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Original Contribution

Free radical-operated proteotoxic stress in macrophages primed with lipopolysaccharide

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ABSTRACT

The free-radical-operated mechanism of death of activated macrophages at sites of inflammation is unclear, but it is important to define it in order to find targets to prevent further tissue dysfunction. A well-defined model of macrophage activation at sites of inflammation is the treatment of RAW 264.7 cells with lipopolysaccharide (LPS), with the resulting production of reactive oxygen species (ROS). ROS and other free radicals can be trapped with the nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), a cell-permeable probe with antioxidant properties, which thus interferes with free-radical-operated oxidation processes. Here we have used immuno-spin trapping to investigate the role of free-radical-operated protein oxidation in LPS-induced cytotoxicity in macrophages. Treatment of RAW 264.7 cells with LPS resulted in increased ROS production, oxidation of proteins, cell morphological changes and cytotoxicity. DMPO was found to trap protein radicals to form protein–DMPO nitrone adducts, to reduce protein carbonyls, and to block LPS-induced cell death. *N*-Acetylcysteine (a source of reduced glutathione), diphenyleneiodonium (an inhibitor of NADPH oxidase), and 2,2′-dipyridyl (a chelator of Fe^{2+}) prevented LPS-induced oxidative stress and cell death and reduced DMPO–nitrone adduct formation, suggesting a critical role of ROS, metals, and protein-radical formation in LPS-induced cell cytotoxicity. We also determined the subcellular localization of protein–DMPO nitrone adducts and identified some candidate proteins for DMPO attachment by LC-MS/MS. The LC-MS/MS data are consistent with glyceraldehyde-3-phosphate dehydrogenase, one of the most abundant, sensitive, and ubiquitous proteins in the cell, becoming labeled with DMPO when the cell is primed with LPS. This information will help find strategies to treat inflammation-associated tissue dysfunction by focusing on preventing free radical-operated proteotoxic stress and death of macrophages.

Introduction

Inflammatory activation of macrophages occurs in atheromatous plaques [1], inflamed adipose tissue in obesity [2,3], and tumors [4]. This activation is associated with the death of macrophages and tissue dysfunction [5], but the role of free-radical-operated processes has not received the attention it deserves—mostly due to the limitations of studying them in biological systems. Treatment of macrophages with bacterial lipopolysaccharide (LPS) is a well-established model for studying the fate of macrophages at sites of inflammation [6].

A number of reactive oxygen species (ROS) are produced inside macrophages primed with LPS. For instance, superoxide radical

Abbreviations: carboxy-H_2DCFDA, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DAPI, 4′-6-diamidino-2-phenylindole; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMSO, dimethyl sulfoxide; DP, 2,2′-dipyridyl; DPI, diphenyleneiodonium; ELISA, enzyme-linked immunosorbent analysis; ESR, electron-spin resonance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; •NO, nitric oxide; NOX-1/2, NADPH oxidase-1 and -2; $^{\bullet}O_2^-$, superoxide radical anion; PBN, alpha-phenyl-*N*-*tert*-butyl nitrone; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

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anion ($^{\bullet}O_2^-$) is produced primarily, but not exclusively, by NADPH oxidases [7–9]. Other recognized sources of $^{\bullet}O_2^-$ in activated macrophages are mitochondria [10], uncoupling of inducible nitric oxide synthase (iNOS) [11], and endoplasmic reticulum stress [12,13]. Another reactive species, nitric oxide ($^{\bullet}NO$), is produced by iNOS in RAW 264.7 cells and primary macrophages primed with LPS [14]. Superoxide can be dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase, or it can react with $^{\bullet}NO$ to form peroxynitrite [15,16]. Peroxynitrite has been shown to nitrate proteins in cells with the intermediacy of transient protein-centered radicals [17].

H_2O_2, a cell-permeable, two-electron oxidant, cannot cause protein-centered radicals due to its low chemical reactivity; however, H_2O_2 reactivity can be enhanced by redox-active transition metals, either free or bound to proteins, to produce hydroxyl radicals [18,19]. Hydroxyl radicals can randomly damage lipids, proteins, and nucleic acids with the formation of macromolecule-centered free radicals as transient intermediates [20,21]. Decay of these intermediates is a complex process that depends on a number of microenvironmental cues [22,23]; one of the most characterized mechanisms is their reaction with molecular dioxygen to form peroxyl radicals [22]. Peroxyl radicals decompose to produce a number of end-oxidation products.

