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Direct, Real-Time Measurement of Shear Stress-Induced Nitric Oxide Produced from Endothelial Cells *In Vitro*

Allison M. Andrews, Dov Jaron, Donald G. Buerk, Patrick L. Kirby, and Kenneth A. Barbee

School of Biomedical Engineering, Science, and Health Systems, Drexel University, 3141 Market St. Philadelphia, PA 19104, USA

Abstract

Nitric oxide (NO) produced by the endothelium is involved in the regulation of vascular tone. Decreased NO production or availability has been linked to endothelial dysfunction in hypercholesterolemia and hypertension. Shear stress-induced NO release is a well-established phenomenon, yet the cellular mechanisms of this response are not completely understood. Experimental limitations have hindered direct, real-time measurements of NO under flow conditions. We have overcome these challenges with a new design for a parallel-plate flow chamber. The chamber consists of two compartments, separated by a Transwell® membrane, which isolates a NO recording electrode located in the upper compartment from flow effects. Endothelial cells are grown on the bottom of the membrane, which is inserted into the chamber flush with the upper plate. We demonstrate for the first time direct real-time NO measurements from endothelial cells with controlled variations in shear stress. Step changes in shear stress from 0.1 dyn/cm² to 6, 10 or 20 dyn/cm² elicited a transient decrease in NO followed by an increase to a new steady state. An analysis of NO transport suggests that the initial decrease is due to the increased removal rate by convection as flow increases. Furthermore, the rate at which the NO concentration approaches the new steady state is related to the time-dependent cellular response rather than transport limitations of the measurement configuration. Our design offers a method for studying the kinetics of the signaling mechanisms linking NO production with shear stress as well as pathological conditions involving changes in NO production or availability.

Keywords

Shear stress; Endothelial Cells; Nitric Oxide; Parallel Plate Flow Chamber

1.1 INTRODUCTION

Release of nitric oxide (NO) from the endothelium is thought to be responsible for shear stress (flow)-dependent vasodilatation [1]. In addition to causing smooth muscle relaxation, NO is important in the inhibition of platelets [2;3], smooth muscle cell proliferation [4] and adhesion of leukocytes to the endothelium [5;6]. Decreased NO production or availability has been linked to endothelial dysfunction in hypercholesterolemia and hypertension [7;8]. NO is expected to be present in nano-micro molar concentration levels, and it reacts rapidly with molecular oxygen to form nitrite such that it has a half-life of 2–30 seconds [9]. Several

Address correspondence to K.A. Barbee, School of Biomedical Engineering, Science, and Health Systems, Drexel University, 3141 Market St. Philadelphia, PA 19104, USA. kab33@drexel.edu.

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direct and indirect techniques have been developed to measure NO. Indirect methods involve measurement of nitrite or nitrate, which is assumed to represent the total NO produced, such as the Griess reagent [10], chemiluminescence NO analyzers [11] and nitrite sensitive electrodes [12]. Other indirect detection methods include chemiluminescence detection of soluble guanylyl cyclase (sGC), which is activated after binding NO [13] and measurement of cGMP production, which is produced by activated sGC, using a sGC derived fluorescence indicator [14]. 4,5-diaminofluorescein diacetate (DAF), a cell permeable fluorescent probe, has also been used to detect NO [15;16] via a change in fluorescence due to nitration by NO-derived nitrosating species (primarily N_2O_3) [15]. Electrodes remain the primary method for direct NO detection [16]. Research on shear stress-induced NO production has been severely limited because of experimental difficulties and detection limitations regarding concentration range and accuracy. NO detection under changes in shear stress due to changes in flow is particularly challenging because of low NO concentrations and many competing phenomena including convection, diffusion, and chemical degradation.

Knowledge of shear stress-induced NO production remains incomplete with few investigators able to directly measure NO produced from shear stress changes. Measurements of NO concentration under varying flow conditions coupled with theoretical simulations can be used to determine the NO production rate and its relationship with shear stress. Buga et al. [1] measured NO produced from endothelial cells grown on microcarrier beads using an *in vitro* bioassay. Microcarrier beads were required to provide sufficient cultivation of large quantities of cells in order to detect changes in NO concentration with their detection assay. In addition, the design of their experiment produced nonuniform laminar flow and thus provided little quantitative information on the relationship between NO and shear stress. Kuchan and Frangos [17] measured NO_x (NO_2^- , NO_3^{2-}) concentrations using the Griess reagent to monitor NO released from cells exposed to laminar flow. Their measurements indicated that NO production is biphasic with an initial rapid increase under 2 hours, followed afterwards by sustained production [17]. However, due to its low sensitivity, the Griess reagent could only measure NO changes over long periods of time (time course of hours). Corson et al. [18] also measured NO_x released from cells exposed to shear stress using a chemiluminescence detector. Their results showed biphasic NO release with an early transient increase within 5 minutes, followed by sustained release [18]. Although both investigators used parallel plate flow chambers and collected fluid samples downstream of the cells, their results differ primarily due to the differences in sensitivity of their NO detection methods. 4,5 Diaminofluorescein diacetate (DAF-2) fluorescence has also been used to monitor NO produced from endothelial cells under shear stress. Qui et al. [19] used DAF-2 to monitor NO produced from endothelial cells grown in microcapillary tubes exposed to laminar flow [19]. However the dye is modified irreversibly by the nitrosating reaction, preventing real-time concentration measurements. Their results showed a gradual increase in NO in response to shear stress (time course of minutes) rather than a rapid increase in NO, which reflects the binding kinetics and low sensitivity of the dye [19].

