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Original Contribution

In vivo detection of free radicals in mouse septic encephalopathy using molecular MRI and immuno-spin trapping

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ABSTRACT

Free radicals are known to play a major role in sepsis. Combined immuno-spin trapping and molecular magnetic resonance imaging (MRI) was used to detect in vivo and in situ levels of free radicals in murine septic encephalopathy after cecal ligation and puncture (CLP). DMPO (5,5-dimethyl pyrroline *N*-oxide) was injected over 6 h after CLP, before administration of an anti-DMPO probe (anti-DMPO antibody bound to albumin–gadolinium–diethylene triamine pentaacetic acid–biotin MRI targeting contrast agent). In vitro assessment of the anti-DMPO probe in oxidatively stressed mouse astrocytes significantly decreased T_1 relaxation ($p < 0.0001$) compared to controls. MRI detected the presence of anti-DMPO adducts via a substantial decrease in % T_1 change within the hippocampus, striatum, occipital, and medial cortex brain regions ($p < 0.01$ for all) in septic animals compared to shams, which was sustained for over 60 min ($p < 0.05$ for all). Fluorescently labeled streptavidin was used to target the anti-DMPO probe biotin, which was elevated in septic brain, liver, and lungs compared to sham. Ex vivo DMPO adducts (qualitative) and oxidative products, including 4-hydroxynonenal and 3-nitrotyrosine (quantitative, $p < 0.05$ for both), were elevated in septic brains compared to shams. This is the first study that has reported on the detection of in vivo and in situ levels of free radicals in murine septic encephalopathy.

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Oxidative stress-generated reactive oxygen (and nitrogen) species (ROS/RNS)[2] play a pathogenic role in many diseases, either as modulators of signal transduction or as causative agents of tissue injury. Understanding the extent and timing of free radical-triggered events in an in vivo environment is of importance to our understanding of these major determinants involved in disease evolution and prognosis. With the combined use of molecular magnetic resonance imaging (mMRI) and immuno-spin trapping (IST) technologies, it is possible for the first time to monitor radicals in vivo and in situ in a mouse model for septic encephalopathy.

Numerous studies indicate that oxidative stress, a result of an imbalance in the levels of ROS and antioxidative defense systems, plays a crucial role in sepsis. Sepsis is associated with the increased generation of ROS [1–5], which leads to multiple organ dysfunctions [6], including encephalopathy and its cognitive consequences [7]. ROS/RNS may directly oxidize nucleic acids, proteins, carbohydrates, and lipids, causing intracellular and intercellular perturbations in homeostasis [8]. As a consequence, high concentrations of lipid-derived electrophilic products readily react with proteins, DNA, and

Abbreviations: anti-DMPO probe, anti-DMPO-biotin-BSA-Gd-DTPA; BBB, blood–brain-barrier; BSA, bovine serum albumin; CLP, cecal ligation and puncture; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; Gd-DTPA, gadolinium diethylene triamine pentaacetic acid; HNE, 4-hydroxynonenal; IST, immuno-spin trapping; mMRI, molecular magnetic resonance imaging; 3-NT, 3-nitrotyrosine; OMRI, Overhauser-enhanced MRI; ROS/RNS, reactive oxygen/nitrogen species

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phospholipids, generating intra- and intermolecular toxic covalent adducts that lead to the propagation and amplification of oxidative stress [8].

Free radicals generated as a result of oxidative stress processes can be tagged by 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) to form DMPO–radical adducts, which can be detected and measured by IST, a method that utilizes an antibody against DMPO adducts [9–12]. To better understand disease pathogenesis, it would be ideal if the formation of oxidation products could be assessed in vivo and in situ, allowing specific cause–consequence relationships to be identified from specific oxidative events. This approach would allow the correlation of the detection of real-time oxidative stress markers with specific longitudinal pathological conditions associated with a particular disease.

There are other imaging methods that have been used to detect radicals, including ESR (electron spin resonance), fluorescence imaging, and Overhauser-enhanced MRI (OMRI). OMRI potentially offers a method of detecting low concentrations of free radical species generated by specific biological processes; however, spatial resolution and general radical detection is limited by sensitivity requirements and only nonspecific probes have been validated [13]. ESR imaging is sensitive, but limited to the detection of an injected paramagnetic probe at the injection site and not the general detection of a broad range of radicals. Fluorescence imaging is restricted to excised tissues or isolated cells. Recently in vivo nitroxide-enhanced MRI, which shows some promise, was done in tumor (neuroblastoma or colon cancer)-bearing mice, in which the appearance or disappearance of the MRI signal indicated a change in the nitroxide (piperidine- or TEMPO-type derivatives) redox cycle, i.e., reduction to the hydroxylamine resulted in the loss of MRI contrast and oxidation resulted in the presence of MRI contrast [14].

