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Superoxide Generation from Nitric Oxide Synthases

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ABSTRACT

Besides nitric oxide (NO), NO synthases (NOS) also produce superoxide ($\cdot O_2^-$), a primary reactive oxygen species involved in both cell injury and signaling. Neuronal NOS was first found to produce $\cdot O_2^-$ *in vitro*. Subsequent studies revealed $\cdot O_2^-$ generation as a common property of all NOS isoforms. Although NOS was originally shown to produce $\cdot O_2^-$ under defined conditions such as substrate or cofactor depletion, recent enzymatic studies found that the reduction of oxygen to $\cdot O_2^-$ is an obligatory step in NO synthesis. Tetrahydrobiopterin appears to play a key role in preventing $\cdot O_2^-$ release from the NOS oxygenase domain. On the other hand, the NOS reductase domain is also capable of producing significant amounts of $\cdot O_2^-$. Increasing evidence demonstrates that $\cdot O_2^-$ generation is involved in both physiological and pathological actions of NOS.
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INTRODUCTION

Nitric oxide (NO), a gaseous free radical, plays a central regulatory role in a variety of physiological and pathological processes, which include cardiovascular regulation, neuronal signal transmission, inflammation, and host defense (5, 19). In animal cells, NO is primarily derived from a cationic amino acid L-arginine via a reaction catalyzed by a family of NO synthases (NOS). To date, three NOS isoforms have been identified as neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III) (21). nNOS and eNOS constitutively exist in cells and their activities were previously thought to be mainly regulated by the levels of intracellular free Ca^{2+}. Upon external stimulation, such as from a neurotransmitter, elevated cytosolic Ca^{2+} combines with calmodulin to form a Ca^{2+}/calmodulin complex, which subsequently activates nNOS or eNOS. In addition to Ca^{2+}, nNOS and eNOS are also critically regulated by posttranslational modifications, protein–protein interactions, and subcellular compartmentations (11, 13). Phosphorylation of certain eNOS residues can fully activate eNOS at basal intracellular Ca^{2+} levels. NO generated by eNOS and nNOS serves as a signaling molecule participating in various physiological processes ranging from regulation of vascular tone to learning and memory development. Abnormalities of NO production in cells and tissues, either too much or too little, have been implicated in the pathogenesis of diseases such as stroke, hypertension, atherosclerosis, and heart attack, (15). In contrast to nNOS and eNOS, iNOS is generally not present in quiescent cells. iNOS expression is often induced by inflammatory substances such as cytokines and microbial endotoxins. Calmodulin is also essential for the catalytic function of iNOS; however, activation of iNOS does not require the elevation of intracellular Ca^{2+}. With a tightly bound calmodulin, iNOS is fully active at basal cytosolic Ca^{2+} concentrations in resting cells (8). The quantity of NO production from iNOS is therefore mainly determined by the amounts of enzyme expressed. Compared to nNOS or eNOS, iNOS is a more potent enzyme with higher L-arginine to NO turnover rates. The constant catalytic activity, along with the high-output enzymatic efficacy, makes iNOS ideal to serve its roles in host defense and inflammation.

In addition to NO, NOSs have also been shown to generate superoxide anion ($\cdot O_2^-$) (16, 24, 30). Compared with the well-established NO synthesis process, $\cdot O_2^-$ formation from NOS was understood in less detail. In the early studies, there were only clues that purified nNOS might produce hydrogen perox-