Electrodes provide a unique advantage in being able to measure local concentrations at the endothelial surface [20]. In addition, they remain the most suitable technique available for direct, real-time measurement of NO at low concentrations. Electrodes have been used to measure NO produced from vessels in response to agonist stimulation *in vivo* [21;22]. The NO response to controlled shear stress changes has been measured with electrodes in an isolated vessel preparation [23]. Nevertheless, there are currently limited data regarding direct *in vitro* measurements of NO produced due to shear stress.

Results from our previously published mathematical modeling of NO produced in a parallel-plate flow chamber suggested that steep concentration gradients exist at the cell surface due to the convective transport which rapidly removes the NO that diffuses into the fluid from the cell surface [24]. These steep gradients and low concentration levels make NO measurements under controlled *in vitro* conditions with electrodes virtually impossible without an extremely precise and controllable positioning system. In addition, placement of the electrodes close to the exposed cell surface can cause disturbances in the flow profile in the vicinity of the cells being monitored. Finally, the electrodes can be sensitive to flow itself. Our design overcomes the limitations involved with using electrodes in a flow environment and is capable of measuring, for the first time, direct, real-time low NO concentration levels produced by endothelial cells exposed to controlled changes in shear stress.

Shear stress produces changes in NO production; however, the nature of that relationship remains largely obscure. To fully understand the shear stress-dependent relationship, the kinetics of the signaling pathways involved must be evaluated using sensitive, direct, real-time measurement of NO concentrations. The purpose of this study was to develop a technique that was capable of direct, real-time measurement of NO produced by endothelial cells under controlled shear stress conditions. Our technique consists of a parallel plate flow chamber device with two compartments. One compartment contains the cells, which are subjected to the flow field. The other compartment, which serves to isolate the recording electrode from the effects of flow, contains an NO sensitive electrode. We demonstrate direct, real-time measurements of NO produced in response to changes in shear stress and an attenuated response with the NO inhibitor N ω -Nitro-L-arginine methyl ester (L-NAME). In addition, we used our previously published mathematical model [24] to evaluate the mass transport characteristics of our current chamber geometries. We show how comparison of simulations with experimental results can be used to estimate the time-dependent NO production of the endothelial cells in response to dynamic changes in shear stress.

1.2 EXPERIMENTAL PROCEDURES

1.2.1 Cell Culture

Bovine Aortic Endothelial Cells (BAECs) were obtained from Dr. Peter Davies' Laboratory (University of Pennsylvania). Endothelial cells (ECs) were cultured in Dulbecco's modified Eagle's medium (Mediatech Cellgro), supplemented with 10% fetal bovine serum (Sigma), 2 mmol/l L-glutamine (Mediatech Cellgro), and penicillin-streptomycin (Mediatech Cellgro) as described previously [25]. Cells were grown to confluency, and then plated to the underside of individual Transwell® membranes (Corning Transwell μ -Permeable Supports; 24 mm diameter culture area, 3 μ m pores, polyester). Membranes were placed in the incubator overnight before inverting and cultured for 1 day before experiments. Membranes were washed 3x with experimental solution then inserted into the flow chamber. The experiment solution was Dulbecco's Phosphate Buffered Saline (PBS) w/calcium/magnesium (Sigma) supplemented with 70 μ M L-arginine (L-arg) (Sigma).

