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COMMENTARY

In Vivo Aspects of Nitric Oxide (NO) Chemistry: Does Peroxynitrite ($^{-}$OONO) Play a Major Role in Cytotoxicity?

Nitric oxide (NO) is a widespread biological mediator that not only represents the pharmacologically active species of nitrovasodilator drugs such as nitroglycerin but is also produced by vascular endothelial cells to regulate blood flow and thrombosis.[1] Although NO has been of significant interest to, among others, environmental and inorganic chemists for a long time, the discovery in the late 1980s of its biosynthesis in mammalian cells and numerous physiological roles led to a virtual explosion of NO-related research. As a tribute to the potential importance of NO in biology, it was chosen as "Molecule of the Year" in 1992 by the editors of *Science* magazine[2] and deemed "biochemistry's unexpected new superstar" on the cover of *Chemical and Engineering News* a year later.[3] Indeed, as remarkable as it may sound, a variety of cells are capable of biosynthesizing nitric oxide (NO) *via* the five-electron oxidation of one of the terminal guanidinium nitrogens on the amino acid arginine[4] (Figure 1).

The physiology of endogenous NO generation has been the subject of extreme interest, and it has thus far been determined that NO plays a key role in, among other things, the vascular system as a vasodilator and inhibitor of platelet function and the central and peripheral nervous systems.[5]

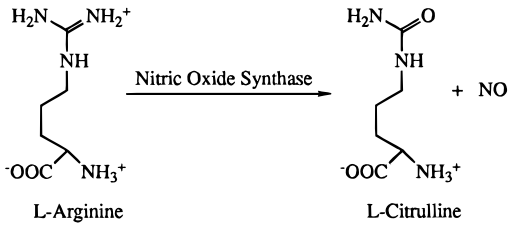

FIGURE 1. Oxidative conversion of L-arginine to NO and L-citrulline by the enzyme nitric oxide synthase.

In these systems, much of the biological activity of NO is due to its direct actions on the enzyme guanylate cyclase. That is, coordination to the enzyme-bound ferrous heme by NO results in a significant increase in intracellular guanylate cyclase-catalyzed generation of cGMP from GTP.[6] cGMP represents the intracellular second messenger that signals the appropriate cellular response, such as vascular smooth muscle relaxation or inhibition of platelet aggregation and adhesion.[6] The increases in cGMP are then responsible for much of the observed biological activity. NO also plays a major role in host defense mechanisms such as acute inflammation and host response to invasion by bacteria, viruses, and parasites. Landmark studies by John Hibbs and co-workers initially established NO as a species generated by phagocytic cells and postulated cytostatic/cytotoxic mechanisms based on its interaction with, for example, metal centers crucial for mitochondrial respiration.[7]

Along with the mechanisms of action associated with NO itself, it has been postulated that oxidized NO species may also play a role in its biological activity (especially with regard to the generation of cytostatic/cytotoxic species). Being a radical species, NO is capable of rapidly reacting with other biologically relevant radicals such as molecular oxygen and superoxide (O_2^{-}). The chemistry and biological significance

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(5) See for example: Nathan, C. *FASEB J.* **1992**, *6*, 3051−3064.
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of NO oxidation by molecular oxygen (both free and metal bound) have been the subject of numerous studies, and it is certain that such reactions are important to the physiology/toxicology of NO.[8] One of the potentially most significant and provocative reactions of NO is with O_2^-. The product of NO and O_2^- is peroxynitrite, $^-$OONO, the conjugate base of peroxynitrous acid, HOONO. The half-life of $^-$OONO under physiological conditions is approximately 1 s as it decomposes spontaneously to give nitrate (NO_3^-) (reaction 1). The

$$NO + O_2^- \rightarrow {}^-OONO \rightarrow NO_3^- \qquad (1)$$

possible biological significance of this reaction was first realized by Beckman and co-workers who pointed out that peroxynitrite may be formed under pathophysiological conditions (where both NO and O_2^- are produced at high rates by phagocytic cells such as macrophages) and that $^-$OONO is a potent oxidant with the potential to destroy critical cellular components.[9]

