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Original Contribution

Development of immunoblotting techniques for DNA radical detection

Fiona A. Summers*, Ronald P. Mason, Marilyn Ehrenshaft

Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

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ABSTRACT

Radical damage to DNA has been implicated in cell death, cellular dysfunction, and cancer. A recently developed method for detecting DNA radicals uses the nitrone spin trap DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) to trap radicals. The trapped radicals then decay into stable nitrone adducts detectable with anti-DMPO antibodies and quantifiable by ELISA or dot-blot assay. However, the sequences of DNA that are damaged are likely to be as important as the total level of damage. Therefore, we have developed immunoblotting methods for detection of DNA nitrone adducts on electrophoretically separated DNA, comparable to Western blotting for proteins. These new techniques not only allow the assessment of relative radical adduct levels, but can reveal specific DNA fragments, and ultimately nucleotides, as radical targets. Moreover, we have determined that denaturation of samples into single-stranded DNA enhances the detection of DNA–DMPO adducts in our new blotting methods and also in ELISA.

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Introduction

Biologically relevant reactive oxygen species (ROS) include radicals such as superoxide radical anion, hydroxyl radical ($^{\bullet}OH$), and peroxyl radical ($ROO^{\bullet}$) and nonradicals such as hydrogen peroxide (H_2O_2) and hypochlorous acid. ROS have been implicated in DNA damage induced by drugs [1–3], environmental hazards such as arsenic [4,5] and ionizing radiation [1], and endogenous processes [6,7]. Unrepaired DNA damage can lead to cell death, cellular dysfunction, and cancer [6,8].

Electron spin resonance (ESR) is used in in vitro studies of a wide range of biological radicals [9–12] but lacks the sensitivity to detect DNA radicals in intact cells. ESR spin trapping involves the use of a "spin trap" that reacts with the free radical to form a more stable radical adduct. Although spin trapping increases the effective lifetime of radicals, thereby enhancing the sensitivity of ESR, it is still not generally applicable in intact cell studies. A more recent advance, termed immuno-spin trapping, enhances the sensitivity of radical detection by orders of magnitude by combining the specificity of spin trapping with the sensitivity of immunological techniques.

Immuno-spin trapping (Scheme 1) comprises two parts: (1) a spin trapping reaction between a radical and the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and (2) immunological detection of the DMPO nitrone adducts (hereafter referred to as DMPO adducts) using an anti-DMPO antibody that recognizes DMPO covalently attached to a macromolecule, such as DNA or protein, at the site of the radical [13,14]. DMPO freely permeates cell membranes and animal organs [15,16] and is nontoxic at concentrations necessary for effective radical trapping. When added to in vitro systems, cell cultures, or animals in which radicals are being generated, DMPO reacts with radicals to form DMPO nitroxide radical adducts, which decay to far longer-lived, ESR-silent nitrone adducts recognized by the anti-DMPO antibody [13,17].

Immuno-spin trapping was first used to study protein radicals [13] but has now been used successfully in DNA radical studies [4,11,14,17]. One disadvantage of immuno-spin trapping is that the chemical structure of the free radical is not identified in this process, but mass spectrometry has been used to identify the structure of a DMPO adduct formed on adenine [18]. Analyses of physiological DNA oxidation products such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (also referred to as 8-hydroxy-2′-deoxyguanosine) by ELISA has been limited because of cross-reactivity of the antibodies with unoxidized 2′-deoxyguanosine [19], which is in high abundance relative to 8-oxo-7,8-dihydro-2′-deoxyguanosine. Analysis of 8-oxo-7,8-dihydro-2′-deoxyguanosine by mass spectrometry is complicated by artifactual oxidation, which can occur easily during DNA extraction and sample workup, leading to discrepancies in the measurement which can vary by as much as 1000-fold depending on which procedure is used [20–22], but this has become less variable with improvements in sample preparation to minimize spurious oxidation [23–26]. In immuno-spin trapping, by contrast, after spin trap reactions are complete, sample processing decreases DMPO concentration to below 1 mM, a level too low to trap radicals [12]. Moreover, anti-DMPO antibodies do not cross-react

* Corresponding author. Fax: (919) 541-1043.
E-mail address: summersfa@niehs.nih.gov (F.A. Summers).

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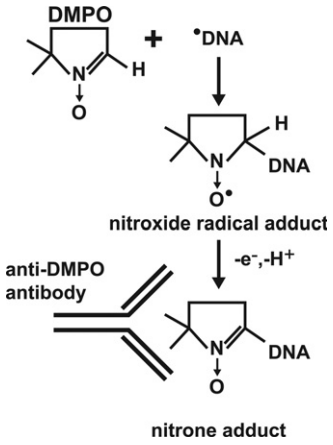

Scheme 1. Reaction of the DMPO spin trap with a DNA radical to form a DNA–DMPO nitrone adduct, which is detectable using an anti-DMPO antibody.

with DNA [14,17], which is a problem that ELISA measurements of 8-oxo-7,8-dihydro-2′-deoxyguanosine have. Relative levels of DNA–DMPO adducts can be measured by ELISA or dot blot [14,17], and differences can then be observed between treatments or over time and can be correlated with a functional effect [4,11]. However, the genes that are damaged are likely to be as important as the total level of damage. The ability to identify specific genes prone to radical damage under specific physiological or developmental regimes would allow connections to be drawn between mutated DNA and health outcomes. Therefore, to more precisely analyze the extent and location of radical-mediated damage throughout the genome, there is a need to extend immuno-spin trapping to detection of DMPO adducts on DNA, analogous to Western blotting.