1.2.2 Electrode

Electrodes and equipment (TBR4100 4-channel Free Radical Analyzer and 200 μ m diameter mini sensors for NO measurements ISO-NOPF) were purchased from World Precision Instruments. Electrodes were frequently recoated with nafion (Sigma) to improve selectivity and re-calibrated. The recoating includes at least two dip/dry sessions. Electrodes were calibrated according to the manufacturer's instructions by the decomposition of a NO donor S-nitroso-N-acetyl-penicillamine (SNAP) using Cu(II). SNAP was prepared by dissolving 5 mg EDTA and 5.0 mg $\pm$ 2.0 mg of SNAP in 250 mL HPLC grade water. The electrode

was immersed in 12mL of 0.1 M copper(II) sulfate in distilled water until the electrode stabilized (1–2hrs). Aliquots of SNAP were sequentially added (10 μ L, 20 μ L, 40 μ L, 80 μ L) after each signal reached a plateau. A multipoint calibration plot was created using Data-Trax software by (WPI). The sampling rate was 10 samples/s. The change in recorded potential was converted to corresponding molarities of NO produced by SNAP addition. The efficiency of the conversion of SNAP to NO is 0.6. Electrode sensitivity was at least 10 pA/nM. Electrodes were tested for sensitivity to nitrite (NO_2^-) after the calibration procedure. Sensitivity was typically 1.5–3% per 100 mM and increased when the electrode sensitivity to NO had decreased significantly below 5pA/nM. The temperature probe supplied by WPI was pre-scaled using a two-point entry of known temperatures (0.03125V = 1°C, 0.625V = 20°C).

1.2.3 Flow Chamber Design

The chamber was made from parallel plates of polycarbonate with a spacer that determines its height. The dimensions (in cm) of the flow channel are 4.57 W $\times$ 12.19 L $\times$ 0.025 H. The flow inlet and outlet were designed with large reservoirs with sampling ports. A glass coverslip covers an opening in the bottom plate, allowing visualization of the cell layer and electrode on an inverted microscope. Endothelial cells are grown on the underside of Transwell® porous membranes, which fit into the chamber flush with the upper plate of the chamber (Figure 1, Figure 2). The chamber design has two compartments separated by the porous Transwell τ membrane. Below the membrane, fluid flows through the parallel plate channel exposing the endothelial cells to uniform shear stress. Above the membrane, a stagnant fluid compartment houses the NO electrode and temperature sensor. This design places the electrode out of the fluid flow and avoids potential flow sensitivities, flow disturbances and chamber leaking due to the electrode insertion site. The electrode is lowered until resting on the inner side of the membrane giving a fixed distance from the ECs, equal to the thickness of the membrane (10 μ m). This configuration allows NO to be measured abluminally from the endothelial layer. Due to the temperature sensitivity of the NO electrode, the flow chamber is enclosed in a water bath at 37°C in order to prevent heat loss and temperature fluctuations. In addition, samples can be taken during experiments from sampling ports and later analyzed for NO concentrations using an NO Analyzer (Sievers NAO280i).

1.2.4 Experimental Setup

The flow chamber was sterilized under UV for 20 minutes before each use. The chamber was flushed with each of the following: 100 mL of 70% ethanol, 100 mL of deionized water and then prepped with 75 mL of the experimental fluid (PBS w/calcium & magnesium supplemented with 70 μ M L-arg). Fluid was pumped using a Reglo-Z Digital pump (Ismatec). Flow rates were calculated based on desired shear stresses using the following formula: $Q = \tau w h^2 / 6\mu$, where w = chamber width, h = chamber height, μ = viscosity, τ = shear stress (dyn/cm²) and Q = flow rate. The chamber height was chosen based on the range of shear stresses we intended to investigate. The pump was controlled using a LabView program, which was adapted from the manufacture's online LabView driver. Using an inverted light microscope (Nikon TE300 Eclipse) under 10x objective, the electrode was lowered using a micrometer until gently resting on the membrane. The chamber was then placed in an enclosed heated water bath (37°C) for an hour without flow until the electrode and temperature in the flow chamber stabilized. 120 mL of the experimental fluid was cycled continuously during the experiment. Cells were exposed to multiple step changes ranging from 0.1 dyn/cm² to 20 dyn/cm² with a 3-minute interval between step changes. The experimental solution was then exchanged with PBS w/calcium/magnesium with 1mM N ω -Nitro-L-arginine methyl ester (L-NAME, pH 7.2, Sigma). The L-NAME solution was

flushed through the chamber and then the flow was turned off for 1 hour prior to a repeating the same sequence of step changes that were performed prior to L-NAME treatment.