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ide (H_2O_2) or $\cdot O_2^-$ in the absence of L-arginine (16). While these observations were intriguing, not much attention was paid to these studies, probably due to the overwhelming interest in the process of NO synthesis and NO-mediated biological effects. Nevertheless, there was an array of important questions associated with $\cdot O_2^-$ generation from nNOS. For example, it was not clear whether $\cdot O_2^-$ production is a general property of all NOS isoforms or just a unique feature of nNOS. It was suspected that $\cdot O_2^-$ generation was probably an *in vitro* phenomenon caused by enzyme purification and only occurred when nNOS function went awry. Little was known about the catalytic process of $\cdot O_2^-$ synthesis from NOS and how this was regulated. Moreover, it was unclear whether NOS-mediated $\cdot O_2^-$ formation occurs in cells or tissues and what role the $\cdot O_2^-$ may play in physiological or pathological processes. One of the obstacles hampering the initial efforts to address these questions was the limited ability to obtain sufficient amounts of pure enzymes to conduct definitive oxygen free radical measurements. Largely owing to the various protein expression systems which enable the preparation of recombinant NOS holoenzyme or truncated domains on a large scale, the mechanisms and potential biological significance of the $\cdot O_2^-$ formation by NOS begin to be unveiled. It is now clear that $\cdot O_2^-$ generation is a common feature of all NOS isoforms (30). Further enzymatic studies demonstrated that the reduction of oxygen to $\cdot O_2^-$ is an intrinsic occurrence in NOS catalysis. NOS-derived $\cdot O_2^-$ appears to play an important role in various physiological processes and diseases.

NOS CATALYSIS

While derived from separate genes, all NOS isoforms have considerable similarities in their structure and function (14). Structure analysis reveals a 50–60% homology in amino acid sequences between any two of the three isoforms. The sequences of substrate- and cofactor-binding regions are highly conserved in these enzymes. Three NOS are all bi-domain enzymes comprising a C-terminal reductase domain and N-terminal oxygenase domain. The reductase domain, structurally resembling the cytochrome P_{450} reductase, contains the NADPH, FAD, and FMN binding sites. The binding sites of heme, L-arginine, as well as tetrahydrobiopterin (BH_4), locate on the oxygenase domain. Calmodulin binds with NOS at a consensus region and serves a critical role to facilitate the electron transfer from reductase to oxygenase domain, where the molecular oxygen is incorporated into the guanidino group of L-arginine, giving rise to NO and L-citrulline. A stepwise electron transfer from NADPH to the heme is involved in the NOS catalytic process, in which L-arginine is converted to NO and L-citrulline through an intermediate product *N*-hydroxyl-L-arginine. FAD, FMN, and BH_4 are the essential cofactors in this enzymatic reaction.

With the progress of stopped-flow and rapid-quench kinetic studies on purified full-length NOS and truncated NOS domains, the details of NO synthesis begin to unravel (31). One important finding is the BH_4 redox cycling in NOS catalysis (32). Upon the binding of calmodulin, the NOS flavoprotein shuttles the electron from NADPH to the heme to form a ferric heme–superoxy intermediate species (Fig. 1). This species is unstable and will decay to release $\cdot O_2^-$ and ferric NOS. However, when NOS is coupled with BH_4, BH_4 donates the second electron to a ferric heme–superoxy species, which then reacts with L-arginine or *N*-hydroxyl-L-arginine to form NO and L-citrulline. This model highlights the critical role of BH_4 in determining the NOS oxygenase domain to produce either NO or $\cdot O_2^-$. However, $\cdot O_2^-$ generation from NOS is also influenced by the L-arginine. Moreover, the FAD and FMN bound at the NOS reductase domain are other important $\cdot O_2^-$-generating sources.

nNOS-MEDIATED $\cdot O_2^-$ GENERATION

The early report suggesting that NOS may produce reactive oxygen species in addition to NO was based on the studies conducted on purified nNOS. With the enzyme isolated from porcine brain, Heinzel *et al*. reported that purified nNOS produced hydrogen peroxide (H_2O_2) at low concentrations of L-arginine or BH_4 (16). They showed that nNOS was switched from NO to H_2O_2 production under the conditions where L-arginine concentrations were $<100\ \mu M$. H_2O_2 formation from nNOS was a Ca^{2+}/calmodulin-dependent process. However, this H_2O_2 formation exhibited distinct responses to different types of NOS blockers. The *N*-nitro L-arginine analogues such as *N*-nitro-L-arginine (L-NNA) and *N*-nitro-L-arginine methyl ester (L-NAME) inhibited H_2O_2 generation from nNOS; whereas the *N*-methyl compounds such as *N*-monomethyl-L-arginine (L-NMMA) did not. Although H_2O_2 was measured from nNOS in this study, it remained uncertain whether H_2O_2 was the primary product of nNOS or secondarily derived from $\cdot O_2^-$. To answer this question, an unambiguous measurement

