We have demonstrated that nitroxides at non-toxic concentrations are effective as *in vitro* and *in vivo* antioxidants when oxidation is induced by superoxide, hydrogen peroxide, organic hydroperoxides, ionizing radiation, or specific DNA-damaging anticancer agents. The protection of oxidative damage in biological systems (both *in vitro* and *in vivo*) by non-toxic levels of nitroxides has several plausible chemical explanations: 1) SOD-mimicking action; 2) oxidation of reduced metals that have potential to generate site specific -OH radicals; 3) termination of free radical chain reactions induced by alkyl, alkoxyl, alkylperoxyl radical species, and detoxifying drug-derived radicals; and 4) detoxification of hypervalent toxic metal species such as ferryl and cupryl ions. Examples of the protection of nitroxides against oxidative stress at the cellular and animal level, proposed chemical mechanisms underlying the protective action(s), and the potential use of nitroxides in clinical settings is presented.

Additionally, the application and feasibility of nitroxides and other paramagnetic probes for *in vivo* Electron Paramagnetic Resonance imaging (EPRI) to study probe uptake, oxygen concentration in tissues, and tissue redox reactivity is discussed. The development of "functional imaging" approaches, in addition to providing the physical architecture of a structure, will provide physiological/metabolic information about the structure. EPRI, a magnetic resonance technique similar to Magnetic Resonance imaging, probes the magnetic properties of species containing unpaired electrons (free radicals, transition metals, etc.). With the availability of exogenous, non-toxic, biologically compatible free radical probes, EPRI has the potential to provide, in a non-invasive manner, valuable physiologic information in three dimensions. For example, nitroxides are redox sensitive probes, which are useful to non-invasively delineate tissue heterogeneity such as that occurring between normal and malignant tissue with respect to distribution, redox status, and oxygen concentration. Measurements using spin label oximetry showed significant hypoxia in tumors compared to normal tissue. These results suggest that tumor hypoxia results in more rapid reduction of nitroxides than in normal tissue, which in turn may explain the selective radioprotection of normal tissue by nitroxides.

1. Samuni A, Krishna CM, Riesz P, Finkelstein E, Russo A. A novel metal-free low molecular weight superoxide dismutase mimic. J Biol Chem **263**:17921–17924, 1988.
2. Mitchell JB, Samuni A, Krishna MC, DeGraff WG, Ahn MS, Samuni U, Russo A. Biologically active metal-independent superoxide dismutase mimics. Biochemistry **29**:2802–2807, 1990.
3. Mitchell JB, DeGraff W, Kaufman D, Krishna MC, Samuni A, Finkelstein E, Ahn MS, Hahn SM, Garrison J, Russo A. Inhibition of oxygen-dependent radiation-induced damage by the nitroxide superoxide dismutase mimic. Tempol Arch Biochem Biophys **289**:62–70, 1991.
4. Krishna MC, Grahame DA, Samuni A, Mitchell JB, Russo A. Oxoarnmonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide. Proc Natl Acad Sci **89**:5537–5541, 1992.
5. DeGraff WG, Krishna CM, Russo A, Mitchell JB. Antimutagenicity of a low molecular weight superoxide dismutase mimic against oxidative mutagens. Environ Molec Mutagen **19**:21–26, 1992.
6. Hahn SM, Tochner Z, Krishna CM, Glass J, Wilson L, Samuni A, Sprague M, Venzon D, Glatstein E, Mitchell JB, Russo A. Tempol, a stable free radical, is a novel murine radiation protector. Cancer Res **52**:1750–1753, 1992.
7. Krishna MC, Samum A, Taira J, Goldstein S, Mitchell JB, Russo A. Stimulation by nitroxides of catalase-like activity of hemeproteins. J Biol Chem **271**:26018–26025, 1996.
8. Hahn SM, Sullivan FJ, DeLuca AM, Krishna MC, Wersto N, Venzon D, Russo A, Mitchell JB. Evaluation of tempol radioprotection in a murine tumor model. Free Radic Blol Med **22**:1211–1216, 1997.
9. Krishna MC, DeGraff W, Hankovszky 0H, Sar CP, Kala IT, Jeko J, Russo A, Mitchell JB, Hideg K. Studies of structure-activity relationship of nitroxide free radicals and their precursors as modifiers against oxidative damage. J Med Chem **41**:3477–3492, 1998.
10. Kuppusamy P, Afeworki M, Shankar RA, Coffin D, Krishna MC, Hahn SM, Mitchell JB, Zweier JL. In vivo electron paramagnetic resonance imaging of tumor heterogeneity and oxygenation in a murine model. Cancer Res **58**:1562–1568, 1998.

