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Simulation of the diffusion and reaction of endogenously produced nitric oxide

(endothelium-derived relaxing factor/autocrine and paracrine effects)

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ABSTRACT **In spite of intense recent investigation of the physiological and pathophysiological roles of endogenously produced nitric oxide (·NO) in mammalian systems, little quantitative information exists concerning the diffusion of this small nonelectrolyte from its source (NO synthase) to its targets of action. I present here a conceptual framework for analyzing the intracellular and intercellular diffusion and reaction of free ·NO, using kinetic modeling and calculations of the diffusibility of ·NO and its reactions in aqueous solution based on published data. If the half-life of ·NO is greater than ≈25 msec and the rates of reaction of ·NO with its targets are slower than its diffusion or reaction with O_2 (for which there is experimental evidence in at least some systems), then (*i*) ·NO acts *in vivo* in a mostly paracrine fashion for a collection of ·NO-producing cells, (*ii*) ·NO diffuses to significant concentrations at distances relatively far removed from a single ·NO-producing cell, and (*iii*) localized sites of vascularization will scavenge ·NO (and thus decrease its actions) at distances many cell diameters away from that site. These conclusions have important implications with regard to the mechanism of endothelium-dependent relaxation, the autocrine vs. paracrine actions of ·NO, and the role of the spatial relationship between specific sites of ·NO formation and neighboring blood vessels in ·NO-effected and -affected neuronal signal transmission.**

Although chemists, biochemists, and microbiologists have examined the unique properties of nitric oxide (·NO) for >200 years, it has only been within the last 7 years that its multiple physiological functions in mammals have been appreciated (reviewed in ref. 1). ·NO is synthesized by specific enzymes in many cell types, in response to inflammatory, neural, or vascular stimuli. In inflammation, ·NO production can be induced by exposure to bacterial products and/or cytokines and functions as a cytostatic/cytotoxic effector for defense against transformed or infected host cells and pathogenic organisms. In neural and endothelial cells, ·NO is an intercellular messenger, effecting signal transduction by stimulation of heme-containing soluble guanylate cyclase.

Intense research has been directed toward characterizing the production of ·NO by the various isoforms of ·NO synthase (2, 3) and the reactions of ·NO with its molecular targets (4). ·NO is a small, neutral, relatively hydrophobic (5, 6) nonelectrolyte in aqueous solution,† consistent with its role as a diffusible intercellular messenger or immune effector. Little attention has been directed toward understanding the quantitative characteristics of this diffusion process and it is frequently characterized as a short-lived, short-range mediator. However, I demonstrate here that kinetic analysis of the production, diffusion, and reaction of ·NO under physiologically significant conditions contradicts these assumptions. The distance of diffusion of ·NO is in fact surprisingly long, and this finding has important implications for its multiple biological actions.

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METHODS

The calculations reported here were performed on a personal computer using simple BASIC programs as described in the text. Copies of these programs and kinetic derivations are available upon request from the author and can be used to simulate the concentration profile of ·NO with virtually any spatial relationship of cellular synthesis and scavenging by the vasculature (illustrated in Fig. 3).

For kinetic simulations of the effects of hemoglobin scavenging of ·NO, two basic models were developed. For simulation of the effects of external scavenging on the intracellular reaction of ·NO in a single cell were based on a steady-state derivation. Out of all possible values over a three-order-of-magnitude range, computer calculations identified those relationships between the rate constants for the production of ·NO and its diffusion and reactions (with intracellular targets, O_2, and external hemoglobin) that result in ≥90% decrease in the reaction of ·NO intracellularly as the rate of external scavenging increases. These calculations revealed that a necessary and sufficient condition for this experimentally observed result (see below) is that the rates of diffusion and reaction with hemoglobin are ≥100-fold more rapid than the rate of intracellular reaction or with O_2.