The idea that $^-$OONO is a cytotoxic species specifically generated in an immune response is predicated on the belief that it is capable of being formed *in vivo* and that it possesses sufficient reactivity to disrupt and/or destroy critical cellular processes. Regarding the possibility of biological $^-$OONO generation, there are currently two primary lines of evidence indicating that it may be formed from the reaction between endogenously generated NO and O_2^-. The first observation consistent with the notion of biological $^-$OONO synthesis was that, in a variety of experiments where the biological activity of NO was being monitored, the addition of superoxide dismutase (SOD) increased its activity.[10] This phenomenon has been attributed to a lowering of steady-state O_2^- levels by SOD which, if the reaction of NO and O_2^- were significant, serves to extend the lifetime and raise the concentration of NO. The second observation consistent with biological $^-$OONO generation was the finding that 3-nitrotyrosine was present in biological fluids (especially under circumstances of expected high levels of NO biosynthesis).[11] Since it has been demonstrated that $^-$OONO is capable of nitrating tyrosine, particularly in the presence of Lewis acid metals such as Cu^{II} or Fe^{III} and metalloproteins such as SOD, this observation has served as an indication of biological $^-$OONO formation. In fact, utilizing the SOD-catalyzed nitration reaction of a phenolic substrate, it has been reported that NO derived from activated macrophages is quantitatively converted to peroxynitrite.[11a] Also, on the basis of the known rate constant for the NO and O_2^- reaction and the presumed concentrations of the two species *in vivo*, it has been theorized that significant $^-$OONO formation should be possible.[12]

The oxidizing capability of $^-$OONO has been investigated and demonstrated in chemical systems. That is, chemically synthesized $^-$OONO was able to oxidize, among other things, sulfhydryls,[13] ascorbate,[14] and α-tocopherol[15] as well as initiate lipid peroxidation.[16] Also, as mentioned above, $^-$OONO has been found to nitrate phenolic compounds, such as tyrosine, in a metal-catalyzed process reported to involve the nitronium cation, NO_2^+. Thus, there appears to be no doubt that $^-$OONO is reactive enough to modify biological molecules under conditions of the above experiments (that is, exposure of the substrates to a relatively high bolus dose of synthetically-derived $^-$OONO).

Although the general idea regarding endogenous $^-$OONO generation and cytotoxicity is an attractive and a seemingly well-supported hypothesis, it is probably premature to universally embrace it. Taking the perspective of the "devil's advocate", it should be realized that the evidence supporting the presence of $^-$OONO in biological systems is indirect and potentially ambiguous. For example, the ability of SOD to enhance the biological activity of NO may not always be due to the ability of SOD to dismutate O_2^-. It has been proposed that this effect, in certain cases, may be due to the ability of SOD to oxidatively convert a reduced metabolite of NO, nitroxyl (HNO or $^-$NO),[17] back to NO and thus increase the overall levels of NO.[18] Also, the formation of nitrotyrosines in biological fluids is not exclusively indicative of $^-$OONO formation since other mechanisms for tyrosine nitration are possible which do not necessarily require the presence of $^-$OONO.[19] Furthermore, the oxidation of crucial biological target molecules by $^-$OONO *in vivo* has not yet been verified, and although there is little doubt that nitration of tyrosine does occur *in vivo*, there is no evidence that this is responsible for any of the adverse effects associated with cellular NO exposure. That is, nitrotyrosine formation may be indicative of NO-mediated cytotoxicity but not the major cause of it. It should also be mentioned that the rate of reaction of $^-$OONO with biological reducing agents such as ascorbate[13] or thiols[14] is actually fairly slow (with second-order rate constants of 235 and $(2-5) \times 10^3$ M^{-1} s^{-1}, respectively) which may indicate that it lacks the inherent kinetic reactivity to be a directly acting cytotoxin.[20] Of course, this argument has another side since the apparent lack of $^-$OONO reactivity with biological antioxidant molecules has been proposed to be significant in $^-$OONO toxicity since it would allow $^-$OONO to diffuse