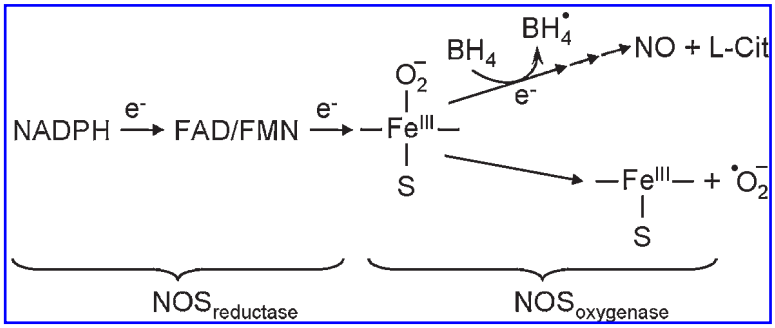

FIG. 1. A model illustrating the role of BH_4 in controlling NO and $\cdot O_2^-$ from NOS. NOS is a bi-domain enzyme with one reductase domain ($NOS_{reductase}$) and one oxygenase domain ($NOS_{oxygenase}$). NOS catalysis involves in an electron transfer from the reductase domain to the oxygenase domain where a ferric heme-superoxy species is formed. Upon receiving the second electron from BH_4, the species will react with L-arginine to form NO and L-citrulline. In the absence of BH_4, the ferric heme-superoxy species decays to generate $\cdot O_2^-$.

of oxygen free radicals on nNOS is needed. Direct evidence demonstrating nNOS-mediated production of oxygen free radicals came from electron paramagnetic resonance (EPR) spin-trapping measurements. While many methods have been used in the detection of oxygen free radicals, EPR remains the definitive technique to directly measure free radicals *per se*. Pou and colleagues carried out EPR measurements of $^{\cdot}O_2^-$ generation from rat nNOS purified from stably transfected cells (24). In the absence of L-arginine, strong $^{\cdot}O_2^-$ signals were detected from nNOS. These signals were abolished by superoxide dismutase (SOD) but not affected by catalase, reconfirming that $^{\cdot}O_2^-$ was the primary product of nNOS when the enzyme was not coupled with L-arginine and BH_4. These findings suggest that the previously reported H_2O_2 formation by nNOS may arise from the dismutation of $^{\cdot}O_2^-$. Both L-arginine and BH_4 affect the process of $^{\cdot}O_2^-$ generation from purified nNOS. The inhibitory action of L-arginine on $^{\cdot}O_2^-$ generation from BH_4-free nNOS was reported to be due to a decrease of NADPH oxidation rate (35). How the binding of L-arginine to the oxygenase domain affects NADPH oxidation at the reductase domain remains unclear. BH_4 can also suppress $^{\cdot}O_2^-$ generation from nNOS in the absence of L-arginine. Rather than preventing one-electron reduction of oxygen, BH_4 was reported to promote nNOS to conduct a two-electron reduction of oxygen leading to H_2O_2 formation (27).