For simulation of scavenging in a multicellular tissue segment with various sites of ·NO production and scavenging (sites of vascularization) (see Figs. 2 and 3), the scheme utilized a 41-cell compartment model where kinetic rates of change of the ·NO concentration in each compartment were defined, with reaction with O_2 occurring in all compartments (based on a half-life of 4 sec; see below) and rates of ·NO production and diffusion chosen to reproduce the experimental findings of Malinski *et al.* (6) using ·NO-selective microsensors and that reproduce the profile predicted from Fick's second law of diffusion (see Fig. 2). Note that the rate constants that satisfied these criteria were unique. The rate of reaction of ·NO with hemoglobin was based on the data of Kelm *et al.* (7), who reported that the reaction of endothelially produced ·NO was complete within 0.1 sec with 4 μM hemoglobin. Since the reaction was 100% complete within this time, this rate constant is almost certainly an underestimate. Numerical integration using Newton's method calculated the time courses for the various configurations, resulting in the steady-state ·NO concentration profile (see Fig. 3). To avoid instabilities in this calculation, the values for

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†Contrary to common reference, in virtually all its biological actions ·NO is not a gas; under biologically relevant conditions (low concentrations in aqueous solution) ·NO is a dissolved (and thus dispersed) nonelectrolyte.

the incremental time increase were chosen so that no intermediate changed in concentration by >1%. A similar approach has been taken by Gally *et al.* (8) in a model of regulation of neuronal development and function, and Goretski and Hollocher (9) also have applied combined kinetic and diffusion calculations to ·NO production by bacteria during denitrification.

RESULTS

Diffusivity of Free ·NO: Paracrine vs. Autocrine Actions. For a molecule such as ·NO, which is constantly reacting and thus disappearing with a half-life of $t_{1/2}$, it can be shown by Fick's second law of diffusion that the amount of ·NO at a distance Δx from a point source of constant production is given by the equation

$$[NO]_x = [NO]_0 \exp[(-\ln 2)(\Delta x)/\sqrt{2Dt_{1/2}}],$$

where D is the diffusion constant, $[NO]_0$ is the concentration of ·NO at the point source, and $[NO]_x$ is the concentration at the distance Δx (10).

Using a value of 3300 μm^2/sec for the diffusion constant of ·NO in aqueous solution (6), Fig. 1*A* shows a calculated profile for the amount of ·NO present at various distances away from a point source (the abscissal origin, normalized concentration = 1) as it diffuses and is simultaneously removed by a uniformly distributed reaction with $t_{1/2}$ of 4 sec (7, 11–14). The units of distance on the abscissa are in 20-μm increments.

It is clear from Fig. 1*A* that even with a seemingly short $t_{1/2}$ (4 sec), appreciable amounts of ·NO will be found at distances far from the cell producing it. Indeed, it is only after diffusing ≈160 μm (8 cell diameters) that the concentration decreases to half the value at the point source of production. Fig. 1*B* presents the individual (squares) and total (dotted line) amounts of ·NO with five ·NO-producing cells and shows that even with a density of only 12% (41 possible at confluence) on average a majority of the total ·NO that affects any cell is actually produced by that cell's neighbors. Defining the average (over all 41 positions) percent of the total ·NO above any cell that it itself produces as "% autocrine," Fig. 1*C* presents this index for $t_{1/2}$ = 4 sec as the density is increased. It is clear from this simple calculation that at any density greater than ≈10%, ·NO will function as mainly a paracrine effector. At 100% density (41 adjacent 20-μm-diameter ·NO-producing cells) on average >94% of the ·NO that affects any cell is actually produced by its neighbors. An illustrative analogy can be applied from atmospheric chemistry. In a polluted city where automobiles are the major source of ·NO, the danger to each motorist is not from the exhaust of the car being driven but rather from the combined effects of all vehicles. As density is increased the effects on each motorist are amplified.

