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Modeling of Biopterin Dependent Pathways of eNOS for Nitric Oxide & Superoxide Production

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Abstract

Endothelial dysfunction is associated with increase in oxidative stress and low NO bioavailability. The eNOS uncoupling is considered an important factor in endothelial cell oxidative stress. Under increased oxidative stress, the eNOS cofactor BH₄ is oxidized to dihydrobiopterin (BH₂), which competes with BH₄ for binding to eNOS, resulting in eNOS uncoupling and reduction in NO production. The importance of the ratio of BH₄ to oxidized biopterins versus absolute levels of total biopterin in determining extent of eNOS uncoupling remains to be determined. We have developed a computational model to simulate the kinetics of the biochemical pathways of eNOS for both NO and O₂^{•−} production to understand the role of BH₄ availability and total biopterin (TBP) concentration on eNOS uncoupling. The downstream reactions of NO, O₂^{•−}, ONOO[−], O₂, CO₂ and BH₄ were also modeled. The model predicted that a lower [BH₄]/[TBP] ratio decreased NO production but increased O₂^{•−} production from eNOS. The NO and O₂^{•−} production rates were independent above 1.5 μM [TBP]. The results indicate that eNOS uncoupling is a result of a decrease in BH₄/TBP ratio and a supplementation of BH₄ might be effective only when the BH₄/TBP ratio increases. The results from the current study will help understand the mechanism of endothelial dysfunction.

Keywords

Mathematical Model; Kinetic Analysis; eNOS uncoupling; Biopterin ratio; NOS Biochemical Pathway

Introduction

Endothelial nitric oxide synthase (eNOS), which is present in endothelial cells of the vasculature, catalyzes the oxidation of L-Arginine to produce NO [1–3]. Under physiological conditions, NO catalyzes the conversion of GTP to cGMP through stimulation of the enzyme soluble guanylate cyclase (sGC) in the vascular smooth muscle cells and dilates the blood vessel [1, 4]. Furthermore, NO prevents platelet aggregation and leukocyte adhesion to endothelium [5–8], thus plays a role in the regulation of vascular permeability [9–11].

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The enzyme eNOS is an oxidoreductase dimer with two domains as shown in Figure 1A [12]. The oxygenase domain contains binding sites for heme, the co-factor biopterin and the substrate L-Arginine [1, 3, 13]. The reductase domain contains binding sites for the flavin co-factors FAD, FMN and the substrate NADPH [1, 3]. The two domains are bound together by a CaM (calmodulin) recognition site [1]. In the presence of Ca^{2+} and CaM, NADPH oxidizes and donates electrons that migrate along the reductase domain (FAD and FMN) into the oxygenase domain [1, 2, 14]. This causes the catalytic reaction between L-Arginine and O_2 to produce NO through a series of biochemical reactions [1]. These reactions can only occur when eNOS is in the dimer form and eNOS is bound to the reduced form of the co-factor biopterin, tetrahydrobiopterin (BH_4) [3, 15–17]. Furthermore, BH_4 promotes the dimerization of eNOS, increases the stability of eNOS and enhances the binding of substrates such as L-Arginine [1].

In pathophysiological conditions including hyperglycemia in diabetes and presence of cardiovascular risk factors, the activity of enzymes such as NADPH oxidases and xanthine oxidase is increased in vascular walls; resulting in an increase in oxidizing species such as superoxide ($\text{O}_2^{\bullet-}$) [3, 12, 18, 19]. $\text{O}_2^{\bullet-}$ reacts with NO to form peroxynitrite (ONOO^-) enhancing endothelial oxidative and nitrosative stress. As shown in Figure 1B, both $\text{O}_2^{\bullet-}$ and ONOO^- reduces the endothelial cell BH_4 levels by oxidizing BH_4 to BH_3 , which further oxidizes to BH_2 [3, 4, 12, 18, 19]. BH_2 can also compete with BH_4 for binding to the oxygenase domain of eNOS [4, 20]. The binding of BH_2 to eNOS causes uncoupling of eNOS. The uncoupling of eNOS is reported to increase the $\text{O}_2^{\bullet-}$ production and decrease the NO production [4, 19, 21]. The uncoupling of eNOS can also occur due to failure of eNOS to form dimers [3]. In such cases, the NADPH donated electrons directly reduces O_2 to $\text{O}_2^{\bullet-}$. However, the $\text{O}_2^{\bullet-}$ generation from monomeric NOS is small in comparison to that seen from the uncoupled dimeric eNOS [3].

Several studies have investigated the role of BH_4 oxidation to BH_2 and subsequent reduction in BH_4 availability on eNOS uncoupling. However, the relative importance between biopterin ratio (the ratio of BH_4 to oxidized biopterins) and absolute levels of total biopterin (TBP) in determining extent of eNOS uncoupling remains unresolved [18]. Vasquez-Vivar et al. [4, 22] and Crabtree et al. [19, 23] reported the importance of biopterin ratio whereas Alp et al. [24] reported that both absolute biopterin levels and biopterin ratio are important to determine the extent of eNOS uncoupling. In addition, it has also been reported that the absolute levels of BH_4 and not the biopterin ratio is the principal determinant of eNOS activity and uncoupling [25].

In vitro BH_4 , BH_2 and sepiapterin supplementation studies performed on either cultured endothelial cells, isolated blood vessel segments and tissues under both normal and diseased conditions have shown an increase in the total biopterin levels (TBP) [23, 26–29]. The biopterin ratio (ratio between reduced (BH_4) and oxidized biopterin (BH_2 and BH_3)) was found to decrease upon supplementation of BH_4 under diseased conditions [23, 26]. Under normal physiological conditions, BH_4 supplementation either had no effect on biopterin ratio or decreased biopterin ratio [23, 26, 29]. Supplementation of BH_2 in cultured endothelial cells under high and low glucose showed an increase in biopterin ratio for high glucose treated endothelial cells. The endothelial cells cultured under normal glucose levels had no effect on the biopterin ratio [23]. In another in-vitro study performed on intestinal epithelial cells under normal conditions, BH_2 supplementation was found to decrease the biopterin ratio [29]. Sepiapterin supplementation led to decrease in biopterin ratio under normal conditions [30]. In disease conditions supplementation of sepiapterin helped in maintaining the biopterin ratio, which was found to decrease when the BH_4 producing enzyme GCH-1 was inhibited [30].

The physiological effects of BH₄, BH₂ and sepiapterin supplementation on biopterin ratio have also been demonstrated in several studies. Studies involving high dose BH₄ supplementation in hypertensive individuals [31] and in patients with heart failure [32] resulted in significant improvement in their forearm blood flow. Other BH₄ supplementation studies involving patients with hypercholesterolemia [33], diabetes [34] and those subjected to cardiac catheterization for complications such as atherosclerosis [35] and hypercholesterolemia [36] showed significant improvement in their endothelial function. In some cases however, prolonged BH₄ supplementation led to worsening of endothelial function as was observed in isolated cerebral arteries [27] and canine basilar arteries [37] and the oxidation of BH₄ to BH₂ was identified as the primary cause of reduced endothelial function. Supplementation of sepiapterin on vessels of apoE-KO mice improved endothelial function and reduced O₂^{•-} production [38]. Incubation of porcine and human atherosclerotic vessels with sepiapterin showed improvement in endothelial vasodilation [39]. On the contrary it was found that incubation of hyperlipidemic rabbit vessels with high concentration of sepiapterin caused an increase in O₂^{•-} production, decrease in NO production and impaired endothelial dependent vasodilation. [40].

Vasquez-Vivar et al. [4] reported that an addition of 2 μM BH₄ to purified BH₄ free eNOS results in 97% of maximal NO production of 145 nmol.min⁻¹.mg eNOS⁻¹. Subsequent addition of 2 μM BH₂ inhibits the NO production to 50% of its maximal value. In addition, the addition of BH₂ at 14 μM increases eNOS based O₂^{•-} production to 50% of maximal O₂^{•-} production of 49 nmol.min⁻¹.mg eNOS⁻¹. In high glucose exposed endothelial cells, Crabtree et al. [23] reported that the addition of BH₄ decreases the biopterin (BH₄:BH₂) ratio and increases the total biopterin levels. They also observed significant decrease in NO production and increase in O₂^{•-} production.

The primary causes of endothelial dysfunction is an increased production of O₂^{•-} and reduced NO availability in endothelial cells. A critical step in our understanding of mechanisms of endothelial dysfunction is to analyze the eNOS biochemical pathways leading to either NO or O₂^{•-} formation. Based on the kinetic approach, the eNOS catalysis mathematical models have been developed [41, 42]. These eNOS catalysis modeling studies focused on the reactions involving the oxidation of L-Arginine for NO production. The important results of these studies include steady state NO production rates ranging from 0.003 μM.s⁻¹ for EC based eNOS concentration of 0.008 μM to 0.034 μM.s⁻¹ corresponding to EC based eNOS concentration of 0.097 μM [41]. The analysis of Santolini et al. [42] showed that the NO concentration increased linearly during the first minute of eNOS catalysis from 0 μM to approximately 0.4 μM for eNOS concentration of 1 μM. Over longer time span (15 minutes), the eNOS concentration of 0.25 μM resulted in a non-linear increase in NO concentration from 0 μM to approximately 8 μM and the steady state is not achieved in the time span.

In order to provide a better insight as to how biopterin forms can affect endothelial dysfunction, it is important to model the O₂^{•-} production pathways for eNOS along with the already established NO production pathways and correlate them with biopterin (BH₄ or BH₂) availability. In this study, a mathematical model has been presented to account for the biochemical pathways of eNOS for both NO and O₂^{•-} production for the first time. In addition, an analysis of biopterin (BH₄ or BH₂) availability to predict the NO and O₂^{•-} production rates is presented. The model also accounts for the reaction involving NO and O₂^{•-} to form ONOO⁻ as well as the oxidation of reduced biopterin (BH₄) by both O₂^{•-} and ONOO⁻. The current study also addresses the relative importance between the biopterin ratio (BH₄:BH₂) and the absolute levels of total biopterin (TBP) on the extent of dimeric eNOS uncoupling.

Materials and Methods

Model Description

Using known biochemical pathway for eNOS, NO production was modeled for a single endothelial cell [41, 42]. For eNOS uncoupling and $O_2^{\bullet-}$ production, we used biochemical pathway described by Berka and co-workers [43, 44]. In brief, the electron transport from reductase to oxygenase domain in eNOS occurs after biopterin binds to the enzyme [1]. Following the binding of biopterin, L-Arginine and CaM to NOS dimer, the heme in the oxygenase domain undergoes a series of redox reactions to produce NO and $O_2^{\bullet-}$ as shown in Figure 2A. The production of NO and $O_2^{\bullet-}$ is dependent on the availability of oxidized biopterin (BH_2) and reduced biopterin (BH_4). Furthermore, NO and $O_2^{\bullet-}$ can react with each other to form $ONOO^-$. $ONOO^-$ can participate in several redox reactions shown in Figure 2B [45].

Overall, these reactions can be classified into three major biochemical pathways of 1) NO production, 2) $O_2^{\bullet-}$ production, and 3) downstream reactions involving NO, $O_2^{\bullet-}$ and $ONOO^-$. The biochemical pathway for eNOS based NO production proceeds when BH_4 is bound to eNOS. The NO production pathway involves oxidation of L-Arginine to N-hydroxyl-L-Arginine (NHA) and subsequent oxidation of NHA to NO and citrulline. Binding of BH_2 to eNOS leads to a separate biochemical pathway leading to production of $O_2^{\bullet-}$. The first three reactions in the biochemical pathway of eNOS based $O_2^{\bullet-}$ production following binding of BH_2 to eNOS are similar to the first three reactions in the biochemical pathway of eNOS based NO production when BH_4 is bound to eNOS. The nomenclature of the different forms of eNOS and the eNOS substrate complexes during NO and $O_2^{\bullet-}$ production is shown in Table 1.

The downstream reactions involving NO, $O_2^{\bullet-}$ and their reaction product $ONOO^-$ include auto-oxidation of NO by O_2 , dissociation of peroxynitrous acid ($ONOOH$), reaction between $ONOO^-$ and CO_2 , reaction between $ONOO^-$ and NO, $O_2^{\bullet-}$ dismutation, oxidation of BH_4 and BH_3 to BH_2 by O_2 and oxidation of BH_4 to BH_3 by $O_2^{\bullet-}$, $ONOO^-$ and radicals formed from the reaction between $ONOO^-$ and CO_2 and dissociation of $ONOOH$. Details of all the reactions for the biochemical pathways of NO and $O_2^{\bullet-}$ production and the downstream reactions involving NO, $O_2^{\bullet-}$ and $ONOO^-$ are shown in Appendix A1.1.

The kinetic model was developed for dimeric eNOS and the production of $O_2^{\bullet-}$ from eNOS monomers was ignored. This is due to the fact that the $O_2^{\bullet-}$ generation from monomeric NOS is small in comparison to that of with uncoupled dimeric eNOS [3]. The large variation in morphology and sizes of the endothelial cells could have an effect on the intracellular concentration of eNOS. We analyzed the effect of eNOS concentration on the NO and $O_2^{\bullet-}$ production rates.