Although these *in vitro* experiments showed that purified nNOS produces $^{\cdot}O_2^-$ under low concentrations of L-arginine, major concerns still remained that this $^{\cdot}O_2^-$ generation might stem from alterations in the property or conformation of this enzyme after isolation. Thus, it was of critical importance to investigate whether nNOS produces $^{\cdot}O_2^-$ inside cells. Under the condition of L-arginine depletion, nNOS-mediated $^{\cdot}O_2^-$ production was measured from intact cells by EPR (37). Culcasi *et al*. also observed that activation of glutamate receptors induced nNOS to produce $^{\cdot}O_2^-$ in L-arginine-depleted granule neurons (9). Since the availability of L-arginine determines whether nNOS produces either NO or $^{\cdot}O_2^-$, suboptimal levels of L-arginine may result in both NO and $^{\cdot}O_2^-$ production from nNOS. This prediction has been demonstrated to be true as both $^{\cdot}O_2^-$ and NO signals were detected by EPR spectroscopy in nNOS-transfected cells after L-arginine depletion (37). Under this condition, decreased L-arginine availability only permits a portion of nNOS to couple with both L-arginine and oxygen to generate NO and L-citrulline. Those L-arginine-uncoupled nNOS will catalyze $^{\cdot}O_2^-$ formation. This immediately raised another question, if these two radicals combine to form another potent oxidant peroxynitrite ($ONOO^-$). It is well known that NO reacts with $^{\cdot}O_2^-$ at a diffusion-limited rate to form $ONOO^-$. $ONOO^-$ is a highly reactive oxidant that causes lipid peroxidation, thiol oxidation, and nitration of the functional groups of amino acids including tyrosine (3, 15). In most of the previous reports, $ONOO^-$ was thought to be formed when the NO generated from NOS reacts with $^{\cdot}O_2^-$ derived from other pathways such as xanthine oxidase, mitochondria, or NADPH oxidase. However, under the condition of L-arginine depletion, concurrent NO and $^{\cdot}O_2^-$ production from nNOS may make this enzyme a sole $ONOO^-$-generating source. Indeed, immunocytochemical staining of nitrotyrosine was found when nNOS was activated in L-arginine-depleted cells but not in normally cultured cells (37). As a result of this $^{\cdot}O_2^-$ and $ONOO^-$ formation, severe cell damage was seen in L-arginine-depleted cells. Thus, nNOS generates $^{\cdot}O_2^-$ and $ONOO^-$ in L-arginine-depleted cells and this leads to cellular injury.

nNOS is known to associate with a number of adaptor proteins such as PSD95. Associations with these proteins target nNOS to a particular subcellular compartment. Whether interactions with these proteins affect $^{\cdot}O_2^-$ generation from nNOS remains to be determined. There are studies showing that $^{\cdot}O_2^-$ generation from nNOS may be modulated by protein–protein interactions. For example, nNOS associates with heat shock protein 90 (Hsp90), and this association enhances nNOS activity (4, 28). Further studies showed that Hsp90 inhibited $^{\cdot}O_2^-$ release from nNOS, and inhibition of $^{\cdot}O_2^-$ generation may be a mechanism by which Hsp90 augments NO synthesis from nNOS (29).

$^{\cdot}O_2^-$ GENERATION FROM iNOS

Early studies reported that iNOS caused much less NADPH oxidation in the absence of L-arginine as compared to nNOS (1). Since molecular oxygen would be the putative acceptor of the electrons from NADPH when L-arginine is not present, these results suggested that iNOS produces little, if any, $^{\cdot}O_2^-$. In fact, there was a perspective that $^{\cdot}O_2^-$ generation is just a unique property of nNOS (16). A highly constrained $^{\cdot}O_2^-$-generating property was deemed to be critical for biological function of iNOS because it was thought that $^{\cdot}O_2^-$ could inactivate NO and thus may perturb iNOS-mediated immune defense actions (2). However, the question regarding whether iNOS generates $^{\cdot}O_2^-$ remained unanswered until direct $^{\cdot}O_2^-$ measurements on iNOS were performed.

The first evidence showing that iNOS does produce significant amounts of $^{\cdot}O_2^-$ came from EPR spin trapping experiments on the mouse macrophage cell line RAW 264.7 (38). L-arginine depletion resulted in $^{\cdot}O_2^-$ generation from RAW 264.7 cells after iNOS induction and this $^{\cdot}O_2^-$ production was inhibited by the NOS blocker L-NAME. The fact that the $^{\cdot}O_2^-$ formation was specifically inhibited by L-NAME also excluded the potential involvement of NADPH oxidase, another potent $^{\cdot}O_2^-$-generating pathway in macrophages. Unequivocal evidence demonstrating that iNOS generates $^{\cdot}O_2^-$ was further obtained by EPR free radical measurements on purified iNOS (39). Since the studies on macrophages showed that L-arginine depletion was required for iNOS-mediated $^{\cdot}O_2^-$ formation, the effect of L-arginine on the $^{\cdot}O_2^-$ formation from purified iNOS was also examined. The results from these experiments turned out to be rather unexpected. The $^{\cdot}O_2^-$ production from iNOS was not affected by low levels of L-arginine. With 100 μM L-arginine present, the concentration that prevented $^{\cdot}O_2^-$ formation from nNOS, iNOS-mediated $^{\cdot}O_2^-$ generation was essentially unchanged. However, L-arginine at high concentrations ($1 \sim 5$ mM) markedly decreased the $^{\cdot}O_2^-$ production. This inhibition was specific because no effect was observed at similar levels of D-arginine, the enantiomer of L-arginine. Since higher levels of L-arginine were required to inhibit $^{\cdot}O_2^-$ production from purified iNOS, iNOS appears to be more prone to produce $^{\cdot}O_2^-$ *in vitro* than *in vivo*. The fact that iNOS continues to catalyze $^{\cdot}O_2^-$ formation even in the presence of significant

