Critical Review

Proteomic Modification by Nitric Oxide

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Received May 3, 2006

Abstract. The role of nitric oxide (NO) in cellular signaling has become one of the most rapidly growing areas in biology during the past two decades. As a gas and free radical with an unshared electron, nitric oxide participates in various biological processes. The interaction between NO and proteins may be roughly divided into two categories. In many instances, NO mediates its biological effects by activating guanylyl cyclase and elevates intracellular cyclic GMP synthesis from GTP. However, the list of cGMP-independent effects of NO is also growing at a rapid rate. In this review, the importance and relevance of nitrotyrosine formation are stressed. The utilization of intact cell cultures, tissues, and cell-free preparations along with the use of pharmacological, biochemical, and molecular biological approaches to characterize, purify, and reconstitute these NO regulatory pathways could lead to the development of new therapies for various pathological conditions that are characterized by unbalanced production of NO.

Keywords: nitric oxide, inducible nitric oxide synthase (iNOS, NOS-2), nitrotyrosine, peroxidase, hemin

1. Introduction

On October 12, 1998, the Nobel Assembly awarded the Nobel Prize in Medicine and Physiology to scientists Robert Furchgott, Louis Ignarro, and Ferid Murad for their discoveries concerning nitric oxide (NO) as a signaling molecule in the cardiovascular system. In contrast with the short research history of the enzymatic synthesis of NO, the introduction of nitrate-containing compounds for medicinal purposes marked its 150th anniversary in 1997. Glyceryl trinitrate (nitroglycerin, GTN) is the first compound of this category. Alfred

Nobel (the founder of Nobel Prize) himself had suffered from angina pectoris and was prescribed nitroglycerin for his chest pain. Almost a century later, research in the NO field has dramatically extended and the role of NO in physiology and pathology has been extensively studied (1, 2). The steady-state concentration and the biological effects of NO are critically determined not only by its rate of formation, but also by its rate of decomposition. Biotransformation of NO and its related N-oxides occurs via different metabolic routes within the body and presents another attractive field for our research as well as for the venture of drug discovery.

2. Generation of NO under pathological conditions

2.1. Enzymatic synthesis of NO and NOS-2

The first nitric oxide synthase (NOS) isoform to be

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Published online in J-STAGE: August 5, 2006

doi: 10.1254/jphs.CRJ06009X

purified was the neuronal or brain NOS or type I NOS (nNOS or NOS-1) (3). This was followed shortly thereafter by inducible NOS, also known as type II NOS (iNOS or NOS-2) (4), and then by endothelial NOS or type III NOS (eNOS or NOS-3) (5). All three isoforms of the enzyme function as a homodimer consisting of two identical monomers, which can be functionally and structurally divided into two major domains: a C-terminal reductase (carboxy) domain and an N-terminal oxygenase (amino) domain (3–5). The catalytically active isoforms exist as homodimers with tetrahydrobiopterin and heme serving to facilitate dimer formation. The carboxy terminal domain has considerable homology between the isoforms and is homologous to cytochrome P450. However, the amino terminal domain has less homology. The homology of the three isoforms is about 50% to 60%, while the homology of a given isoform between species can be as great as 85% to 92%.

NOS converts L-arginine to L-hydroxyarginine and subsequently to nitric oxide and citrulline. The NOS enzyme catalyzes the 5 electron oxidation of one N^w -atom of the guanidino of L-arginine to NO and L-citrulline through cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH_4) (6). Despite the research progress that has been made, the precise role of each cofactor remains unresolved. This area still attracts attention for further investigation. Current X-ray crystallography studies with enzyme fragments should help elucidate the role of the cofactors and enzyme mechanism.