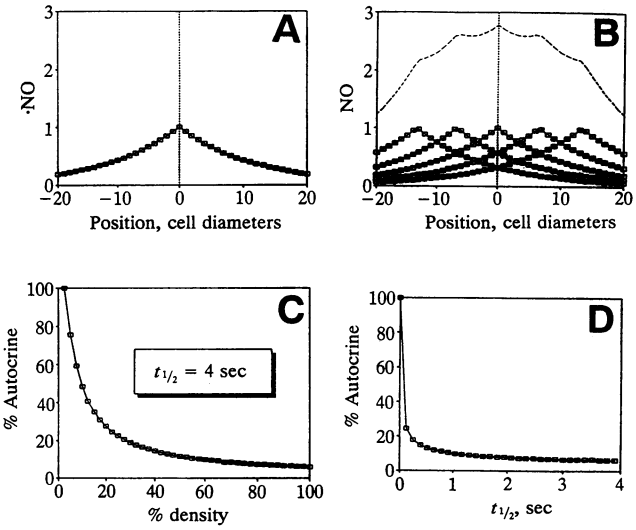

FIG. 1. Spatial distribution of ·NO as it diffuses [D = 3300 $\mu m^2 \cdot sec^{-1}$ (6)] and simultaneously disappears, according to Fick's second law of diffusion. (*A* and *B*): $t_{1/2}$ for ·NO is 4 sec, and cell density is increased from 1 to 5 cells out of a total of 41 at confluence. Total NO concentration above each location is denoted by the dotted line and the concentration of ·NO produced by individual cells, by squares. The concentration of ·NO above each ·NO-producing cell is normalized to 1, and the units on the abscissa are in 20-μm increments. (*C* and *D*) Effect of increasing cell density (for $t_{1/2}$ of 4 sec for ·NO) (*C*) and of $t_{1/2}$ of ·NO (at 100% cell density) on the percent of the total ·NO (*D*) which affects any cell that the cell itself produces ("% autocrine").

What if $t_{1/2}$ of ·NO in tissues is <4 sec? For example, Kelm and Schrader (14) reported that the $t_{1/2}$ of ·NO in heart tissue was 0.1 sec. In this case, ·NO will disappear more rapidly, thus increasing its percent autocrine actions. This relationship between $t_{1/2}$ and percent autocrine is presented in Fig. 1*D* for a density of 100%. Using these simple calculations, it is clear that the actions of ·NO would be mostly paracrine for any $t_{1/2}$ greater than ≈25 msec, a very rapid reaction.

Are there any experimental data to support this suggestion that ·NO acts in mainly a paracrine manner? In the vasculature the actions of ·NO clearly involve intercellular diffusion between two cell types, the endothelium (·NO producer) and smooth muscle cell (·NO target); indeed, prevention by hemoglobin of vascular ring relaxation in cascade perfusion experiments provided initial evidence that intercellular (paracrine) ·NO can effect signaling. In several instances, however, extracellular scavenging of ·NO by hemoglobin can also prevent the actions of ·NO even when the same cell that produces it is also a target (15–21). In the isolated rat hepatocyte, for example, reaction of ·NO with intracellular iron is prevented by coculture with erythrocytes, where the ·NO must diffuse out of the ·NO-producing hepatocyte and into the erythrocyte in order to be scavenged (22). As described in *Methods*, simple steady-state kinetic modeling demonstrates that the only way this can occur is if the phenomenological rate for diffusion of ·NO is appreciably faster than the rates of reaction with its intracellular targets. From combination with (*a*) the relatively wide diffusibility of ·NO as determined by the calculations presented here (as well as experimental measurements using ·NO-selective porphyrinic microsensors, described below) and (*b*) the appreciable overlap of ·NO concentration from individual cells (Fig. 1), it thus seems inescapable that the actions of ·NO will be relatively long-range and that it will act in a mainly paracrine manner for a collection of ·NO-producing cells.

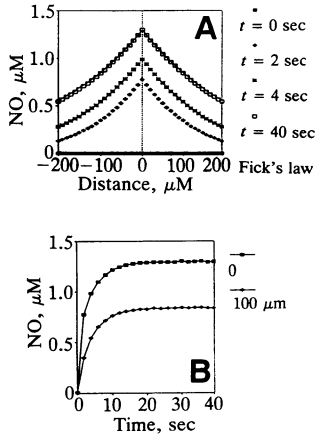

FIG. 2. Formation, diffusion, and reaction of ·NO for one endothelial cell. (*A*) Symbols are concentration profiles for ·NO calculated at 0, 2, 4, and 40 sec after initiation of ·NO synthesis, and the solid line is the profile calculated from Fick's second law, normalized to a concentration of 1.3 μM at the origin. (*B*) Increase in ·NO in the endothelial cell ("0") and 100 μm away from this ·NO-producing cell.

