Nitric oxide synthase (NOS) catalyzes both coupled and uncoupled reactions, which generate nitric oxide and reactive oxygen species (ROS). Oxygen is often the overlooked substrate, and the oxygen metabolism catalyzed by NOS has been poorly defined. In this paper we focus on the oxygen stoichiometry and effects of substrate/cofactor binding on the endothelial NOS isoform (eNOS).

In the presence of both L-arginine and tetrahydrobiopterin, eNOS is highly coupled (>90%), and the measured stoichiometry of O₂/NADPH is very close to the theoretical value. We report, for the first time, that the presence of L-arginine stimulates oxygen uptake by eNOS. The fact that non-hydrolyzable L-arginine analogs are not stimulatory indicates that the occurrence of the coupled reaction, rather than the accelerated uncoupled reaction, is responsible for the L-arginine-dependent stimulation. The presence of 5,6,7,8-tetrahydrobiopterin quenched the uncoupled reactions and resulted in much less ROS formation, while the presence of redox-incompetent 7,8-dihydrobiopterin demonstrates little quenching effect. These results reveal different mechanisms for oxygen metabolism for eNOS as opposed to nNOS and, perhaps, partially explain their functional differences.

Nitric oxide synthases (NOS) catalyze the formation of NO from L-arginine in two consecutive monooxygenation reactions, which include ω-hydroxy-L-arginine (NOHA) as a tightly bound intermediate (1-3).

Arginine + O₂ + (NADPH + H⁺) → NOHA + H₂O + NADP⁺

**Reaction 1** (the first step)

NOHA + O₂ + ½ (NADPH + H⁺) → NO + L-Citrulline + H₂O + ½ NADP⁺

**Reaction 2** (the second step)

Arginine + 2 O₂ + 3/2 (NADPH + H⁺) → NO + Citrulline + 3/2 NADP⁺ + 2 H₂O

**Reaction 3** (the overall reaction)

In addition to the reactions listed above, NOS also catalyzes the formation of reactive oxygen species such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) (4-6):

2 O₂ + NADPH + H⁺ → 2 O₂⁻ + NADP⁺ + 2 H⁺

**Reaction 4**

and/or

O₂ + NADPH + H⁺ → H₂O₂ + NADP⁺

**Reaction 5**

Unlike **Reaction 3**, where activated molecular oxygen is split to oxidize L-arginine to citrulline, NO and H₂O, **Reactions 4 and 5** do not require the cleavage of the O-O bond. NOS is said to be “coupled” when all the electrons from NADPH are utilized in the formation of monooxygenated products and H₂O (**Reaction 1, 2, and 3**), and “uncoupled” when electrons leak to molecular oxygen to form ROS (**Reactions 4 and 5**).
Spontaneous dismutation (Reaction 6) of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) \( (k = 2 \times 10^5 \text{ M}^{-1}\text{sec}^{-1} \) at pH 7.4) produces \( \text{H}_2\text{O}_2 \) at a detectable level whether or not the NOS-catalyzed two-electron reduction of oxygen to form \( \text{H}_2\text{O}_2 \) as a direct product occurs:

\[
2 \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

**Reaction 6**

Diacetyldeuteroheme-substituted horseradish peroxidase, or dHRP, is an artificial trapping agent first used by Ishimura’s group to distinguish superoxide and hydrogen peroxide formation (7,8). dHRP rapidly \( (k > 10^6 \text{ M}^{-1}\text{sec}^{-1}) \) reacts with \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) to form compound III and compound II, respectively, according the equations below:

\[
d\text{HRP(Fe}^{\text{III}}) + \text{O}_2^- \rightarrow d\text{HRP (Fe}^{\text{II}} \text{O}_2)
\]

**Reaction 7**

\[
d\text{HRP(Fe}^{\text{III}}) + \text{H}_2\text{O}_2 \rightarrow d\text{HRP (Fe}^{\text{IV}}\text{O}) + \text{H}_2\text{O}
\]

**Reaction 8**

thereby effectively competing with Reaction 6 for the spontaneous mutation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \).

Compounds II and III are chemically stable and spectrally distinguishable from each other; thus, both \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) can be trapped, and the initial product(s) of the uncoupled reaction(s) can be determined. We have modified the original dHRP trapping protocol for NADPH-consuming enzymes such as NOS, and reported the successful combination of the dHRP trapping system with an NADPH regeneration system (9). Maintaining a constant low concentration of NADPH not only keeps NADPH from being oxidized by dHRP compound I, but also keeps NADP+ “feedback inhibition” negligible. In this paper, we applied this modified dHRP trapping protocol to study the products of the eNOS reactions, both qualitatively and quantitatively.

