APPLICATION OF CARBON FIBER MICROELECTRODES FOR MEASUREMENT OF KINETIC CONSTANTS OF NITRIC OXIDE DECAY IN BLOOD

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ABSTRACT

Endogenous nitric oxide (NO) is a potent vasodilator that regulates vascular tone. There is evidence that cigarette smoking increases superoxide production in blood vessels by rapidly inactivating NO. This impaired NO bioavailability is a critical cause of endothelial dysfunction and a major risk factor for vascular disease, such as hypertension and atherosclerosis. Quantitative measurement of the NO decay rate in the blood of cigarette smoke treated animals is important for understanding the effect of cigarette smoking on NO decay kinetics in the vasculature. Carbon fiber microelectrodes (CFM) have been used for measurements of NO concentration. However, the time course of recorded current changes (*I-t* curves) by a CFM is different from the actual time course of NO concentration changes (*c-t* curves) due to CFM's response time (several seconds). This complicates the determination of rate constants for NO decay from the *I-t* curves. To find a simple method for analyzing experimental data, we present a mathematical model to describe the relationship between the recorded currents at the CFM and the NO concentrations in the solution. Using computer simulations based on the mathematical model, an approximation method was developed for determining the rate constants of NO decay from *I-t* curves, and the measurement accuracy was determined. This method was tested in several simple reaction systems with known rate constants, and applied to measure the rate constants of NO decay in blood samples of cigarette smoke exposed and control unexposed mice. These measurements demonstrate that smoking exposure increases the rate of NO decay in blood due to leukocyte activation with superoxide generation.

NTRODUCTION

The physiological role of nitric oxide (NO) is closely related to its bioavailability, whereas NO bioavailability is dependent on the rate of NO generation, metabolism, and diffusion. Like studies of the NO generation process, studies of the NO metabolism process have provided important information for better understanding the physiological function of NO. Various techniques have been used in studying NO metabolism process. Among these methods, electrochemical NO sensors or electrodes have some special advantages because of their ability to directly measure NO concentration in solution. Two types of commercial NO electrodes have been generally used in laboratories. One is the Clark-type electrode, the other is the CFM. The response time of these electrodes to a change of NO concentration is from milliseconds to seconds. In a previous paper, we have described how to use a Clark-type NO electrode to measure a fast reaction in which NO half-life is less than the response time of the NO electrode by solving diffusion-reaction equations. In this study, we focus on the application of CFM in measuring relatively slow NO reactions, and an approximate but accurate method is developed to determine kinetics and rate constants of NO decay without solving diffusion-reaction equations. Measurement errors are estimated based on theoretical analysis and computer simulations.

METHODS

***Electrochemical Measurements of NO Concentration*—The** electrochemical system for measuring NO consisted of a CFM (ISO-NOPF200 from World Precision Instruments (WPI), Sarasota, FL), a 4-port water-jacketed electrochemical chamber (NOCHM-4 from WPI, FL), a magnetic stirrer (WPI, FL), a Haake DC10-P5/U circulating bath, and an Apollo 4000 free radical analyzer (WPI, FL). The electrochemical chamber contained 2 mL of phosphate-buffered saline (37 ℃). The solution was stirred at a constant speed with the magnetic bar. NO was added into the solution with a Hamilton syringe (Hamilton Company, NV) by a bolus injection.

***Animal model and preparation of Blood Cells*—**Male C57BL/6J mice, 11-12 weeks of age were housed at a 23 ± 2 ℃, with 12h day-night cycle. After a week of acclimatization, mice were exposed to whole body mainstream and side stream cigarette smoke using the TE-10 cigarette smoking machine and 3R4F reference research cigarettes that deliver 9.4 mg tar/0.726 mg nicotine per cigarette under the standard Cambridge filter smoking condition. The smoking machine was programmed to give 3 sets of exposure. In each set, the machine puffs smoke/air mixture over a period of ~24 min, followed by a break of fresh air for ~ 20 min. The total exposure time was ~72 min per day 6 days per week for 32 weeks. Age-matched, air exposed mice served as controls. Blood was drawn from control and smoke-exposed mice after 24 hours from the last exposure into heparinized tubes. Blood samples were centrifuged, the supernatant containing the plasma was discarded, and the RBC/WBC pellet was washed three times with phosphate buffered saline.