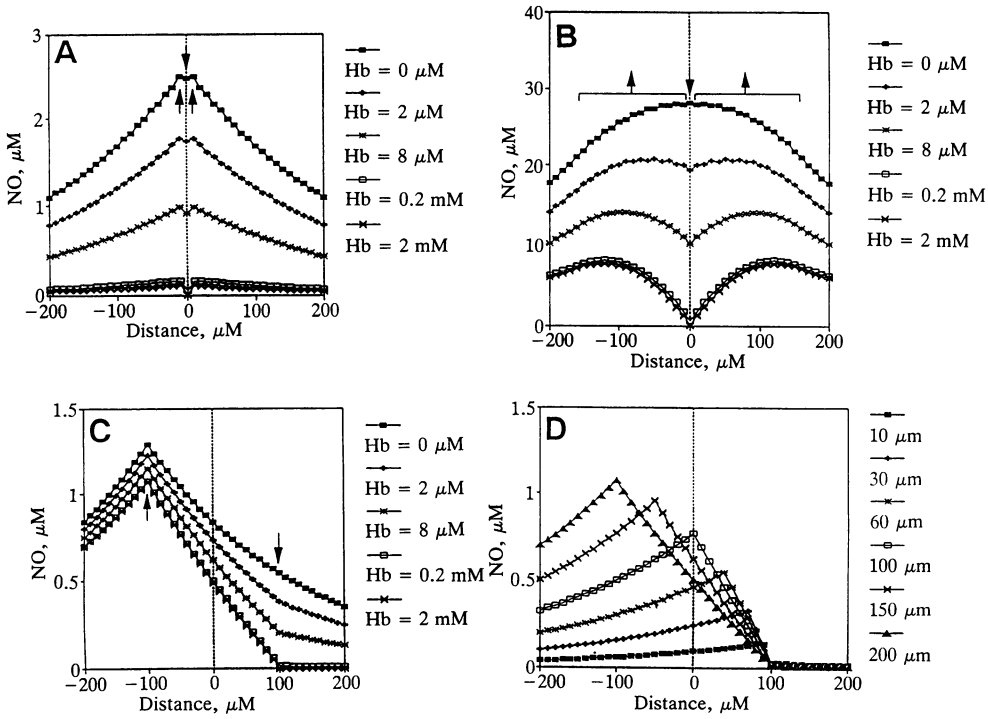

FIG. 3. Steady-state profiles of ·NO concentration above a 400-μm distance. For *A–C* sites of ·NO formation are designated by the upward arrows and scavenging by hemoglobin by the downward arrows. Hemoglobin concentrations at these sites are varied from 0 to 2 mM, the approximate value for whole blood. For *D*, a single site of ·NO production is placed at the designated distances from 10 to 200 μm away from a single site of scavenging (hemoglobin concentration at that site, the 100-μm point, is 2 mM).

Long-Range Scavenging of Free ·NO by the Vasculature. If the rate of diffusion of ·NO out of the cell is more rapid than its intracellular reactions, and substantial diffusion of ·NO over large distances occurs after its exit from the cell, might a rapid scavenging mechanism at one location effectively lower the concentration of free ·NO at relatively distant locations, thus decreasing its actions? Physiologically this possibility has important implications. Specifically, this suggests that sites of vascularization may be capable of effective removal of ·NO at locations very distant from that site.