Cytotoxicity Related to Oxidative and Nitrosative Stress by Nitric Oxide

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The role of nitric oxide (NO) in toxicology and pathophysiology has received considerable attention since its discovery as an endogenous mediator of different physiological functions. As additional biological aspects of NO have been discovered, speculation has emerged that this diatomic radical plays quintessential roles in various pathophysiological mechanisms. This has lead to considerable effort to understand the underlying mechanisms involving NO during pathophysiological events, to possibly point to therapeutic strategies.

The chemistry is the most important determinant of NO in biological systems. However, deciphering the chemistry

of NO in biological systems can be complex with respect to its diverse biological effects and numerous potential chemical reactions. This can leave a large number of possible mechanistic explanations. To reduce these possibilities to a manageable level, we have formulated a scheme called "the chemical biology of NO." This scheme describes the relevant chemical reactions of NO and predicts where they may occur *in vivo.*

There are two distinct categories in the chemical biology of NO: direct and indirect effects. Direct effects are those chemical reactions where NO reacts directly with its biological target. Conversely, the indirect effects are mediated by reactive nitrogen oxide species (RNOS) which are derived from NO metabolism. The direct effects are very rapid reactions that occur at low NO concentrations (< I uM) and generally involve heme proteins such as guanylate cyclase, cytochrome P450, and hemoglobin. The indirect effects require that NO is first activated by superoxide (O_2–) or oxygen to form RNOS which then undergo further reactions with the respective biological target. These RNOS are highly reactive with biological macromolecules such as protein, lipids, and DNA and are thought to be responsible for NO-mediated cell death. These reactions are relevant only when the concentrations of NO are high locally (> 1 uM) for prolonged periods of time, such as in the vicinity of activated leukocytes or other cells sensitive to stimulation by cytokines or pathogenic products.

The indirect effects can be further subdivided into nitrosative and oxidative stress. Traditionally, oxidative stress has referred to conditions where oxygen radical species mediate deleterious effects *in vivo.* Although enzymes such as cytochrome P450 form powerful oxidants under normal physiological conditions, these chemical reactions are confined to a protein active site. Yet, when oxidants such as O_2– and H_2O_2 reach fluxes that begin to overwhelm the endogenous defense systems, chemical alterations of biological molecules can occur such that the biological system becomes stressed. Recently, it has been shown that RNOS can mediate some of the same kind of chemistry as reactive oxygen species (ROS). This implies that NO may be metabolized to species that mediate oxidative stress.

There are two primary chemical pathways from which oxidative stress is derived: reactive oxygen species or reactive nitrogen oxide species. ROS are formed from O_2– and H_2O_2 through a series of reactions which are referred to as Haber-Wiess chemistry. Specific transition metals, such as iron, react with peroxides/superoxide to form powerful metal-oxo species or hydroxyl radicals. In contrast, oxidative chemistry mediated by RNOS are mediated primarily by two nitrogen oxide species, peroxynitrite (ONOO–) and nitroxyl (NO–). Peroxynitrite originates from the reaction between NO and O_2–, while NO– can result from a variety of chemical pathways.