Even though Reactions 4 and 5 appear to be simple chemical reactions, quantitative analysis has always been a challenge given the spontaneous/catalyzed dismutation of \( \text{O}_2^- \). Electron paramagnetic resonance (EPR) is the logical analytical tool for studying the formation of small radicals such as \( \text{O}_2^- \); however, quantification of \( \text{O}_2^- \) to determine stoichiometry, and determination of rates are difficult or impossible using EPR (5,10). Our modified dHRP trapping protocol has been shown to be compatible with simultaneous measurement of oxygen consumption and dHRP compound III formation, revealing the uncoupled stoichiometry of eNOS.

Both NO and ROS are highly reactive chemical entities in aqueous solution; the complexity of their coexistence in a reaction mixture makes it difficult to determine stoichiometry (11-13). To simplify the system, superoxide dismutase (SOD) and catalase can be added to suppress accumulation of ROS, and recycle partially reduced oxygen to either \( \text{H}_2\text{O} \) or \( \text{O}_2 \). The presence of SOD accelerates Reaction 6 to a rate that is diffusion-limited (14), and catalase catalyzes the breakdown of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) and \( \text{O}_2 \) (15):

\[
2 \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

**Reaction 9**

In a system that contains sufficient amounts of both SOD and catalase, oxygen in the uncoupled reaction is fully reduced to water. Regardless of whether \( \text{O}_2^- \) and/or \( \text{H}_2\text{O}_2 \) is the initial product, the overall uncoupled reaction will be:

\[
\text{O}_2 + 2\text{NADPH} + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O} + 2\text{NADP}^+
\]

**Reaction 10**

Considering all the reactions listed above, it is apparent that molecular oxygen is the ultimate oxidizing reagent in both the coupled and uncoupled reactions by NOS; NADPH, on the other hand, is the ultimate electron donor that initiates all subsequent redox processes. The ratio of \([\text{O}_2]/[\text{NADPH}]\) varies in accordance with substrate/cofactor binding, and serves as an important parameter to study coupling/uncoupling of NOS.

The fully coupled NOS reaction with L-arginine as substrate produces the theoretical value of \([\text{O}_2]/[\text{NADPH}] = 2/1.5 = 1.33\) (Reaction 3). When NOHA is added as the substrate, the theoretical value becomes \([\text{O}_2]/[\text{NADPH}] = 2.0\) (Reaction 2). In the
absence of L-arginine and NOHA, NOS is totally uncoupled and the theoretical value becomes $[O_2]/[NADPH] = 1/2 = 0.5$ (Reaction 10). In the absence of SOD and catalase, we calculate the theoretical value of $[O_2]/[O_2^-]$ to be 1/1, when $O_2$ is the initial product of the uncoupled reaction (Reaction 4).

Tetrahydrobiopterin (BH$_4$) is an essential cofactor in the oxygenase domain, and is proposed to have multiple roles in all three mammalian NOS isoforms (16-18). One of the presently accepted functions of BH$_4$ is to act as a 1-electron donor during reductive activation of the oxyferrous complex of the heme (18-21). Although there are many publications about the roles of BH$_4$ in eNOS, and how the absence of BH$_4$ promotes eNOS uncoupling (22-24), there are little quantitative data regarding its effect on oxygen metabolism. In this study, we report a decreased oxygen consumption rate, as well as a lower $[O_2]/[NADPH]$ ratio when BH$_4$ is absent from the fully coupled eNOS reaction.

Experimental Procedures

Chemicals - NADPH was purchased from Alexis (San Diego, CA). 5,6,7,8-tetrahydrobiopterin and 7,8-dihydrobiopterin (BH$_2$) were from Research Biochemicals International (Natick, MA). Fe(III) 2,4-diacetyl deuteroporphyrin IX chloride was purchased from Porphyrin (now Frontier Scientific, Inc. Logan, UT). Trizma base, L-arginine, cytochrome c (horse heart), K$_2$Fe(CN)$_6$, DL-isocitric acid trisodium salt, L-arginine and other commercial chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Enzymes – eNOS was expressed in Escherichia coli and purified as described by Martásek et al. (25). The enzyme preparation was concentrated to 50 µM after being purified by a 2′5′-ADP-Sepharose 4B column, and then dialyzed against 50 mM Tris-HCl (pH 7.4, with 100 mM NaCl and 5% glycerol) to remove 2′-AMP, arginine and BH$_4$. NO Formation rate (hemoglobin capture assay) of the purified eNOS was determined to be 120 ± 10 nmol/min/mg, using extinction coefficients of 60 mM$^{-1}$cm$^{-1}$ at 401 nm.