Previous studies have shown that majority of the intracellular eNOS is located on endothelial cell membrane [14, 46, 47]. Cytosolic eNOS, if at all present, is inactive [14]. We assumed that only membrane bound eNOS was responsible for production of NO and $O_2^{\bullet-}$. Species (NO and $O_2^{\bullet-}$) transport within and through the EC was not taken into consideration. An earlier study by Chen and Popel [41] of computational analysis for the kinetics of eNOS based NO production has shown that the NO production rate is independent of the geometrical location of eNOS within the cell. Thus, the kinetics of eNOS based reactions for the biochemical pathways of NO and $O_2^{\bullet-}$ production is reasonable approximation for predictions of eNOS related NO and $O_2^{\bullet-}$ production in endothelial cells.

Model Formulation

The model developed in this study accounts for the mass conservation of the enzyme (eNOS), which was a simplification in earlier kinetic modeling studies related to the biochemical pathways of eNOS [41, 42]. Furthermore, the total biopterin (BH₄, BH₃ and BH₂) is conserved. To account for the conservation of eNOS and total biopterin, one of the intermediate rate equations for eNOS complex and the rate equation for BH₂ is in the form of algebraic equations. Apart from the two algebraic equations, there are 32 different rate expressions for the 32 chemical species in the biochemical and downstream pathways involving NO and O₂^{•−} production. The rate equations are in the form of ordinary differential equations (a total of 32 differential equations). Appendix A1.2 lists the justifications behind the use of 34 model equations along with the model equations in their mathematical form.

Model Parameters

The model parameters of concentrations and rate constants are shown in Table 2 and 3, respectively. The eNOS concentration of 0.097 μM was assumed, which is based on the reported eNOS concentration of 5137 pg/10⁶ cells in HUVECs [41] and a single EC volume of 400 μm³ [41]. Werner-Felmayer et al. [48] measured the BH₄ concentration of 2.6 pmol/10⁶ cells in HUVEC cells under normal physiological conditions. Thus, the BH₄ concentration was calculated to be 6.5 μM using the volume of a single EC. A small fraction (5–10 %) of total biopterin (TBP) is in oxidized form under normal physiological conditions [23, 25]. The concentration of total biopterin (TBP) was assumed to be 7 μM. In the current study, the concentrations of BH₄ and TBP were constant for the entire time span. Thus, the simulation had a constant BH₄/total oxidized biopterin (sum of BH₂ and BH₃) ratio. Though the total oxidized biopterin was constant for a simulation, BH₂ and BH₃ concentrations were varying with time.

Oxidation of BH₄ by oxidants such as O₂^{•−}, ONOO[−] and radicals such as •OH, •NO₂ and CO₃^{•−} convert BH₄ to an intermediate radical known as BH₃. BH₃ and BH₄ undergo autooxidation in a two step process. In the first step BH₄ or BH₃ is oxidized to an intermediate form called quinonoid BH₂ (qBH₂) [20, 49]. The qBH₂ depending upon pH and temperature can either undergo self rearrangement to form a more stable isomer 7,8 dihydro-L-biopterin (7,8-BH₂) or eliminate the side chain in 6 position to form 7,8-dihydropterin (7,8-PH₂), which can be rapidly hydrated to form other species including dihydroxanthopterin [49]. Previous studies have shown that the different isoforms of BH₂ bind to NOS with similar affinities [4, 20, 50] and increase NOS based superoxide production [4, 22]. We thus assume a generalized form of dihydrobiopterin (BH₂) as the final product of BH₄ oxidation, which accounts for 7,8 BH₂, 7,8-PH₂ and dihydroxanthopterin.

The L-Arginine concentration was assumed to be 100 μM, which is the lower limit of the reported intracellular L-Arginine concentration of 0.1 to 1 mM in EC's [51]. The O₂ concentration was assumed to be 140 μM [52]. We assumed CO₂ concentration to be 1.1 mM [53]. The SOD concentration was assumed to be 10 μM based on the intracellular SOD concentration of 4–10 μM reported by Beckmann and Koppenol [45]. The NADPH concentration was calculated to be 166–295 μM based on the reported value of 66.3–117.9 pmol/10⁶ EC's in rat endothelial cells by Meininger et al. [54]. The K_m of NADPH is 0.65 μM [55]. Persechini and Stemmer [56] reported the concentration of CaM in EC's to be approximately 5 μM, which is several orders of magnitude above its EC₅₀ value of 0.009 μM [55]. Wickham et al. [57] reported the Ca²⁺ concentration of 0.218 μM in HUVEC cells, which closely matches its EC₅₀ value of 0.11 μM [55]. Therefore, we assumed an excess of NADPH, CaM and Ca²⁺.

The rate constant for most of the reactions were obtained from literature. The rate constants measured at lower temperatures were scaled up to the physiologically relevant temperature of 37°C using the Arrhenius equation as shown below

$$k(T_2)=k(T_1) \exp \left[-E_a \left(\frac{T_1 - T_2}{RT_1 T_2} \right) \right] \quad (1)$$

where, $k(T_2)$ represents the rate constant calculated at the scaled up temperature (T_2) (usually 37°C or 310 K) and $k(T_1)$ represents the rate constant at the temperature at which it was measured (T_1). R represents the universal gas constant ($8.314 \text{ J.mol}^{-1}.\text{K}^{-1}$). E_a is the activation energy (J.mol^{-1}). When the rate constants were available at two distinct temperatures, the activation energy was obtained using Arrhenius equation. The estimated activation energy can then be used for scaling up the respective rate constant corresponding to 37°C. Chen and Popel [41] reported that when either L-Arginine or NHA is bound to eNOS, the eNOS complexes pair including the O_2 binding to ferrous (Fe^{2+}) heme, O_2 dissociation from the heme dioxy complex ($\text{Fe}^{3+}\text{-O}_2^-$) and conversion of the heme back to ferric (Fe^{3+}) state are structurally similar. Therefore, the following pairs of rate constants k_{a3} and k_{b3} , k_{a-3} , k_{b-3} and k_{c-6} , k_{a5} and k_{b5} , and k_{a6} and k_{b6} were assumed to have similar activation energies. There were insufficient experimental rate constant data for k_{a2} , k_{a8} , k_{b2} , k_{b-7} , k_{b8} , k_{b9} , k_{b10} , k_{b-10} , k_{c5} , k_{16} , k_{23} , k_{24} , k_{25} , k_{26} , k_{28} , k_{31} and k_{32} . The activation energy for these rate constants was assumed to be $27690.79 \text{ J.mol}^{-1}$, which is the average of the other known activation energies used in this study.

Numerical Solution

The rate equations as shown in the Appendix A1.2 along with the appropriate initial conditions were solved numerically using the MATLAB (Mathworks Inc, Natick, MA) ODE (ordinary differential equation) solver *ode15s*. The relative and absolute error tolerance values were set at $1e^{-10}$ and $1e^{-15}$ respectively. The simulations were run for 1×10^4 s. To obtain the steady state values, the model was run for $3\text{--}5 \times 10^4$ s in some cases.

Results

NO production decreases and $\text{O}_2^{\bullet-}$ production increases nonlinearly with a reduction in tetrahydrobiopterin availability

In normal physiological conditions, a small amount (5–10%) of total biopterin is in oxidized biopterin (BH_2 and BH_3) form [23, 25]. However, the amount of oxidized biopterin can increase as much as 90% in endothelial cell dysfunction [23, 25]. To understand the impact of BH_4 availability on the NO and $\text{O}_2^{\bullet-}$ productions from eNOS; six cases were simulated with $[\text{BH}_4]/[\text{TBP}]$ ratio from 0.99 to 0.05. A high value for the ratio indicates that the majority of total biopterin (TBP) is in the reduced form BH_4 (normal function) whereas a low value for the ratio indicates that the majority of total biopterin is in the oxidized forms BH_3 and BH_2 (dysfunction). The concentrations of eNOS, TBP, L-Arginine and O_2 were 0.097, 7, 100 and 140 μM , respectively. The SOD and CO_2 concentrations were set at 10 μM and 1.1 mM, respectively. Figure 3A and B show the NO and $\text{O}_2^{\bullet-}$ productions, respectively for $[\text{BH}_4]/[\text{TBP}]$ ratios of 0.99, 0.90, 0.70, 0.5, 0.25 and 0.05. A decrease in $[\text{BH}_4]/[\text{TBP}]$ ratio decreased the NO production and increased the $\text{O}_2^{\bullet-}$ production. For $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99, 0.9, 0.7, 0.5, 0.25 and 0.05 at 10,000 s, the NO production was 26.0, 25.6, 24.4, 22.2, 15.9 and 4.0 nM.s^{-1} , respectively and the $\text{O}_2^{\bullet-}$ production was 0.02, 0.3, 1.0, 2.2, 6.1 and 15.1 nM.s^{-1} , respectively. The decrease in $[\text{BH}_4]/[\text{TBP}]$ ratio also increased the time required to attain steady state. The steady state NO and $\text{O}_2^{\bullet-}$ productions are shown in Figure 3C with respect to absolute BH_4 concentration. The increase in BH_4

concentration in Figure 3C also represents the increase in $[\text{BH}_4]/[\text{TBP}]$ ratio since the total biopterin concentration ($[\text{TBP}]$) is maintained at $7\text{ }\mu\text{M}$. As seen, both the decrease in the NO production and increase in $\text{O}_2^{\bullet-}$ production are nonlinear with respect to BH_4 concentration. For BH_4 concentrations of 6.93, 6.3, 4.9, 3.5, 1.75, 0.35, 0.07, 0.007 and $0.0007\text{ }\mu\text{M}$, respectively, which correspond to biopterin ratios of 0.99, 0.9, 0.7, 0.5, 0.25, 0.05, 0.01, 0.001 and 0.0001, the steady state NO production rates were 26.5, 26.1, 25.0, 23.3, 18.4, 6.73, 1.55, 0.14 and $0.013\text{ nM}\cdot\text{s}^{-1}$, respectively and the steady state $\text{O}_2^{\bullet-}$ production rates were 0.02, 0.2, 0.9, 2.0, 5.0, 13.2, 17.7, 19.1 and $19.29\text{ nM}\cdot\text{s}^{-1}$, respectively. We also ran the simulations by changing the rate constant of the reaction between NO and $\text{O}_2^{\bullet-}$ to $10^{10}\text{ M}^{-1}\text{s}^{-1}$. The maximum percentage change in the results of NO and superoxide production rates were less than 2 % using the rate constant of $10^{10}\text{ M}^{-1}\text{s}^{-1}$ for the reaction between NO and $\text{O}_2^{\bullet-}$.

A reduction in $[\text{BH}_4]/[\text{TBP}]$ ratio increases the peroxynitrite concentration

Figure 4 shows the time dependent ONOO^- concentration profiles for simulation of varying $[\text{BH}_4]/[\text{TBP}]$ ratio in the previous sub-section. The ONOO^- concentrations reached a peak value within 3000 s and the peak values increased as the $[\text{BH}_4]/[\text{TBP}]$ ratio decreased except for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.05. For $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.05, the ONOO^- concentration increased slowly but reached a higher value than all $[\text{BH}_4]/[\text{TBP}]$ ratios at 10000 s. A possible reason for this may be a higher production of $\text{O}_2^{\bullet-}$ and a lower but increasing production of NO for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.05. The maximum ONOO^- concentrations were 0.7, 7.3, 21.0, 32.2, 38.0 and 39.0 pM for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99, 0.9, 0.7, 0.5, 0.25 and 0.05, respectively. Furthermore, the time required to achieve the maximum ONOO^- concentration decreased with increasing $[\text{BH}_4]/[\text{TBP}]$ ratio. The steady state ONOO^- concentrations were 0.13, 1.4, 5.2, 11.2, 25.3 and 39.0 pM for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99, 0.9, 0.7, 0.5, 0.25 and 0.05, respectively.

Total biopterin concentration above $1.5\text{ }\mu\text{M}$ have no effect on NO and $\text{O}_2^{\bullet-}$ production for a constant $[\text{BH}_4]/[\text{TBP}]$ ratio

It is known that BH_4 supplementation increases the total biopterin levels ($[\text{TBP}]$) in endothelial cells [23]. Studies investigating the effects of BH_4 supplementation on restoration of endothelial function have showed mixed results; in some cases endothelial dysfunction improved [19, 20, 22, 58] while in others endothelial dysfunction remained unchanged or deteriorated [18, 22, 23]. These studies indicate that there may be a possible correlation among BH_4 supplementation, total biopterin levels and oxidation of BH_4 by oxidizing agents such as $\text{O}_2^{\bullet-}$, ONOO^- and O_2 . It is thus important to understand the effect of total biopterin concentration on the NO and $\text{O}_2^{\bullet-}$ production rates. To understand how total biopterin concentrations affect the NO and $\text{O}_2^{\bullet-}$ production rates, we varied the total biopterin concentration from $0\text{--}70\text{ }\mu\text{M}$ for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99, 0.9, 0.5 and 0.05. The concentrations of eNOS, L-Arginine, O_2 , CO_2 and SOD were $0.097\text{ }\mu\text{M}$, $100\text{ }\mu\text{M}$, $140\text{ }\mu\text{M}$, 1.1 mM and $10\text{ }\mu\text{M}$ respectively. As seen in Figure 5A and B, the NO and $\text{O}_2^{\bullet-}$ production rates increased rapidly with increasing total biopterin concentration and remained constant above total biopterin concentration of $1.5\text{ }\mu\text{M}$ for all ratios. The NO and $\text{O}_2^{\bullet-}$ production rates dependence on $[\text{BH}_4]/[\text{TBP}]$ ratio showed that the maximum NO production was achieved at $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99 and the maximum $\text{O}_2^{\bullet-}$ production was achieved at $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.05. For $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99, 0.9, 0.5 and 0.05 μM and at total biopterin concentration of $1.5\text{ }\mu\text{M}$, the steady state NO production rates were 26.451, 26.077, 23.161 and $6.675\text{ nM}\cdot\text{s}^{-1}$, respectively and the steady state $\text{O}_2^{\bullet-}$ production rates were 0.022, 0.241, 1.969 and $13.069\text{ nM}\cdot\text{s}^{-1}$, respectively.