Mathematical Model

The sensing part of the CFM is the micro-cylinder with a diameter of 200 μm and a length of 5 mm. The surface of the CFM is coated with multi-layered NO-selective membrane. Since the solution is stirred, the NO concentration almost uniformly distributes in the whole solution except the space near the surface of cylinder CFM, where a NO concentration gradient exists. The NO gradient layer consists of the selective membrane and a thin solution layer near the electrodes. We use the concept of effective diffusion layer to simplify the diffusion process. This concept assumes that the diffusion through multiple layers is approximately considered diffusion problem through a single layer, and an apparent NO diffusion coefficient can be used to describe NO diffusion process in this single layer. By solving a time-dependent diffusion equation, we can find NO concentration gradient at the surface of the CFM and calculated the normalized current at the CFM from the following equation:

$$i_n(t) = \frac{i}{i_d} = \frac{\left.\frac{\partial u(t)}{\partial R}\right|_{R=R_0}}{\left.\frac{\partial u(t_\infty)}{\partial R}\right|_{R=R_0}} = A\left.\frac{\partial u(t)}{\partial R}\right|_{R=R_0} \qquad (1)$$

If NO concentration in the bulk solution, $[NO]_b$, is known or given, then NO diffusion coefficient D and the diffusion layer thickness L, or the apparent parameters L^2/D and R_0 can be determined by finding the following Least-Square method:

$$f(L,D) = \sum_t [i_n - I_n]^2 \geq 0 \qquad (2)$$

Where I_n is the normalized current that is measured experimentally and i_n is the normalized current that is calculated from Eq. (1).

RESULTS

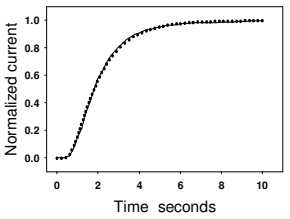

Figure 1. Response current of the CFM with a radius of 100 μm to a bolus injection of 2 μM NO in the chamber containing 2 mL deaerated solution at 37 ° C (solid line: recorded by the electrode, dotted line: the best fitting curve using Eqs. (2). The apparent parameter L^2/D determined from the best-fitting curves is 11.5±1.7 s (n=5). The response time of the CFM at $T_{95\%}$ is 4.7±0.7 s.

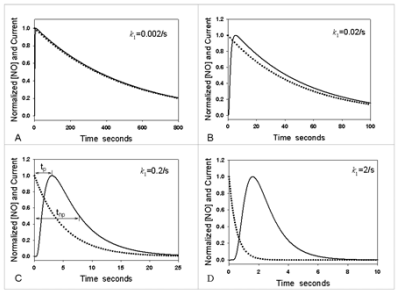

Figure 2. Computer-simulated normalized NO concentrations (dotted lines) that decay in the solution with first-order kinetics at four different rates and the corresponding normalized currents (solid line) at the CFMs. A: k_1=2x10^{-3} s^{-1}, B: k_1=2x10^{-2} s^{-1}, C: k_1=0.2 s-1, and D: k_1=2 s^{-1}. A significant difference between the normalized NO concentration (dotted line) and the normalized current exists when k_1≥2x10^{-2} s^{-1}.

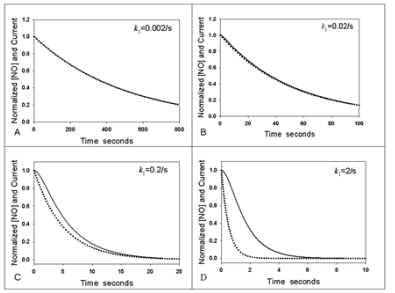

Figure 3. Shift of solid lines (normalized currents) in Fig. 2 to the left to overlap their peaks with peaks of the normalized NO concentrations (dotted lines). A: $k1$=2x10^{-3} s^{-1}, B: $k1$=2x10^{-2} s^{-1}, C: k_1=0.2 s^{-1}, and D: k_1=2 s^{-1}. Solid lines match the dotted lines pretty well as k_1 is up to 0.02 s^{-1} (A & B). When k_1 increases to 0.2/s, the main part of the solid line is still parallel to the dotted line except the curve part near the peak (C). A big difference is seen between the solid line and the dotted line at k_1=2/s (D).