Cytosolic NADP$^+$-dependent isocitrate dehydrogenase (IDP-2) was prepared as described by Ding et al. (26,27) with help from Dr. Lee McAlister-Henn’s lab (The University of Texas Health Science Center at San Antonio). The His-tagged protein was purified from a nickel column and concentrated to 8 mg/ml. Activity was tested by monitoring NADPH formation at 340nm. Purified IDP-2 has stoichiometrically bound NADP$^+$. The activity of the IDP-2, measured by monitoring the formation of NADPH at 340 nm with an extinction coefficient of 6220 M$^{-1}$cm$^{-1}$, was calculated to be 40 µmol/min/mg.

dHRP was prepared using the method described by Makino et al. (7,28) Native HRP was bought from Toyobo Inc. (Japan). Calmodulin (CaM) was prepared by the method of Zhang and Vogel (29); superoxide dismutase (SOD) and catalase were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

dHRP Experiments - dHRP trapping of superoxide anion and hydrogen peroxide was monitored by an Agilent 8453 diode array UV-Vis spectrophotometer. The standard reaction buffer consists of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 200 µM CaCl$_2$, and 50 µM diethylene triamine pentaacetic acid (DPTA). The reaction was carried out in a glass cuvette and measurements were taken every 10 seconds. A typical reaction mixture contained the standard reaction buffer and 100 µM dHRP, 200 µM MgCl$_2$, 0.65 µM eNOS (BH$_4$- and arginine-free), 2 µM IDP-2, and 10 mM DL-isocitric acid (trisodium salt) was added to start the reaction immediately prior to the first optical measurement. Where needed, CaM (7 µM) and SOD (23 µg/ml) were added as described in the figure legends. Control experiments were performed in the absence of eNOS.

Quantitative analysis of the uncoupled $[O_2]/[O_2^-]$ ratio was performed using simultaneous measurements of oxygen consumption and abovementioned dHRP trapping of superoxide anion. The reaction was carried out in an air-tight cuvette (2.22 ml), and the oxygenated standard reaction buffer was premixed with 160 or 100 µM dHRP, 200 µM MgCl$_2$, 2.3 µM eNOS (BH$_4$- and arginine-free), and 2 µM IDP-2; 10 mM DL-isocitric acid was added to start the reaction. The rate of compound III accumulation was calculated using
the 583 nm delta extinction coefficient of 3.8 mM\(^{-1}\) reported by Makino et al., and the corresponding oxygen consumption was recorded by the oxygen sensing system described below.

**Oxygen Consumption** – The uptake of molecular oxygen was measured at 24 °C using a fluorescence-based oxygen sensing system consisting of an SD2000 dual channel spectrophotometer combined with an LS-450 Blue LED light source and a Foxyl-18G probe, all from Ocean Optics (Dunedin FL). The reaction was carried out in an air-tight cuvette (1.80 ml), designed and fabricated in the laboratory. The standard reaction buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 200 µM CaCl\(_2\), and 50 µM DPTA) was stirred gently overnight to ensure full oxygenation. A typical reaction mixture contained 7 µM CaM, 23 µg/ml SOD, 1.7 µM catalase, 5.5 µM BH\(_4\) and 0.3~2 µM eNOS. To study the coupled reaction, either 500 µM L-arginine or NOHA may be added. The reaction was started by the addition of NADPH to a final concentration of 440 µM. Determination of the \([\text{O}_2]/[\text{NADPH}]\) ratio was performed by injecting a small volume of NADPH quantitatively and monitoring the oxygen concentration trace. The reaction was carried out in the abovementioned oxygen apparatus. Standard reaction buffer containing 7 µM CaM, 23 µg/ml SOD, 1.7 µM catalase, 5.5 µM BH\(_4\) & 0.3~0.5 µM eNOS was mixed before multiple stepwise injections of NADPH, which started the reaction. Each injection contained a precisely determined amount of NADPH, and could only reduce a certain amount of oxygen. The \([\text{O}_2]/[\text{NADPH}]\) ratio was determined under three conditions: (1) in the totally uncoupled reaction in the absence of L-arginine or NOHA, (2) in the presence of 500 µM L-arginine or NOHA as substrate, and (3) in the presence of 500 µM NOHA as substrate.

It is noteworthy that excessive amounts of SOD and catalase were present in the oxygen consumption experiments to avoid accumulation of ROS. Each experiment contains multiple injections of NADPH and the results were averaged. The resulting \(\text{O}_2\) uptake at each addition was within ±3%. At the end of each experiment, an excessive amount of sodium dithionite was added to ensure depletion of oxygen. At least two different preparations of eNOS were used to confirm stoichiometry in each instance.

In the absence of L-arginine or NOHA, the reaction was totally uncoupled. When either L-arginine or NOHA was added, eNOS was partially uncoupled (more in the discussion section), and the overall oxygen consumption equals the weighted sum of both the coupled and uncoupled reactions. Percent coupling is calculated by comparing the theoretical values of \([\text{O}_2]/[\text{NADPH}]\) to the experimental values.

**RESULTS**

**The Uncoupled Reaction and the Effect of CaM.** The basic uncoupled reaction, performed in the presence of dHRP, CaM, and eNOS, free of BH\(_4\) and L-arginine, showed the formation of compound III of dHRP (Figure 1A), indicating \(\text{O}_2^-\) formation by eNOS. Sharp isosbestic points at 481, 530 and 605 nm were observed, indicating that the transition from ferric dHRP (\(\lambda_{\text{max}} = 507 \text{ and } 640 \text{ nm}\)) to dHRP compound III is the only reaction occurring, and that \(\text{O}_2^-\) was the only product formed (~4 min\(^{-1}\)) under such conditions. The control experiment, performed in the absence of eNOS, showed no spectral changes over 10 minutes.

