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Reviews

Nitric oxide: a synchronizing chemical messenger

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Abstract. Nitric oxide (NO) has been recognized as a ubiquitous chemical messenger in a large number of different biological systems. Its chemical properties make it less specific and less controllable than practically any other neurotransmitter or hormone. In view of this, its extensive biological role as a chemical messenger seems surprising. It is suggested that the biological function of NO evolved early in the anaerobic stage of evolution. In view of its low molecular weight, limited interaction with water, and its electrical neutrality, which allow it to diffuse rapidly through the cytoplasm and biomembranes, it is suggested that the need for NO has been retained by and maintained in eukaryote cells because of its ability to affect many biochemical functions simultaneously, acting primarily as an intracellular synchronizing chemical messenger.

Key words. Nitric oxide; chemical messenger; autocatalysis; metallo-enzymes; intracellular synchronization; anaerobic microorganisms; evolution of eukaryotes; chemical evolution.

Since the discovery of its vasodilatory effect[45], nitric oxide (NO) has generated considerable interest in its role as a chemical messenger in numerous biological systems[30]. NO, which is produced in the membranes of the endoplasmic reticulum of neurons[61], acts as a neurotransmitter in the brain[41], allowing both synaptic and non-synaptic communication[40]. While in the central nervous system NO may control the release of vasopressin[44], it is unambiguously a major vasodilator[47], enhancing the perfusion of the brain[13]. It was also shown to be produced as an active messenger in different parts of the autonomic nervous system[30], notable for its vasodilatory effect on the peripheral vasculature, including both arteries and veins[47]. Evidence for endogenous NO modulation of acetylcholine release was obtained when NO synthase inhibition modified atropine-sensitive, nerve-mediated contractile responses[60]. Further, NO is a modulator of peripheral pain sensitivity and of the transmission of pain signals to the brain[52,62].

However, NO is not only a neurotransmitter and vasotonic modulator. It is also an immune and autoimmune modulator[28,54]. Produced by macrophages[29], it enhances their phagocytic activity against bacteria[27] and neoplastic cells[23,34]. NO has also been shown to have cytotoxic effects of its own, being capable of killing neurons[33], phagocytized bacteria[53], and neoplastic cells[55]. On the other hand, certain neoplastic cells were reported to produce NO[49], exhibiting one of the many positive and negative feedback loops involving NO. Furthermore, NO has also been implicated as a carcinogen[32,42]. Finally, NO was shown to induce modification of gene expression[32], probably by binding to mRNA iron regulatory factors[58]. Since most of the research cited has been published in the last three years, it is very likely that additional physiological or biochemical functions of NO will be discovered in the near future.

Many actions of nitric oxide are mediated by stimulation of soluble guanylate cyclase and the associated increase of the intracellular concentration of 3′,5′-cyclic guanosine monophosphate (cGMP)[18,40]. NO's neurological effects may involve modulation of Ca^{2+}-dependent neurotransmission[5,25]. However, the enhancement of cGMP production is by no means the only mode of action of NO. For instance, it has been shown that NO *directly* affects the permeability of Ca^{++} dependent potassium channels[6]. NO can affect the enzymatic activity of most iron-binding enzymes (e.g., the cytochrome metabolic chain, peroxidase, catalase, xanthine and aldehyde oxidases, NADH and succinate dehydrogenases) by formation of a nitrosyl complex which may alter the charge and binding affinity of the complexed iron[22,58]. Nitric oxide also reacts directly with DNA to inhibit its replication[20]. The cytotoxic, carcinogenic and gene expression effects of NO may be related to this mechanism.

The enzymatic synthesis of NO which produces nitroso-L-arginine as an intermediate is directly dependent on oxygen concentration[38]. A certain synthetic pathway may require superoxide ions, hydrogen peroxide and probably hydroxyl radicals as oxidative intermediates[37]. Different cell lines produce NO using genetically different isoforms of iron-binding NO synthase[4,50], only some of which are Ca^{2+}-dependent, demonstrating the antiquity and biological importance of nitric oxide. NO can also be produced enzymatically by oxidation of strongly basic amines other than L-arginine[8], or non-enzymatically by direct oxidation of arginine or other primary amines to their nitroso analogs by hydroxyl radicals formed by the classic Haber-Weiss Fe^{+2}-Fe^{+3} peroxidative mechanism[19]. The enzymatic pathways allow the highly oxidative process needed to generate NO to proceed in the non-oxidative intracellular milieu. A unique biochemical activity of NO is its ability to release Fe^{+2} from iron-binding protein[48, 57], probably by forming a nitrosyl-iron complex, similar to the one formed between NO and hemoglobin[14]. The Fe^{+2} released may induce further production of NO by the Fe^{+2}-Fe^{+3} peroxidative mechanism, constituting an alternative, autocatalytic pathway for NO production[2]. On the other hand, the same oxidative environment that is conducive to its production also limits the survival of NO, which is readily oxidized non-enzymatically to nitrite. This makes modeling the chemical kinetics of NO in the cytoplasm extremely difficult.

