Diffusion and Reactions of Nitric Oxide, Oxygen, and Superoxide In Cells and Culture Media

By

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Abstract

As part of the non-specific immune response to infection, activated macrophages synthesize nitric oxide (NO) at relatively high rates, thereby creating local concentrations of NO that are toxic to the invading microorganisms. However, high rates of NO synthesis can also damage host tissues, especially if prolonged (as in chronic inflammations). Although NO can act directly, much of its cytotoxicity and genotoxicity may be due to peroxynitrite ($ONOO^-$), formed by the rapid reaction of NO with superoxide (O_2^-). Superoxide is a byproduct of mitochondrial respiration and of certain cytosolic reactions, and it is present in virtually all cells. Thus intracellular $ONOO^-$ formation is inevitable when cells are exposed to NO, which diffuses readily across cell membranes. Superoxide is generated also by an NADPH oxidase on the external membrane surface of macrophages, thereby delivering it to the extracellular fluid at significant rates. The research in this thesis aimed to determine the concentration profiles of NO and related species in cell cultures and tissues. Toward that end, the diffusion and reactions of NO, O_2 and O_2^- were studied by mathematical modeling and by experimental work with macrophage cultures. The results obtained will be useful in designing NO toxicity experiments and determining potentially toxic conditions in body tissues.

A mathematical model was developed to predict the intracellular concentrations of NO, O_2^-, and $ONOO^-$ in suspension cell cultures exposed to NO and/or peroxynitrite. Steady state concentrations were computed as a function of radial position within an idealized spherical cell, with a distinction being made between cytosolic and mitochondrial values. Potential sources of peroxynitrite include intracellular generation in mitochondria and cytosol and (depending on the type of experiment) diffusion of extracellular peroxynitrite into the cell. The relative importance of extracellular and intracellular sources was estimated for a wide variety of conditions. The calculated mitochondrial concentrations were generally 5-10 times higher than the cytosolic values. For the baseline conditions, including an NO concentration of 1 μM and no peroxynitrite in the medium, the cytosolic peroxynitrite concentration was estimated as ~2 nM. The extracellular peroxynitrite concentration required to double the cytosolic level was ~25 nM, and an extracellular concentration of ~100 nM was needed to effect a five-fold increase.

Another approach for studying NO and $ONOO^-$ toxicity involves the co-culture of activated macrophages (producers of NO and O_2^-) with "target cells" (which make O_2^- but not NO). Because peroxynitrite concentrations in such experiments are too small and localized to measure, reaction-diffusion models were developed for situations ranging from isolated cells to many cells randomly distributed on a plate. The average concentration inside randomly distributed target cells increased with increasing macrophage number density, as extry of extracellular peroxynitrite grew in importance relative to intracellular formation. For high cell densities, large peroxynitrite membrane permeabilities, and low rates of intracellular synthesis, the surrounding macrophages were calculated to double or triple the peroxynitrite concentration in an average target cell. It was estimated that a macrophage enveloping a target cell could cause as much as a ten-fold increase in the concentration inside the target cell.

The interactions between NO and O_2 in activated macrophages were analyzed by incorporating previous cell culture and enzyme kinetic results into a novel reaction-diffusion model for plate cultures. The kinetic factors considered were: (i) the effect of on NO production by inducible NO synthase (iNOS); (ii) the effect of NO on NO synthesis by iNOS; (iii) the effect of NO on respiratory and other O_2 consumption; and (iv) the effects of NO and O_2 on NO consumption by a possible NO dioxygenase (NOD). Published data obtained by varying the liquid depth in macrophage cultures were compared with the modeling results. The model predicted that the rate of NO_2^- production should be nearly constant, and that the net rate of NO production should decline sharply with increases in liquid depth, in excellent agreement with the experimental findings. The main reason for the decrease in NO production with increasing liquid depth was the modulation of NO synthesis by NO, with O_2 availability playing only a minor role. The model suggested that it was the ability of iNOS to consume NO, as well as to synthesize it, that created very sensitive feedback control, setting an upper bound on the NO concentration of ~ 1 μM.

Experiments were performed to measure NO, O2, and O_2^- formation and consumption rates in murine macrophage-like RAW 264.7 cells. Cellular kinetic parameters for NO and O_2 were obtained by continuous monitoring of their time-dependent concentrations in a special closed chamber, and net cellular synthesis of O_2^- was quantified from ferricytochrome c reduction in cultures where NO synthesis was inhibited. Also measured was the photosensitive generation of O_2^- in the culture media. Unactivated cells, where NO synthesis was absent, had an O_2 consumption rate of 32 ± 3 pmol s^{-1} $(10^6$ cells$)^{-1}$, a level typical of mammalian cells. Also typical was that adding NO rapidly and reversibly inhibited respiration. Activated cells, which synthesized NO at a rate of 4.9 ± 0.6 pmol s^{-1} $(10^6$ cells$)^{-1}$, consumed O_2 at more than three times the rate of unactivated cells (108 ± 17 pmol s^{-1} $(10^6$ cells$)^{-1}$). Surprisingly, NO consumption by activated as well as unactivated cells was found to be negligible, and was insurricient to constitute a strong feedback control mechanism for NO as postulated in the modeling study. Accounting for O_2^- generation in the culture media resulted in net rates of cellular O_2^- synthesis smaller than previously reported; the rate was 6% of NO synthesis in activated cells and was undetectable in unactivated cells.

Finally the results from the experiments were used in a reaction-diffusion model developed to predict the concentrations of NO and O_2 in tissues containing macrophages. Steady-state concentrations were computed as a function of radial position within an

idealized spherical tissue region with different localizations of macrophages. The two situations considered were: (i) macrophages present in perivascular positions (i.e., lining the blood vessels), and (ii) an aggregate of macrophages at the center of the spherical tissue. As a result of inhibition of respiration of NO, spatial variations in the concentrations of O_2 were found to be negligible. For the baseline conditions, an NO concentration in tissue cells of ~ 0.2 – 0.3 μM was achieved in the two model configurations. Potential conditions leading to toxic levels of NO (0.5 μM) were also identified.

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