The abovementioned reaction was then repeated in the absence of CaM. Exactly the same optical changes were observed (spectra not shown) but at much slower rate (~1 min\(^{-1}\)). A plot of spectral changes at 583 nm is shown in Figure 1B; in the first two minutes, the reaction rates in the presence and absence of CaM were both linear, but the (+) CaM rate is about three to four times faster than the (−) CaM rate. Our results are consistent with the CaM effect reported by Masters and co-workers using EPR spectroscopy (30).

Quantitative analysis of the uncoupled reaction was performed by simultaneous measurement of oxygen consumption and \(\text{O}_2^-\) formation (9), and the uncoupled \([\text{O}_2]/[\text{O}_2^-]\) stoichiometry was obtained by comparing the rate of oxygen consumption to the rate of \(\text{O}_2^-\) formation. While the oxygen concentration was continuously monitored by the oxygen sensing system,...
system described in the Experimental Procedures, a diode array UV-visible spectrophotometer recorded a series of spectra of the reaction mixture every 10 seconds. When a spectrum was recorded, the burst of light from the diode array spectrophotometer interfered with the oxygen sensor, leaving ticks visible on the oxygen concentration trace at 10-second intervals (Figure 2A); time alignment was achieved using these small ticks as an internal reference.

Difference spectra of dHRP compound III were produced by subtracting the spectrum of ferric dHRP from those recorded every 10 seconds after the reaction started (Figure 2B). The sharp isosbestic points in Figure 2B confirm that compound III formation (Reaction 7) is the only reaction occurring during the initial 2-minute time course. The oxygen consumption trace, recorded simultaneously on the same sample, is shown in Figure 2A. The rates of both O₂⁺ formation and O₂ consumption were calculated to be 5.4 µM/min, reflecting a stoichiometry of [O₂]/[O₂⁺] = 1/1 (Reaction 4).

The Arginine Effect. L-arginine was found to stimulate the oxygen consumption rate by eNOS ~10 fold, depending on the enzyme preparation. This is a surprising finding given our previous publication (9) that indicates the oxygen consumption rate by neuronal nitric oxide synthase (nNOS) is strongly inhibited by L-arginine. A comparison is shown in Figure 3A, in which the oxygen consumption rate by eNOS was stimulated from ~4 min⁻¹ to ~40 min⁻¹, whereas the oxygen consumption rate by nNOS was inhibited from ~500 min⁻¹ to ~140 min⁻¹.

In contrast, non-hydrolyzable L-arginine analogs, such as Nω-nitro-L-arginine methylster (L-NAME), N⁵-monomethyl L-arginine citrate (L-NMMA) and L-citrulline (the product of Reaction 3) did not stimulate oxygen consumption by eNOS (Figure 3B and 3C). The significance of these experiments and the comparison of the L-arginine effect on eNOS and nNOS will be addressed in detail in the Discussion.

The Tetrahydrobiopterin Effect. BH₄ stimulates the oxygen consumption rate of eNOS by ~3 fold in the presence of L-arginine (Figure 4A), but has no effect on oxygen consumption in the absence of L-arginine (data not shown). To evaluate the amount of ROS generated in a reaction mixture, SOD and catalase were introduced sequentially to the reaction mixture while oxygen concentration was monitored. For example, the reaction could start in the presence of SOD, which keeps ROS in the form of H₂O₂ (Reaction 6); after ~100 µM of oxygen was consumed, catalase could be introduced to the reaction mixture and H₂O₂ would be rapidly converted to molecular oxygen (Reaction 9), which can be monitored/recorded by the oxygen sensing system. BH₄ almost totally inhibited ROS formation by eNOS, but ROS formation occurs in the absence of BH₄ or the presence of BH₂, a redox-incompetent BH₄ analog (Figure 4B and 4C).

Figure 4B indicates that a significant amount of ROS was formed by BH₄-free eNOS even in the presence of L-arginine (detailed conditions are indicated in the figure legend). Upon the addition of catalase, the evolution of oxygen was observed to be 20–30 µM. Repeating the experiment with BH₂-bound eNOS produced a similar result (Figure 4C), but repeating the experiment with BH₄-replete eNOS yielded a negligible amount of oxygen evolution (Figure 4D). Although these experiments are not strictly quantitative, they present a qualitative measure of ROS formation under different conditions.

Because BH₄ itself is a strong reducing reagent, and may react with ROS directly, the experiment presented in Figure 4D was repeated with a range of BH₄ concentrations (500 µM to 10 mM). The same result was obtained (data not shown), indicating that the diminished formation of ROS by BH₄-replete eNOS, rather than the ROS-scavenging activity by BH₄ itself, is responsible for the striking differences in the comparison of Figures 4B and 4C to 4D. The literature indicates that free BH₂ and eNOS-bound BH₄ degrade at neutral pH at 0.017 min⁻¹ and 0.006 min⁻¹ respectively (31), rates that are insufficient to compete with eNOS-catalyzed oxygen consumption.