In a novel approach, we have combined a Gd-DTPA-albumin-based contrast agent for signal detection with the specificity of antibodies (Abs) for DMPO radicals (anti-DMPO probe) with the desired morphological image resolution of mMRI to detect in vivo free radicals (see Fig. 1). The anti-DMPO probe was used in this study to assess free-radical formation in the brain, liver, and lung tissues in a mouse model of sepsis.

Materials and methods

Synthesis of DMPO-specific MRI contrast agents

To recognize the DMPO–protein/lipid radicals, a mouse monoclonal anti-DMPO antibody bound to a contrast agent was used. The macromolecular contrast material, biotin-BSA-Gd-DTPA, was prepared using a modification of the method of Dafni et al. [15]. The biotin moiety in the contrast material was added to allow histological localization. Biotin-BSA-Gd-DTPA was synthesized as described in Towner et al. [16,17]. A solution of biotin-BSA-Gd-DTPA was added directly to the solution of antibody (anti-DMPO, 200 μg/ml) for conjugation through a sulfo-NHS-EDC link between albumin and antibody according to the protocol of Hermanson [18]. The product was lyophilized and subsequently stored at 4 °C and reconstituted to the desired concentration for injections in phosphate-buffered saline (PBS). The final amount of the product, anti-DMPO-biotin-BSA-Gd-DTPA, that was injected into the mice was estimated to be 20 μg anti-DMPO Ab/injection and 10 mg biotin-BSA-Gd-DTPA/injection. The estimated molecular weight of the anti-DMPO-biotin-BSA-Gd-DTPA probe was estimated to be 232 kDa.

In vitro characterization of anti-DMPO probe

Vials were prepared containing water, primary mouse astrocytes alone, astrocytes with the anti-DMPO probe, astrocytes + DMPO + anti-DMPO probe, astrocytes with the oxidant hydrogen peroxide (H_2O_2) and DMPO, or astrocytes with H_2O_2 + DMPO + anti-DMPO probe ($n = 5$ for each group). Cells (mouse astrocytes, CC-3187, Lonza Walkersville, Walkersville, MD, USA) were grown to confluency in flasks in complete growth medium (ABM basal medium, Lonza Walkersville). Two to three hours before treatment, the growth medium was replaced with serum-free medium. DMPO (40 mM) was added to the appropriate vials, and after 15 min to reach equilibrium, H_2O_2 (50 μM) was added to the appropriate vials. In the samples that were treated with the anti-DMPO probe, the probe was added (2 μg, based on antibody

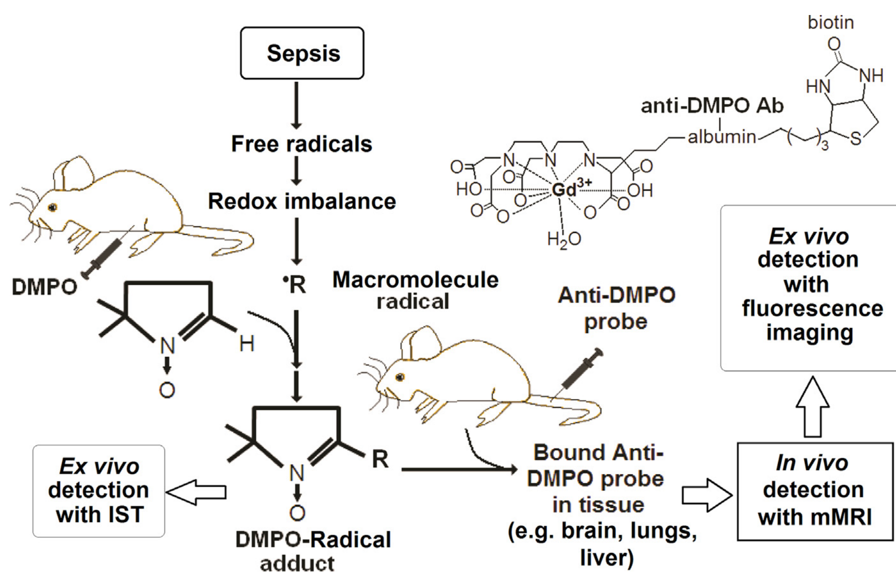

Fig. 1. Approach for combined in vivo mMRI and IST. Immuno-spin trapping of macromolecular radicals (•R) with anti-DMPO mMRI probe (anti-DMPO–albumin–Gd–DTPA–biotin mMRI probe). DMPO is injected ip to trap macromolecular radicals and generate DMPO–radical adducts that decay to DMPO nitrone adducts. Anti-DMPO is injected iv to target DMPO–radical nitrone adducts, which can be visualized by mMRI.

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