Using isotope dilution mass spectrometry, the author of this paper demonstrated for the first time that humans and other mammals maintain a finite steady state concentration of nitric oxide in their circulation[14]. The same level of NO was found in different individuals and in different mammalian species, under a variety of environmental conditions[14]. It appeared, therefore, that NO is an *endogenic* substance. To follow up on that research, this short paper presents some new ideas about the role and evolution of NO in living organisms. Since, as described above, nitric oxide is a ubiquitous biochemical entity, these ideas should be of interest to anyone involved in biological or medical research.

Nitric oxide is a simple, highly reactive two-atom free-radical molecule that readily oxidizes to form nitrite or nitrate ions. Unlike other neurotransmitters, NO is a small uncharged species that diffuses easily through both hydrophobic and hydrophilic media. Consequently, once NO is produced at one site, it may readily diffuse throughout the surrounding tissues, inside and outside the vascular system, and induce a *variety* of physiological or biochemical changes depending on the function of the particular receptor. NO's effects include vasodilation wherever it reaches the appropriate binding sites in the endothelium of arteries or arterioles. NO may also readily diffuse in the extracellular fluid, permeating macrophages and inducing in them biomechanical and biochemical phagocytic activities. The most likely receptors of NO are iron-carrying metalloproteins, where NO binds as a nitrosyl ligand and alters the active site of such enzymes. This is most probably the mechanism of NO-mediated inhibition of NO-synthase, which carries both a heme and a cytochrome Fe^{2+} group[4], providing a feedback that regulates NO production. Nitric oxide may also intercalate with π bonds of aromatic compounds, including purines, changing their orientation vis-à-vis their neighbors or biochemical reactants. This may explain some of the effects of NO on DNA functions.

One may ask, what is the advantage of having nitric oxide act as a chemical messenger? The biological effects of nitric oxide must have existed early in the evolution of eukaryotes, since its production from arginine has been demonstrated also in arthropods[12], molluscs[16], starfish[36], certain slime moulds[59], as well as in bacteria[7]. While NO formed as an intermediate in denitrification by bacteria and plants can be considered merely a byproduct, NO formed from arginine must be a chemical messenger. Nevertheless, NO is far from being an ideal chemical messenger for several reasons:

1) It is not highly specific – the simplicity of this compound compromises the information content of the message. Living organisms have developed many more complex biochemical entities (of much lower entropy) that act as more *specific* chemical messengers – hormones or neurotransmitters. Because of its high reactivity, NO reacts with or attaches to many different biomolecules, metallo-enzymes in particular. This low specificity implies a tremendous over-production of this chemical compared with a more specific messenger. Moreover, its concentration at a given receptor site is poorly predictable because of its dependence on so many competing sinks and binding sites. The free energy expenditure per message using NO as a messenger is, therefore, higher than for most other chemical messengers.

2) NO has been evolutionarily selected and maintained despite the difficulty of its biosynthesis. The biosynthesis of NO requires a complex amino acid (L-arginine) as precursor, very reactive and unstable oxidants, or a number of complex protein molecules as the oxidative catalysts (NO synthases)[4, 50], all of which are developmentally advanced entities that can operate today solely under aerobic conditions.

3) Compared with other chemical messengers, NO has a long half-life in the biological milieu. A short lifetime is a major requirement for any chemical messenger that cannot be permitted to accumulate once it reaches its target. In this respect NO is at a significant disadvantage; since its oxidation to nitrite is not enzyme mediated, it is not as effective and specific as the deactivation of other neurotransmitters, e.g., acetylcholine or serotonin. The controlled deactivation of chemical messengers provides an additional negative feedback control of their concentration near the receptors; this feedback control seems to be lacking in the case of nitric oxide.

4) Because of its ease of diffusion, NO cannot be contained in a vesicle like most other chemical messengers, to be released on demand. NO must, instead, be rapidly synthesized de novo when needed. This means that both the risetime and the rate of decay of the NO signal at

the target site are much slower than those of other neurotransmitters. Given all these shortcomings, one might have expected the chemical messenger role of NO to be eliminated by biological evolution in favor of messengers that are more specific and easier controllable.