Stoichiometry and eNOS Coupling. The determination of the [O₂]/[NADPH] ratio is shown in Figure 5. In the absence of L-arginine or NOHA, the reaction was fully uncoupled, and the measured [O₂]/[NADPH] ratio was 0.50, the
same as the theoretical value (Table I). The same ratio measured with L-arginine or NOHA was slightly lower than the theoretical value (Table I), indicating that eNOS was highly coupled under our experimental conditions. Repeating these experiments proved that our enzyme preparations consistently exhibited 92%~96% coupling in the presence of L-arginine, or 88%~91% in the presence of NOHA. Oxygen consumption rates are calculated from the initial slope of oxygen traces in Figure 5, and listed in Table II. Unlike the \( \left[ O_2 \right]/[\text{NADPH}] \) ratio, enzyme preparations vary in terms of turnover numbers in a ±10% range.

DISCUSSION

The Uncoupled Reaction of eNOS.

eNOS uncoupling is known to be controlled by substrate/cofactor availability (30,32,33), and the uncoupled reactions play important roles under various physiological/pathological conditions, such as atherosclerosis and septic shock (34-38). We applied the dHRP trapping technique with an NADPH regeneration system (9) to identify the initial product of the eNOS uncoupled reaction. \( O_2^- \) is the direct product detected in the absence of substrate (L-arginine and/or NOHA). CaM accelerates eNOS-mediated \( O_2^- \) generation by a factor of 3, which is consistent with the results reported by Masters and co-workers (30). In the absence of a trapping agent, \( H_2O_2 \) formed by spontaneous dismutation of \( O_2^- \) will also be a detectable product.

In the absence of L-arginine, oxygen uptake by eNOS is very slow, ~4 min\(^{-1}\) in the presence of CaM, and even slower (~1 min\(^{-1}\)) in its absence. NADPH-cytochrome P-450 reductase, a flavin enzyme that shares ~60% sequence identity with the eNOS reductase domain, has an uncoupled oxygen consumption rate of ~1 min\(^{-1}\) (unpublished observation measured in the present system). These results indicate that: (1) flavins in the NADPH-cytochrome P-450 reductase and eNOS are poor electron donors for molecular oxygen; and (2) albeit slow, there are probably two sites for oxygen activation in eNOS, one in the reductase domain (presumably the flavins) (39) and the other in the oxygenase domain (presumably the heme), as binding of CaM to eNOS enables electron transfer from the reductase domain to oxygenase domain (1,40), and activates overall oxygen consumption.

\textit{L-Arginine and BH}_4\textit{ effects.} The oxygen chemistry on the heme site of NOS has been generally assumed to follow that of cytochrome P450 monoxygenases (1,41-43). A simplified list of intermediates in the initial steps of heme-oxygen interaction can be represented as:

\[
\begin{align*}
\text{Fe}^{III} & \leftrightarrow \text{Fe}^{II} \leftrightarrow \text{Fe}^{III}O_2^- \leftrightarrow \text{Fe}^{III}O_2^-^+ \\
\text{Reaction 11}
\end{align*}
\]

After these initial steps, activated oxygen can either heterolytically dissociate from heme to form superoxide anion:

\[
\begin{align*}
\text{Fe}^{III}O_2^- & \leftrightarrow \text{Fe}^{III} + O_2^- \\
\text{Reaction 12}
\end{align*}
\]

or follow the cytochrome P450 scheme to complete the monoxygenation of L-arginine:

\[
\begin{align*}
\text{Fe}^{III}O_2^- & \leftrightarrow \text{Fe}^{III}O_2^-^+ \leftrightarrow \text{Fe}^{IV}=O \leftrightarrow \text{Fe}^{III} \\
\text{Reaction 13}
\end{align*}
\]

The intermediates in \textit{Reaction 11} have been intensively studied with optical absorption spectroscopy, by stabilizing them under cryogenic conditions or by capturing them in real-time with time-resolved measurements (19-21,31,44-47). With the heme known as the center for oxygen chemistry in NOS, we assume \textit{Reaction 12} is the major source for \( O_2^- \) formation from the uncoupled eNOS in the presence of CaM (5,9,10,30,34,39). However, the two intermediates (\( \text{Fe}^{III}O_2^-^2 \) and \( \text{Fe}^{IV}=O \)) in \textit{Reaction 13} have not been convincingly observed to date, and it is unclear whether different NOS isoforms follow the same scheme in \textit{Reaction 13}.