1.3 RESULTS

1.3.1 Shear Stress-Induced NO response

Step changes in flow elicited reproducible changes in NO concentration, and cells could be repeatedly stimulated without diminution of the response. The magnitudes of the responses were consistent within an experiment but varied among cultures. The chamber was continuously perfused at a low flow rate of 0.25 mL/min (corresponding to a wall shear stress of 0.1 dyn/cm²) to prevent the accumulation of NO due to the basal (unstimulated) production. A series of step changes in flow rate corresponding to shear stresses of 1, 6, 10, or 20 dyn/cm² were applied, always returning to 0.1 dyn/cm² between stimuli (Figure 3). The steady-state NO concentration at 0.1 dyn/cm² was offset to zero in order to show the change in NO concentration in response to each step change in shear stress (shown to occur at 50s). The responses include a sharp, transient decrease in NO concentration upon step initiation followed by an increase in NO concentration until a steady state was reached. The change in steady state was calculated as the difference between the baseline prior to the step change and the steady-state values following the step change (Figure 4A). The differences were calculated using the average concentration over a 13 second interval prior to the step change and the average concentration at the new steady state over a 9 second interval. The steady state change averaged -6 nM for a step change to 1 dyn/cm², 25 nM for a step change to 6 or to 10 dyn/cm² and 45 nM for a step change to 20 dyn/cm². In addition, the magnitude of the initial decrease was found to be shear-stress dependent (data not shown). The time course of the NO concentration profiles following a step change was analyzed using an exponential fit, to calculate the time constant (t_c) (Figure 4B). Average t_c values were 64 s, 41 s, and 21 s for 6, 10 and 20 dyn/cm² respectively. Because the steady-state changes in NO concentration for the step change from 0.1 to 1 dyn/cm were small, time constants could not be reliably determined for this group.

1.3.2 Effect of L-NAME on Shear Stress-Induced NO Response

Shear stress-induced NO responses were compared before and after L-NAME treatment. Following stimulation at multiple shear stresses, the experimental fluid was exchanged with 1 mM L-NAME (pH 7.2). Measurements with L-NAME were made under the same protocol as prior to treatment with L-NAME with the shear stress starting at a 0.1 dyn/cm² and then increasing in step changes to 1, 6, 10 or 20 dyn/cm². In the sample traces (Figure 5) this step change occurs at 50s. The steady-state concentration at 0.1 dyn/cm² was offset to zero in order to show the individual NO response due to the step change. Steady-state changes after treatment with L-NAME were reduced by approximately 40% and were statistically significant for all changes in shear stress except for 0.1 to 1 dyn/cm² (Figure 6 * $p < 0.05$; ** $p < 0.01$; for paired one-tailed t-test). We observed a decrease in the baseline concentration following treatment with L-NAME that is not reflected in the data because our measurements only examine relative changes in NO concentration. Thus, the effects of L-NAME treatment on the absolute concentrations of NO were more profound than suggested by the 40% reduction of the shear stress response.

1.3.3 Comparison with Mathematical Modeling

The measured concentrations depend on the production rate of NO (R_{NO}) as well as the convective and diffusive mass transport processes occurring in our flow chamber. It is therefore necessary to analyze the transport processes in order to properly interpret the measurements. We utilized our previously published mathematical model of NO transport [24] to compare transient and steady state predictions with our experimental data. The

development of the model is described in detail elsewhere [24]. Briefly, within the flow domain (described by plane Poiseuille flow) the convection-diffusion equations for the transport of NO are solved using finite element analysis. Production of NO occurs in the 5 μm thick endothelial layer where mass transport is by diffusion only. An auto-oxidation reaction is included in both domains with the oxygen concentration taken to be a constant throughout the chamber. For the simulations presented here, the dimensions and configurations of the chamber were modified to incorporate the porous membrane (10 μm) and stagnant compartment above the cell layer of the current experimental chamber.