All NOS isoforms have a calmodulin-binding site in their midregion, which is important for the transfer of electrons from the reductase region bound FMN to the oxygenase region heme iron in order to initiate the catalytic machinery (7). The endothelial and neuronal isoforms of NOS are expressed constitutively (cNOS), and the production of NO by cNOS is regulated on a moment-to-moment basis by calmodulin binding, triggered by transient elevations in intracellular-free calcium levels. In contrast, NOS-2 is unique because 1) it requires *de novo* synthesis in most cells; 2) upon exposure to stimuli such as endotoxin (LPS) and proinflammatory cytokines, it is rapidly expressed and results in the production of much larger quantities of NO relative to the two other isoforms; and 3) it is widely distributed in various cell types (8–10). NOS-2 does not appear to be present under normal conditions in most cells. The activation of NOS-2 and the subsequent production of large amounts of free radical gas NO is an important anti-infectious and anti-tumor mechanism of innate immunity. However, overproduction of NO has been implicated in several pathological conditions that

include (but are not limited to) the following: 1) tissue injury and various inflammatory disorders (11, 12); 2) neuronal disease (13, 14); 3) auto-immune diseases (15, 16); 4) cancer or tumor cell proliferation (17, 18); 5) angiogenesis and related pathological changes (19, 20); and 6) diabetes mellitus (21, 22).

2.2. NOS function regulation and NOS-2 selective inhibition

Thus, selective inhibition of NOS-2 may have therapeutic potential for treatment of diseases mediated by the overproduction of NO (23). In contrast to NOS-2, NOS-1 and NOS-3 are constitutively expressed and are important physiological regulators in various organ systems, including the cardiovascular, nervous, and gastrointestinal system. It is not surprising that non-selective inhibition of NOS, which blocks constitutive isoforms of NOS as well as NOS-2, has deleterious effects. It follows then that selective inhibitors of NOS-2 will have considerable therapeutic potential. So far, two major categories of NOS-2 inhibitors have been developed: 1) L-arginine analogues, which show limited isoform selectivity and affect the substrate for all NOS enzyme (24, 25), and 2) guanidine inhibitor (aminoguanidine), which has some selectivity towards NOS-2 inhibition, but has low efficacy and causes severe side effects (25, 26). The recent discovery made by us indicates that an alternative approach is possible (10, 27). We have found NOS-2 expression can be selectively down regulated by *Trichinella spiralis* (*T. spiralis*, a helminth) infection (10, 27). The features of this inhibition include the following: a) Systemic: local jejunal infection by *T. spiralis* induces systemic inhibition of NOS-2 expression in the ileum, colon, kidney, lung and uterus; b) mRNA level inhibition: inhibition of NOS-2 expression appears to be regulated at gene transcriptional level. This serves as a very attractive mechanism by which we can develop a novel and selective NOS-2 inhibitor since expression of NOS-2 requires *de novo* synthesis in most cells and is primarily regulated at the transcriptional level; c) Potent: the effect of inhibition can override endotoxin (LPS)-stimulated NOS-2 expression that is the major cause of septic shock and multiple organ failure (MOF); d) Selective: the inhibition does not extend to the expression of other isoforms of NOS, the housekeeper protein paxillin, or to cyclooxygenase-2 (COX-2), another inducible protein by proinflammatory cytokines (5). More recently, our study with a variety of genetically modified mice has shed new light on our path to identify this selective NOS-2 inhibitor. We have demonstrated (10, 27) that inhibition of NOS-2 expression by *T. spiralis* infection is dependent on the signaling

pathway that includes the interleukin (IL)-4 receptor α subunit, receptor-associated kinases, Janus tyrosine kinase, and Stat6 in the suppression of NOS-2. It is surprising that activation of the IL4R α /Stat6 pathway is T-cell-independent, and is not affected by lack of endogenous IL-4. Furthermore, the serum levels of IL-13 during infection are not consistent with the change of NOS-2. Thus, we propose that a yet undefined signal or alternative IL4R α ligand could be involved in the IL-4R α /Stat6 stimulating pathway that plays an important role in helminth provoked host immunoresponses. Further elucidation of this pathway could lead to the development of new therapies for inflammatory conditions characterized by overproduction of nitric oxide, but could also offer more information to the hygiene hypothesis that has been very influential in directing strategies to prevent allergic diseases.