We report that the most striking difference between the oxygen metabolism of eNOS and nNOS is the L-arginine effect, wherein L-arginine strongly stimulates oxygen consumption of eNOS, and inhibits that of nNOS (Figure 3A). It is well-known that recombinant nNOS is 5~7 times faster than eNOS in terms of NO production, and the difference is generally attributed to the rates at which the reductase domains provide electrons to the heme (25,48). However, a comparison of
the rates listed in Table II reveals a huge difference between eNOS and nNOS in oxygen consumption in the absence of L-arginine. So the question is: why is L-arginine-free eNOS so slow, and L-arginine-free nNOS so fast?

The slow oxygen consumption rate (~4 min⁻¹) reported in the current study is either the result of inefficient Fe⁴O₂ formation in the absence of L-arginine, or a stabilized Fe⁴O₂ complex that doesn’t easily dissociate to form O₂⁻ (Reactions 11 and 12). Tsai’s group has studied the L-arginine effects on oxygen binding to eNOS in great detail, and their observations highly support the first hypothesis (31). Monitored at 427 nm, the accumulation of the FeIIO₂ complex was shown to be much higher in the reaction with L-arginine than without (31), meaning that the presence of L-arginine facilitates the binding of molecular oxygen to ferrous heme in eNOS. More detailed kinetic data demonstrate a 7~8-fold slower association constant, as well as a 20~30-fold slower dissociation constant of oxygen in the presence of L-arginine, helping to explain the accumulation of FeIIO₂ complex.

These kinetic data, while self-sufficient, are in agreement with the paradoxical L-arginine effects reported by other groups: On the one hand, the binding of L-arginine impedes the binding of diatomic molecules such as NO and CO as demonstrated by a variety of methods, including EPR (49-51), and it is logical to expect anti-cooperative binding of L-arginine and oxygen. On the other hand, L-arginine conversely stabilizes the FeIIO₂ complex and slows down oxygen dissociation (52).

Up to now, there is no crystal structure for the O₂-bound derivative of any of the NOS isoforms; thus some details of oxygen binding geometry are still missing. Nevertheless, different groups have reported isoform-specific environments in the binding of diatomic molecules to nNOS and eNOS, using infrared/Raman spectroscopy and EPR (50,53-57). All these studies agree that, despite of the general similarity in structures, there are small differences among NOS isoforms that allow limited isoform specificity in the interaction between L-arginine and diatomic molecules, such as NO, CO or O₂.

A resonance Raman study by Rousseau and coworkers (58) presented supportive evidence to the differences in oxygen metabolism between eNOS and nNOS reported here. By monitoring the Fe-CO and C-O stretching modes of CO-ferrous complexes with bound L-arginine, the authors intriguingly showed that nNOS has a much more open substrate-binding pocket than eNOS. Regardless of the two possible interpretations proposed by this paper, the conclusion highly supports the tighter coupling of eNOS (Table I) and the faster dissociation of superoxide from nNOS (Table II).

Complementary electron-nuclear double resonance studies was performed by Hoffman’s and Masters’ groups, generating a series of publications regarding substrate binding to all three NOS isoforms (59-62). Based on spectral differences, the authors concluded that nNOS provides greater shielding of the charge on the guanidino nitrogen than eNOS does (63), which is one of the two interpretations in the previously mentioned resonance Raman study (58).

This differential L-arginine effect could be attributable to BH₄. In the L-arginine-free eNOS, BH₄ does not accelerate superoxide dissociation (Figure 3A), but with nNOS, it was reported that BH₄ accelerated the decay of the FeIIO₂ complex by a factor of 70, irrespective of the presence of L-arginine (52).

Even though BH₄ does not seem to directly affect oxygen binding in eNOS (31,50), it controls the coupling of eNOS and, to some extent, the fate of oxygen reduction. The imperfect coupling of eNOS in the presence of substrate and absence of BH₄ is evidenced not only in this study (Figure 4), but also by other research groups using different approaches (33,64,65). In the presence of both substrate and BH₄, however, eNOS is highly coupled (30,64). The results presented herein (Figure 4) confirm that BH₄ is the “coupling switch” of eNOS; our approach is unique in that we quantitatively monitor oxygen consumption and utilize chemical reactions to convert accumulated ROS to visible and measurable molecular oxygen (Reactions 4, 6, 9 and 10).

BH₂, a redox-incompetent BH₄ analog, is unable to inhibit ROS formation by eNOS.
Our results confirm the view that BH₄ takes a redox role in the catalytic cycle, most likely by providing the second electron to the heme (21). Electron transfer from BH₄ is proposed to be faster than that from the reductase domain and, thus, is consistent with the increased rate of oxygen uptake in the presence of BH₄ over that in its absence (Figure 4A). Under pathological conditions, the limited availability of BH₄ has been shown to trigger eNOS uncoupling, and increase the risk of having cardiovascular diseases including atherosclerosis, coronary artery disease and hypertension (66-73). In addition, external stimuli, such as the chemicals in cigarettes, can convert BH₄ to BH₂ in the human body (74-76). Our results support the view that the introduction or stabilization of BH₄ may be used for the treatment of endothelial dysfunction (70-72,77).