To develop this method, we used an in vitro system consisting of DNA, copper(II), and H_2O_2 to generate DNA radicals in the presence of DMPO. Under these conditions, no assignable ESR spectrum has been obtained [18]. H_2O_2, a nonradical oxidant, does not react with DNA but can react with iron and copper through Fenton-type reactions to produce $^{\bullet}OH$ that can react with DNA at a diffusion-limited rate [9,27–29]. Copper ions bind preferentially to the N7 of guanine and to a lesser extent the N7 of adenine [30–32]. Hydroxyl radical scavengers are relatively ineffective at inhibiting Cu-mediated damage, suggesting that scavengers in bulk solution cannot effectively compete when hydroxyl radical is formed at the damage site [14,27,29]. A less likely alternative is that the DNA radical damage may be due to a species closely related to the hydroxyl radical that does not react with hydroxyl radical scavengers.

Although the copper–Fenton system is an in vitro model of DNA damage, it may have physiological relevance. Wilson disease, for example, is due to a mutation that blocks copper efflux from the liver, resulting in copper accumulation and liver cirrhosis. The bulky DNA lesions detected in liver DNA extracted from Wilson disease patients are similar to the bulky DNA lesions formed in vitro by a copper–Fenton system [33]. Moreover, penicillamine and triethylenetetramine, drugs used to treat patients with Wilson disease, can chelate copper and inhibit radical formation as measured by ESR [9].

This work was undertaken to expand the utility of DNA immuno-spin trapping through development of a blotting technique for spin-trapped DNA comparable to Western blotting. Examination of a number of standard techniques for nucleic acid transfer allowed us to identify reproducible methods for immunoblotting of both high- and low-molecular-weight DNA.

Material and methods

Materials

Nitrocellulose and AG 501-X8 resin were from Bio-Rad. DMPO was from Dojindo Molecular Technologies. Immobilon-FL polyvinylidene difluoride (PVDF) membrane was from Millipore. The LumiGLO peroxidase chemiluminescence substrate kit was from KPL, Inc. Reacti-Bind DNA coating solution, stabilized goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase (HRP), and rabbit anti-chicken IgY (H+L) conjugated to HRP were from Pierce Scientific. Donkey anti-chicken IgG-800 (H+L) IRDye 800CW, donkey anti-mouse IgG-800 (H+L) IRDye 800CW, and 10× orange loading dye were obtained from Li-Cor Biotechnology. Low IgG fetal bovine serum, Iscove's modified Dulbecco's medium, 6% (wt/vol) DNA retardation polyacrylamide gels, Novex TBE running buffer, and SYTO 60 red fluorescent nucleic acid stain were from Invitrogen Life Technologies. Calf thymus DNA, copper(II) chloride, casein, diethylenetriaminepentaacetic acid (DTPA), polydeoxyguanylic acid · polydeoxycytidylic acid sodium salt (poly(dG) · poly(dC)), poly(deoxyguanylic–deoxycytidylic) acid sodium salt (poly(dG-dC) · poly(dG-dC)), polydeoxyadenylic acid · polythymidylic acid sodium salt (poly(dA) · (dT)), poly(deoxyadenylic–thymidylic) acid sodium salt (poly(dA-dT) · poly(dA-dT)), glyoxal trimer dihydrate, and all other chemicals were from Sigma Chemical Co. The chicken polyclonal antibody to anti-5,5-dimethyl-2-(8-octonoic acid)-1-pyrrolone-*N*-oxide conjugated to bovine serum albumin (anti-DMPO adduct) was made by Aves Labs in a way similar to that described previously for polyclonal rabbit anti-DMPO adduct antibody [13]. The hybridoma clone N1664A that produces mouse anti-DMPO adduct monoclonal antibody was grown in a humidified incubator in 5% CO_2 at 37 °C in 10% (vol/vol) low IgG fetal bovine serum, 90% (vol/vol) Iscove's modified Dulbecco's medium supplemented with 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. The monoclonal antibody was purified by protein G chromatography in-house, but this antibody can be obtained commercially.

Preparation of DNA radicals and spin trapping with DMPO

DNA was incubated at 37 °C with copper(II) chloride, hydrogen peroxide, and DMPO in phosphate-buffered saline (PBS; 2 mM potassium phosphate, 8 mM sodium phosphate, 2.7 mM potassium chloride, and 137 mM sodium chloride, pH 7.4) with DMPO being added last. After 1 h, DTPA was added to a final concentration of 1 mM to terminate the reaction. The DNA was precipitated with 1/10 volume 3 M sodium acetate, pH 5.2, and 2 volumes ice-cold ethanol and incubated for 10 min at room temperature (RT) because the DMPO precipitated in this mixture if incubated at 4 °C. The DNA was centrifuged at 13,000 rpm for 15 min at RT, washed with 70% (vol/vol) ethanol, and redissolved in 10 mM Tris, 1 mM EDTA, pH 8.0.

DNA electrophoresis

DNA was denatured immediately before electrophoresis by adding deionized formamide to a final concentration of 60% (vol/vol), with 1/10 volume 10× orange loading dye and 1 μl 5 μM SYTO 60 (for sample volumes ranging from 10 to 30 μl). The samples were denatured by heating for 5 min at 65 °C, followed by immediate chilling on ice for 5 min before loading onto the gel. DNA to be run under native conditions was mixed with 1/5 volume 10× orange loading dye and 1 μl 5 μM SYTO 60 and incubated for 5 min at RT. DNA (5 μg/lane) was electrophoresed on either 1% (wt/vol) agarose gels in TAE (40 mM Tris–acetate, 1 mM EDTA) for 45 min at 90 V or on 6% (wt/vol) DNA retardation