In the case of protein-centered radicals, the end products include aldehydes and carbonyls [22]. Although carbonyl formation in proteins is considered a marker of protein oxidation by free radical processes [18], a number of reactive lipid oxidation-derived carbonyl products (e.g., 4-hydroxynonenal) can form adducts with proteins, resulting in increased protein carbonylation [24,25]. This class of reactions compromises the specificity of carbonyls as markers of free radical-mediated protein oxidation processes. Therefore, the detection of protein-centered radicals is a critical proof of protein oxidation by one-electron or free-radical-operated oxidation mechanisms.

Characterization of protein-centered radicals in biochemistry has been possible since the development of electron spin resonance (ESR), the conventional technique that is used to study free radicals [26]. However, because free radicals are unstable and highly reactive species, their detection in cells was a challenge until the development of spin trapping [27]. Spin trapping makes use of organic compounds to react with the free radicals to produce radical adducts with longer half-lives and thus facilitate their detection by ESR [28]. 5,5-Dimethy-l-pyrroline *N*-oxide (DMPO), a nitrone spin trap, passes across cell membranes, effectively traps a number of small and large free radicals, and possesses low toxicity [27], making it a good spin trap for investigating free radicals in biological systems.

We have previously found that protein-centered radicals that are trapped by DMPO as nitrone adducts are protected from further oxidation [21]. Herein, we hypothesized that if free radical operated processes are involved in LPS-induced cell damage, then DMPO should be able to affect this process and at the same time "tag" specific macromolecules undergoing one-electron oxidation, which might or might not be directly involved in the death process. To test our hypothesis, we have here used the DMPO-based immuno-spin trapping technique ([29] and references therein). This technique is based on the use of DMPO, which traps protein-, lipid-, carbohydrate-, and nucleic acid-centered radicals to form radical adducts which then decay to stable nitrone adducts. Protein– and DNA–nitrone adducts can be subsequently determined by immunoassays using an anti-DMPO serum and mass spectrometry methods [30,31].

We found that DMPO prevented LPS-induced proteotoxic damage in macrophages and tagged a number of proteins inside the cell. We corroborated the identity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), one of the most ubiquitous, abundant, and redox-sensitive proteins in cells [32], as one of the major targets of free radical-mediated oxidation in cells primed with LPS. The information gathered herein will help in understanding how free radical-operated protein oxidation occurs during macrophage death at sites of inflammation, and thus will provide new avenues for intervention in subsequent tissue dysfunction processes.

Materials and methods

Materials

Murine macrophage-like RAW 264.7 cells were purchased from the American Type Culture Collection (TIB-71, Rockville, MD). DMPO ($\varepsilon_{228}=7800\ M^{-1}\ cm^{-1}$) and rabbit polyclonal anti-DMPO antibody were purchased from Alexis Biochemicals (San Diego, CA). LPS (*Escherichia coli* serotype 055:B5, L2637), *N*-acetylcysteine (NAC), and rabbit anti-dinitrophenyl were from Sigma (St. Louis, MO). The goat anti-rabbit IgG and goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatments with LPS and DMPO

RAW 264.7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Sigma, D-5796) supplemented with 10% fetal bovine serum (Advantage FBS, Atlanta Biologicals, Lawrenceville, GA), referred to as complete medium. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and split 2–3 times weekly. Cells between passages 3 and 30 were used in this study. Before the treatments with LPS and DMPO, cells were allowed to stabilize overnight. Medium was then removed and replaced by the indicated concentrations of LPS and/or DMPO in complete medium. Typically, cells were incubated for 24 h in complete medium containing 1 ng/ml LPS and 50 mM DMPO.

Cytotoxicity assays

Cell viability was determined by assaying mitochondrial reduction of a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to purple formazan crystals [33]. We also assessed the release of lactate dehydrogenase (LDH) to the culture medium as a marker of membrane damage by using a commercial kit (Bioassay Systems, Hayward, CA). Alternatively, we measured cell viability by assessing total cell protein and DNA remaining bound to culture plates after the described treatments and three washes with PBS to remove unattached (nonviable) macrophages. Briefly, cells were grown and treated with LPS and DMPO in black-walled, clear-bottom, 96-well plates (BD Biosciences, San Jose, CA), and DNA was quantified using a CyQUANT cell proliferation assay kit (Invitrogen) and bacteriophage λ DNA as a standard. In another experiment, after a rinse of the monolayers with PBS, proteins bound to the plate were dissolved in 50 µl of 1 M NaOH followed by determination of proteins using a BCA (bicinchoninic acid) protein assay kit (Thermo Fisher Scientific, Rockford, IL).

Measurement of ROS production

Intracellular ROS production was estimated using carboxy-H_2DCFDA, which becomes fluorescent in living cells in response to various ROS including $^{\bullet}OH$, H_2O_2, and $ONOO^-$ [34]. Cells were incubated with 25 µM carboxy-H_2DCFDA for 30 min, washed twice, and then treated with 0 to 1 ng/ml LPS for another