Stoichiometry. We report for the first time the quantitative measurement of eNOS oxygen stoichiometry. In the absence of L-arginine, the stoichiometry is simple, and the experimental values match the theoretical values (Table I). Assessing the results from the simultaneous measurements (Figure 2) and from the oxygen consumption experiment (Figure 5A), it is clear that eNOS utilizes 1 mole of NADPH to provide electrons to two moles of molecular oxygen, generating two moles of superoxide anion as the product of the uncoupled reaction.

When L-arginine or NOHA is added in the presence of BH₄, however, the eNOS reaction is highly coupled. A comparison with nNOS data (9) illustrates two points: (1) eNOS is more coupled than nNOS, with either L-arginine or NOHA as substrate; (2) in both eNOS and nNOS, the enzymes are more coupled with L-arginine as substrate than with NOHA. The first point can be easily understood, given the physiological importance of the precise control of blood pressure (41), and the second one is at least partially due to the substrate-specific H-bonding interactions with the terminal oxygen, as recently reported by Li et al. (53).

In conclusion, we have demonstrated the CaM, L-arginine and BH₄ effects on eNOS oxygen metabolism. We report, for the first time, the quantitative measurement of superoxide anion formation from eNOS. Our recombinant eNOS is 92-96% coupled in the presence of L-arginine, which strongly stimulates oxygen uptake. In the presence of either L-arginine or NOHA, the availability of BH₄ controls eNOS coupling, and diminishes ROS formation by eNOS. This exquisite control is necessitated by its role in the vascular system in which the production of ROS must be prevented. It is verified that oxygen consumption by eNOS in the absence of substrate is extremely low and, only when substrate is supplied does NO production proceed in a highly coupled, physiologically significant process.

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1 The abbreviations used are: NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; BH₄, tetrahydrobiopterin; BH₂, 7,8-dihydrobiopterin; dHRP, diacetyldeuteroheme-substituted horseradish peroxidase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive (or reduced) oxygen species; NOHA, Nω-hydroxy-L-arginine; IDP-2, cytosolic NADP⁺-dependent isocitrate dehydrogenase; EPR, electron paramagnetic resonance; CaM, calmodulin; SOD, superoxide dismutase; DPTA, diethylene triamine pentaacetic acid.

FIGURE LEGENDS

Figure 1. Qualitative analysis of eNOS uncoupled reactions and the CaM effect. The reaction mixture contained 400 µl reaction buffer, 100 µM dHRP, 200 µM MgCl₂, 5 µM eNOS (BH₄- and arginine-free), 2 µM IDP-2; and 10 mM DL-isocitric acid. (1A): dHRP compound III formation in the presence of 7 µM CaM introduced to the reaction mixture before the reaction started; optical measurement was taken every 10 seconds for 2 minutes. (1B) CaM effect: a plot of 583 nm absorption v.s. time in the presence and absence of 7 µM CaM.

Figure 2. Simultaneous measurement of oxygen consumption and dHRP compound III formation. The reaction mixture contained oxygenated reaction buffer, 100 µM dHRP, 200 µM MgCl₂, 1.2 µM eNOS (BH₄- and arginine-free), 2 µM IDP-2; and 10 mM DL-isocitric acid. (2A) Oxygen concentration trace. (2B) Difference spectra of compound III accumulation every 10 seconds; solid arrow indicates elapse of time; and empty arrows indicate isosbestic points.

Figure 3. L-arginine effect of oxygen uptake. The nNOS trace shown in this picture was taken from our previous publication for comparison (9). Oxygenated standard reaction buffer and 7µM CaM, 23 µg/ml SOD, 1.7 µM catalase, 5.5 µM BH₄ and 1.2 µM eNOS were mixed before addition of 440 µM NADPH. (3A) L-arginine effect (500 µM L-arginine after addition). (3B) L-NAME and L-NMMA were introduced after the reaction started; no stimulation was observed. (3C) Two additions of L-citrulline were introduced after the reaction started; no stimulation was observed.

Figure 4. BH₄ effect. Reaction mixture contained 1800 µl reaction buffer, 500 µM L-arginine, 440 µM NADPH, 23 µg/ml SOD, 0.5 µM eNOS (BH₄-free), (4A) BH₄ was introduced after the reaction started; (4B) catalase was introduced after the reaction started; (4C) Reaction was started in the presence of 5.5 µM BH₂, and catalase was introduced after the reaction started; (4D) Reaction was started in the presence of 5.5 µM BH₄, and catalase was introduced after the reaction started.