It has long been known that ROS derived from hydrogen peroxide (H_2O_2), O_2–, and alkylhydroperoxide result in powerful oxidants that can mediate cell death. Recently, there has been some discussion as to whether NO augments or abates ROS toxicity. It has been argued that ONOO– formed from the interaction of NO and O_2– is a powerful oxidant, and thus must also be a potent cytotoxin . However, comparing the cytotoxicity of ROS and RNOS in fibroblast cells, it shows potency as follows: H_2O_2, > t-alkylhydroperoxide ~ HNO/NO– >> ONOO– (intermediates such as ONOO– formed from the NO/O_2– reaction are orders of magnitude less toxic). Moreover, NO is a potent antioxidant and protects cells from ROS mediated cell death. Examination of the effect of NO on ROS levels generated during ischemic reperfusion injury shows that NO dramatically reduces oxidative stress and could provide a potential therapeutic strategy.

Nitroxyl has recently been proposed as an important component of NO metabolism and may be a product of NOS. NO– can oxidize various substrates but does not mediate nitrosative chemistry. Examination of NO– chemistry showed it to be a powerful cytotoxin that mediated double stranded DNA breaks. Further studies showed that interaction between NO– and oxygen is required for cytotoxicity. The chemistry of NO– suggests that the oxidant is not ONOO– but a hydrated species (O(O)NOO_3'–).

Nitrosative stress occurs when intermediates are produced from nitrosate thiol, hydroxy and amine groups. Unlike the variety of possible intermediates that could mediate oxidative stress, the primary initiator of nitrosative stress under physiological conditions is N_2O_3. This species is not as potent an oxidant as those that mediate oxidative stress. Rather, its primary reaction is to donate an equivalent of NO+ to different biological molecules. N_2O_3 is formed primarily at neutral pH either from the NO/O_2 reaction or the NO/O_2– reaction when the flux of NO is higher than that of O_2–. Due to the rate laws of these and competing reactions, nitrosative stress can only occur in the presence of high localized fluxes of NO. Although at first glance the kinetic rate laws may suggest that nitrosative stress does not occur *in vivo* there are numerous reports that show nitrosation of amines under inflammatory conditions. Thus nitrosative stress (i.e., N_2O_3 formation) does exist *in vivo.*

When mammalian cells were exposed to nitrosative stress by addition of NO donor little toxicity was observed (< 20–40%). Nitrosating intermediates have the greatest affinity for thiols such as glutathione (GSH) suggesting that they are a primary target under nitrosative stress conditions. Cells depleted of GSH were dramatically more susceptible to toxicity from nitrosative stress. In addition, proteins rich in thiols such as metallothionein were also shown to be protective against N_2O_3. However, if toxic metals such as cadmium are sequestered in metal I othionein, nitrosative stress resulted in release of metal and a subsequent marked enhancement of metal mediated toxicity. It has been reported using Raman spectroscopy that a RSNO adduct is formed upon exposure to NO in an aerobic environment. From these studies, it was suggested that the formation of

S-nitrosothiol adducts under conditions of nitrosative stress play a key role in various toxicological mechanisms.

In several studies, the formation of S-nitrosothiol adducts with protein has been suggested as the important step in the inhibition of a variety of enzymes. For example, S-nitrosothiol adduct stimulates ADP ribosylation of glyceraldehyde phosphate dehydrogenase. Additionally, S-nitrosation inhibits DNA alkyl transferase activity both *in vitro* and *in vivo* and results in the potentiation of the toxicity of alkylating agents such as chemotherapeutic agents. Proteins containing zinc finger motifs lose their structural integrity upon exposure to NO, resulting in inhibition of enzyme activity.

1. Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB. The multifaceted roles of nitric oxide in cancer. Carcinogenesis **19:**711–721, 1998
2. Wink DA, Mitchell JB. The Chemical Biology of Nitric Oxide: Insights into Regulatory, Cytotoxic and Cytoprotective Mechanisms of Nitric Oxide. Free Rad Biol Med **25:**434–456, 1998
3. Grisharn MB, Jourd'Heuil D, Wink DA. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites: implications in inflammation. Am J Physiol **276:**G315–G321, 1999