Our model allows us to relate the steady-state NO concentration at the position of the electrode to the production rate within the endothelial layer. This relationship is approximately linear with the slope strongly dependent of the shear rate (Figure 7). These relationships demonstrate that the size of a stimulated change in production rate, represented by the measured $\Delta[\text{NO}]$, depends on the baseline concentration. At present, our measurement technique only allows relative changes in $[\text{NO}]$ to be determined. However, using our computational model for the flow chamber, we can estimate the basal production rate of NO based on an analysis of the steady-state concentration changes for a range of shear stress step changes. We investigated three relationships for shear stress-dependent R_{NO} and found the best fit to our steady state experimental results for each relationship, as shown in Figure 8. The simplest model tested for the NO production rate was linear with shear stress ($R_{\text{NO}} = R_{\text{basal}} + A\tau$; black bars) where R_{basal} is the basal production rate and A is the slope. We also investigated two nonlinear relationships for R_{NO} as a function of shear stress: a hyperbolic model ($R_{\text{NO}} = R_{\text{basal}} + R_{\text{max}}\tau/(\tau+A)$; gray bars), and a sigmoidal model ($R_{\text{NO}} = R_{\text{max}}/(1+A\exp^{-B\tau})$; hatched bars). The sigmoidal function is similar to that used recently in a flow chamber model by Plata *et al.* [26]. The basal rate for the sigmoidal model can be calculated from $R_{\text{basal}} = R_{\text{max}}/(1+A)$. Predicted steady state NO values at each shear stress for each relationship were obtained from the computational model for the flow chamber, allowing the steady state difference in NO ($\Delta[\text{NO}]$) between any two shear stress levels to be determined. Comparisons for the best fit of these models to our experimental data (mean $\pm$ SE, open bars) are shown (Figure 8A). The linear model (black bars) provided the worst fit. The two nonlinear models appear to provide excellent fits to most of the experimental data, although both underestimate ΔNO for the change in shear stress from 0.1 to 6 dyn/cm^2 . The hyperbolic model (gray bars) provided the closest match to the data. Although the sigmoidal function could be made to fit the discrete data points fairly well, the production rate is nearly constant within the plateau phase of the curve while shear stress (and thus convective transport) is increasing. This would lead to an unphysiological situation in which $[\text{NO}]$ decreases at shear stress values higher than a local maximum occurring between 10 and 20 dyn/cm^2 . Calculated values for the basal NO production rate (R_{basal}) at zero shear stress and R_{NO} at different shear rates based on parameters that provided the best fit to the experimental data for each relationship are summarized in Table 1 and shown graphically in Figure 8B. The three models for shear stress-dependent R_{NO} were also fit to steady state ΔNO data obtained from L-NAME studies. The hyperbolic model again provided the best fit and the linear model the worst fit, and all models underestimated experimental data for the 0.1 to 6 dyn/cm^2 change in shear stress (not shown). The analysis found that L-NAME partially inhibited R_{NO} under the experimental conditions of our study (L-arg also in perfusate). At the highest shear stress change (0.1 to 20 dyn/cm^2), R_{NO} was 57.3%, 57.6% and 58.3% of R_{NO} estimated for untreated ECs using linear, hyperbolic, and sigmoidal models, respectively.

Our computational model also allows us to investigate the role of mass transport in the dynamics of the transient response. Simulations for transient changes in NO concentrations were evaluated at the electrode location for a step change in shear stress from 0.1 dyn/cm^2 to 20 dyn/cm^2 (Figure 9). When R_{NO} was assumed to depend on shear stress alone with no

time lag between change in shear stress and the increase in production rate, the NO concentration reached its new steady state almost instantaneously. In contrast, when we simulated a gradual increase in production rate (linear with time) following a step change in shear stress, it was possible to mimic the time-dependent changes in NO concentration observed experimentally, including the transient decrease and subsequent slower rise to the new steady state.

1.4 DISCUSSION

We report for the first time direct, real-time measurement of NO concentration changes due to flow-induced shear stress stimulation of endothelial cells *in vitro*. The concentration changes were partially attenuated by the endothelial nitric oxide synthase (eNOS) inhibitor L-NAME. The failure of L-NAME to completely abolish the NO response could be in part be due to the incomplete removal of L-arg from the upper compartment. However, the level of inhibition we observed is consistent with previous studies with L-NAME treatment of *in vivo* vessels [27]. This study found that treatment with L-NAME or L-NNA individually inhibited vessel relaxation to acetylcholine stimulation by 30–40%. Several other investigators have also reported incomplete inhibition as listed in Cohen et al. [27].

NO-sensitive microelectrodes have been available for over ten years, but the rapid diffusion and short half-life of NO produces sharp gradients in concentration near the source of production. Thus, precise, reproducible positioning of the electrode near the source becomes a critical factor limiting the utility of this method. These observations are supported by Isik et al. [28] who demonstrated on static cell culture that the magnitude of the NO response to bradykinin decreased significantly with increased distance away from the cells. Furthermore, their experiments showed that the electrode could not detect changes in NO concentrations greater than 25 μm in response to 80 nM of bradykinin. The decreased NO detection at greater distances from the NO source was attributed to the rapid chemical oxidation of NO.

Measuring NO concentration under flow conditions presents even more severe challenges. The convective mass transport of NO produces much steeper concentration gradients near the surface of the NO-producing cells and reduces the NO in the endothelium. Our recent results using computer simulations demonstrated that NO concentration gradients at the cell surface are so sharp that positioning the electrode near enough to the exposed surface of the cells with the precision needed for accurate and reproducible measurements was technically unfeasible [24]. Furthermore, the electrodes used for NO measurements can be sensitive to flow, distorting or masking the NO signal. Finally, the placement of the electrode in the flow field can create disturbances in the flow profile making it difficult to relate flow to shear stress.