3. NO overproduction and tyrosine nitration

3.1. Life sciences where the ROS and the RNS meet

As the consequence of aerobic metabolism, the biological body is constantly generating reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($\bullet OH$), and nonradical hydrogen peroxide (H_2O_2). The discovery of nitric oxide further

drew attention to the reactive nitrogen species (RNS), including $\bullet NO$ (nitrogen monoxide) that can under going interconversion to form NO^+ (nitrosonium) and NO^- (nitroxyl anion) in certain cellular conditions. Induction of NO synthesis during inflammatory processes represents a defense mechanism, but excessive formation of NO has also been implicated in host tissue injury. For example, $\bullet NO$ reacts with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$) that can further form its acid form, peroxynitrous acid ($ONOOH$), a very unstable and reactive oxidizing species. Involvement of $ONOO^-$ in inflammatory diseases has been determined by detection of nitrotyrosine (NO_2 -Tyr) formation in various inflamed tissues (Fig. 1) (9, 28, 29). Nitrite (NO_2^-) is another major oxidation product derived from NO, and it can be oxidized by myeloperoxidase (MPO), lactoperoxidase (LPO), or horseradish peroxidase to form a reactive nitrogen intermediate(s) such as nitrogen dioxide ($NO_2^{\bullet}$) that is capable of nitrating tyrosine (Fig. 1). MPO and other peroxidase are also able to use halides and pseudohalides as co-substrates to generate reactive intermediate hypochlorous acid ($HOCl$), which further forms nitryl chloride (NO_2Cl) that results in formation of NO_2 -Tyr. Thus, it is likely that multiple pathways participate in tyrosine nitration (Fig. 1) (30, 31).

For a period of time, protein nitration was thought to

Fig. 1. The biological pathways toward protein nitration: 1) peroxynitrite ($ONOO^-$) mediated protein nitration (pathway I); 2) in the presence of H_2O_2 , peroxidases (enzymic) induced nitration (pathway II); and 3) a non-enzymic pathway that is heme (iron) dependent, which also requires the presence of H_2O_2 (pathway III).

be the evidence of the existence of ONOO^- and hence protein nitration was the foot print of ONOO^- formation in biological systems. However, the nitration of proteins has been postulated to alter a protein's conformation and structure, catalytic activity, and/or susceptibility to protease digestion (2, 32). It has also been shown that tyrosine nitration can diminish a protein's effectiveness as a substrate for tyrosine kinases (33).

3.2. Protein tyrosine nitration, role of peroxidase

During the past years, detection of NO_2 -Tyr-containing proteins has been reported in more than 60 human diseases and animal or cellular models of disease (34–37) in over 700 publications. While all tyrosine residues in proteins may theoretically be targets for nitration, presumably the efficiency of tyrosine nitration is dependent on various biological conditions such as the local production and concentration of the reactive species, the existence and availability of antioxidants and scavengers, the accumulation of inflammatory cell and the presence of pro-inflammatory cytokines, as well as the proximity and compartmentation of these components.

In the above-mentioned inflammatory disorders, immunochemical studies have revealed a very close association between the formation of NO_2 -Tyr and the presence of activated granulocytes which containing significant amount of peroxidases, such as MPO. However, several studies (38, 39) have tried to establish an in vivo NO_2 -Tyr formation model in which ONOOH is the most likely mechanism underlying the nitration. The major explanation that insists the importance of ONOO^- and ONOOH is based on the scenario that $\text{NO}_2^{\cdot}$ and nitryl chloride are 1 electron oxidants that are not powerful enough to overcome endogenous antioxidants, while ONOOH can catalyze 2 electron oxidation and is not completely inhibited by these antioxidants (40). In order to provide evidence for the contributions of MPO to in vivo NO_2 -Tyr formation, we have developed an animal intestinal inflammatory model following infection with *T. spiralis*, a one host helminth (41, 42). Our studies have revealed that severe inflammatory histological changes occurred in *T. spiralis*-infected jejunum, including shortened villi, elongated crypts, and a greatly thickened smooth muscle. These prominent tissue changes are paralleled by increases in MPO activity (reflecting the presence of polymorphonuclear neutrophils and monocytes) that is maximally elevated at 7–10 days post infection. However, a strikingly different induction of NOS-2 was demonstrated in the inflamed jejunum. NOS-2 expression was detected at 1 and 3 days post infection,