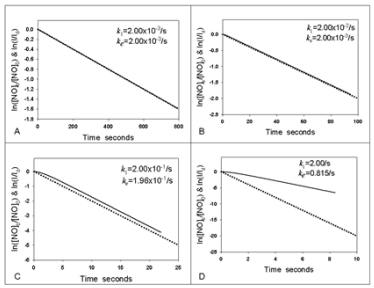

Figure 4. Plots of ln(I_n) (solid lines) and ln{[NO]$_n$} (dotted lines) vs time t. The values of k_1 determined from the slope of each plot of ln{[NO]$_n$} vs t are: A: 2x10^{-3} s^{-1}, B: 2x10^{-2} s^{-1}, C: 0.2 s^{-1}, and D: 2 s^{-1}. Correspondingly, The values of k_1 determined from the slope of each plot of ln(I_n) vs t are: A: 2x10^{-3} s^{-1}, B: 2x10^{-2} s^{-1}, C: 0.196 s^{-1}, and D: 0.815 s^{-1}, respectively.

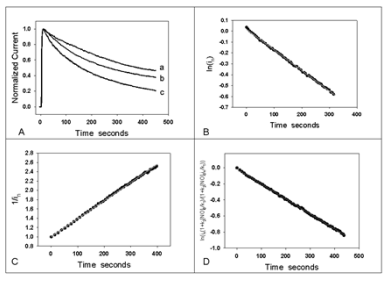

Figure 5. Application of the CFCM in determining kinetic orders or rate constants of NO decay in solution. A: The NO decay curves recorded by the CFM in the deaerated solution (a), in the aerated solution without (b) and with (c) headspace. (B) Plot of ln (I_n) vs t using data from the right side of the peak of curve a. (C) Plot of ($1/I_n$) vs t using data from the right side of the peak of curve b. (D) Plot of $\ln\frac{I_n(1+\frac{k_2}{k_1}[NO]_b)}{(I_n+\frac{k_2}{k_1}[NO]_b)}$ vs t using data from the right side of the peak of curve c.

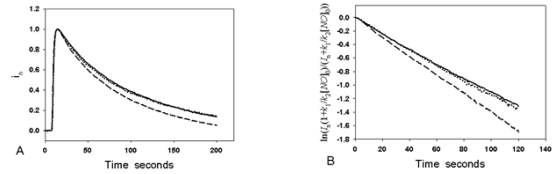

Figure 6. Application of the CFCM in determining NO decay rate constants in diluted blood samples from mice in smoking and control groups. A: Normalized currents recorded from the CFM in the presence of diluted blood sample from smoking group (---), smoking blood sample + 10 μM DPI (…), and control group (—). B: Plots of $\frac{\ln(I_n(1+\frac{k_2}{k_1}[NO]_b))}{(I_n+\frac{k_2}{k_1}[NO]_b)}$ vs t are straight lines, indicating that NO decay rates follow mixed first and second order kinetics. The values of k1 determined from the slope of the plots are (9.9±0.7)x10^{-3} s^{-1} (control, —), (1.21±0.09)x10^{-2} s^{-1} (smoking, ---), and (10.3±0.4)x10^{-3} s^{-1} (smoking +DPI, …), respectively.

Discussion

For a CFM with a response time of several seconds, computer simulations show that the relative difference between I_n and [NO]$_n$ is dependent on rate constants of NO decay. However, if we shift the current peaks to the left as shown in Fig. 3, we can see that I_n is very close to [NO]$_n$ in Figs. 3A-3C until NO decay rate is too big (Fig. 3D). Using computer simulations, we observed that the "measurement error" of rate constants with this approximate method is less than 5% when t_{hp}>3t_p or t_p<(t_h-t_p)/2 for the CFMs. Our results show that this approximation method accurately determined the kinetic characteristic for the NO volatilization from solution to gas phase, the second order kinetic rate constants for NO autoxidation in solution, and the rate constants for the mixed first order and second order kinetics (Fig. 5). We then applied this approximate method to determine NO decay in the presence of blood cells from mice with/without smoking treatment. It was observed that after hemoglobin was pre-oxidized by extra amount of NO, the rate of NO decay in the presence of blood cells from mice in smoking group is appreciably increased compared to mice in the control group. The increased NO decay rate can be inhibited by 10 μM DPI, suggesting that superoxide production from leakage of NADPH oxidase and/or other flavin enzymes is increased in smoking mice. The increased superoxide production reduces NO bioavailability and contributes to endothelial dysfunction, resulting in cardiovascular diseases.

Conclusions

We present an approximation method for measuring NO decay constants by a carbon fiber NO microelectrode with a response time of several seconds. Our results show that it is possible to determine NO decay rate constants directly from the normalized current data recorded by the NO electrode within an acceptable measurement error when the condition t_{hp}>3t_p is met.