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A New Hammer in the Redox Toolbox: High-Purity Peroxynitrite for Cell Signaling and Toxicology Studies

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Abstract

A new chemical synthesis has been disclosed for the oxidizing and nitrating species peroxynitrite. This nitric oxide and superoxide-derived cell signaling mediator can also serve as a toxicant at higher rates of generation. The new synthetic strategy reported by Sturzbecher-Höhne and colleagues overcomes long-standing issues with purity and quality of peroxynitrite preparations and should lay a foundation for nailing new insight into the redox reactions of peroxynitrite and its products.

For almost two decades, peroxynitrite (ONOO^-) has been a source of both exciting discovery and vibrant debate within the broad community of chemically oriented biologists. First synthesized in the early 1900s and then appreciated as a biological mediator of cell signaling and toxicity in 1990, investigation of this product of superoxide and nitric oxide reaction has expanded our insight into how both the basal and the inflammatory production of these species converge on a panoply of levels (1). Because of the almost diffusion-limited reaction rate of its ubiquitous precursors, one can expect to always contend with the direct and secondary reactions of ONOO^- . The reactions of ONOO^- are dictated by rates of precursor production, anatomic location, and critical characteristics of the local milieu that include proton, thiol, carbon dioxide, and antioxidant enzyme concentration. This local milieu defines both the steady-state concentration of ONOO^- and its spectrum of products including peroxynitrous acid, nitrogen dioxide, hydroxyl radical, and carbonate radical (Figure 1).

An abundance of support underscores the formation and clinically significant actions of ONOO^- and its products in biological systems. This support stems from (1) computational predictions, (2) the suppression of superoxide and nitric oxide concentrations, and (3) the use of probes of varying specificity. Work in this area has solidly established the contribution of ONOO^- to the fundamental regulation of redox-dependent cell signaling, hemostasis, and host defense. Also, when xenobiotic exposure and inflammatory responses accelerate the generation of superoxide and nitric oxide, ONOO^- further contributes to autoimmune, neurodegenerative, apoptotic, genotoxic, and an abundance of target molecule reactions that affect all aspects of cellular existence (2).

While stabilized as an anion at high pH, even the purest ONOO^- has a relatively short half-life under physiological conditions due to rapid reaction with biological targets and unimolecular decomposition via rearrangement or homolytic scission. Critics have

suggested that bolus injection of ONOO^- into reaction systems or infusion into in vivo models is not physiologically relevant, as ONOO^- is more plausibly formed in specific microenvironments at low steady-state concentrations, with the local chemical milieu also influencing downstream reactions. Furthermore, detection of ONOO^- in cells relies entirely on the analysis of secondary products, sometimes at the mercy of endogenous scavengers and alternative reactions. The most useful markers for ONOO^- formation in this context are nitration and hydroxylation products and the dimerization of tyrosine residues. Mass spectrometric and immunodetection of nitrotyrosine is a detection method typically applied for the presence of biological ONOO^- formation. The use of synthetic ONOO^- in model systems and the rigorous use of controls in more biological systems (e.g., ONOO^- scavengers and suppression of superoxide and nitric oxide concentrations) have provided a solid foundation of knowledge that encourages the significance of this species as a dynamic redox signaling mediator and, at higher rates of production, a toxicant.

Why has there been such a robust debate over the extents of biological generation and the actions of ONOO^- ? By virtue of our training and first-hand experience as investigators unraveling the biochemistry and toxicology of evanescent species, we know one hard-earned truth—Definitive and quantitative answers to questions in redox biology are often lacking when one grapples with short-lived and promiscuously reactive species. Because of the overlapping molecular targets and products of many biological “redox mediators” and the multiplicity of reactive species present in any cellular milieu, it becomes imprudent to ascribe a particular product or differentiated cell response to any one specific reaction or mediator. Importantly, the experimental models and reagents that we employ in redox biology are frequently subject to an interpretation colored by our biases and the vagaries of both detection systems and reagents. Thus, debate becomes a natural and necessary part of the scientific process in this area. Good fortune in all of these regards now comes our way with the landmark observation reported by Sturzbecher-Höhne, Kissner, Nauser, and Koppenol in this issue of *Chemical Research in Toxicology* (3). This report eliminates a key imponderable, the purity of ONOO^- used in in vitro reactions, allowing for future discoveries that are less clouded by unappreciated ancillary reactions.