Effect of eNOS concentration on NO and $O_2^{\bullet-}$ production rates

Intracellular eNOS concentration range of 0.007–0.097 μM has been reported for endothelial cells as shown in Table 2. It has also been reported that eNOS expression and concentration is significantly elevated under oxidative stress conditions [12, 18, 23, 25]. Figures 6 and 7 show the effect of eNOS concentration on NO and $O_2^{\bullet-}$ production rates, respectively for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99 (Panel A) and 0.05 (Panel B). The concentrations of total biopterin, L-Arginine, O_2 , SOD and CO_2 were 7 μM , 100 μM , 140 μM , 10 μM and 1.1 mM, respectively. Increased eNOS concentration led to increased production of both NO and $O_2^{\bullet-}$ for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99 and 0.05. However, the increase in steady state production of NO with eNOS concentration was higher for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99 as compared to 0.05, whereas for $O_2^{\bullet-}$ the increase in steady state production was higher at the ratio of 0.05.

For $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99, the NO production rates were 3.0, 10.6, 26.0, 188.1 and 450.0 $\text{nM}\cdot\text{s}^{-1}$ for eNOS concentrations of 0.015, 0.045, 0.097, 0.5 and 1 μM , respectively at 10,000 s. The $O_2^{\bullet-}$ production rates were 0.003, 0.010, 0.022, 0.115 and 0.230 $\text{nM}\cdot\text{s}^{-1}$ for eNOS concentrations of 0.015, 0.045, 0.097, 0.5 and 1 μM , respectively at 10,000 s. The steady state NO production rates were 3.15, 11.0, 26.5, 188.1 and 450 $\text{nM}\cdot\text{s}^{-1}$ while the steady state $O_2^{\bullet-}$ production rates were 0.004, 0.01, 0.02, 0.11 and 0.23 $\text{nM}\cdot\text{s}^{-1}$ for eNOS concentrations of 0.015, 0.045, 0.097, 0.5 and 1 μM , respectively.

For $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.05, the NO production rates were 0.6, 1.8, 4.0, 23.0 and 48.0 $\text{nM}\cdot\text{s}^{-1}$, for eNOS concentrations of 0.015, 0.045, 0.097, 0.5 and 1 μM , respectively at 10,000 s. The $O_2^{\bullet-}$ production rates were 2.3, 7.0, 15.1, 78.0 and 156.0 $\text{nM}\cdot\text{s}^{-1}$, respectively at 10,000 s. The steady state NO production rates were 0.9, 2.95, 6.73, 37.6 and 77.2 $\text{nM}\cdot\text{s}^{-1}$ while the steady state $O_2^{\bullet-}$ production rates were 2.1, 6.1, 13.2, 68.1 and 136.0 $\text{nM}\cdot\text{s}^{-1}$ for eNOS concentrations of 0.015, 0.045, 0.097, 0.5 and 1 μM , respectively.

Impact of feedback inhibition on NO production rate and concentration

The reaction schematic (Figure 2A) shows that the competitive binding of NO to eNOS based ferric or ferrous heme. The ferric heme NO complex (E_{b5}) dissociates in a reversible manner to form NO with the enzyme reverting back to its co-factor bound native state (E). Furthermore, the ferric heme NO complex (E_{b5}) can be reduced to ferrous heme NO complex (E_{b6}). This complex also reversibly dissociates to form NO and ferrous heme (E_{b7}). The backward reaction in these reactions involves consumption of NO. We investigated the effect of the feedback inhibition on the NO production rates and concentration by shutting down (equal to 0) either or both rate constants k_{b-7} and k_{b-10} .

Figure 8A and B shows the NO production rates and NO concentration, respectively in the presence and absence of the feedback inhibition. While there were no significant changes in the NO concentration profiles, the NO production rates were affected by the feedback inhibition process for the reactions involving the ferric heme NO complex (E_{b5}), NO and the co-factor bound ferric heme (E). Upon shutting down the backward reaction involving co-factor bound ferric heme (E) and NO, the NO production rate drops from 25 $\text{nM}\cdot\text{s}^{-1}$ to 15 $\text{nM}\cdot\text{s}^{-1}$. The $O_2^{\bullet-}$ production rates and concentrations remain unaffected by the feedback inhibition process (results not shown).

Effect of glutathione concentration on eNOS based NO and $O_2^{\bullet-}$ production

Another important constituent, which is present in mM concentration within endothelial cells is the thiol glutathione (GSH). GSH reacts with ONOO^- and N_2O_3 to form nitroso-glutathione (GSNO) [59]. GSH also forms oxidized glutathione (GSSG) by its reaction with peroxynitrite [59, 60]. GSNO reacts with $O_2^{\bullet-}$ to form NO [59]. GSSG can be reduced back

to GSH by glutathione reductase [59]. The kinetics of these reactions were incorporated into the current model separately from the earlier analysis along with the mass conservation of all the thiol species. GSH concentrations used were 0.1, 1 and 10 mM. The concentrations of total bipterin ([TBP]), L-Arginine, O₂, SOD, CO₂, and eNOS were 7 μM, 100 μM, 140 μM, 10 μM, 1.1 mM, and 0.097 μM, respectively. The temporal change in NO and O₂^{•−} production rates and GSH and GSSG concentrations were obtained with respect to [BH₄]/[TBP] ratio for each of the different initial GSH concentrations ([GSH]₀). For all three initial GSH concentrations ([GSH]₀), the NO and O₂^{•−} production rates were affected less than 1% from those observed in the absence of GSH (results not shown but similar to Figure 3A and B).

The temporal change in GSH concentration with respect to [BH₄]/[TBP] ratios and for different initial GSH concentrations ([GSH]₀) are shown in Figure 9A. The figures on the left, centre and right in Figure 9A represent [GSH]₀ values of 0.1, 1 and 10 mM, respectively. For all the different initial GSH concentrations, the GSH concentration was found to reduce with time for all [BH₄]/[TBP] ratios. For initial GSH concentration of 0.1 mM, the GSH concentration at 10,000 s reduced to zero for [BH₄]/[TBP] ratios of 0.99, 0.9, 0.7 and 0.5. For [BH₄]/[TBP] ratios of 0.25 and 0.05 they were 0.04 and 0.09 mM, respectively. For initial GSH concentration of 1 mM, the GSH concentration at 10,000 s was between 0.85 to 0.9 mM for all [BH₄]/[TBP] ratios. For initial GSH concentration of 10 mM, the GSH concentration at 10,000 s was 9.9 mM for all [BH₄]/[TBP] ratios.

The temporal change in GSSG concentration for different [BH₄]/[TBP] ratios and for different initial GSH concentrations are shown in Figure 9B. The figures on the left, centre and right in Figure 9B represent initial GSH concentrations ([GSH]₀) of 0.1, 1 and 10 mM, respectively. For any given initial GSH concentration, the GSSG concentration was found to increase with time, and independent of initial GSH concentration ([GSH]₀). For initial GSH concentration of 0.1, 1 and 10 mM, the GSSG concentration at 10,000 s corresponding to [BH₄]/[TBP] ratio of 0.99, 0.9, 0.7, 0.5, 0.25 and 0.05 were 0.88×10^{-3} , 8.9×10^{-3} , 27.617×10^{-3} , 43.44×10^{-3} , 36.7×10^{-3} and 0.82×10^{-3} pM, respectively.

Discussion

In this study, we developed a computational model for eNOS biochemical pathways for the production of NO and O₂^{•−} to understand the eNOS uncoupling and related endothelial dysfunction mechanism. We analyzed the effects of [BH₄]/[TBP] ratio, total bipterin, eNOS concentrations and feedback inhibition of NO consumption on NO and O₂^{•−} production.

eNOS uncoupling, endothelial cell dysfunction and the importance of [BH₄]/[TBP] ratio for the NO and O₂^{•−} production rates

Endothelial dysfunction is usually associated with increased oxidative stress in the endothelial region [18, 22–24, 40, 61]. The increased oxidative stress promotes production of more O₂^{•−}, which can react with the eNOS generated NO to produce ONOO[−] [3, 18, 19, 61]. Together, both O₂^{•−} and ONOO[−] aid in the oxidation of the eNOS co-factor BH₄ into BH₂ [3, 18, 23–25]. BH₂ is known to competitively bind with eNOS and increases endothelial O₂^{•−} production by uncoupling of eNOS, which also reduces NO production levels [4, 18, 23, 25]. Several studies were performed for analyzing the effect of absolute BH₄ levels as well as the bipterin ratio on the extent of eNOS uncoupling [4, 23–25]. However most of these studies could not ascertain accurately whether absolute BH₄/bipterin levels or bipterin ratio was more important for controlling eNOS uncoupling. One of these experimental studies [23] also emphasized the need for developing a comprehensive computational model, which could define the boundary conditions associated with BH₄ and

BH₂ levels on the extent of eNOS uncoupling. The current study has been able to achieve these objectives.

There is question regarding which one from the biopterin ratio ([BH₄]/[TBP] or BH₄:BH₂) or absolute level of BH₄ is important for the extent of eNOS uncoupling [12, 18, 23, 25]. In this study, the biopterin ratio is expressed as [BH₄]/[TBP]. In this notation, a high or low [BH₄]/[TBP] ratio indicates that a majority of the biopterin is in the reduced form BH₄, or in the oxidized forms BH₃ and BH₂, respectively. We predicted that NO and O₂^{•-} production rates were independent of total biopterin at concentrations above 1.5 μM for a given [BH₄]/[TBP] ratio (see Figure 5). With a decreasing [BH₄]/[TBP] ratio from 0.99 to 0.05, we predicted that the NO production rate decreased 4 fold and the O₂^{•-} production rates increased 594 fold. The increase in O₂^{•-} production is more noticeable because of the fact that the primary function of eNOS is to produce NO. Thus, these results indicate that at total biopterin concentration above 1.5 μM, the extent of eNOS uncoupling is only dependent on the [BH₄]/[TBP] ratio. At total biopterin concentration above 1.5 μM, supplementation of BH₄ can increase eNOS based NO production and reduce eNOS based O₂^{•-} production only if the ratio of BH₄ to TBP increases.

These model predictions can explain some of the experimental observations. The NO production rate increases with BH₄ concentration but is independent of BH₄ concentration beyond 2 μM for NO production in the in vitro study of bovine eNOS supplemented by BH₄ [4]. Crabtree et al. [23] measured NO and O₂^{•-} production rates after the supplementation with BH₄ and BH₂ in high glucose endothelial cells. They reported that the BH₄/BH₂ ratio reduced from 1:1 to 1:6 for the supplementation of BH₄ but did not change for BH₂ supplementation in high glucose cells as compared to normal glucose cells. The reason for the reduction in BH₄/BH₂ ratio was conversion of BH₄ to BH₂ in high glucose cells. In addition, the NO production rate decrease 40% and O₂^{•-} production rate increase 200% in BH₄ supplemented high glucose cells. They concluded that the extent of eNOS coupling correlates inversely with the ratio of intracellular BH₄ to BH₂, but not absolute levels of intracellular BH₄.

Using the GTPCH overexpressed (BH₄ producing enzyme) normal and diabetic mouse aortas, Alp et al. [25] observed that the total biopterin levels remains constant whereas BH₄ levels reduce from 80% to 10% in the diabetic aortas. In addition, the endothelial O₂^{•-} production increases 3 fold in the diabetic aortas. Vasquez-Vivar et al. [40] reported that the BH₂/total biopterin ratio increases from 0.3 to 0.63 in the aortas of rabbits on normal cholesterol and high cholesterol diet, respectively, resulting in a 1.3 fold reduction in the endothelial dependent relaxation for high cholesterol diet. Alp et al. [24] reported a [BH₄]/[TBP] ratio of 0.25 and 0.80 for an ApoE-KO mouse aorta and a GTPCH overexpressed ApoE-KO mouse aorta, respectively. A 2 fold reduction in endothelial O₂^{•-} production was observed in GTPCH overexpressed ApoE-KO mouse aorta.

Comparison of predicted NO production rates with experimental results

Several experimental studies have quantified NO production rates from endothelial cells [55, 62–65] in a number of systems and cell sources. In addition, the NO production rates were reported for isolated vessel segments [66] and for purified eNOS in in vitro systems [4]. The reported NO production rates in endothelial cell lines are 2.19 pM.s⁻¹ [62], 415 nM.s⁻¹ [63], 0.117 nM.s⁻¹ [64] and 2.97 μM.s⁻¹ [65], respectively based on cell volume of 400 μm³, endothelial layer thickness of 4 μm and endothelial cell eNOS concentration of 0.097 μM. In isolated vessels, the NO production rate of 26.53 μM.s⁻¹ is reported by Lu and Kassab [66]. The predicted NO production rates are either several orders of magnitude higher [65, 66] or lower [62, 64] than the NO production rates in endothelial cells or isolated vessels. Possible reasons for the discrepancy include a contribution of NO from other

isoforms of NOS and over-estimation or under-estimation of the endothelial cell size or volume leading to incorrect estimation of total eNOS concentration. As seen from Figure 6A, an increase in eNOS concentration to 1 μM predicts NO production rate of 450 nM.s^{-1} similar to the reported value of 415 nM.s^{-1} by Hood et al. [63]. In addition, the NO production rates in purified eNOS [4] is similar to the prediction from the current study. Vasquez-Vivar et al. [4] reported experimental NO production rate of 322.7 nM.s^{-1} for an eNOS concentration of $0.97 \mu\text{M}$, which is similar to the model prediction at 1 μM eNOS concentration.