Our current design addresses these challenges by controlling the distance from the cell surface and by measuring NO within a thin stagnant compartment. In our system, the electrode is placed out of the path of the fluid, thus preventing any disturbances in the fluid flow profile, and eliminating the potential effects of the flow on the electrode itself. Just as important, the electrode distance from the cells is controlled in a precise and reproducible manner because it always rests on the Transwell $\surd$ membrane, fixing the distance at 10 μm . The 3 μm pore size is large enough that diffusion of NO within the pores will not be hindered, so the permeability of the membrane depends primarily on the area fraction of the pores (0.14 for the membrane used). Computational simulations on the membrane permeability showed negligible effects on the time to reach steady state for this porosity (data not shown). Furthermore, the thickness of the stagnant compartment is small, with a zero flux condition at the other boundaries. Because of this and the short diffusion distance

through the membrane, the concentration of NO in this compartment will be nearly uniform and will rapidly equilibrate with the concentration in the cell layer.

Our experimental results indicate that following a step change from a low shear stress of 0.1 dyn/cm² to higher shear stresses, the NO concentration at the electrode first transiently decreases and then increases to a steady-state concentration that is higher than the initial steady-state value except for a shear stress change to 1 dyn/cm². Our simulations suggest that this initial decrease is due to convective washout whose effect is immediate. Since the simulated production rate is actually the net release of NO from the cell (production minus any consumption by the cell), we cannot distinguish between changes in production and changes in reactions consuming NO within the cell. Thus, we cannot rule out the possibility that the lag in release is due in part to increased production accompanied by a simultaneous increase in NO consumption through rapid reactions with reactive species such as superoxide or lipid peroxyl radicals. When we simulated an instantaneous increase in production rate, we predicted a very rapid increase in NO concentration with no transient decrease. In addition, the magnitude of the initial decrease in our experimental results was found to be shear stress dependent (data not shown), supporting the idea that the transient decrease was related to the convective effect of the step change in flow.

Following the initial decrease, the concentration increases to a new steady state that is higher than the pre-stimulus level. The ability to measure changes in NO concentration in real-time allows us to analyze the kinetics of the responses of endothelial cells to changes in shear stress. The time constants characterizing the rate at which the concentration approached the steady state decreased significantly as the size of the step change in shear stress increased. This suggests that in addition to the steady state production rate being dependent on shear stress, the rate at which the signaling processes leading to increased production are activated is also dependent on the size of the shear stress stimulus. Our simulation of the time course of NO concentration changes due to an instantaneous increase in production in response to a step in shear stress indicates that there is negligible transport lag in our measurement system. Therefore, the time dependence of the concentration changes reflects the dynamics of the cellular response.

The measured concentrations of NO depend on the geometry of our chamber, the placement of the electrode, and the transport and production of NO. For any experimental apparatus of this kind, it is necessary to account for the transport effects in the chamber in order to properly interpret the measured concentration changes. For the steady-state concentration to increase with increased shear stress, the stimulated NO production rate must exceed the rate of removal by the increased convective transport effects. Our data and analysis shows that for small increases in shear stress, the concentration goes down despite an increase in production rate. This is consistent with the predictions of our previously published analysis [24]. Furthermore, we have shown that even though the steady-state concentration changes for 6 and 10 dyn/cm² were similar, the change in production rate at 10 dyn/cm² was much greater than at 6 dyn/cm² (Table I and Figure 8B).

The relationships between steady state [NO] and production rate for different flow conditions presented in Figure 7 provides a template for interpreting the measured responses in terms of stimulated changes in production. However, use of this template requires knowledge of the absolute concentration in the baseline condition. This could potentially be accomplished through the development of an in situ calibration procedure. Alternatively, we have outlined a procedure whereby the basal production rate can be estimated by fitting a theoretical relationship between production rate and shear stress to the measured NO changes for a range of shear stress step changes.

The highest values for R_{NO} occurred at $\tau = 20 \text{ dyn/cm}^2$ in our study (Table 1), above the range determined theoretically (5 to 100 nM/s) by Chen and Popel [29] from available data in the literature. A previous study with BAEC by Kanai et al. [16] reported a very low value for $R_{NO} = 2.23 \text{ nM/s}$, which is similar to the R_{basal} values in our study. However, a NO microelectrode used in their study was placed in the flow stream at a distance stated to be around 100 μm from endothelial cells, where our mass transport model predicts that NO concentrations are very low. A 500-fold higher value for $R_{NO} = 10.4 \text{ } \mu\text{M/s}$, depending on the length of time of exposure to shear, was determined from HUVEC data reported by Kuchan and Frangos [17] based on indirect measurements of NO metabolites (nitrite/nitrate). In their study, R_{NO} at a given shear rate increased rapidly for approximately the first 3 hr of shear exposure, with additional but smaller increases in R_{NO} with longer shear exposure times up to 15 hrs. Our technique allowed us to observe the dynamics of the response on a much smaller time scale. In contrast to our results, Kanai, et al. [16] reported only transient increases in NO in response to shear stress changes. In addition, we did not see any oscillations in NO under steady flow conditions as reported by Kanai, et al. [16].