The model used to simulate vascular scavenging for a collection of cells is described in *Methods* and is illustrated in Fig. 2*A*, where a single ·NO-producing cell is located at the origin, no vascular scavenging is present, and $t_{1/2}$ of ·NO is 4 sec. Using a unique set of phenomenological rate constants for production (10.3 μM·sec^{-1}) and diffusion (91 sec^{-1}), this calculation results in a profile of ·NO concentration at selected times after initiation of ·NO synthesis that increases to reach (within 10–20 sec) a steady-state concentration of 1.3 μM at the ·NO-producing cell and 0.85 μM at a distance 100 μm away (Fig. 2*B*). With this unique set of rate constants, this model closely approximates the experimental data of Malinski *et al.* (6) using ·NO-selective microsensors. Solution of the equation of diffusion above based on Fick's second law using these data results in a $t_{1/2}$ of 4 sec [within the range of most previously reported values (7, 11, 12, 14)]. The validity of this model is demonstrated by the close similarity of the steady-state profile (at t = 40 sec; □, Fig. 2*A*) to both the data of Malinski *et al.* (6) and the profile predicted from the equation of diffusion (solid line; see also Fig. 1).

The usefulness of this model is the ability to mimic the spatial distribution of [NO] with various configurations of sites of ·NO production and scavenging. Fig. 3*A* presents the steady-state profiles of [NO] with two ·NO-producing endothelial cells at positions −10 and +10 μm (upward arrows) and a single site of scavenging between (position 0, downward arrow). This model approximates a blood vessel 20 μm in diameter.

As shown in Fig. 3*A*, in the absence of hemoglobin, endothelial-produced ·NO is widely diffusible, extending to appreciable distances on either side of the blood vessel. This is consistent with the results of Malinski *et al.* (6), who showed that ·NO diffused identically on either side of the producing endothelial cell. The total concentration of ·NO at the location of each endothelial cell is approximately twice that for a single cell (Fig. 2*A*), due to the overlap of the profiles for these two neighboring cells. When only small amounts of hemoglobin are present between the two endothelial cells, there is a marked decrease in the concentration of ·NO at all locations, and at a quite low hemoglobin concentration (0.2 mM, equivalent to only about 10% the concentration in normal blood), >93% of the ·NO present immediately surrounding the blood vessel (and also at all other locations) is scavenged.‡ One important overall conclusion from these simulations is that because of the rapid diffusibility of ·NO a single site of vascular scavenging will result in a dramatic lowering of the concentrations of endothelial-produced ·NO over a very large distance on either side of that site, as opposed to scavenging confined within the vascular lumen.

Fig. 3*B* is a simulation of steady-state ·NO concentration by two collections of 15 adjacent ·NO-producing cells (each cell 10 μm in diameter, denoted by the upward arrows) on either side of a single site of vascular scavenging in between (the downward arrow). This simulation mimics parenchymal cell ·NO production under conditions such as sepsis. Because of the overlap of the concentration profiles of ·NO from each ·NO-producing cell, the total concentrations at each location are much higher than for only two cells (compare the ordinate scales for Fig. 3 *A* and *B*). As for only two ·NO-producing cells (Fig. 3*A*), increasing hemoglobin also significantly decreases the overall ·NO concentration, and the decrease is most dramatic immediately adjacent to the scavenging site. However (although not shown), because of substantial ·NO production at positions distal to that of the scavenging site,

‡Like oxyhemoglobin, oxymyoglobin rapidly reacts with ·NO to produce NO_3^- and metmyoglobin (23). Thus, in muscle tissues producing ·NO, such as heart (24) and skeletal muscle (25), its actions may be confined to localized areas through this mechanism. This suggests a novel physiological function for myoglobin, as well as for metmyoglobin reductase, which will be required to regenerate oxyferromyoglobin (26). Indeed, this may explain the short $t_{1/2}$ of ·NO when exposed to heart tissue as reported by Kelm and Schrader (14).

even at high (2 mM) concentrations of hemoglobin the ·NO concentration immediately adjacent to the vascular lumen is significantly (>10-fold) higher than when ·NO is produced solely by the endothelial cells. Thus, even in the face of rapid luminal scavenging, the ready diffusibility of ·NO for a collection of ·NO-producing parenchymal cells in the vicinity of the blood vessel may be a major contributor to hypotensive conditions such as sepsis and inflammation.