Comparison of predicted $\text{O}_2^{\bullet-}$ production rates with experimental results

Quantitative studies for measurement of endothelial cell based $\text{O}_2^{\bullet-}$ production are limited. Arnal et al. [62] and Potdar and Kavdia [65] reported the $\text{O}_2^{\bullet-}$ production rates of 0.043 nM.s^{-1} and $2.425 \mu\text{M.s}^{-1}$, respectively from endothelial cell lines. The $\text{O}_2^{\bullet-}$ production rate reported by Arnal et al. [62], is close to the predicted steady state $\text{O}_2^{\bullet-}$ production rate of 0.02 nM.s^{-1} for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99. Possible reason for several orders of magnitude higher $\text{O}_2^{\bullet-}$ production rate reported by Potdar and Kavdia [65] may be NADPH oxidase, mitochondrial electron transport chain as the $\text{O}_2^{\bullet-}$ sources apart from eNOS. For purified eNOS, Vasquez-Vivar et al. [4] measured the maximum $\text{O}_2^{\bullet-}$ production rate of 217.5 nM.s^{-1} at eNOS concentration of $0.97 \mu\text{M}$, which is similar to the predicted maximum $\text{O}_2^{\bullet-}$ production rate 184 nM.s^{-1} for eNOS concentration of 1 μM for $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.05.

Comparison of model prediction to existing computational models

Past kinetic modeling studies for eNOS catalysis focused only on the biochemical pathway involving NO synthesis [41, 42]. These studies consider an excess of the co-factor BH_4 . The NO production rate predicted by Chen and Popel [41] ranged from 3 to 34 nM.s^{-1} for the eNOS concentration of 0.008 to $0.097 \mu\text{M}$, respectively. For $0.097 \mu\text{M}$ eNOS concentration with $[\text{BH}_4]/[\text{TBP}]$ ratio of 0.99, the steady state NO production rate was 26.5 nM.s^{-1} in this study. Vaughn et al. [67] developed a mathematical model to estimate NO production rate by utilizing the experimental data of Malinski et al. [68] for NO release from rabbit aorta endothelium. They reported NO production rate of $5.4 \times 10^{-12} \text{ mol.cm}^{-2}.\text{s}^{-1}$, which is equivalent to $13.5 \mu\text{M.s}^{-1}$ for endothelial layer thickness of 4 μm . Thus, the NO production rates from our and Chen and Popel [41] models are significantly lower. The possible reasons for this discrepancy are described in the above Section.

Importance of feedback inhibition

As seen in Figure 8A, the NO production rates were reduced by the feedback inhibition process for the reactions involving the ferric heme NO complex (E_{b5}), NO and the co-factor bound ferric heme (E). Shutting down the backward reaction involving co-factor bound ferric heme (E) and NO causes the NO production rate to drop from 25 nM.s^{-1} to 15 nM.s^{-1} . This can be explained by looking at the production and consumption pathways of the complex E_{b5} . Figure 2A shows that E_{b5} can be produced from E_{b4} ($k_{b6} [\text{E}_{b4}]$) and through combination of NO and E ($k_{b-7} [\text{NO}] [\text{E}]$). On the other hand E_{b5} can dissociate from E and NO ($k_{b7} [\text{E}_{b5}]$) and also reduce to ferrous heme NO complex ($k_{b8} [\text{E}_{b5}]$). Our analysis revealed that the production rate and consumption rate of E_{b5} are exactly balanced throughout the simulated timespan. Thus, the removal of the reversible reaction to generate E_{b5} leads to a faster consumption of E_{b5} in comparison to its production. Since dissociation of the complex E_{b5} is responsible for majority of the NO generated, mitigation in its production rate will eventually reduce the production rate of NO itself. Shutting down the reaction involving NO and the ferrous heme complex (E_{b7}) did not seem to affect the NO production rate. This is due to the extremely slow dissociation rate of the ferrous heme NO complex (E_{b6}) as experimentally evidenced by Santolini et al. [42].

In conclusion, a biochemical pathway model for eNOS uncoupling and NO and $O_2^{\bullet-}$ production was developed. The model results indicate that the extent of eNOS uncoupling is dependent on the ratio of BH_4 to TBP. Supplementation of BH_4 can only reduce eNOS uncoupling if the ratio of BH_4 to TBP increases. It is envisaged that the results from the current study will help understand the mechanisms of endothelial dysfunction and could also aid in understanding the effect of therapeutics for treatment of endothelial dysfunction.

Incorporation of glutathione into the eNOS biochemical pathway

Incorporation of glutathione (GSH) based kinetics into the pre-existing eNOS biochemical pathway did not cause any significant changes in the NO and $O_2^{\bullet-}$ production rates for different $[BH_4]/[TBP]$ ratios. The temporal GSH concentration profiles as observed in Figure 9A does not drop significantly with time for initial GSH concentrations ($[GSH]_0$) at the physiologically relevant range (1–10 mM). At concentration below physiological range (0.1 mM), the GSH concentration drops almost to zero at $[BH_4]/[TBP]$ ratios of 0.99, 0.9, 0.7 and 0.5 suggesting that glutathione reductase is ineffective in restoration of glutathione levels if there is sufficient production of NO and its oxidation products $ONOO^-$ and N_2O_3 .

The concentration of oxidized glutathione (GSSG) formed as a result of reaction between $ONOO^-$ and GSH was maximum at $[BH_4]/[TBP]$ ratio of 0.5 as observed in Figure 9B. This could be due to the fact that at $[BH_4]/[TBP]$ ratio of 0.5, there is sufficient production of NO and $O_2^{\bullet-}$ which can counteract the enzymatic reduction of GSSG to GSH by glutathione reductase and oxidation of NO to form N_2O_3 . The GSSG concentration profiles shown below are formed as a result of $ONOO^-$ formed from reaction between eNOS based NO and $O_2^{\bullet-}$. Since there are other sources of NO and $O_2^{\bullet-}$ apart from eNOS, the GSH and GSSG concentration profiles shown in Figure 9A and B, respectively can be much different under actual physiological conditions. This is certainly worth investigating but is beyond the scope of the current study.

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Appendix A1.1

Description of Biochemical Pathways and Downstream Reactions

The three major pathways viz. 1) biochemical pathway for NO production, 2) biochemical pathway for $O_2^{\bullet-}$ production, and 3) downstream reactions involving NO, $O_2^{\bullet-}$ and $ONOO^-$ are described below. Table 1 shows the nomenclature of the different forms of eNOS and the eNOS substrate complexes.

1) Biochemical pathway for NO production

eNOS catalyzes the reaction involving L-Arginine, O_2 and NADPH (all substrates) to generate NO and citrulline [1]. The reaction pathway involves two distinct oxidation reaction cycles [41, 69, 70]. These oxidation reaction cycles are oxidation of L-Arginine to generate NHA and subsequent oxidation of NHA to produce NO and citrulline.

Oxidation of L-Arginine to generate NHA—The resting state of eNOS is E_{-1} (E_{-1} , eNOS-(Fe^{III})). The heme (Fe^{3+}), which is in the oxygenase domain, is in the resting state of eNOS [1]. The heme in ferric form has a lower redox potential compared to the flavin containing reductase domain. The binding of BH_4 to eNOS (E , eNOS-(Fe^{III})- BH_4) can increase the redox potential of the heme sufficiently to facilitate transfer of electrons from the reductase to the oxygenase domain [1]. Next, L-Arginine binds to eNOS-(Fe^{III})- BH_4 in a

reversible manner (E_{a1} , eNOS-(Fe^{III})-BH₄-Arg) increasing its redox potential significantly [1]. We ignored the flavin reduction in the reductase domain as the rate of electron transfer from NADPH to flavin is very fast when CaM is bound to eNOS [71]. Thereafter, the transfer of electron from the reductase domain reduces the heme in the oxygenase domain to ferrous (Fe²⁺) form (E_{a2} , eNOS-(Fe^{II})-BH₄-Arg). The reduced heme (Fe²⁺) then binds with O₂ reversibly to form a heme-dioxy intermediate (eNOS-[Fe^{II}-O₂]-BH₄-Arg) that is instantaneously converted into a ferric superoxy complex (eNOS-[Fe^{III}-O₂⁻]-BH₄-Arg) [1, 41, 72]. The kinetics of this rapid transition was ignored and both these species were considered the same (E_{a3} , eNOS-[Fe^{II}-O₂]-BH₄-Arg or eNOS-[Fe^{III}-O₂⁻]-BH₄-Arg).

In the ferric superoxy complex (E_{a3}), the bound BH₄ donates an electron to form heme peroxo (II) species (E_{a4} , eNOS-[Fe^{III}-OOH]-BH₃-Arg) [70]. The heme peroxo species E_{a4} then protonates resulting in the irreversible scission of the oxygen-oxygen bond to form a ferryl iron with eNOS bound cation radical (E_{a5} , eNOS-[Fe^{IV}-O]-BH₃-Arg) [1, 70]. The source of protonation could be arginine itself [1, 13]. The ferryl iron species is highly oxidizing and rapidly oxidizes L-Arginine to NHA and the heme is regenerated back to ferric state (E_{a6} , eNOS-(Fe^{III})-BH₃-NHA). The NHA and the oxidized biopterin (BH₃) are still bound to the enzyme (E_{a6}). The oxidized biopterin is reduced back to BH₄ by an electron from the flavin of the reductase domain of the other eNOS monomer (E_{b1}) [3, 18, 73] resulting in the formation of N-hydroxyl-L-Arginine enzyme complex (E_{b1} , eNOS-(Fe^{III})-BH₄-NHA).

Oxidation of NHA to produce NO and citrulline—The heme in the E_{b1} is reduced to ferrous form (E_{b2} , eNOS-(Fe^{II})-BH₄-NHA) by electron transfer from the reductase domain. In addition, the E_{b1} can reversibly dissociate to generate free NHA and the enzyme is returned to its co-factor bound native state E [41]. The enzyme complex E_{b2} can reversibly bind to O₂ to form intermediate oxy/superoxy complex E_{b3} (E_{b3} , eNOS-[Fe^{II}-O₂]-BH₄-NHA or eNOS-[Fe^{III}-O₂⁻]-BH₄-NHA) similar to the oxidation of L arginine to NHA pathway [1]. In addition, the E_{b2} can dissociate to release NHA and form E_{b7} (E_{b7} , eNOS-(Fe^{II})-BH₄) [41]. In the E_{b3} , the bound BH₄ donates an electron to form heme peroxo (II) species (E_{b4} , eNOS-[Fe^{III}-OOH]-BH₃-NHA) [73, 74].

The peroxo complex in the E_{b4} oxidizes the NHA bound to the enzyme into NHA radical and converts into a hydroperoxy complex that reacts almost immediately with each other in a radical rebound mechanism to produce ferric-NO complex (E_{b5} , eNOS-(Fe^{III})-NO-BH₄) and citrulline [1, 42]. In a reversible reaction, E_{b5} releases bound NO and converts the enzyme in co-factor bound native state (E). The E_{b5} is reduced to ferrous-NO complex (E_{b6} , eNOS-(Fe^{II})-NO-BH₄), which can then be oxidized by O₂ to form nitrate with the enzyme returned to its co-factor bound native state (E) [42]. The E_{b6} also dissociates reversibly to release NO with the enzyme converted into E_{b7} . This reaction occurs at a much slower rate in comparison to decomposition of E_{b5} [42]. The E_{b7} can also reversibly react with L-Arginine to generate the E_{a2} [41].

2) Biochemical pathway for O₂^{•-} production

Oxidized form of biopterin, dihydrobiopterin (BH₂), competes with BH₄ for the binding to eNOS [4, 23, 75]. The binding of BH₄ to eNOS results in the biochemical pathway leading to NO production, whereas the binding of BH₂ creates a separate biochemical pathway leading to O₂^{•-} production. The first three steps following the binding of BH₂ to eNOS are similar to those following BH₄ binding to eNOS. The binding of BH₂ with the enzyme in resting state (E_{-1}), converts the enzyme to an activated state (E_{c1} , eNOS-(Fe^{III})-BH₂) [43, 50]. Subsequently, L-Arginine binds reversibly to the enzyme to form the enzyme substrate complex E_{c2} (E_{c2} , eNOS-(Fe^{III})-BH₂-Arg) [44, 50]. The heme in the E_{c2} is reduced to form

ferrous state complex (E_{c3} , eNOS-(Fe^{II})-BH₂-Arg) by electron transfer from the flavin domain as experimentally evidenced by Presta et al. [50].

The O₂ binds reversibly with the enzyme complex E_{c3} to form a heme dioxy intermediate complex (E_{c4} , eNOS-[Fe^{II}-O₂]-BH₂-Arg or eNOS-[Fe^{III}-O₂⁻]-BH₂-Arg). Berka et al. [43] showed that the rate of enzyme-oxygen binding is independent of biopterin form using full length eNOS and eNOS oxygenase domain in the presence of L-Arginine. Experimental studies have reported that the E_{c4} dissociates to form O₂^{•-} and E_{c2} [21, 43, 72].

3) Downstream reactions involving NO, O₂^{•-} and their reaction product peroxynitrite (ONOO⁻)

The NO and O₂^{•-} produced by eNOS react with each other to form a potent oxidizing agent peroxynitrite (ONOO⁻) [45]. NO, O₂^{•-} and ONOO⁻ participates in several redox reactions that can affect the NO or O₂^{•-} production rates. The description of important reactions used in this study follows.