but by day 7, NOS-2 was abolished and remained suppressed for up to 20 days (10). We have evaluated intestinal protein for NO_2 -Tyr formation at different time points after parasite infection. At 7 to 10 days post infection, 5 distinct bands of NO_2 -Tyr were detected in the jejunum. To further eliminate the involvement of high concentration of NO in our in vivo NO_2 -Tyr formation model, we have utilized NOS-2 knockout (KO) mice for *T. spiralis*-induced high MPO inflammation study. Despite the failure to induce mass production of NO after parasite challenge, significant levels of tyrosine nitration can be found in NOS-2 KO mice, which are comparable to the levels in control (CF-1) mice (43). In summary, NO_2 -Tyr formation is observed following *T. spiralis* infection in jejunum, and the degree of tyrosine nitration is correlated with the expression of MPO, but not with NOS-2. It has been postulated that in addition to 1 electron reaction, MPO theoretically can catalyze 2 electron oxidation with the formation of either $\text{NO}_2^{\cdot}$ or ONOO^- . While the extremely brief half life of $\text{NO}_2^{\cdot}$ (approx. equal to 1 ns) made it unlikely to perform its function under the physiological condition (44), there is not enough direct evidence that supports the formation of ONOO^- by MPO. Thus, NO_2 -Tyr may be formed in vivo even with less concentration of NO, and effective suppression of MPO and other peroxidase (Fig. 1) activity may be important in preventing NO_2 -Tyr formation.

3.3. Nonenzymatic (transition metal) pathway of protein nitration

As we mentioned above, substantial evidence has emerged that revealed a very close association between the formation of NO_2 -Tyr and the presence of activated granulocytes containing peroxidases such as MPO. Two radical intermediates have been proposed to play a key role in peroxidase catalyzed nitration of phenolic rings (2). First, on addition of H_2O_2 , MPO (ground state with ferric form) can be oxidized to form compound I (ferryl cation radical) that is readily reduced to form HOCl in the presence of physiological concentrations of chloride. The reaction could then proceed by Cl transfer from HOCl to NO_2^- to generate NO_2Cl as a reaction intermediate. The second radical intermediate is $\text{NO}_2^{\cdot}$, which can be generated via either compound I- or II-catalyzed oxidation of NO_2^- . In addition, it has been suggested that physiological concentrations of NO_2^- can be oxidized to $\text{NO}_2^{\cdot}$ by the Fenton reaction (45), a Fe^{2+} -dependent reaction during which decomposition of H_2O_2 forms $\text{OH}^{\cdot}$ that oxidizes NO_2^- to $\text{NO}_2^{\cdot}$.

Peroxidases are enzymes that use H_2O_2 to oxidize substrates. In addition to MPO, the family of human peroxidases includes eosinophil peroxidase, LPO,