The only two advantages one can see in nitric oxide as a *chemical* messenger are a) its ability to easily diffuse into and out of cellular compartments or subcompartments (organelles), and b) its relative non-specificity which allows it to activate *simultaneously* several *unrelated* biochemical processes that may have to act *in concert* in different parts of the system (the cell or the organ). In other words, the uniqueness of NO may be its function as a *synchronizing* messenger. Its low molecular weight, small size, electric neutrality, minimal polarizability, and minimal hydration, allow it to diffuse rapidly through both hydrophobic and hydrophilic media. NO diffuses faster than practically any other stable chemical species, except H_2, CH_4, and N_2, none of which can act as a chemical messenger because of their chemical inertness in the biological milieu. Molecular oxygen could perhaps be a chemical messenger, but apart from photosynthesis in chloroplasts no other biogenic O_2 is known. NO can affect the nucleus and the different cytoplasmic organelles at practically the same time on a biochemical time scale, making it well suited for a *synchronizing* role. As discussed below, CO may act similarly to NO but its biological role has not yet been studied as extensively.

For example, if we wished to modulate the cytochrome chain we could inhibit the activity of any one of these iron-binding enzymes, but then substrate will be consumed and products will accumulate at other stages of the chain, resulting in a delayed restart. If, however, NO is the modulator, it will inhibit all the members of the chain at the same time, and the chain will restart smoothly and synchronously when NO's concentration diminishes. Such synchronization may thus occur in bacteria and in mitochondria alike. In eukaryote cells, NO may coordinatedly inhibit ATP production in the mitochondria, mRNA-dependent protein synthesis on the ribosomes and DNA gene transcription in the nucleus. Since the intracellular level of NO is expected to oscillate as a result of the autocatalysis associated with NO production[2], NO may provide a biological clock that paces intracellular biochemistry.

Multicellular organisms can also benefit from NO as a synergistic synchronizing chemical messenger. For example, the modulation of neuronal processes in the brain involves synchronous vasodilation (oxygen supply) and release of calcium (long term potentiation)[40,41]. Another example is the synergistic synchronization of the immune response – the NO-induced vasodilation increases the blood supply through arterioles to the affected area and with it the influx of monocytes and macrophages plus the oxygen and nutrients needed to enhance their function. The vasodilation of veins minimizes backup of blood and the consequent acidosis and buildup of other waste products. At the same time, nitric oxide enhances the motility of the macrophages and therefore the phagocytic process which can combine with the bactericidal or tumoricidal effect of NO[2].

Notwithstanding these valid reasons for the evolutionary retention of nitric oxide as an intracellular chemical messenger in eukaryotes, the intracellular functions of NO seem to be far more fundamental. Let me speculate about the evolution of the biological role of nitric oxide. Nitric oxide must have been present at low concentrations when life first evolved in a partially oxidizing environment, long before the evolvement of atmospheric O_2. NO was probably produced photochemically from CO_2 and N_2 in the upper atmosphere, and by cavitation from N_2 and water in the oceans[1]. In the absence of free O_2, NO reached substantially higher ambient concentrations than in today's atmosphere. Still its concentration was fairly low, because of the kinetic limitations on its production. Primordial living systems, at that time, most probably contained purines and pyrimidines, oligopeptides, porphyrins and iron. Due to its binding to iron and to aromatic π electrons, NO must have exerted some effect on many different aspects of early biochemistry, possibly including replication, transcription, as well as biosynthesis of certain constituents. Even under anaerobic conditions, NO was oxidized to nitrite by electron transfer, in parallel with other bio-oxidative processes that take place also under anoxic conditions. Since its concentration in primordial cells was modulated by its oxidation, nitric oxide, which at that time was probably wholly exogenous, may have become a regulatory factor in the biochemistry of early prokaryotic life forms. NO might have thus synchronized different processes that went on in different parts of the early anaerobic cell.

When aerobic, eukaryote cells evolved, with their many and different organelles, the function of NO as a synchronizer must have become more important. One might go so far as to suggest that the unique function of NO as an intracellular synchronizing messenger is absolutely necessary for the survival of eukaryote cells, the complexity of which requires *synchronized* intracellular information transfer. This unique function may be the reason for the fact that this primordial chemical messenger was not replaced by more elaborate biochemicals.

In an O_2 dominated environment NO was not available exogenously, and therefore enzymatic production processes evolved. Different synthetic processes may have evolved in parallel in different organisms and in different cell lines[4,50]. Notwithstanding our current fascination with the multitude of activities of NO as an intercellular messenger, these may still be of secondary importance, from the standpoint of fundamental biological processes, to its *intracellular* function.