- a. Auto-oxidation of NO by O₂ is represented by the following chemical reaction [65]:

- b. Reaction between NO and O₂^{•-} to form ONOO⁻ is shown in equation (A2). The ONOO⁻ formed is in rapid equilibrium with peroxynitrous acid (ONOOH) [60, 65].

The fraction of total peroxynitrite (ONOO⁻+ONOOH) present in anion form (ONOO⁻) is calculated as follows [76]:

$$f = \frac{1}{1 + 10^{\text{pK}_{\text{per}} - \text{pH}}} = \frac{[\text{ONOO}^-]}{[\text{ONOO}^-] + [\text{ONOOH}]} \quad (\text{A3})$$

The acid dissociation constant (pK_{per}) is 6.75 [65, 77]. The pH value was assumed to be 7.4. The peroxynitrous acid dissociates to form NO₃⁻ and •NO₂ through the following chemical reactions [60, 65]:

ONOO⁻ rapidly reacts with CO₂ to form approximately 33% •NO₂+CO₃^{•-} and the remaining as NO₃⁻+CO₂ according to the following reaction [60]:

- c. NO can react with ONOO^- or ONOOH to form NO_2^- according to the following reaction [78]:

- d. The $\text{O}_2^{\bullet-}$ can undergo dismutation catalyzed by superoxide dismutase (Eq 9) or can self dismutate (Eq 10) to form H_2O_2 and O_2 in the following manner [60]:

The superoxide is in acid base equilibrium with hydroperoxyl (HO_2) radical with an acid dissociation constant (pK) of 4.8 [77]. The concentration of hydroperoxyl radical is related to the anion form of superoxide ($\text{O}_2^{\bullet-}$) as

$$[\text{HO}_2] = 0.0025[\text{O}_2^{\bullet-}] \quad (\text{A11})$$

- e. The $\text{CO}_3^{\bullet-}$ radical formed from the reaction between ONOO^- and CO_2 can react with NO and $\text{O}_2^{\bullet-}$ as shown below [60].

- f. Oxidation of BH_4 can occur by $\text{O}_2^{\bullet-}$, ONOO^- and O_2 according to the reactions shown below [20, 22, 79, 80].

BH_4 can also be oxidized from radicals formed including $\bullet\text{OH}$, $\bullet\text{NO}_2$ and $\text{CO}_3^{\bullet-}$ from the decomposition of peroxynitrous acid and reaction of ONOO^- with CO_2 [81].

- g. The oxidized form of biopterin, BH_3 , can be oxidized by O_2 to form BH_2 [79].

Furthermore, the BH_3 can undergo a self-combination reaction to form the reduced biopterin (BH_4) and BH_2 [79, 81].

Appendix A1.2

Based on the *Description of Biochemical Pathways and Downstream Reactions* in Appendix A1.1 and a closer look at the reaction pathways (Figure 2A and B), the justification for the use of 34 model equations is shown below

- a. The oxidation of L-Arginine to NHA involves the native enzyme (E_{-1}) and eight enzyme substrate complexes (E to E_{b1}), which results in a total of 9 distinct chemical species.
- b. The oxidation of NHA to produce NO and citrulline involves six enzyme substrate complexes (E_{b2} to E_{b7}) resulting in 6 additional chemical species.
- c. The $\text{O}_2^{\bullet-}$ production pathway involves the native enzyme (E_{-1}) and four enzyme substrate complexes (E_{c1} to E_{c4}). This adds four distinct chemical species excluding the native enzyme (E_{-1}).
- d. The major reaction products add another five chemical species. These include NHA, NO, citrulline, NO_3^- and $\text{O}_2^{\bullet-}$. The oxidation of L-Arginine produces NHA and the oxidation of NHA produces reaction products NO, citrulline, and NO_3^- . The $\text{O}_2^{\bullet-}$ is produced from the $\text{O}_2^{\bullet-}$ production pathway.
- e. A look at the downstream reaction pathway for reactions involving NO, $\text{O}_2^{\bullet-}$ and their reaction product peroxynitrite indicates major reaction products of BH_3 , ONOO^- , H_2O_2 , NO_2 , $\bullet\text{OH}$, $\bullet\text{NO}_2$, $\text{CO}_3^{\bullet-}$, CO_3^{2-} and OH^- . Amongst these, the first seven participate in other reactions whereas the CO_3^{2-} and OH^- are reaction end-products. These reaction end-products were ignored in the kinetic analysis. This adds another seven chemical species.
- f. One of the key objectives of this model is to estimate the production rates of NO and $\text{O}_2^{\bullet-}$, two rate expressions for their production were incorporated.
- g. Furthermore, BH_2 binds to the native enzyme (E_{-1}). The concentration of BH_2 is variable and was accounted in the kinetic analysis.

On the basis of the above statements, the rate equations can be mathematically represented as follows:

$$\frac{d[E_{-1}]}{dt} = k_{c-3}[E_{c1}] + k_{c-2}[E] - k_{c3}[BH_2][E_{-1}] - k_{c2}[E_{-1}][BH_4] \quad (A22)$$

$$\frac{d[E_{c1}]}{dt} = k_{c-4}[E_{c2}] + k_{c3}[BH_2][E_{-1}] - k_{c4}[E_{c1}][Arg] - k_{c-3}[E_{c1}] \quad (A23)$$

$$\frac{d[E_{c2}]}{dt} = k_{c4}[E_{c1}][Arg] + k_{c8}[E_{c4}] - k_{c-4}[E_{c2}] - k_{c5}[E_{c2}] \quad (A24)$$

$$\frac{d[E_{c3}]}{dt} = k_{c5}[E_{c2}] + k_{c-6}[E_{c4}] - k_{c6}[E_{c3}][O_2] \quad (A25)$$

$$\frac{d[E_{c4}]}{dt} = k_{c6}[E_{c3}][O_2] - k_{c-6}[E_{c4}] - k_{c8}[E_{c4}] \quad (A26)$$

$$\begin{aligned} \frac{d[E]}{dt} = & k_{c2}[E_{-1}][BH_4] \\ & + k_{a-1}[E_{a1}] \\ & + k_{b1}[E_{b1}] \\ & + k_{b7}[E_{b5}] \\ & + k_{b9}[E_{b5}] \\ & + k_{b9}[E_{b6}] - k_{c-2}[E] \\ & - k_{a1}[E][Arg] \\ & - k_{b-1}[NHA][E] \\ & - k_{b-7}[E][NO] \end{aligned} \quad (A27)$$

$$\frac{d[E_{a1}]}{dt} = k_{a1}[E][Arg] - k_{a-1}[E_{a1}] - k_{a2}[E_{a1}] \quad (A28)$$

$$\frac{d[E_{a2}]}{dt} = k_{a2}[E_{a1}] + k_{a-3}[E_{a3}] + k_{13}[Arg][E_{b7}] - k_{-13}[E_{a2}] - k_{a3}[E_{a2}][O_2] \quad (A29)$$

$$\frac{d[E_{a3}]}{dt} = k_{a3}[E_{a2}][O_2] - k_{a-3}[E_{a3}] - k_{a5}[E_{a3}] \quad (A30)$$

$$\frac{d[E_{a4}]}{dt} = k_{a5}[E_{a3}] - k_{a6}[E_{a4}] \quad (A31)$$

$$\begin{aligned} \frac{d[E_{a5}]}{dt} = & 0 = [E_{-1}] \\ & + [E_{c1}] \\ & + [E_{c2}] \\ & + [E_{c3}] \\ & + [E_{c4}] \\ & + [E] \\ & + [E_{a1}] \\ & + [E_{a2}] \\ & + [E_{a3}] \\ & + [E_{a4}] + [E_{a5}] \\ & + [E_{a6}] \\ & + [E_{b1}] \\ & + [E_{b2}] \\ & + [E_{b3}] \\ & + [E_{b4}] \\ & + [E_{b5}] \\ & + [E_{b6}] \\ & + [E_{b7}] - [eNOS] \end{aligned} \quad (A32)$$

$$\frac{d[E_{a6}]}{dt} = k_{a7}[E_{a5}] - k_{a8}[E_{a6}] \quad (A33)$$

$$\frac{d[E_{b1}]}{dt} = k_{a8}[E_{a6}] + k_{b-1}[NHA][E] - k_{b2}[E_{b1}] - k_{b1}[E_{b1}] \quad (A34)$$

$$\frac{d[E_{b2}]}{dt} = k_{b2}[E_{b1}] + k_{b-3}[E_{b3}] + k_{b-12}[E_{b7}][NHA] - k_{b3}[E_{b2}][O_2] - k_{b12}[E_{b2}] \quad (A35)$$

$$\frac{d[E_{b3}]}{dt} = k_{b3}[E_{b2}][O_2] - k_{b-3}[E_{b3}] - k_{b5}[E_{b3}] \quad (A36)$$

$$\frac{d[E_{b4}]}{dt} = k_{b5}[E_{b3}] - k_{b6}[E_{b4}] \quad (A17)$$

$$\frac{d[E_{b5}]}{dt} = k_{b6}[E_{b4}] + k_{b-7}[E][NO] - k_{b8}[E_{b5}] - k_{b7}[E_{b5}] \quad (A38)$$

$$\frac{d[E_{b6}]}{dt} = k_{b8}[E_{b5}] + k_{b-10}[NO][E_{b7}] - k_{b10}[E_{b6}] - k_{b9}[E_{b6}] \quad (A39)$$

$$\frac{d[E_{b7}]}{dt} = k_{b10}[E_{b6}] + k_{-13}[E_{a2}] + k_{b12}[E_{b2}] - k_{b-10}[E_{b7}][NO] - k_{b-12}[E_{b7}][NHA] - k_{13}[E_{b7}][Arg] \quad (A40)$$

$$\begin{aligned} \frac{d[NO]}{dt} = & k_{b7}[E_{b5}] \\ & + k_{b10}[E_{b6}] \\ & - k_{b-7}[NO][E] \\ & - k_{b-10}[NO][E_{b7}] \\ & - 4k_{15}[NO]^2[O_2] \\ & - k_{14}[NO][O_2^{\bullet-}] \\ & - 0.22k_{27}[ONOO^-][NO] \\ & - k_{32}[NO][CO_3^{\bullet-}] \end{aligned} \quad (A41)$$

$$\frac{d[Cit]}{dt} = k_{b6}[E_{b4}] \quad (A42)$$

$$\frac{d[NHA]}{dt} = k_{b1}[E_{b1}] + k_{b12}[E_{b2}] - k_{b-1}[NHA][E] - k_{b-12}[E_{b7}][NHA] \quad (A43)$$

$$\frac{d[NO_3^-]}{dt} = k_{b9}[E_{b6}] + k_{25}[ONOO^-] + k_{29}[ONOO^-][CO_2] \quad (A44)$$

$$\frac{d[O_2^{\bullet-}]}{dt} = k_{c8}[E_{c4}] - k_{14}[NO][O_2^{\bullet-}] - k_{16}[SOD][O_2^{\bullet-}] - 0.0025k_{28}[O_2^{\bullet-}]^2 - k_{31}[O_2^{\bullet-}][CO_3^{\bullet-}] - k_{18}[BH_4][O_2^{\bullet-}] \quad (A45)$$

$$\frac{d[H_2O_2]}{dt} = k_{16}[SOD][O_2^{\bullet-}] + 0.0025k_{28}[O_2^{\bullet-}]^2 + k_{18}[BH_4][O_2^{\bullet-}] \quad (A46)$$

$$\begin{aligned} \frac{d[\text{BH}_3]}{dt} = & k_{33}[\bullet\text{OH}][\text{BH}_4] \\ & + k_{34}[\bullet\text{NO}_2][\text{BH}_4] \\ & + k_{35}[\text{CO}_3^{\bullet-}][\text{BH}_4] + k_{22}[\text{BH}_4][\text{ONOO}^-] \\ & + k_{18}[\text{BH}_4][\text{O}_2^{\bullet-}] \\ & - 2k_{19}[\text{BH}_3]^2 \\ & - k_{36}[\text{BH}_3][\text{O}_2] \end{aligned} \quad (\text{A47})$$

$$\frac{d[\text{BH}_2]}{dt} = 0 = [\text{BH}_4] + [\text{BH}_3] + [\text{BH}_2] - [\text{TBP}] \quad (\text{A48})$$

$$\begin{aligned} \frac{d[\text{ONOO}^-]}{dt} = & k_{14}[\text{NO}][\text{O}_2^{\bullet-}] \\ & - k_{25}[\text{ONOO}^-] \\ & - k_{26}[\text{ONOO}^-] \\ & - k_{29}[\text{ONOO}^-][\text{CO}_2] - k_{30}[\text{ONOO}^-][\text{CO}_2] \\ & - 0.22k_{27}[\text{ONOO}^-][\text{NO}] \\ & - k_{22}[\text{BH}_4][\text{ONOO}^-] \end{aligned} \quad (\text{A49})$$

$$R_{\text{NO}} = k_{b7}[E_{b5}] + k_{b10}[E_{b6}] \quad (\text{A50})$$

$$R_{\text{O}_2^{\bullet-}} = k_{c8}[E_{c4}] \quad (\text{A51})$$

$$\frac{d[\text{NO}_2^-]}{dt} = 4k_{15}[\text{NO}]^2[\text{O}_2] + 0.22k_{27}[\text{NO}][\text{ONOO}^-] + k_{32}[\text{CO}_3^{\bullet-}][\text{NO}] + k_{34}[\bullet\text{NO}_2][\text{BH}_4] \quad (\text{A52})$$

$$\frac{d[\bullet\text{OH}]}{dt} = k_{26}[\text{ONOO}^-] - k_{33}[\bullet\text{OH}][\text{BH}_4] \quad (\text{A53})$$

$$\frac{d[\bullet\text{NO}_2]}{dt} = k_{26}[\text{ONOO}^-] + k_{30}[\text{ONOO}^-][\text{CO}_2] + 0.22k_{27}[\text{ONOO}^-][\text{NO}] - k_{34}[\bullet\text{NO}_2][\text{BH}_4] \quad (\text{A54})$$

$$\frac{d[\text{CO}_3^{\bullet-}]}{dt} = k_{30}[\text{ONOO}^-][\text{CO}_2] - k_{31}[\text{CO}_3^{\bullet-}][\text{O}_2^{\bullet-}] - k_{32}[\text{CO}_3^{\bullet-}][\text{NO}] - k_{35}[\text{CO}_3^{\bullet-}][\text{BH}_4] \quad (\text{A55})$$

Equation (A22) to equation (A55) represents the rate equations used in the current model. Table 1 shows the chemical composition of the enzyme (such as E_{-1} and E) and the enzyme substrate complexes (such as E_{c1} , E_{a1} etc.) associated with the rate equations. The terms

[Arg], [eNOS], [NHA] and [Cit] represents the concentration of L-Arginine, eNOS, N-hydroxy L-Arginine and citrulline, respectively. Equation (A32) represents the mass conservation of the enzyme (eNOS) and equation (A48) represents the mass conservation of biopterin.