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(17) With regard to the possible physiological existence and fate of $^-$NO, it should be mentioned that $^-$NO is isoelectronic with molecular oxygen and, therefore, has available to it singlet and triplet spin states. Although triplet $^-$NO has been reported to react with O_2 to generate $^-$OONO, the singlet spin state does not appear to react rapidly with O_2 (Donald, C. E.; Hughes, M. N.; Thompson, J. M.; Bonner, F. T. *Inorg. Chem.* **1986**, *25*, 2676−2677). Thus, physiological formation of nitroxyl does not necessarily mean that conversion to $^-$OONO by reaction with O_2 is imminent since it may be dependent on the spin state. The physiological significance of $^-$NO remains to be determined.

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(20) It should be noted, however, that rate constants may not be the best indicator of cytotoxic potential. That is, the rate of reaction of the cytotoxic entity with a crucial cellular component might be slow, but killing by a NO-mediated mechanism is also slow (a point taken from a private discussion with Dr. Toshio Nakaki of Keio University).

through cellular defenses to reach critical targets (such as mitochondria, DNA, tyrosines, etc.). However, this phenomenon remains to be demonstrated.

If the reaction between NO and O_2^- does occur *in vivo*, it may simply be a way of regulating the biological activity associated with NO itself (since the thermodynamic product of $^-$OONO decomposition, nitrate (NO_3^-), is biologically inactive). Thus, $^-$OONO may be nothing more than an inactive metabolic decomposition product of NO (at least inactive at the steady-state concentrations that may exist biologically). This view is consistent with the fact that the rate constant of the reaction between NO and O_2^- is nearly diffusion limited[21,22] ($4.3-6.7) \times 10^9$ M^{-1} s^{-1}), and if given the opportunity (i.e., the concentrations of the two reactants are significant), this interaction could represent the principal biological mechanism for terminating the action of NO. The idea that the reaction of O_2^- with NO is a detoxification mechanism for NO has been questioned since the destruction of a weak oxidant, NO, to give a stronger oxidant, $^-$OONO, seems unsound.[23] However, this argument assumes that the cellular damage caused by NO is due to its action as an oxidant. This is not necessarily the case. In fact, the original cytotoxic mechanisms proposed by Hibbs and co-workers[7] do not require that NO react as an oxidant but merely a ligand capable of binding vital metal centers.

The generation of $^-$OONO from phagocytic cells such as macrophages or neutrophils is, presumably, a result of the simultaneous generation of O_2^- and NO by these cells (when in the activated state). Therefore, the formation of $^-$OONO *in vivo* is dependent on NO and O_2^- being generated in the same place and at the same time. The major source of O_2^- in these cells is the enzyme system NADPH oxidase. Interestingly, several reports have indicated that this enzyme system is markedly inhibited by NO.[24] Therefore, it appears that O_2^- generation may actually be attenuated by NO, thus decreasing the likelihood of significant *in vivo* $^-$OONO formation. This may represent a biological mechanism by which NO enhances its own actions, indirectly, by limiting O_2^- production and thereby prolonging the biological lifetime. Also, it may be significant that O_2^- production by macrophages occurs through a direct activation of NADPH oxidase and therefore occurs rapidly (over minutes)[25] whereas NO production by macrophages requires *de novo* protein synthesis and occurs only after several hours. Therefore, if peroxynitrite were the primary cytotoxic species generated by macrophages, NO and O_2^- should be generated simultaneously, yet it appears that they are not.

