to overcome many of the limitations outlined earlier. However, HPLC-MS, is not suitable for measuring the frequency of the lesions within the range of a few modifications per 10^7 to 10^9 normal nucleosides [11]. HPLC-MS/MS is now considered to be the gold standard for the purpose of studying oxidatively damaged DNA. It should be added that even HPLC-MS^3 is necessary for the detection of modified nucleosides whose frequency is around a few lesions per 10^9 nucleosides [10,11].

Some immunological assays [21,22] have also been designed for the measurement of oxidative DNA base damage, but they have been plagued by a lack of specificity. Several attempts have been made to use antibodies raised against 8-oxodGuo, but it has been difficult to obtain a highly specific antibody. 8-OxodGuo differs from 2′-deoxyguanosine by a single oxygen atom, thereby challenging the specificity of the antibody. The method's applications have been limited by cross-reactivity of the antibodies with normal DNA bases and other abundant biological constituents [23].

The study of primary radicals is critical to understanding DNA damage. However, the detection of these primary transients is difficult because of their short lifetimes. To address this difficulty, a method developed in our laboratory, immuno-spin trapping (IST), combines the specificity of spin trapping with the sensitivity of an antigen–antibody-based assay [24,25]. This method has been very successful in detecting radicals in a number of macromolecules such as proteins [26–32] and in DNA [33–36]. IST allows sensitive, reliable, and economical detection of radical-generated damage. Its major limitation is that the radical is not identified by this procedure. Thus, an analytical question that is often posed is, "What is ELISA detecting?" [23]. Because the detection of DNA is limited to dot blots and enzyme-linked immunosorbent assays (ELISAs), the identification of a radical as DNA-derived and not a contaminant (protein-derived) depends on the absolute purity of the DNA as achieved by traditional DNA purification methods. The possible structural identification of DNA radicals detected by ELISA in previous work [33–36] is further complicated by the limited investigation of DNA radical adducts by electron spin resonance (ESR) or the corresponding DNA nitrone adducts by MS. The use of LC-MS to detect 5,5 dimethyl-1-pyrroline N-oxide (DMPO) adducts of radicals derived from nucleosides has been reported for radicals generated photochemically from the photolysis of 5-halo-2′-deoxyuridines, 5-thiophenylmethyl-2′-deoxyuridine, and thymidine (which are well-known photo precursors of nucleoside free radicals), with menadione bisulfite as the photosensitizer, and a new uridine radical was detected in RNA [37].

In our present work, coupling of the very sensitive IST with ESR, HPLC, MS, and MS/MS provides a very useful technique for determining the primary DNA radical damage rather than the stable end product that forms after a possibly complex sequence of events. The initial DNA spin-trapped radical adducts are enzymatically digested to 2′-deoxyribonucleosides. The resulting digestion mixture is separated by reverse-phase liquid chromatography and analyzed by MS and MS/MS, which allows the unequivocal identification of the radical trapped by the spin trap. Furthermore, because additional nitrone adduct formation is impossible once the DNA is separated from DMPO (even by dilution), artifactual DNA oxidation during extraction and subsequent hydrolysis cannot lead to nitrone adduct formation. Dilution of DMPO by 100-fold is sufficient to prevent artifactual nitrone formation because at DMPO concentrations below 1 mM, DNA radicals will decay before they can be trapped. No attempt has been made to quantitate DNA–DMPO adducts, which is beyond the scope of this study.

Here we have studied the damage to DNA induced by a Cu(II)–H_2O_2 oxidizing system with the combined use of ESR, IST, HPLC, MS, and MS/MS. The data presented here were reproduced with the Fe(II)–H_2O_2 system (the more classical Fenton hydroxyl radical-generating system) as well. The hydroxyl radical reacts at diffusion-controlled rates with virtually any macromolecule, including DNA. Using this combined approach, we have detected and identified a nitrone adduct on the 2′-deoxyadenosine moiety in nucleosides, pure DNA, and cellular DNA.

Presentations and discussions have been limited to Cu(II)–H_2O_2-induced, oxidatively generated DNA damage, which is pathophysiologically important because copper associates with DNA bases in the nuclei of mammalian cells [38]. Copper-mediated ROS damage assumes significance because of the increased evidence of elevated levels of copper in tumor growth and angiogenesis [39–41]. There have been several reports of copper concentration being significantly higher in cancer patients [39–41], whereas the concentrations of other elements such as zinc, iron, and selenium were significantly lower [39]. Copper chelation or copper depletion is under intense investigation for therapeutic purposes [39,40].

Experimental procedures

Reagents

2′-Deoxycytidine, 2′-deoxyguanosine, thymidine, 2′-deoxyadenosine (dAdo), and inosine were purchased from MP Biomedicals (Irvine, CA, USA). Calf thymus DNA, nuclease P1 (from *Penicillium citrinum*), 7-deazaadenosine, and 3-deazaadenosine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Snake venom phosphodiesterase was obtained from Worthington Chemicals (Freehold, NJ, USA). Cupric chloride was purchased from Alfa Aesar (Ward Hill, MA, USA). Calf intestinal alkaline phosphatase was purchased from Invitrogen. The 2′-deoxyadenosine isotopes $^{15}N_5$ and $^{13}C_{10}{}^{15}N_5$ were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The spin trap DMPO was purchased from Alexis Biochemicals (San Diego, CA, USA), purified twice by vacuum distillation at room temperature, and stored under argon at −80 °C. The DMPO concentration was measured at 228 nm assuming a molar absorption coefficient of 7800 M^{-1} cm^{-1}. Hydrogen peroxide was obtained from Fisher Scientific (Fairlawn, NJ, USA). The hydrogen peroxide concentration was verified using UV absorption at 240 nm ($\varepsilon_{240nm} = 43.6\ M^{-1}\ cm^{-1}$). All buffers used were treated with Chelex 100 ion-exchange resin (Bio-Rad Laboratories, Hercules, CA, USA) to avoid transition metal-catalyzed reactions.

Chemical reactions

Production of DNA–nitrone adducts in nucleosides and calf thymus DNA

Typically, reaction mixtures contained 2.5–5 mM nucleoside, 300 μM $CuCl_2$, 100 μM H_2O_2, and 100 mM DMPO in 100 mM Chelex-treated phosphate buffer (pH 7.4) and were incubated for 1 h at 37 °C. For 2′-deoxyguanosine experiments, a saturated solution of the nucleoside was used. Calf thymus DNA (250 μg/ml) was reacted with $CuCl_2$ (300 μM), H_2O_2 (100 μM), and 100 mM DMPO in Chelex-treated 100 mM phosphate buffer (pH 7.4) and incubated at 37 °C for 1 h. For ELISA, the nitrone DNA reaction mixtures were diluted to 5 μg/