Incorporation of GSH in the analysis adds another four chemical species into the mathematical model, which are GSH, GSNO, N_2O_3 and GSSG. Furthermore, due to incorporation of GSH, there are additional reactions which includes reaction between $ONOO^-$ and GSH and between GSNO and $O_2^{\bullet-}$ to produce NO. Hence equation (A41), equation (A45), equation (A49) and equation (A50) had to be modified during the GSH based analysis to account for these reactions. The modified rate equations for NO, $O_2^{\bullet-}$, $ONOO^-$ and NO production are as follows:

$$\begin{aligned} \frac{d[NO]}{dt} = & k_{b7}[E_{b5}] \\ & + k_{b10}[E_{b6}] \\ & + k_{37}[GSNO]^2[O_2] \\ & - k_{b-7}[NO][E] \\ & - k_{b-10}[NO][E_{b7}] - 4k_{15}[NO]^2[O_2] \\ & - k_{14}[NO][O_2^{\bullet-}] \\ & - 0.22k_{27}[ONOO^-][NO] \\ & - k_{32}[NO][CO_3^{\bullet-}] \end{aligned} \quad (A56)$$

$$\begin{aligned} \frac{d[O_2^{\bullet-}]}{dt} = & k_{c8}[E_{c4}] \\ & - k_{14}[NO][O_2^{\bullet-}] \\ & - k_{16}[SOD][O_2^{\bullet-}] - 0.0025k_{28}[O_2^{\bullet-}]^2 - k_{31}[O_2^{\bullet-}][CO_3^{\bullet-}] \\ & - k_{18}[BH_4][O_2^{\bullet-}] \\ & - k_{38}[GSNO]^2[O_2^{\bullet-}] \end{aligned} \quad (A57)$$

$$\begin{aligned} \frac{d[ONOO^-]}{dt} = & k_{14}[NO][O_2^{\bullet-}] \\ & - k_{25}[ONOO^-] \\ & - k_{26}[ONOO^-] \\ & - k_{29}[ONOO^-][CO_2] \\ & - k_{30}[ONOO^-][CO_2] \\ & - 0.22k_{27}[ONOO^-][NO] \\ & - k_{22}[BH_4][ONOO^-] \\ & - k_{38}[ONOO^-][GSH] \end{aligned} \quad (A58)$$

$$R_{NO} = k_{b7}[E_{b5}] + k_{b10}[E_{b6}] + k_{37}[GSNO]^2[O_2^{\bullet-}] \quad (A59)$$

In addition, the total thiol species also had to be conserved. Hence the rate equation for GSSG was in the form of an algebraic equation. Therefore in total, the GSH based analysis

led to an addition of three ordinary differential equations and one algebraic equation. They are as follows:

$$\frac{d[\text{GSNO}]}{dt} = k_{38}[\text{ONOO}^-][\text{GSH}] - k_{39}[\text{N}_2\text{O}_3][\text{GSH}] - k_{37}[\text{GSNO}]^2[\text{O}_2^{\bullet-}] \quad (\text{A60})$$

$$\frac{d[\text{N}_2\text{O}_3]}{dt} = 4k_{15}[\text{NO}]^2[\text{O}_2] - k_{39}[\text{N}_2\text{O}_3][\text{GSH}] \quad (\text{A61})$$

$$\frac{d[\text{GSH}]}{dt} = \frac{V_{m,\text{GR}}[\text{GSSG}]}{K_{m,\text{GR}} + [\text{GSSG}]} - k_{39}[\text{N}_2\text{O}_3][\text{GSH}] - k_{38}[\text{ONOO}^-][\text{GSH}] \quad (\text{A62})$$

$$[\text{GSSG}] = [\text{GSH}]_0 - [\text{GSH}] - [\text{GSNO}] \quad (\text{A63})$$

Equation (A60) to equation (A63) represents the rate equations of the additional species incorporated in the model during the GSH based analysis. Equation (A63) represents the mass conservation of thiols. $V_{m,\text{GR}}$ and $K_{m,\text{GR}}$ represents the enzymatic kinetic parameters related to the enzyme glutathione reductase and are shown in Table 3.

References

1. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J.* 2001; 357:593–615. [PubMed: 11463332]
2. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J.* 1994; 298(Pt 2):249–258. [PubMed: 7510950]
3. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation.* 2006; 113:1708–1714. [PubMed: 16585403]
4. Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, Kalyanaraman B. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J.* 2002; 362:733–739. [PubMed: 11879202]
5. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev.* 1991; 43:109–142. [PubMed: 1852778]
6. Suematsu M, Tamatani T, Delano FA, Miyasaka M, Forrest M, Suzuki H, Schmid-Schonbein GW. Microvascular oxidative stress preceding leukocyte activation elicited by in vivo nitric oxide suppression. *Am J Physiol.* 1994; 266:H2410–H2415. [PubMed: 8024002]
7. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A.* 1991; 88:4651–4655. [PubMed: 1675786]
8. Kashiwagi S, Kajimura M, Yoshimura Y, Suematsu M. Nonendothelial source of nitric oxide in arterioles but not in venules: alternative source revealed in vivo by diaminofluorescein microfluorography. *Circ Res.* 2002; 91:e55–e64. [PubMed: 12480826]
9. Kajimura M, Michel CC. Flow modulates the transport of K⁺ through the walls of single perfused mesenteric venules in anaesthetised rats. *J Physiol.* 1999; 521(Pt 3):665–677. [PubMed: 10601497]
10. Young EW, Watson MW, Srigunapalan S, Wheeler AR, Simmons CA. Technique for real-time measurements of endothelial permeability in a microfluidic membrane chip using laser-induced fluorescence detection. *Anal Chem.* 82:808–816. [PubMed: 20050596]
11. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. *Physiol Rev.* 2006; 86:279–367. [PubMed: 16371600]

12. Channon KM. Tetrahydrobiopterin: regulator of endothelial nitric oxide synthase in vascular disease. *Trends Cardiovasc Med*. 2004; 14:323–327. [PubMed: 15596110]
13. Crane BR, Arvai AS, Ghosh DK, Wu C, Getzoff ED, Stuehr DJ, Tainer JA. Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science*. 1998; 279:2121–2126. [PubMed: 9516116]
14. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem*. 1994; 63:175–195. [PubMed: 7526779]
15. Wei CC, Crane BR, Stuehr DJ. Tetrahydrobiopterin radical enzymology. *Chem Rev*. 2003; 103:2365–2383. [PubMed: 12797834]
16. Wei CC, Wang ZQ, Arvai AS, Hemann C, Hille R, Getzoff ED, Stuehr DJ. Structure of tetrahydrobiopterin tunes its electron transfer to the heme-dioxy intermediate in nitric oxide synthase. *Biochemistry*. 2003; 42:1969–1977. [PubMed: 12590583]
17. Hurshman AR, Krebs C, Edmondson DE, Marletta MA. Ability of tetrahydrobiopterin analogues to support catalysis by inducible nitric oxide synthase: formation of a pterin radical is required for enzyme activity. *Biochemistry*. 2003; 42:13287–13303. [PubMed: 14609340]
18. Alp NJ, Channon KM. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arterioscler Thromb Vasc Biol*. 2004; 24:413–420. [PubMed: 14656731]
19. Crabtree MJ, Tatham AL, Al-Wakeel Y, Warrick N, Hale AB, Cai S, Channon KM, Alp NJ. Quantitative regulation of intracellular endothelial nitric-oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biopterin redox status: insights from cells with tet-regulated GTP cyclohydrolase I expression. *J Biol Chem*. 2009; 284:1136–1144. [PubMed: 19011239]
20. Vasquez-Vivar J. Tetrahydrobiopterin, superoxide, and vascular dysfunction. *Free Radic Biol Med*. 2009; 47:1108–1119. [PubMed: 19628033]
21. Wei CC, Wang ZQ, Wang Q, Meade AL, Hemann C, Hille R, Stuehr DJ. Rapid kinetic studies link tetrahydrobiopterin radical formation to heme-dioxy reduction and arginine hydroxylation in inducible nitric-oxide synthase. *J Biol Chem*. 2001; 276:315–319. [PubMed: 11020389]
22. Vasquez-Vivar J, Kalyanaraman B, Martasek P. The role of tetrahydrobiopterin in superoxide generation from eNOS: enzymology and physiological implications. *Free Radic Res*. 2003; 37:121–127. [PubMed: 12653200]
23. Crabtree MJ, Smith CL, Lam G, Goligorsky MS, Gross SS. Ratio of 5,6,7,8-tetrahydrobiopterin to 7,8-dihydrobiopterin in endothelial cells determines glucose-elicited changes in NO vs. superoxide production by eNOS. *Am J Physiol Heart Circ Physiol*. 2008; 294:H1530–H1540. [PubMed: 18192221]
24. Alp NJ, McAteer MA, Khoo J, Choudhury RP, Channon KM. Increased endothelial tetrahydrobiopterin synthesis by targeted transgenic GTP-cyclohydrolase I overexpression reduces endothelial dysfunction and atherosclerosis in ApoE-knockout mice. *Arterioscler Thromb Vasc Biol*. 2004; 24:445–450. [PubMed: 14707037]
25. Alp NJ, Mussa J, Cai S, Guzik T, Jefferson A, Goh N, Rockett KA, Channon KM. Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J Clin Invest*. 2003; 112:725–735. [PubMed: 12952921]
26. Li L, Chen W, Rezvan A, Jo H, Harrison DG. Tetrahydrobiopterin Deficiency and Nitric Oxide Synthase Uncoupling Contribute to Atherosclerosis Induced by Disturbed Flow. *Arterioscler Thromb Vasc Biol*.
27. Tsutsui M, Milstien S, Katusic ZS. Effect of tetrahydrobiopterin on endothelial function in canine middle cerebral arteries. *Circ Res*. 1996; 79:336–342. [PubMed: 8756013]
28. Hattori Y, Hattori S, Wang X, Satoh H, Nakanishi N, Kasai K. Oral administration of tetrahydrobiopterin slows the progression of atherosclerosis in apolipoprotein E-knockout mice. *Arterioscler Thromb Vasc Biol*. 2007; 27:865–870. [PubMed: 17272747]
29. Sawabe K, Suetake Y, Nakanishi N, Wakasugi KO, Hasegawa H. Cellular accumulation of tetrahydrobiopterin following its administration is mediated by two different processes; direct uptake and indirect uptake mediated by a methotrexate-sensitive process. *Mol Genet Metab*. 2005; 86 Suppl 1:S133–S138. [PubMed: 16165391]