Our measurements combined with mathematical modeling of the transport processes also allows the dynamic changes in NO production by the cells to be determined. Therefore, our measurement system can be used to evaluate simulations in which specific signaling events are explicitly modeled. Furthermore, the signaling pathways can be studied in detail by simulating the effects of inhibitors or other interventions and comparing the effects on the dynamics of the NO response.

Our innovative technique offers a method for studying the mechanisms linking NO production with shear stress as well as pathological conditions involving changes in NO production or availability. *In vitro* experiments offer a significant advantage by providing the ability to control shear stresses and determine the NO concentration changes in real time. However, the measured NO concentrations reflect a combination of NO production by the cells and convective and diffusive mass transport effects of the system. Therefore, experimental data should be coupled with mathematical modeling to properly interpret the results and to relate NO production to shear stress. Furthermore, experimental measurements with our technique can be used to explore the mechanisms that determine NO production and to evaluate different theoretical models (reviewed by Buerk [30] & Tsoukias [31]) which have previously been limited by the paucity of quantitative data regarding production and transport of NO.

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Figure 1.

Schematic of the flow chamber used for NO measurements. A) The endothelial cells are grown on the bottom of polyester Transwell® inserts (24 mm diameter, 3 μm pores, thickness 10 μm, 4.67 cm² area) and then inserted into the chamber flush with the upper plate. The electrode, which is located in the upper compartment, is lowered until resting on the Transwell® membrane. Fluid is pumped through the lower compartment to produce laminar flow at desired shear stresses at the cell surface. The chamber is enclosed in a water bath at 37°C to ensure constant temperature during the experiments. A temperature probe is also located in the upper compartment to monitor the temperature during the experiment. B) Close up of the cell-membrane-electrode interface. (Not to scale)

Figure 2.

Assembly of chamber parts. The flow chamber is constrained by the top and bottom chamber plates and a gasket, which determines the lateral dimension. The Transwell insert fits into the chamber top flush with the upper plate. The chamber dimensions (in cm) are $4.57 \text{ W} \times 12.19 \text{ L} \times 0.025 \text{ H}$. The electrode and temperature probe are attached to the electrode holder, which fits into the Transwell insert and lowered using a micrometer until resting gently on the membrane. (Not to scale)

Figure 3.

Sample traces of the shear stress-induced NO response. Experiments began at a low flow rate, which corresponds to a shear stress of 0.1 dyn/cm^2 that was then increased to higher flow rates corresponding to 1, 6, 10, or 20 dyn/cm^2 . The steady-state concentration at 0.1 dyn/cm^2 was offset to zero in order to show the individual NO response due to the step change. Responses show a transient decrease in NO concentration following a step change (applied at 50s), followed by an increase to a steady-state value above the initial baseline except for a step change from 0.1 to 1 dyn/cm^2 , which reached a steady-state value below the initial baseline. The magnitude of the transient decrease in NO concentration following a step change was shear stress dependent.

Figure 4.

Analysis of the NO response curves in response to shear stress changes. Experiments started at a low shear stress of 0.1 dyn/cm², which was then increased by a step change to 1, 6, 10, or 20 dyn/cm². A) Changes in the steady-state value were calculated based on the difference between the steady-state concentrations before and after a step change. The steady state change at 20 dyn/cm² was statistically different from both the change observed in response to 6 or 10 dyn/cm² but was not statistically significant between 6 and 10 dyn/cm². The steady-state change from 0.1 to 1 dyn/cm² was statistically different from step changes to all the other shear stresses. Concentration changes ranged from -21–9nM, 19–53nM, 20–47nM, and 24 to 62nM for a step change to 1, 6, 10, and 20 dyn/cm² respectively. B) To characterize the time course of NO concentration following a step change, concentration profiles were fitted with an exponential curve and time constants (t_c) were calculated. Exponential curves did not accurately reflect the time courses for a step change from 0.1 to 1 dyn/cm². (Mean and SE were plotted and statistics were calculated using the paired two-tailed t-test n= 8 for 1, 6 and 10 dyn/cm² and n=6 for 20 dyn/cm², *p<0.05. For time constants, one value for 6 dyn/cm² and for 10 dyn/cm² were significant outliers and were excluded using Grubb's test, α=0.05)

Figure 5.