In addition to its role in the vasculature, ·NO is also produced by neuronal cells and acts as an intercellular messenger (27). By using the simulations presented here, it is possible to ask how far away might neuronally produced ·NO be scavenged by a blood vessel in the vicinity of the ·NO-producing neuron? Fig. 3*C* shows that 200 μm away from a single ·NO-producing cell (upward arrow), increasing the concentration of hemoglobin (downward arrow) results in efficient decrease of the ·NO in the vicinity but has relatively little effect on the ·NO concentration close to the neuron. Additionally, the rapid scavenging prevents ·NO from diffusing across the site of scavenging. As shown in Fig. 3*D*, with 2 mM hemoglobin at position +100 μm, progressively more of the ·NO is scavenged when the ·NO-producing cell is moved closer to the blood vessel, from a distance of 200 μm to 10 μm. This result suggests that the long-range effects of ·NO as a diffusible neurotransmitter are very sensitive to the spatial relationship between the ·NO-producing neuron and the sites of vascularization.

DISCUSSION

In this paper I provide a conceptual framework within which to analyze the dynamics in time and in space of the long-range interactions of ·NO with its targets. Making certain simplifying assumptions and based on published data, I demonstrate that if ·NO alone (unmodified) is produced, the $t_{1/2}$ of ·NO is greater than ≈25 msec, and the rates of reaction of ·NO with its targets are slower than its diffusion or reaction with O_2 [as supported experimentally (15–22)], then (*i*) in a collection of ·NO-producing contiguous cells ·NO acts in a mostly paracrine fashion (i.e., for any given cell the ·NO that *does* act intracellularly actually originated from that cell's neighbors) and (*ii*) within spatial limits a localized site of vascularization will scavenge ·NO at appreciable distances away from that site.

In vivo, since blood is constantly flowing immediately adjacent to the source of endothelium-derived relaxing factor (the endothelial cell) why isn't the factor rapidly scavenged? Although it is common to assume that only half of endothelially produced ·NO (the "half" that diffuses luminally) is scavenged, this explanation is untenable because diffusion at any point is isotropic (i.e., equally likely in all directions) and net movement in any direction is dictated solely by the existence of concentration gradients, which in this case will be directed toward the vascular lumen.

There are two most obvious explanations for this apparent paradox. First, although luminal hemoglobin may indeed scavenge virtually all of the ·NO, the amount remaining, although small, may still be sufficient to induce vascular smooth muscle relaxation. Indeed, the apparent K_m for ·NO for guanylate cyclase is in the nanomolar range (28), much lower than the amount measured by ·NO-selective microsensors at a smooth muscle cell fully 100 μm away from an ·NO-producing endothelial cell (6). However, this seems intrinsically wasteful, since it means that only a very small minority of the free ·NO produced by the endothelial cell actually induces relaxation (the rest would be scavenged). In addition, it has been shown that extracellular scavenging of ·NO by hemoglobin (15–21) prevents cGMP increase in several cultured cell types, even when the same cell that produces ·NO also produces cGMP, demonstrating that at least in these cases the amount of ·NO remaining after scavenging is not sufficient to stimulate guanylate cyclase.

The second possible explanation is that the endothelium-derived relaxing factor that acts on adjacent smooth muscle cells is not free ·NO [for which there is experimental evidence (29–31)]. However, in light of the simulations here this would mean that the reason for postulating such a stabilized form of ·NO is to actually decrease its diffusibility (in contrast to the more common notion that a $t_{1/2}$ in the range of seconds may indicate actions that are too short-range spatially). It is also possible that both free ·NO and a "packaged" form(s) functions effectively *in vivo* for vasodilation, but that the packaged form prevents the complete scavenging by the vasculature. It may be, for example, that the 1.5-sec delay in the appearance of ·NO in a smooth muscle cell 100 μm away from an ·NO-producing endothelial cell described by Malinski *et al.* (6) corresponds to the formation of such a packaged form.