30. An J, Du J, Wei N, Xu H, Pritchard KA Jr, Shi Y. Role of tetrahydrobiopterin in resistance to myocardial ischemia in Brown Norway and Dahl S rats. *Am J Physiol Heart Circ Physiol*. 2009; 297:H1783–H1791. [PubMed: 19717731]
31. Higashi Y, Sasaki S, Nakagawa K, Fukuda Y, Matsuura H, Oshima T, Chayama K. Tetrahydrobiopterin enhances forearm vascular response to acetylcholine in both normotensive and hypertensive individuals. *Am J Hypertens*. 2002; 15:326–332. [PubMed: 11991218]
32. Setoguchi S, Hirooka Y, Eshima K, Shimokawa H, Takeshita A. Tetrahydrobiopterin improves impaired endothelium-dependent forearm vasodilation in patients with heart failure. *J Cardiovasc Pharmacol*. 2002; 39:363–368. [PubMed: 11862115]
33. Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, Rabelink T. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest*. 1997; 99:41–46. [PubMed: 9011574]
34. Heitzer T, Krohn K, Albers S, Meinertz T. Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetes mellitus. *Diabetologia*. 2000; 43:1435–1438. [PubMed: 11126415]
35. Maier W, Cosentino F, Lutolf RB, Fleisch M, Seiler C, Hess OM, Meier B, Luscher TF. Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease. *J Cardiovasc Pharmacol*. 2000; 35:173–178. [PubMed: 10672847]
36. Fukuda Y, Teragawa H, Matsuda K, Yamagata T, Matsuura H, Chayama K. Tetrahydrobiopterin restores endothelial function of coronary arteries in patients with hypercholesterolaemia. *Heart*. 2002; 87:264–269. [PubMed: 11847169]
37. Kinoshita H, Katusic ZS. Exogenous tetrahydrobiopterin causes endothelium-dependent contractions in isolated canine basilar artery. *Am J Physiol*. 1996; 271:H738–H743. [PubMed: 8770117]
38. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T, Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation*. 2001; 103:1282–1288. [PubMed: 11238274]
39. Tiefenbacher CP, Bleeke T, Vahl C, Amann K, Vogt A, Kubler W. Endothelial dysfunction of coronary resistance arteries is improved by tetrahydrobiopterin in atherosclerosis. *Circulation*. 2000; 102:2172–2179. [PubMed: 11056088]
40. Vazquez-Vivar J, Duquaine D, Whitsett J, Kalyanaraman B, Rajagopalan S. Altered tetrahydrobiopterin metabolism in atherosclerosis: implications for use of oxidized tetrahydrobiopterin analogues and thiol antioxidants. *Arterioscler Thromb Vasc Biol*. 2002; 22:1655–1661. [PubMed: 12377745]
41. Chen K, Popel AS. Theoretical analysis of biochemical pathways of nitric oxide release from vascular endothelial cells. *Free Radic Biol Med*. 2006; 41:668–680. [PubMed: 16864000]
42. Santolini J, Meade AL, Stuehr DJ. Differences in three kinetic parameters underpin the unique catalytic profiles of nitric-oxide synthases I, II, and III. *J Biol Chem*. 2001; 276:48887–48898. [PubMed: 11684690]
43. Berka V, Yeh HC, Gao D, Kiran F, Tsai AL. Redox function of tetrahydrobiopterin and effect of L-arginine on oxygen binding in endothelial nitric oxide synthase. *Biochemistry*. 2004; 43:13137–13148. [PubMed: 15476407]
44. Berka V, Tsai AL. Characterization of interactions among the heme center, tetrahydrobiopterin, and L-arginine binding sites of ferric eNOS using imidazole, cyanide, and nitric oxide as probes. *Biochemistry*. 2000; 39:9373–9383. [PubMed: 10924132]
45. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*. 1996; 271:C1424–C1437. [PubMed: 8944624]
46. Figueroa XF, Martinez AD, Gonzalez DR, Jara PI, Ayala S, Boric MP. In vivo assessment of microvascular nitric oxide production and its relation with blood flow. *Am J Physiol Heart Circ Physiol*. 2001; 280:H1222–H1231. [PubMed: 11179067]
47. Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, Murad F. Purification and characterization of particulate endothelium-derived relaxing factor synthase from

- cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A*. 1991; 88:10480–10484. [PubMed: 1720542]
48. Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Schmidt K, Weiss G, Wachter H. Pteridine biosynthesis in human endothelial cells. Impact on nitric oxide-mediated formation of cyclic GMP. *J Biol Chem*. 1993; 268:1842–1846. [PubMed: 7678411]
 49. Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun*. 1999; 263:681–684. [PubMed: 10512739]
 50. Presta A, Siddhanta U, Wu C, Sennequier N, Huang L, Abu-Soud HM, Erzurum S, Stuehr DJ. Comparative functioning of dihydro- and tetrahydropterins in supporting electron transfer, catalysis, and subunit dimerization in inducible nitric oxide synthase. *Biochemistry*. 1998; 37:298–310. [PubMed: 9425051]
 51. Wu G, Morris SM Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J*. 1998; 336(Pt 1): 1–17. [PubMed: 9806879]
 52. Popel AS. Theory of oxygen transport to tissue. *Crit Rev Biomed Eng*. 1989; 17:257–321. [PubMed: 2673661]
 53. Denicola A, Freeman BA, Trujillo M, Radi R. Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated oxidations. *Arch Biochem Biophys*. 1996; 333:49–58. [PubMed: 8806753]
 54. Meininger CJ, Marinos RS, Hatakeyama K, Martinez-Zaguilan R, Rojas JD, Kelly KA, Wu G. Impaired nitric oxide production in coronary endothelial cells of the spontaneously diabetic BB rat is due to tetrahydrobiopterin deficiency. *Biochem J*. 2000; 349:353–356. [PubMed: 10861247]
 55. Liu J, Garcia-Cardena G, Sessa WC. Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: implications for caveolae localization. *Biochemistry*. 1996; 35:13277–13281. [PubMed: 8873592]
 56. Persechini A, Stemmer PM. Calmodulin is a limiting factor in the cell. *Trends Cardiovasc Med*. 2002; 12:32–37. [PubMed: 11796242]
 57. Wickham NW, Vercellotti GM, Moldow CF, Visser MR, Jacob HS. Measurement of intracellular calcium concentration in intact monolayers of human endothelial cells. *J Lab Clin Med*. 1988; 112:157–167. [PubMed: 2840476]
 58. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. *J Biol Chem*. 2003; 278:22546–22554. [PubMed: 12692136]
 59. Hu TM, Hayton WL, Mallery SR. Kinetic modeling of nitric-oxide-associated reaction network. *Pharm Res*. 2006; 23:1702–1711. [PubMed: 16850266]
 60. Lancaster JR Jr. Nitroxidative, nitrosative, and nitrative stress: kinetic predictions of reactive nitrogen species chemistry under biological conditions. *Chem Res Toxicol*. 2006; 19:1160–1174. [PubMed: 16978020]
 61. Brown DI, Griendling KK. Nox proteins in signal transduction. *Free Radic Biol Med*. 2009; 47:1239–1253. [PubMed: 19628035]
 62. Arnal JF, Clamens S, Pechet C, Negre-Salvayre A, Allera C, Girolami JP, Salvayre R, Bayard F. Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. *Proc Natl Acad Sci U S A*. 1996; 93:4108–4113. [PubMed: 8633024]
 63. Hood JD, Meininger CJ, Ziche M, Granger HJ. VEGF upregulates eNOS message, protein, and NO production in human endothelial cells. *Am J Physiol*. 1998; 274:H1054–H1058. [PubMed: 9530221]
 64. Kuchan MJ, Frangos JA. Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *Am J Physiol*. 1994; 266:C628–C636. [PubMed: 8166225]
 65. Potdar S, Kavdia M. NO/peroxynitrite dynamics of high glucose-exposed HUVECs: chemiluminescent measurement and computational model. *Microvasc Res*. 2009; 78:191–198. [PubMed: 19362569]
 66. Lu X, Kassab GS. Nitric oxide is significantly reduced in ex vivo porcine arteries during reverse flow because of increased superoxide production. *J Physiol*. 2004; 561:575–582. [PubMed: 15579542]

67. Vaughn MW, Kuo L, Liao JC. Estimation of nitric oxide production and reaction rates in tissue by use of a mathematical model. *Am J Physiol*. 1998; 274:H2163–H2176. [PubMed: 9841542]
68. Malinski T, Taha Z, Grunfeld S, Patton S, Kapturczak M, Tomboulis P. Diffusion of nitric oxide in the aorta wall monitored in situ by porphyrinic microensors. *Biochem Biophys Res Commun*. 1993; 193:1076–1082. [PubMed: 8323533]
69. Santolini J, Adak S, Curran CM, Stuehr DJ. A kinetic simulation model that describes catalysis and regulation in nitric-oxide synthase. *J Biol Chem*. 2001; 276:1233–1243. [PubMed: 11038356]
70. Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S. Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem*. 2004; 279:36167–36170. [PubMed: 15133020]
71. Abu-Soud HM, Ichimori K, Presta A, Stuehr DJ. Electron transfer, oxygen binding, and nitric oxide feedback inhibition in endothelial nitric-oxide synthase. *J Biol Chem*. 2000; 275:17349–17357. [PubMed: 10749853]
72. Marchal S, Gorren AC, Sorlie M, Andersson KK, Mayer B, Lange R. Evidence of two distinct oxygen complexes of reduced endothelial nitric oxide synthase. *J Biol Chem*. 2004; 279:19824–19831. [PubMed: 15004019]
73. Wei CC, Wang ZQ, Tejero J, Yang YP, Hemann C, Hille R, Stuehr DJ. Catalytic reduction of a tetrahydrobiopterin radical within nitric-oxide synthase. *J Biol Chem*. 2008; 283:11734–11742. [PubMed: 18283102]
74. Wei CC, Wang ZQ, Hemann C, Hille R, Stuehr DJ. A tetrahydrobiopterin radical forms and then becomes reduced during Nomega-hydroxyarginine oxidation by nitric oxide synthase. *J Biol Chem*. 2003; 278:46668–46673. [PubMed: 14504282]
75. Klatt P, Schmid M, Leopold E, Schmidt K, Werner ER, Mayer B. The pteridine binding site of brain nitric oxide synthase. Tetrahydrobiopterin binding kinetics, specificity, and allosteric interaction with the substrate domain. *J Biol Chem*. 1994; 269:13861–13866. [PubMed: 7514595]
76. Nalwaya N, Deen WM. Analysis of cellular exposure to peroxynitrite in suspension cultures. *Chem Res Toxicol*. 2003; 16:920–932. [PubMed: 12870895]
77. Kavdia M, Stanfield JL, Lewis RS. Nitric oxide, superoxide, and peroxynitrite effects on the insulin secretion and viability of betaTC3 cells. *Ann Biomed Eng*. 2000; 28:102–109. [PubMed: 10645793]
78. Pfeiffer S, Gorren AC, Schmidt K, Werner ER, Hansert B, Bohle DS, Mayer B. Metabolic fate of peroxynitrite in aqueous solution. Reaction with nitric oxide and pH-dependent decomposition to nitrite and oxygen in a 2: 1 stoichiometry. *J Biol Chem*. 1997; 272:3465–3470. [PubMed: 9013592]
79. Kirsch M, Korth HG, Stenert V, Sustmann R, de Groot H. The autoxidation of tetrahydrobiopterin revisited. Proof of superoxide formation from reaction of tetrahydrobiopterin with molecular oxygen. *J Biol Chem*. 2003; 278:24481–24490. [PubMed: 12714605]
80. Vasquez-Vivar J, Whitsett J, Martasek P, Hogg N, Kalyanaraman B. Reaction of tetrahydrobiopterin with superoxide: EPR-kinetic analysis and characterization of the pteridine radical. *Free Radic Biol Med*. 2001; 31:975–985. [PubMed: 11595382]
81. Patel KB, Stratford MR, Wardman P, Everett SA. Oxidation of tetrahydrobiopterin by biological radicals and scavenging of the trihydrobiopterin radical by ascorbate. *Free Radic Biol Med*. 2002; 32:203–211. [PubMed: 11827745]
82. Lubrano V, Vassalle C, Blandizzi C, Del Tacca M, Palombo C, L'Abbate A, Baldi S, Natali A. The effect of lipoproteins on endothelial nitric oxide synthase is modulated by lipoperoxides. *Eur J Clin Invest*. 2003; 33:117–125. [PubMed: 12588285]
83. Rengasamy A, Johns RA. Determination of Km for oxygen of nitric oxide synthase isoforms. *J Pharmacol Exp Ther*. 1996; 276:30–33. [PubMed: 8558447]
84. Berka V, Chen PF, Tsai AL. Spatial relationship between L-arginine and heme binding sites of endothelial nitric-oxide synthase. *J Biol Chem*. 1996; 271:33293–33300. [PubMed: 8969188]
85. Wei CC, Wang ZQ, Durra D, Hemann C, Hille R, Garcin ED, Getzoff ED, Stuehr DJ. The three nitric-oxide synthases differ in their kinetics of tetrahydrobiopterin radical formation, heme-dioxy reduction, and arginine hydroxylation. *J Biol Chem*. 2005; 280:8929–8935. [PubMed: 15632185]

Figure 1. eNOS structure and [BH₄]/[TBP] dependent NO and O₂^{•-} production

Panel A: Structure of eNOS showing the reductase and oxygenase domain with co-factors (light green), substrates L-Arginine and O₂ (yellow) and products L-citrulline (blue), NO (red) and O₂^{•-} (purple). **Panel B:** The effects of binding of reduced bioperin (BH₄) and oxidized bioperin (BH₂) to eNOS on the products of catalytic cycle of eNOS. eNOS binding of BH₄ causes NO production and that of BH₂ causes production of O₂^{•-}. The NO and O₂^{•-} formed can react with each other to form ONOO⁻, which along with O₂^{•-} can oxidize BH₄ to BH₃. The BH₃ formed can be oxidized to BH₂ by O₂.

Figure 2. Schematic of the reaction pathways associated with eNOS

Panel A shows the biochemical pathways of eNOS related to the production of NO by oxidation of L-Arginine upon binding of BH₄ to eNOS as well as the biochemical pathway associated with the production of O₂^{•-} upon binding of BH₂ to eNOS. Green represents the co-factors, grey the enzyme, orange the enzyme substrate complexes, yellow the substrates, blue, red and purple represent the products of eNOS catalysis i.e. NHA (blue), L-citrulline (blue), NO (red) and O₂^{•-} (purple). **Panel B** shows the reactions associated with NO, O₂^{•-} and their mutual reaction product peroxynitrite (ONOO⁻) as well the reactions associated with O₂ and CO₂ and the different forms of biopterin (BH₄, BH₃ and BH₂).