thyroid peroxidase, and intestinal peroxidase (46), all of which share heme-containing homology and can catalyze oxidative reactions. In addition, due to structural similarities, several hemoproteins such as cytochrome P450 isoenzymes, prostaglandin endoperoxide synthase (prostaglandin H synthase), cytochrome c, and hemoglobin are expected to act more or less as an oxidant (45). The biological role of heme has been reviewed extensively (47, 48), and the heme molecule has been shown to promote most biological oxidation processes involved in oxygen transport, mitochondrial respiration, drug metabolism, steroid biosynthesis, cellular antioxidant defenses, and signal transduction processes (49, 50). The presence of heme and peroxides may modulate vascular cell survival and oxidative stress adaptation, thus providing a possible mechanism for the evolution of atherosclerotic lesions (51). Heme is a complex of protoporphyrin IX with iron that is penta-coordinated and in the high-spin Fe (III) oxidation state. Iron has the capacity to accept and donate electrons readily and to interconvert between ferric (Fe^{3+}) and ferrous (Fe^{2+}), which makes it a useful component of hemoproteins or metalloproteins. However, as a transition metal, iron is able to reduce (active) molecular oxygen and to catalyze the conversion of superoxide and H_2O_2 to free radical species. Thus, the iron-containing structure of heme is an oxidant in several model systems where the prooxidant effects of free iron, heme, and hemoprotein may be attributed to the formation of free radical species and hypervalent states of the heme iron that attack cellular components. Recently, we have tested the hypothesis that both free heme and iron play a key role in NO_2 -Tyr formation and demonstrated that isolated heme and free metals are capable of tyrosine nitration in the presence of H_2O_2 and NO_2^- . Our studies (2, 41) have yielded several important observations about the mechanisms underlying the hemin/iron catalyzed nitration:

1) *Heme-catalyzed protein nitration*: Incubation of bovine serum albumin (BSA) with various combinations of heme, NO_2^- , and H_2O_2 in phosphate buffer at physiological pH for 30 min can result in BSA nitration. All components of the heme/ NO_2^- / H_2O_2 system are critical for NO_2 -Tyr formation. To further confirm the requirements of the reaction, we used glucose/glucose oxidase (10 mM/100 units) to generate the H_2O_2 (41), which also induced BSA NO_2 -Tyr formation. Our experiment indicated that the heme (25 μM) catalyzed BSA tyrosine nitration is comparable with the nitration induced by 5 mM ONOO^- .

2) *The role of H_2O_2* : To further elucidate the biochemical basis of protein nitration by the heme/ NO_2^- / H_2O_2 system, we investigated the carbonyl formation

and NO_2 -Tyr formation as markers of protein oxidation and nitration, respectively, and revealed that nitration and oxidation are two distinguishable processes. By dissecting those two reactions, we were able to conclude that despite the fundamental important role of H_2O_2 in heme (or Fe) catalyzed nitration, high concentrations of H_2O_2 markedly reduced the level of protein nitration by hemin while exerting less effect on BSA oxidation. We also stated that a decreased extent of nitration may reflect the degradation of heme by higher concentrations of H_2O_2 since without the heme structure, iron catalyzed BSA nitration was positively correlated with the increased concentration of H_2O_2 .

3) *The role of NO_2^-* : NO_2^- has been recognized as one of the key oxidation products of NO. NO_2^- is a small molecule with high affinity for the heme center in peroxidase, which has been reported to enhance the oxidizing capacity of peroxidase/ H_2O_2 (34). In the presence of heme and H_2O_2 , we observed a strong NO_2^- -concentration-dependent nitration ($r = 0.98$) of BSA. In contrast, carbonyl formation showed no relationship ($r = 0$) with NO_2^- concentration.

4) *Explore the mechanism responsible for the heme (iron)-mediated nitration*: Heme-containing enzymes are involved in both reduction and activation of oxygen. In the heme molecule, five of the coordination sites of iron are occupied by intrinsic ligands, and the sixth is able to bind O_2 or other extrinsic ligands. The strong and symmetrical coordination of the axial ligands enhances the interaction between O_2 and iron-porphyrin and results in the formation of $\text{O}_2^{\cdot-}$ by reduction of O_2 (52). As we know, superoxide dismutase (SOD) interferes with the ROS and reactive nitrogen species generating system by converting $\text{O}_2^{\cdot-}$ into H_2O_2 . This will reduce the formation of ONOO^- but increase the concentration of H_2O_2 , which, in the presence of transition metals such as iron can generate a strong oxidant, $\cdot\text{OH}$, via the Fenton reaction. However, another heme-containing enzyme catalase can degrade H_2O_2 to water and oxygen, thus inhibiting $\cdot\text{OH}$ formation. To evaluate the effect of SOD and catalase on protein nitration, we used heme as well as MPO to catalyze BSA NO_2 -Tyr formation. Treatment with SOD had no significant effect on BSA nitration. In contrast, the application of catalase abolished both heme and MPO-induced protein tyrosine nitration. Thus, the above results further confirmed the requirement for H_2O_2 in protein nitration by the heme/ $\text{O}_2^{\cdot-}$ / H_2O_2 system. Although the inhibitory effect of catalase on heme- or Fe-catalyzed nitration may suggest the involvement of the Fenton reaction as suggested by Thomas et al. (53), our data have revealed that a potent $\cdot\text{OH}$ scavenger, mannitol together with EtOH, had no effect on Fe-catalyzed BSA tyrosine