Peroxynitrite generated and shared by investigator laboratories often falls short of purity. Contaminants of ONOO^- produced by typical methods include nitrite, nitrate, peroxide, and azide (4). All of these species can display biological effects that obscure the development of cause and effect relationships specifically related to ONOO^- . Even ONOO^- generation using the most reliable of methods still requires extensive controls to account for the presence of impurities. Fortunately, ONOO^- can be identified and quantified spectrophotometrically when in pure form and stored for reasonable lengths of time under proper conditions, and many of the confounding impurities can also be quantified if not eliminated. Still, debate over the stability, purity, and actions of ONOO^- continues.

The more facile synthesis (5) of ONOO^- uses hydrogen peroxide oxidation of sodium nitrite followed by rapid addition of base to afford stable sodium peroxynitrite as an aqueous solution, preferably by means of an elaborate flow-reactor structure of syringe pumps, tubing, and experienced timing. The product is expected to contain significant chloride, nitrite, nitrate (both of the latter detectable by the Griess reaction), and residual hydrogen

peroxide, which can be decomposed by manganese dioxide addition. Freeze fractionation can also provide a degree of additional concentration.

The major alternative (6) method for ONOO^- preparation requires significant dedicated equipment. This two-step procedure begins with potassium superoxide, which is milled (an explosive hazard) and metathesized to tetramethylammonium superoxide. This superoxide salt is reacted in liquid ammonia with freshly generated nitric oxide. Exciting work to be sure! The product ONOO^- is analytically pure, containing no metal ions, nitrite, nitrate, or peroxides. Fortunately, this procedure has been adapted to commercial production of pure tetramethylammonium peroxynitrite. Unfortunately, the unnatural tetramethylammonium counterion may introduce problems of its own.

As evidence of the great creativity within the field, recent alternatives take a more radical approach: the use of chemicals that spontaneously decompose within the cellular milieu to yield peroxynitrite. This includes combining chemical or enzymatic NO donors such as the NONOates (diazoniumdiolates) with superoxide coming from enzymatic sources, hyponitrites or SIN-1 (3-morpholinosyndodimine)—the latter of which generates both precursors of ONOO^- in a manner unfortunately complicated by cellular electron transfer reactions. These approaches have partially, but not fully, alleviated spatial distribution issues regarding sites of reactive species generation and transport into cells but still present significant challenges with respect to adventitious impurities. The intracellular concentrations of ONOO^- precursors and products can only be estimated by relative kinetic rates and include an expected amount of potentially complicating decomposition byproducts.

Thanks to the fundamental significance of discoveries in redox signaling and the pathobiology of reactive species, the synthetic and logistical challenges inherent in studying the actions of ONOO^- will be in constant need of revisiting and revision. One major development in this regard has been the appreciation of nitrite itself as a physiological mediator, since recent work has indicated a much more prominent role by nitrite as a source of nitric oxide via heme reduction under oxygen-depleted conditions—thus revealing nitrite as a bioavailable reservoir for nitric oxide and its secondary products (7). This insight may catalyze a reevaluation of ascribed effects of ONOO^- and further expand an appreciation of the contributions of ONOO^- to cell regulation and injury. As an example, early observations showing a putative vasodilatory effect of peroxynitrite may be reinterpreted in the light of subsequent work to be largely an effect of nitrite, itself a significant vasodilator. This, however, does not rule out still-possible direct and indirect vasodilatory signaling actions of ONOO^- . Clearly, the study of the formation and reactions of ONOO^- remains a vibrant area for discovery.

In this new report, Koppenol and colleagues give us a valuable and straightforward approach to addressing many of the vexing problems inherent in studying nitric oxide-derived reactive species. The ion-exchange column metathesis of commercially available, analytically pure tetramethylammonium salt to a biocompatible lithium or sodium salt quickly and easily affords useful amounts of ONOO^- without introducing new complications from high concentrations of unnatural counterions. Now, we have the best combination of conditions

when working with “pre-formed” ONOO⁻: clean material, simplified production, and biocompatibility.

The development of additional insight into the actions of ONOO⁻ relies on the purity of materials, the clarity of observations, and the tenacity and resourcefulness of the investigators. Future areas of discovery may include the development of novel intracellular production strategies for ONOO⁻ precursors to compare with bolus addition of ONOO⁻ that can even be modulated by mechanical syringe pumps. Perhaps more specific in vivo detection of ONOO⁻ formation and concentrations can be lent by the use of redox-sensitive fluorescent probes that display a high degree of discrimination, thus further supplementing the use of nitrotyrosine detection strategies. Our newfound ability to now work with “clean” ONOO⁻ should thus propel new discoveries and allow some controversies to be more affirmatively resolved.

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Figure 1.