Figure 3. [BH₄]/[TBP] and [BH₄] dependent NO and O₂^{•-} production rate by eNOS
 The biopterin ratio ([BH₄]/[TBP]) was varied from 0.99, 0.9, 0.7, 0.5, 0.25 and 0.05. **Panel A**, and **B** represents the time dependent profiles of NO production rate, and the O₂^{•-} production rates, respectively with respect to [BH₄]/[TBP] ratio. **Panel C** represents the steady state production rates of NO and O₂^{•-} with respect to concentration of BH₄. The concentration of total biopterin ([TBP]) was set at a constant value of 7 μM for all the different biopterin ratios simulated. The concentrations of L-Arginine, O₂, SOD, CO₂ and eNOS were set at constant values of 100 μM, 140 μM, 10 μM, 1.1 mM and 0.097 μM, respectively for all the cases simulated.

Figure 4. Peroxynitrite concentration profile for $[BH_4]/[TBP]$ ratio of 0.99, 0.9, 0.7, 0.5, 0.25 and 0.05

The peroxynitrite generation was only from the eNOS produced NO and $O_2^{\bullet-}$. The concentrations of total biopterin ([TBP]), L-Arginine, O_2 , SOD, CO_2 and eNOS were 7 μM , 100 μM , 140 μM , 10 μM , 1.1 mM and 0.097 μM , respectively.

Figure 5. Effect of total bioprotein concentration

Panel A and **B** show the eNOS related NO and $\text{O}_2^{\bullet-}$ production rates, respectively for [BH₄]/[TBP] ratio of 0.99, 0.9, 0.5 and 0.05. The concentrations of L-Arginine, O_2 , SOD, CO_2 and eNOS were 100 μM , 140 μM , 10 μM , 1.1 mM and 0.097 μM , respectively.

Figure 6. Effect of eNOS concentration on the NO production rate

Panel A and **B** show the effect the NO production rates for biopterin ratio ($[BH_4]/[TBP]$) of 0.99 and 0.05, respectively. The concentrations of total biopterin ($[TBP]$), L-Arginine, O_2 , SOD, and CO_2 were 7 μM , 100 μM , 140 μM , 10 μM , and 1.1 mM, respectively.

Figure 7. Effect of eNOS concentration on the $O_2^{\bullet-}$ production rate
Panel A and **B** show the effect the $O_2^{\bullet-}$ production rates for biopterin ratio ($[BH_4]/[TBP]$) of 0.99 and 0.05, respectively. The concentrations of total biopterin ($[TBP]$), L-Arginine, O_2 , SOD, and CO_2 were 7 μM , 100 μM , 140 μM , 10 μM , and 1.1 mM, respectively.

Figure 8. Effects of feedback inhibition pathways for consumption of NO

Panel A shows the production rate of NO and **Panel B** shows the temporal NO concentration profiles. The concentrations of total biopterin ([TBP]), L-Arginine, O₂, SOD, CO₂ and eNOS were 7 μ M, 100 μ M, 140 μ M, 10 μ M, 1.1 mM and 0.097 μ M, respectively. The biopterin ratio [BH₄]/[TBP] was 0.99.

Figure 9. Effect of incorporation of GSH in the eNOS biochemical pathway

Panel A shows the temporal variation in the GSH concentration for biopterin ratios of 0.99, 0.9, 0.7, 0.5, 0.25 and 0.05, respectively at initial GSH concentrations ($[GSH]_0$) of 0.1 mM (left), 1 mM (center) and 10 mM (right), respectively. **Panel B** shows the temporal variation in the GSSG concentration for biopterin ratios of 0.99, 0.9, 0.7, 0.5, 0.25 and 0.05, respectively at initial GSH concentrations ($[GSH]_0$) of 0.1 mM (left), 1 mM (center) and 10 mM (right), respectively. The concentrations of total biopterin ([TBP]), L-Arginine, O_2 , SOD, CO_2 , and eNOS were 7 μM , 100 μM , 140 μM , 10 μM , 1.1 mM and 0.097 μM , respectively.

Table 1

Enzyme substrate complex nomenclature for eNOS

Nomenclature	Enzyme/Enzyme Substrate complex
E ₋₁	eNOS-(Fe ^{III})
E _{c1}	eNOS-(Fe ^{III})-BH ₂
E _{c2}	eNOS-(Fe ^{III})-BH ₂ -Arg
E _{c3}	eNOS-(Fe ^{II})-BH ₂ -Arg
E _{c4}	eNOS-[Fe ^{II} -O ₂]-BH ₂ -Arg / eNOS-[Fe ^{III} -O ₂ ⁻]-BH ₂ -Arg
E	eNOS-(Fe ^{III})-BH ₄
E _{a1}	eNOS-(Fe ^{III})-BH ₄ -Arg
E _{a2}	eNOS-(Fe ^{II})-BH ₄ -Arg
E _{a3}	eNOS-[Fe ^{II} -O ₂]-BH ₄ -Arg or eNOS-[Fe ^{III} -O ₂ ⁻]-BH ₄ -Arg
E _{a4}	eNOS-[Fe ^{III} -OOH]-BH ₃ -Arg
E _{a5}	eNOS-[Fe ^{IV} -O]-BH ₃ -Arg
E _{a6}	eNOS-(Fe ^{III})-BH ₃ -NHA
E _{b1}	eNOS-(Fe ^{III})-BH ₄ -NHA
E _{b2}	eNOS-(Fe ^{II})-BH ₄ -NHA
E _{b3}	eNOS-[Fe ^{II} -O ₂]-BH ₄ -NHA / eNOS-[Fe ^{III} -O ₂ ⁻]-BH ₄ -NHA
E _{b4}	eNOS-[Fe ^{III} -OOH]-BH ₃ -NHA
E _{b5}	eNOS-(Fe ^{III})-NO-BH ₄
E _{b6}	eNOS-(Fe ^{II})-NO-BH ₄
E _{b7}	eNOS-(Fe ^{II})-BH ₄

Table 2Parameters related to eNOS kinetics for NO/O₂⁻ production

Variable/Constant	Values	Units	References
V_{max}	0.585	μmol/min/mg	[55]
[Ca ²⁺]	0.013–0.280	μM	[54, 57]
[eNOS]	0.007–0.097	μM	[41, 82]
[NADPH]	166–295	μM	[54]
[CaM]	5	μM	[56]
[L-Arginine]	0.1–1	mM	[51]
[O ₂] (arterioles)	127–172	μM	[41, 52]
[O ₂] (venules)	50–80	μM	[41, 52]
K _m (NADPH)	0.65	μM	[55]
K _m (O ₂)	7.7	μM	[83]
K _m (L-Arginine)	2.9–5	μM	[51, 55]
EC ₅₀ (Ca ²⁺)	0.11	μM	[55]
EC ₅₀ (CaM)	0.009	μM	[55]

Table 3Rate constants for biochemical reactions for production and interactions of NO and $O_2^{\bullet-}$

Constant	Reported Values	Experimental Conditions	Value at 37°C	References
$k_{a1} (M^{-1}.s^{-1})$	2×10^5 8×10^5	4°C 23°C	1.19×10^6	[44, 84]
$k_{a-1} (s^{-1})$	0.08 1.6	4°C 23°C	3.77	[44, 84]
$k_{a2} (s^{-1})$	0.17	10°C	0.474	[42]
$k_{a3} (M^{-1}.s^{-1})$	2.65×10^5 3.4×10^5	7°C 10°C	8.20×10^5	[71, 72]
$k_{a-3} (s^{-1})$	24 28	7°C 10°C	48.3	[71, 72]
$k_{a5} (s^{-1})$	6.2 6.5 ± 1.5	7°C 10°C	7.68	[72, 85]
$k_{a6} (s^{-1})$	$\geq k_{a5}$	Same as k_{a5}	7.68	[16]
$k_{a7} (s^{-1})$	5.8 ± 1.7	10°C	6.85	[85]
$k_{a8} (s^{-1})$	1.3	10°C	3.62	[73]
$k_{b1} (s^{-1})$	0.08 1.6	4°C 23°C	0.1	[84]
$k_{b-1} (M^{-1}.s^{-1})$	2×10^5 8×10^5	4°C 23°C	1×10^5	[84]
$k_{b2} (s^{-1})$	0.17	10°C	0.474	[42]
$k_{b3} (M^{-1}.s^{-1})$	3.42×10^5	7°C	9.19×10^5	[72]
$k_{b-3} (s^{-1})$	22	7°C	40.5	[72]
$k_{b5} (s^{-1})$	31 ± 3.2	10°C	36.6	[74]
$k_{b6} (s^{-1})$	8	10°C	9.45	[74]
$k_{b7} (s^{-1})$	2.5 3.5 ± 0.5	7°C 10°C	11.5	[42, 72]
$k_{b-7} (M^{-1}.s^{-1})$	6.1×10^5	10°C	1.7×10^6	[71]
$k_{b8} (s^{-1})$	$(2.8 \pm 0.4) \times 10^{-3}$	10°C	7.8×10^{-3}	[42]
$k_{b9} (s^{-1})$	0.63 ± 0.05	10°C	1.76	[42]
$k_{b10} (s^{-1})$	$(6.3 \pm 1.0) \times 10^{-4}$	10°C	1.76×10^{-3}	[42]
$k_{b-10} (M^{-1}.s^{-1})$	1.1×10^6	10°C	3.07×10^6	[71]
$k_{b12} (s^{-1})$	0.08 1.6	4°C 23°C	3.66	[84]
$k_{b-12} (M^{-1}.s^{-1})$	2×10^5 8×10^5	4°C 23°C	1.09×10^6	[84]
$k_{13} (M^{-1}.s^{-1})$	2×10^5 8×10^5	4°C 23°C	1.19×10^6	[41, 84]
$k_{-13} (s^{-1})$	0.08 1.6	4°C 23°C	3.77	[41, 84]
$k_{c2} (M^{-1}.s^{-1})$	0.22×10^5	37°C	2.20×10^4	[75]

Constant	Reported Values	Experimental Conditions	Value at 37°C	References
$k_{c-2} (s^{-1})$	0.005	37°C	0.005	[75]
$k_{c3} (M^{-1}.s^{-1})$	0.22×10^5	37°C	2.20×10^4	[75]
$k_{c-3} (s^{-1})$	0.047	37°C	0.047	[75]
$k_{c4} (M^{-1}.s^{-1})$	2×10^5 8×10^5	4°C 23°C	1.19×10^6	[44, 84]
$k_{c-4} (s^{-1})$	0.08 1.6	4°C 23°C	3.77	[44, 84]
$k_{c5} (s^{-1})$	0.17	10°C	0.474	[21, 50]
$k_{c6} (M^{-1}.s^{-1})$	$(3.2 \pm 1.4) \times 10^5$ 4.52×10^5	4°C 7°C	1.73×10^6	[43, 72]
$k_{c-6} (s^{-1})$	7.2 ± 3.1	4°C	14.2	[43]
$k_{c8} (s^{-1})$	0.15 0.181	4°C 7°C	0.375	[43, 72]
$k_{14} (M^{-1}.s^{-1})$	6.7×10^9	37°C	6.7×10^9	[65]
$k_{15} (M^{-2}.s^{-1})$	2.4×10^6	37°C	2.4×10^6	[60]
$k_{16} (M^{-1}.s^{-1})$	2.5×10^9	25°C	3.85×10^9	[65]
$k_{17} (M^{-1}.s^{-1})$	0.60 ± 0.03	37°C	0.6	[20, 79]
$k_{18} (M^{-1}.s^{-1})$	3.9×10^5	37°C	3.9×10^5	[79]
$k_{19} (M^{-1}.s^{-1})$	4.65×10^4	37°C	4.65×10^4	[79, 81]
$k_{22} (M^{-1}.s^{-1})$	6×10^3	37°C	6×10^3	[58]
$k_{25} (s^{-1})$	0.568	22°C	0.981	[60]
$k_{26} (s^{-1})$	0.232	22°C	0.401	[60]
$k_{27} (M^{-1}.s^{-1})$	9.1×10^4	37°C	9.1×10^4	[65]
$k_{28} (M^{-1}.s^{-1})$	2.54×10^5	25°C	3.57×10^5	[60]
$k_{29} (M^{-1}.s^{-1})$	3.886×10^4	37°C	3.886×10^4	[60]
$k_{30} (M^{-1}.s^{-1})$	1.914×10^4	37°C	1.914×10^4	[60]
$k_{31} (M^{-1}.s^{-1})$	4×10^8	23°C	6.65×10^8	[60]
$k_{32} (M^{-1}.s^{-1})$	3.5×10^9	23°C	5.82×10^9	[60]
$k_{33} (M^{-1}.s^{-1})$	8.8×10^9	37°C	8.8×10^9	[81]
$k_{34} (M^{-1}.s^{-1})$	9.4×10^8	37°C	9.4×10^8	[81]
$k_{35} (M^{-1}.s^{-1})$	4.6×10^9	37°C	4.6×10^9	[81]
$k_{36} (M^{-1}.s^{-1})$	3.2×10^3	37°C	3.2×10^3	[79]
$k_{37} (M^{-2}.s^{-1})$	6×10^8	37°C	6×10^8	[59]
$k_{38} (M^{-1}.s^{-1})$	1.35×10^3	37°C	1.35×10^3	[59]
$k_{39} (M^{-1}.s^{-1})$	6.6×10^7	37°C	6.6×10^7	[59]
$K_{m,GR} (\mu M)$	50	37°C	50	[59]
$V_{m,GR} (M.s^{-1})$	3.2×10^{-4}	37°C	3.2×10^{-4}	[59]