As mentioned above, it has been proposed that NO generation from activated macrophages, which also generate O_2^-, can result in a near quantitative conversion to $^-$OONO.[11a] If this were indeed the case, activated macrophages should generate exclusively NO_3^- (the exclusive decomposition product of $^-$OONO). However, several studies indicate that activated macrophages, instead, generate significant levels of NO_2^- (the primary decomposition product of NO after reaction with O_2 in aerobic solution[26]).[27,28] Also, in general, cytotoxicity and/or cytostasis associated with activated macrophages correlates with NO_2^- generation. Therefore, it appears unlikely that $^-$OONO is the sole reactive nitrogen species generated by activated macrophages and that other potentially toxic entities must be considered. Furthermore, an early study by Nathan and co-workers[29] found that the cytotoxicity associated with activated macrophages could be enhanced by the addition of SOD and blocked by the addition of catalase (an H_2O_2-scavenging enzyme) to the medium. Since SOD will not cross cell membranes and therefore cannot act as a nitration catalyst within the target cells, it is likely that cytotoxic enhancement was due to scavenging of O_2^- with a consequent decrease in $^-$OONO formation.

Of special note, a recent report indicates that the relative fluxes of NO and O_2^- are important in determining the lifetime of $^-$OONO.[30] That is, excess NO or O_2^- can react with $^-$OONO. The exact identity and/or nature of the products of this reaction have not yet been fully characterized, but it was demonstrated that certain oxidative processes are decreased significantly. This finding may indicate that significant *in vivo* $^-$OONO generation (and lifetime) requires that NO and O_2^- are synthesized at not only the same place and same time but similar rates as well.

One aspect of NO-mediated cytotoxicity which has been seemingly ignored is the fact that cells are differentially susceptible to the adverse effects of NO. For example, phagocytic cells are able to destroy target cells in a time frame where they themselves remain active. Therefore, any mechanism for NO-mediated cellular toxicity must be able to accommodate a corresponding mechanism of cellular resistance. If $^-$OONO were directly toxic by virtue of its ability to indiscriminately oxidize critical cellular components or nitrate aromatic functionalities, then it would be expected that the cells responsible for $^-$OONO formation would be killed first. After all, the highest concentration of $^-$OONO should be at the point of origin. It may be argued that $^-$OONO is itself not the ultimate toxic species, but is enzymatically converted, for example, by SOD, to a reactive and toxic species (such as NO_2^+). Moreover, it is possible that enzymatic destruction of $^-$OONO to innocuous species, by a dismutase or peroxidase of some type, may prevent such chemistry from occurring. Thus, variation in the levels of an "$^-$OONO dismutase" may explain differential susceptibility. However, this type of activity has yet to be identified in any protein, and we are, for the time being, left with the enigmatic issue of cellular resistance and/or susceptibility.

The arguments presented above raise some doubts regarding both the *in vivo* formation of $^-$OONO as well as its possible role as a cytotoxic agent in immune response. The purpose of this Commentary is to point out that $^-$OONO has yet to be established as a predominate species associated with NO-mediated cytotoxicity *in vivo* and that other possible mechanisms need to be sought after and considered. In all likelihood, the mechanism of NO-mediated cytotoxicity is going to be multifaceted and highly dependent on the cellular environment. That is, multiple mechanisms will be possible, and the predominance of one over another will depend on

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the physiological and redox status of the target cell. Moreover, $^{-}$OONO may not cause cytotoxicity at all concentrations, in all cells, or in any biological environment. In some cases, $^{-}$OONO may merely represent a metabolic inactivation product of NO and/or O_2^{-}. In any event, the area of NO-mediated cytotoxicity (especially with regard to its relationship to O_2-derived toxicity) is far from understood, and it will take considerably more work to elucidate all the mechanistic intricacies. To be sure, these types of discussions are not uncommon to the field of oxygen or free radical biology. That is, the merits or validity of hypotheses regarding the potential role of, for example, O_2^{-}, H_2O_2, $^1O_2$, and $HO^{\bullet}$ in biological toxicity is constantly being scrutinized and reevaluated.

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