Sample traces of the shear stress-induced NO response before and after treatment with 1mM L-NAME (pH 7.2). The steady-state concentration at 0.1 dyn/cm² was offset to zero in order to show the individual NO response due to the step change occurring at 50s. The solid line represents the NO response prior to L-NAME treatment and the dotted line represents the NO response after L-NAME treatment. A) Sample response from a step change from 0.1 to 1 dyn/cm². B) Sample response from a step change from 0.1 to 6 dyn/cm². C) Sample response to a step change from 0.1 to 10 dyn/cm². D) Sample response to a step change from 0.1 to 20 dyn/cm².

Figure 6.

Comparisons of the steady-state NO concentration changes in response to a step change before and after treatment with L-NAME. These changes were calculated between steady-state concentrations before and after application of a step change in shear stress. Comparison of the steady state changes between untreated and L-NAME treated responses were found to be statistically significant between step changes from 0.1 to 6, 10 and 20 dyn/cm² but not for 0.1 to 1 dyn/cm² (Mean and SE were plotted, n=8 for 6 and 10 dyn/cm², n=6 for 20 dyn/cm² * p<0.05; ** p<0.01; for paired one-tailed t-test). Steady-state changes after treatment with L-NAME averaged 60% of the untreated values. One value for 6 dyn/cm² was a significant outlier and was excluded using Grubb's test $\alpha=0.01$).

Figure 7.

Steady-state [NO] v. R_{NO} . At steady state, the concentration of NO is proportional to the production rate for a given shear rate. This relationship for each of the shear stress values used in our study is indicated by the solid lines. If the baseline concentration (at 0.1 dyn/cm²) is known or can be estimated, then the measured change in [NO] can be related to the change in production rate. By fitting an expression for the shear-stress dependent production of NO to the measured changes in [NO] in response to a range of shear stress steps, we were able to estimate the basal production rate. The symbols represent the [NO] and R_{NO} values determined by the best fit hyperbolic function relating R_{NO} to shear stress. Note that for a change in shear stress from 0.1 to 1 dyn/cm², there is an increase in production, yet the concentration decreases owing to the increased convective washout. This phenomenon was also described in our previously published model [24].

Figure 8.

A. Comparison of experimental results for changes in shear stress (open bars, mean $\pm$ SE) with steady state ΔNO predicted from 3 different models for shear stress-dependent rate of NO production ($R_{NO}(\tau)$): linear = black bars, hyperbolic = gray bars, sigmoidal = hatched bars). B. NO production rate (left axis) and corresponding NO release rate (right axis) as function of shear stress (τ) with best fit parameters for each model. Calculated R_{NO} and release rates for experimental shear stresses are shown for the hyperbolic model (gray squares). Insert: detail at low $\tau < 1$ dyn/cm².

Figure 9.

Comparison of experimental results and mathematical simulations. Measured NO (solid line) depicts NO concentration as a result of a step change (at 50s) from 0.1 to 20 dyn/cm². Note the initial decrease in NO concentration followed by an increase to an elevated steady-state concentration. Two mathematical simulations are shown utilizing either a time-dependent (ramp) (dashed line) or time-independent (instantaneous step) relationship (dotted line) between NO production and shear stress. Simulations were performed for a shear stress change from 0.1 dyn/cm² to 20 dyn/cm², which occurred at 50s. The time-independent model demonstrated that following a step change a steady-state concentration was reached almost instantaneously. The time-dependent model utilized a linearly increasing production rate in response to the initiation of shear stress. This produced an initial decrease in NO concentration in response to the step change followed by an increase until a new steady-state concentration was reached.

Table 1
Analysis of experimental results from flow chamber using different models of shear stress dependent NO production

τ (dyn/cm ²) =	0	0.1	1	6	10	20
Model	R_{basal}	$R_{\text{NO}} (\tau)$	(nM/s)			
$R_{\text{NO}} = R_{\text{basal}} + A\tau$	1.74	2.49	9.26	48.9	76.9	152
$R_{\text{NO}} = R_{\text{basal}} + R_{\text{max}} \tau/(\tau + A)$	2.13	3.43	14.8	69.1	104	168
$R_{\text{NO}} = R_{\text{max}}/(1 + A\exp^{-B\tau})$	1.12	1.17	1.83	19.3	73.0	129

Model Parameters

Linear, $A = 7.52 \text{ (nM/s)/(dyn/cm}^2\text{)}$
Hyperbolic, $R_{\text{max}} = 457.5 \text{ nM/s}$, $A = 35 \text{ cm}^2\text{/dyn}$
Sigmoidal, $R_{\text{max}} = 129.5 \text{ nM/s}$, $A = 115$, $B = 0.5 \text{ cm}^2\text{/dyn}$