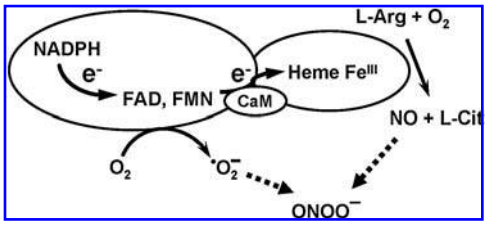

FIG. 2. A schematic diagram illustrating $^{\cdot}O_2^-$ generation from the flavin sites of iNOS reductase domain. *Solid arrows* denote the electron flow in iNOS catalysis. In the presence of certain concentrations of L-arginine, concurrent $^{\cdot}O_2^-$ and NO syntheses occur at the reductase and oxygenase domain, respectively. These two radicals may further combine to form $ONOO^-$ (*dashed arrows*).

amount of L-arginine (over 100 μM) raised the question whether $^{\cdot}O_2^-$ was synthesized at the heme center of the oxygenase domain or the flavins bound at the reductase domain. Further studies using the flavoprotein inhibitor diphenyleneiodonium (DPI) and heme blocker sodium cyanide (NaCN) showed that $^{\cdot}O_2^-$ generation from iNOS was completely blocked by DPI but not affected by NaCN. These findings suggest that iNOS-catalyzed $^{\cdot}O_2^-$ synthesis primarily occurs at the flavin-binding sites of its reductase domain, not the heme center of its oxygenase domain (Fig. 2). Indeed, the studies using expressed iNOS constructs demonstrated that the isolated iNOS reductase domain (residues 499–1144) alone catalyzed $^{\cdot}O_2^-$ formation (39).

These studies revealed an important difference between iNOS and nNOS in their $^{\cdot}O_2^-$ generation processes. The enzyme sites where $^{\cdot}O_2^-$ are synthesized and the responses to L-arginine differ between nNOS and iNOS. nNOS produces $^{\cdot}O_2^-$ from the heme of its oxygenase domain when L-arginine is not present. iNOS-mediated $^{\cdot}O_2^-$ formation primarily occurs at the flavins of its reductase domain. The $^{\cdot}O_2^-$ generation from nNOS is more sensitive to L-arginine than that from iNOS. It remains puzzling that large amounts of L-arginine are required to inhibit the $^{\cdot}O_2^-$ generated from the reductase domain, especially in view of the low K_m value for L-arginine with iNOS (1–10 μM). A nonspecific effect of L-arginine due to the high concentrations is unlikely since identical levels of D-arginine had no effect. It is speculated that higher concentrations of L-arginine may suppress the $^{\cdot}O_2^-$ generation from iNOS either by altering the conformation of the protein and accessibility of the flavin, or by rendering the flavins in a more oxidized state due to more rapid electron transfer to the heme.