One possible clue in favor of this hypothesis is the mode of interaction of macrophages with bacteria. We do know that macrophages engulf bacteria and kill them by exposure to endogenic NO[9]. We also know that this effect, which proceeds at a relatively slow rate – comparable to the replication time of the microorganism – involves interaction of NO with iron-binding enzymes inside the prokaryote[46]. The same mechanism seems to operate in the interaction of macrophages with parasitic protozoa[9,56]. Since NO per se is not toxic to bacteria, which produce it endogenously[3,7,17], one mechanism of the slow killing of engulfed microorganisms by macrophages would be the lack of synchronicity between the cyclic phases of production of NO in the macrophage and the phases of NO production inside the prokaryote; this results in the metabolic exhaustion of the microorganism, a process that takes considerable time. A corollary of this suggestion is that the symbiotic incorporation of certain bacteria into the primordial eukaryote, forming the progenitors of mitochondria[35], was by selection of microorganisms that synchronized their NO-mediated biological clocks. The observed oscillatory behavior of NO concentration in the medium of certain denitrifying bacteria with partially inhibited nitric oxide reductase[24], which can be inhibited also by NO, suggests again the possibility of a synchronizing function of NO, at least in those prokaryotes.

In brief, what makes NO unique is the combination of its small size (allowing a fast rate of diffusion), its electrical neutrality (allowing its easy movement through biological membranes and penetration into an active site inside a folded metalloprotein), and its high reactivity (instant attachment to iron and rapid oxidation to nitrite). Carbon monoxide (CO) could simulate the synchronizing role of NO, in view of its recently reported function as a neurotransmitter in the brain[10]. However, unlike NO it cannot be oxidized without the help of an enzyme. Without an oxidation pathway it cannot act as a chemical messenger, which must be readily removed to allow the next message to come through. Carbon monoxide can be biologically oxidized by carbon monoxide dehydrogenase, as demonstrated in several primitive anaerobic bacteria[15,26,31]. Although not yet isolated, an enzyme with a similar function is predicted to exist in mammalian tissue. The similarity between NO and CO suggests similar intracellular functions in eukaryotes as well.

Methyl bromide, which is produced biogenically in the sea[51], is a *potential* synchronizing chemical messenger – it is highly reactive, small, and neutral. It has also been shown to be enzymatically oxidized to CO_2 by methanotrophic bacteria[43]. Unlike MeBr, no biogenic MeCl or MeI have been reported so far. Methyl chloride is probably too inert to act as a messenger, and methyl iodide is too hydrophobic and too reactive. Moreover, MeI is unlikely to be available in quantities needed for a ubiquitous synchronizing messenger like NO. Although it may be worthwhile to look for endogenic methyl bromide in mammalian tissue, there are two major differences between NO and CO on one hand, and MeBr on the other: 1) There are intrinsic chemical differences – NO and CO can react *reversibly* with metalloenzymes, whereas CH_3Br reacts irreversibly with sulfhydryls and will be eliminated by the same process. This accounts for the well-known toxic effects of MeBr. 2) Unlike NO or CO, abiotic methyl bromide does not exist in nature in sufficient amounts to have had a role in the early evolution of life. We still do not know what biological function methyl bromide may have today in marine biology, but most probably it appeared relatively late in biological evolution.

This communication is not intended simply to present a few speculative ideas. Its goal is to provoke thought and to challenge researchers to prove or disprove the notions presented. However, some of the tools used to study the systemic effects of NO may not be appropriate to explore its intracellular functions. The conventional introduction of antimetabolites to NO synthase, even if they affect the intercellular activity of NO, may not reach sufficient concentrations to interfere significantly with intracellular processes. One possible way might be to expose cells to pulses, at different frequencies, of O_2 followed by complete anoxia, to find out the effect on the synchronization of cell replication or metabolism. Similar pulses of NO-enriched air could also be used. Another possible approach is to use EPR to follow the intracellular modulation of NO level and compare it with the modulation of other biochemical activities inside the cell. This are just two suggestions; I am sure that some of the readers of this paper will come up with additional, probably more effective ways to test my hypothesis. If NO synthase could be isolated from different tissues and from different organisms, it would be highly instructive to study its biochemical phylogeny. It would not be surprising to find utterly different synthetic pathways in different organisms, especially in prokaryotes. The study of the effect of NO on prokaryotes and the search for endogenic NO in bacteria, especially in anaerobic and facultative aerobic microorganisms, will undoubtedly be highly rewarding.

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