·NO is also an intercellular messenger for neural signal transmission (32). Relatively long-range effects of ·NO in the brain are indicated by the coupling of neural activity to cerebral blood flow (33, 34), suggesting that the ·NO produced by synaptic transmission is capable of diffusion to neighboring blood vessels and induction of vasodilation. Indeed, recent studies with hippocampal slices have suggested that neuronally produced ·NO is capable of relatively long-range intercellular diffusion with induction of effects on synaptic transmission (35). As shown in Fig. 3 *C* and *D*, within spatial limits (10–100 μm) specific sites of vascularization are capable of substantially long-range scavenging of ·NO produced by a single cell, such as an ·NO-producing neuron. This indicates that the actions of ·NO as an intercellular mediator of neuronal signal transmission may be greatly affected by the spatial relationships between the neuron(s) and sites of vascularization in the vicinity. In addition, this rapid scavenging results in a spatial barrier beyond which ·NO will not diffuse (Fig. 3 *C* and *D*). This effect may confine the effects of ·NO in the brain [including strengthening synaptic transmission in discreet tissue volume segments (36)] to locations in between these vascular sites. It is even possible that communication between ·NO-producing neurons and adjacent sites of vascularization may be "two-way"; i.e., ·NO-induced increased vascular flow may in turn result in increased ·NO scavenging. In addition, the overlap of ·NO concentration profiles in a collection of ·NO-producing cells (Fig. 1*B*) indicates that as more and more neurons begin to produce ·NO they will respond more and more to the cumulative ·NO produced rather than to specific neighboring cells, thus potentially providing a mechanism whereby signaling occurs by integration of total neural activity within the tissue segment defined by sites of vascularization at the boundaries.

The free diffusibility of ·NO and its paracrine vs. autocrine actions may also help explain the puzzling observation that in amino acid excitotoxicity [believed by some (15, 17, 37, 38) but not others (16, 39–41) to be due to ·NO production], the cells staining most intensely for ·NO synthase activity are selectively spared from toxicity while surrounding nonproducing cells are injured (32). The concepts presented here suggest that the responsiveness or resistance of any given cell to ·NO is not necessarily due to whether it is an ·NO producer but rather due to the relative rates of reaction of ·NO with its intracellular targets. If ·NO-producing cells are relatively nonreactive or possess mechanisms for resistance, any surrounding cells may be selectively responsive even though they do not themselves produce ·NO.

The models presented here deal exclusively with systems where a constant, relatively sustained production of ·NO is occurring. When ·NO is produced as a signal molecule, it is possible that in at least some circumstances it is synthesized

as a "burst" in response to fluctuating cytosolic Ca^{2+} concentration (42–45). A potentially important result will be that neighboring cells will thus also be exposed to ·NO as a "pulse," and so those reactions that are more rapid (i.e., binding to heme to stimulate guanylate cyclase) will be more responsive to this transient signal than slower (possibly toxic) reactions such as inhibition of iron-protein enzyme function. In the case of nonheme iron-sulfur clusters, this inhibition may be second order with respect to ·NO [since the EPR spectrum resembles the spectra of iron dinitrosyl complexes (46)], and so the rate of formation would decrease exponentially as the NO concentration decreases. In the event of sustained ·NO production, such as during response to excess excitatory amino acid release as a result of tissue damage [stroke, ischemia, trauma (32, 47)], these toxic, kinetically slower reactions may become significant.

Finally, these results raise an important cautionary note in the interpretation of results from certain types of experiments designed to test whether ·NO acts as an intercellular agent. Specifically, since it is possible to scavenge ·NO externally and prevent its actions even when the source cell is also a target, it may not be appropriate to conclude that ·NO acts by intercellular diffusion when extracellular scavenging prevents signal transmission. For example, it would still be possible that an ·NO-producing cell is also a sensitive target for its actions and that this action causes the production of a second agent that is the actual intercellular messenger. This illustrates an important overall conclusion from the concepts presented here—that, within spatial limits, the control of the localized actions of ·NO may be dictated not by the proximity of the target to the source, but rather by the preprogrammed relative kinetic rates of cellular and extracellular scavenging and reactivity at specific locations.

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