The fact that substantial $^{\cdot}O_2^-$ generation is sustained even in the presence of L-arginine strongly suggests that $^{\cdot}O_2^-$ and NO synthesis may occur simultaneously at iNOS. These create a situation favoring $ONOO^-$ formation. Indeed, two lines of evidence demonstrated that $^{\cdot}O_2^-$ and NO generated by iNOS combined to form $ONOO^-$ in L-arginine-depleted macrophages (38). $ONOO^-$-induced chemiluminescence was measured in L-arginine-depleted macrophages and this was demonstrated to arise from iNOS. Also, the $ONOO^-$ nitration product nitrotyrosine was found in L-arginine-depleted macrophages and this was prevented by iNOS blockade. Conditional iNOS overexpression in mouse myocardium resulted in $ONOO^-$ formation and sudden death (20). iNOS-mediated $ONOO^-$ formation is rather unique because $ONOO^-$ is generally thought to derive from the combination of NO and $^{\cdot}O_2^-$ generated from distinct enzymatic pathways. Here, NO and $^{\cdot}O_2^-$ stem from the different domains of a single enzyme. Figure 2 illustrates that under certain levels of L-arginine, $^{\cdot}O_2^-$ and NO generated from iNOS reductase and oxygenase domain combine to form $ONOO^-$.

There was evidence suggesting that $^{\cdot}O_2^-$ and $ONOO^-$ production from iNOS may participate in the immune function of macrophages. Both $^{\cdot}O_2^-$ and NO have been individually shown to be cytotoxic and play roles in the antimicrobial action of macrophages (2, 26). NO is produced by iNOS and $^{\cdot}O_2^-$ was previously thought to be primarily synthesized from NADPH oxidase in macrophages. These two pathways were thought to be differentially regulated to avoid simultaneous activation. However, reports also showed that NO alone exerted minimal activity to kill bacteria. NO was reported to markedly potentiate the toxicity of other oxidants such as H_2O_2 (23). NO reacts with $^{\cdot}O_2^-$ to form $ONOO^-$, which is a more potent oxidant and possesses strong bactericidal effects (41). With cytokine-treated human neutrophils, abundant nitrotyrosine formations were detected around the phagocytosed bacteria, indicating that $ONOO^-$, rather than NO *per se*, likely conveyed the bactericidal action in these cells (10). In fact, $ONOO^-$ formation appeared to enhance the host defense action of iNOS in macrophages. For example, the macrophages activated in low L-arginine medium, where both $^{\cdot}O_2^-$ and $ONOO^-$ were generated, exhibited more than twofold increased inhibitory effect on bacterial growth than the cells activated in normal medium, where only NO was produced (38).

eNOS-CATALYZED $^{\cdot}O_2^-$ FORMATION

In view of the structural and functional similarities among the NOS isoforms, it may not be a surprise that eNOS was found to produce $^{\cdot}O_2^-$ as the other two isoforms (34, 40). However, the controlling mechanism underlying $^{\cdot}O_2^-$ generation from eNOS is distinctive from that of nNOS or iNOS. $^{\cdot}O_2^-$ generation from eNOS is also a Ca^{2+}/calmodulin dependent process and can be blocked by L-NAME. Blockade of heme by cyanide or imidazole prevented $^{\cdot}O_2^-$ generation from eNOS, indicating that $^{\cdot}O_2^-$ synthesis occurs at the heme center of its oxygenase domain. Unlike nNOS whose $^{\cdot}O_2^-$ generating process is influenced by both BH_4 and L-arginine, $^{\cdot}O_2^-$ generation from eNOS is exclusively controlled by the concentration of BH_4. Under BH_4-free conditions, activated eNOS catalyzes $^{\cdot}O_2^-$ formation even in the presence of high concentrations of L-arginine (1 mM) (31). Recent enzyme kinetic studies have shed some light on why eNOS is prone to produce $^{\cdot}O_2^-$ at lower levels of BH_4. Stuehr and colleagues compared the formation and decay rates of BH_4 radicals among three NOS isoforms (36). They found that eNOS displayed the lowest BH_4 radical-forming rate. On the other hand, eNOS exhibited the fastest decay rate of BH_4 radicals. This intrinsic feature implies that when exogenous BH_4 concentrations decrease, eNOS is more prone to be uncoupled and release the $^{\cdot}O_2^-$ from the heme-superoxy intermediate species. The structural basis for this unique kinetic property of eNOS remains to be determined.