Figure 5. Stoichiometry of the eNOS reaction in the presence and absence of substrate. Standard reaction buffer and 7µM CaM, 23 µg/ml SOD, 1.7 µM catalase, 5.5 µM BH₄ & 1.5~5 µM eNOS were mixed before multiple injections of NADPH. (5A) No L-arginine or NOHA, 84.5 µM NADPH per step. (5B) 500 µM L-arginine as substrate, 84.5 µM NADPH per step. (5C) 500 µM NOHA as substrate after the carry-over L-arginine was consumed, 35.6 µM NADPH per step.
Table I

*Oxygen Stoichiometry of eNOS*. a.

| Substrate / Cofactor | [O₂] / [NADPH] Ratio | Experimental Value | Theoretical Value | eNOS Coupling |
|----------------------|-----------------------|--------------------|------------------|--------------|
| (-) L-arginine, (-) NOHA / (+) BH₄ | 0.50 ± 0.01 | 0.50 | 100% Uncoupled |
| (+) L-arginine / (+) BH₄ | 1.28 ± 0.03 | 1.33 | 92 ~ 96% Coupled |
| (+) L-arginine / (-) BH₄ | 1.03 ± 0.03 | N/A  b | Uncoupled |
| (+) L-arginine / (+) BH₂ | 0.94 ± 0.03 | N/A | Uncoupled |
| (+) NOHA / (+) BH₄ | 1.84 ± 0.02 | 2.00 | 88 ~ 91% Coupled |

a. Experiments were performed in the presence of excessive amounts of SOD and catalase. Theoretical values were derived from equations listed in the introduction, and experimental values are averaged from multiple measurements of multiple eNOS holoenzyme preparations. Note that the [O₂] / [NADPH] Ratio is extremely close to theoretical value in the absence of substrate, and eNOS is highly coupled in the presence of either L-arginine or NOHA.

b. N/A: Not applicable
Table II

*Oxygenase Activities of eNOS and nNOS.*

|                                | eNOS   | nNOS   |
|--------------------------------|--------|--------|
| NO formation rate (min⁻¹)      | 15 ~ 20| 60 ~ 80|
| O₂ consumption rate (min⁻¹) in the presence of L-arginine | 35 ~ 40| 130 ~ 150|
| O₂ consumption rate (min⁻¹) in the absence of L-arginine or NOHA | 3 ~ 4   | 420 ~ 540|

nNOS data are obtained from previous publications by Gao *et. al.* (9), eNOS data were derived from the experiments shown in Figure 5. At least four different enzyme preparations were tested for each isoform to give the range of activities.
Figure 1A

A dHRP Compound (III) Formation

Absorbance

Wavelength (nm)

460 480 500 520 540 560 580 600 620 640 660 680 700

481 nm 507 nm 530 nm 605 nm 550 nm 640 nm 585 nm
Figure 1B

Absorbance at 583 nm

Time (second)

+ Calmodulin

- Calmodulin

eNOS – Arg
Figure 2A

Add NADPH

Interference from UV-Vis Spectrophotometer

Oxygen (µM)

Time (min)
Figure 2B

[Graph showing spectral change per 10 seconds as a function of wavelength (nm) with time indicated along the vertical axis. Key wavelengths marked: 481 nm, 530 nm, and 605 nm.]
Figure 3A

- **nNOS**
- **eNOS**
- **100% Velocity**
- **Addition of L-Arginine**
- **30% Velocity**

- **O₂ (µM)**
- **Time (min)**
Figure 3B

![Graph showing the effects of different concentrations of L-NAME and L-NMMA on oxygen levels over time.](image-url)
Figure 3C

- NADPH
- 1.25 mM Citrulline
- 1.25 mM Citrulline
- 500 µM L-Arginine
Figure 4A

[Graph showing the concentration of Oxygen (µM) over time (min)].

- NADPH
- BH₄
- 33% Velocity
- 100% Velocity
Figure 4B

- L-arginine – BH$_4$

| Time (min) | Oxygen (µM) |
|------------|-------------|
| 0          | 300         |
| 2          | 275         |
| 4          | 250         |
| 6          | 225         |
| 8          | 200         |
| 10         | 175         |
| 12         | 150         |
| 14         | 125         |
| 16         | 100         |
| 18         | 75          |
| 20         | 50          |

NADPH

Catalase
Figure 4C

+ L-arginine + BH$_2$

Oxygen (µM)

Catalase

NADPH

Time (min)
Figure 4D

+ L-arginine + BH$_4$

Oxygen (µM)

100% Velocity

97% Velocity

Catalase

NADPH

Time (min)

10 11 12 13 14 15 16 17 18

100 150 200 250 300
Figure 5A

![Graph showing oxygen depletion over time with various conditions: L-arginine depletion, NADPH, and Dithionite.](http://www.jbc.org/)

- Time (min): 0 5 10 15 20 25 30 35 40
- Oxygen (µM): 0 50 100 150 200 250 300
- L-arginine Depletion
- NADPH
- Dithionite

No Substrate
Figure 5B

[Graph showing time (min) on the x-axis and oxygen (µM) on the y-axis. The graph depicts the effect of NADPH and Dithionite on oxygen levels, with peaks at 281, 170, and 54 µM, and a note indicating the addition of L-arginine.]
Figure 5C

Oxygen (µM)

Time (min)

L-arginine Depletion

NADPH Depletion

NADPH

NADPH

+ NOHA

0 5 10 15 20 25 30 35 40

0 50 100 150 200 250 300
