Diffusion and Reaction of Nitric Oxide in Cell Cultures

by

Bo Chen

Submitted to the Department of Chemical Engineering
in July 2001 in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy

Abstract

Nitric oxide (NO) is synthesized throughout the body and its intercellular signaling and other functions are necessary for health. However its mutagenic effects make it a potential cause of cancer, if it is produced at a high enough rate for a sufficient time. Sustained overproduction of NO may occur, for example, in macrophages as part of the immune response to certain chronic infections. The mutagenic effects of NO are thought to be due largely to trace compounds such as nitrous anhydride (N_2O_3) and peroxynitrite (ONOO-), formed during its reaction with molecular oxygen or superoxide anion (O_2-). It is not technically feasible to monitor the concentrations of these species under most conditions of interest, yet assessing the amounts of these species reaching target cells is essential to correlate levels of toxicity and to extrapolate to pathological situations in the body. Accordingly mathematical models are needed to predict the concentrations of NO and related compounds in cell cultures.

A reaction-diffusion model was developed to predict the fate of NO released by activated macrophages attached to microcarrier beads suspended in a stirred vessel, where experimental data were obtained previously. In the analysis the reactor was divided into a "stagnant film" with position-dependent concentrations and a well-mixed bulk solution. Model prediction based on independently determined kinetics and mass transfer parameters were found to be in good agreement with the experimental results for the concentration of NO in bulk solution, nitrite and nitrate accumulation rate, as well as the NO loss to the head space. The coupling between reaction and diffusion was found to greatly influence the spatial distributions of NO, O_2-, as well as N_2O and peroxynitrite, the potential mediators of cytotoxicity and mutagenicity. Superoxide and peroxynitrite were predicted to be present only within very localized regioins, whereas NO and N_2O_3 were found to distribute everywhere, more or less uniformly. The CO_2 pathway was shown to be the most important pathway for peroxynitrite decomposition and the reaction involving HCO_3- was suggested to appreciably reduce the level of N_2O_3 and therefore the formation of N-nitroso compounds.

Reaction-diffusion models were employed also to describe the spatiotemporal behavior of NO and related species in "plate cultures", a configuration that is employed frequently to examine the toxicity and/or mutagenicitiy of NO. In such experiments macrophages are maintained on the bottom of culture dishes in submonolayer amounts, typically with 1-4 mm of medium separating the cells from a head space. The reaction-

diffusion analysis revealed that there exists in the culture medium a distinctive spatial segregation among the key reactions, which leads to important simplifications in the kinetics and suggests relatively simple approximations to the concentration fields on both the macroscopic (culture medium depth) and microscopic (cell diameter or cell spacing) length scales. Transient effects were found to be unimportant. In contrast to the usual assumption made by those who have used this type of culture system, the calculations showed that NO loss to the head space is not negligible. Thus the usual approach of using the nonvolatile end products of NO oxidation (NO_2^- and NO_3^-) to infer the rate of NO production by the cells significantly underestimates that rate. Moreover it was found that varying the cell number density has very different influences on the relative exposures of the cells to NO, O_2^-, N_2O_3 and ONOO-. These results suggest that previously reported decreases in macrophage viability with increasing cell number density are much more likely to be the result of autoexposure of N_2O_3 and/or NO than to O_2^- or ONOO-.

The effect of liquid depth on the synthesis of NO and O_2^- was studied in murine macrophage-like RAW 264.7 cells activated by bacterial lipopolysaccharide and interferon-γ. Rates of NO_2^- and NO_3^- accumulation were determined by the rates of concentration increase 8-11 h after stimulation. The rate of NO synthesis was computed by using the plate culture model to correct NO_2^- and NO_3^- accumulation for physical loss of NO, whereas O_2^- synthesis was equated with NO_3^- formation. Rates of O_2^- synthesis determined by a spectrophotometric (cytochrome c) assay were in good agreement with those from NO_3^- accumulation, and showed production of O2- to be detectable immediately, in contrast to the ≈6 h time lag for NO. The assumption that NO_2^- and NO_3^- are stable end-products of the extracellular oxidation of NO by O_2 and O_2^-, respectively, was supported by the fact that NO_2^- and NO_3^- concentrations remained constant in the presence of unstimulated cells or stimulated cells where NO synthesis was inhibited. Data were obtained for depths of stagnant media ranging from 1 to 4 mm. The physical loss of NO was found to be quite significant, exceeding NO_2^- and NO_3^- accumulation by an order of magnitude at the smallest depth. The principal finding was that the rates of NO_2^- and NO_3^- accumulation each remained constant over the four-fold range of liquid depths. Because greater depths should greatly facilitate the trapping of NO as NO_2^-, this implies that NO synthesis decreased markedly with increasing depth. In contrast O_2^- synthesis remained constant. Oxygen availability is likely to have affected NO synthesis, in that diffusional limitation will yield the lowest O_2 concentrations at the cells when the liquid depth is greatest, and NO synthesis is known to decrease when O_2 levels are reduced. Concentrations of NO near the cells were calculated to remain at ≈1 μM for all conditions examined, raising the possibility that regulation of NO synthase activity by NO might also have mediated the effect of liquid depth.

Thesis Supervisor: William M. Deen
Title: Carbon P. Dubbs Professor of Chemical Engineering, MIT.