BH_4 is a potent reducing agent prone to be inactivated by ox-

idants. For this reason, there have been tremendous amounts of interest in investigating whether BH_4 oxidation may switch eNOS from NO to $^{\cdot}O_2^-$ production in disease conditions, particularly in the study on the mechanism of endothelial dysfunction (17). Endothelial dysfunction is the key initiating event in the development of most cardiovascular diseases. Decrease of NO production and excessive $^{\cdot}O_2^-$ formation are the characteristic changes in dysfunctional endothelial cells (6, 17). There are increasing numbers of functional evidence suggesting that eNOS-mediated $^{\cdot}O_2^-$ generation occurs in a variety of cardiovascular diseases such as hypertension, hypercholesterolemia, atherosclerosis, heart failure, diabetes, and reperfusion injury (6, 33) There is an intensive interest to develop therapeutic strategies targeting on BH_4. Indeed, supplementation with BH_4 or its precursors has been reported to improve endothelial function in a variety of cardiovascular disease models (12, 22, 33).

In addition to BH_4, $^{\cdot}O_2^-$ generation from eNOS is also modulated by protein–protein interactions and protein phosphorylation. For example, eNOS couples with Hsp90 in cells. Inhibition of Hsp90 function has been reported to promote $^{\cdot}O_2^-$ generation from eNOS (25). Mutating eNOS threonine 497 to mimic dephosphorylation resulted in increased $^{\cdot}O_2^-$ production from eNOS in transfected cells, suggesting that threonine 497 phosphorylation might suppress $^{\cdot}O_2^-$ generation from eNOS (18). On the other hand, purified S1179D eNOS, a gain-of-function mutant, exhibited increased $^{\cdot}O_2^-$-generating potency in the absence of BH_4 (7).

CONCLUSIONS

The research over the last decade has established $^{\cdot}O_2^-$ generation as a common feature of all NOS isoforms. However, each NOS isoform possesses distinctive characteristics regarding the enzyme site responsible for $^{\cdot}O_2^-$ formation and controlling mechanisms. Significant progress has been made in understanding the action of BH_4 on $^{\cdot}O_2^-$ generation from NOS. On the other hand, the mechanism underlying the effect of L-arginine is understood in less detail. In addition to BH_4 and L-arginine, future studies are also needed to elucidate the regulation of $^{\cdot}O_2^-$ generation from NOS by other mechanisms such as protein–protein interactions and posttranslational modifications. Since NOS participates in various physiological processes, the roles of $^{\cdot}O_2^-$ generation from uncoupled NOS in diseases will remain an exuberant research subject in the future. Particularly in cardiovascular research, the endeavors to recouple eNOS function via enhancing BH_4 bioavailability or biostability may yield important therapeutic implications.

Elucidating the process and regulation of $^{\cdot}O_2^-$ formation from NOS may provide some insights into why evolution favors the existence of different isoforms. For example, while iNOS and nNOS structurally resemble each other and both produce $^{\cdot}O_2^-$, there are significant differences between the mechanism and regulation of their $^{\cdot}O_2^-$ formation. nNOS produces $^{\cdot}O_2^-$ primarily from the oxygenase domain and is sensitive to L-arginine inhibition, whereas iNOS releases $^{\cdot}O_2^-$ from its reductase domain and is not sensitive to L-arginine. These different properties appear to be tailored to suit their biological functions. nNOS mainly generates NO that serves as a neurotransmitter. Because of the relatively high L-arginine levels in cytosol (200–800 μM), $^{\cdot}O_2^-$ generation would rarely occur from nNOS under physiological conditions. This will ensure that nNOS only produces NO and operates properly in signal transduction in neurons. On the other hand, host defense is the main function of iNOS. Concurrent generation of NO and $^{\cdot}O_2^-$ may be more beneficial than NO formation alone, because these two free radicals interact to form the more toxic $ONOO^-$. Moreover, the cross reaction of $^{\cdot}O_2^-$ and NO prevents the feedback inhibition on iNOS caused by NO or $^{\cdot}O_2^-$. This can promote sustained NO and $^{\cdot}O_2^-$ generation, which eventually enhance the immune defense function of iNOS.

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ABBREVIATIONS

BH_4, tetrahydrobiopterin; eNOS, endothelial NOS; EPR, electron paramagnetic resonance; iNOS, inducible NOS; NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NOS; $^{\cdot}O_2^-$, superoxide; $ONOO^-$, peroxynitrite.

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