nitration. Thus, heme/iron-mediated protein nitration does not depend on the formation of $\cdot\text{OH}$. In addition, the Fenton reaction effectively produces $\cdot\text{OH}$ in several seconds; however, our study showed that a 16-h incubation is needed for iron to induce BSA nitration. Thus, the inefficiency of the iron/ H_2O_2 / NO_2^- /system in protein nitration casts further doubt on the role of hydroxyl radical in iron-catalyzed tyrosine nitration.

5) *Formation of a Fe(IV) hypervalent compound may be essential for heme(Fe) catalyzed nitration*: The release of iron in an oxidative manner from heme-containing proteins has received consideration recently (54). As a transition element, iron is able to exist in several oxidative states in response to different liganding environments (55). Therefore, we tested whether iron or chelated iron has a catalytic effect on tyrosine nitration. According to our study, both ferrous and ferric ions are capable of catalyzing the nitration of BSA at tyrosine residues when incubated with BSA in the presence of NO_2^- and H_2O_2 . To study the mechanism, cyanide (CN^-) and EDTA were used as chelators. When iron was added first into the BSA containing mixture, followed by adding of CN^- or EDTA, significant BSA nitration was detected. Nitration also was detected if the iron was mixed with EDTA before addition to the BSA-containing reaction buffer. However, if the iron was first mixed with CN^- and then added to the BSA-containing buffer, no protein nitration was observed. Of interest, addition of EDTA partly reversed the inhibited nitration by the premixture of iron with CN^- , whereas the addition of CN^- into an iron-EDTA mixture markedly enhanced NO_2 -Tyr formation. Thus, the sequential addition of iron and CN^- and the mixture of iron and CN^- first before application into the BSA-containing buffer created completely opposite results in terms of protein nitration. As a potent iron-chelating agent, CN^- can occupy all of the binding sites of iron in a 6:1 ratio and completely block the catalytic effect of iron on BSA nitration. Incomplete binding characteristics of either EDTA or BSA (56) are useful for iron to form its redox status $[\text{Fe(IV)}]$ (55) that may further be stabilized by addition of CN^- . Summarizing current information, the catalytic effect of heme or chelated iron on the BSA tyrosine nitration could be described by following reaction (Equations 1–4):

Briefly, hemin or chelated iron reacts with H_2O_2 to form the ferryl π -cation radical complex, which then

oxidizes both NO_2^- and tyrosine to form nitric dioxide radical ($\cdot\text{NO}_2$) and tyrosine radical ($\text{Tyr}^\cdot$), respectively. These nitrating species nitrate tyrosyl residues in proteins to form NO_2 -Tyr.

6) *Cardiovascular system, a major target for protein nitration*: The average adult human body contains approx. 3–4 g of iron, and 65% of it is bound to hemoglobin. Ten percent is a constituent of myoglobin, cytochromes, and iron-containing enzymes, and the rest is bound to the iron storage proteins (57). The higher contents of iron (heme) in certain tissues such as heart and vascular smooth muscle cells could serve as a biological base for iron toxicity on free radical-mediated tissue damage, including formation of NO_2 -Tyr. The first evidence (58) that muscle contraction can be altered by nitration of key proteins is suggested by the study of skeletal muscle sarcoplasmic-reticulum (SR) Ca^{2+} -ATPase isoforms 2 (SERCA2a), which suggests that tyrosine nitration may affect Ca^{2+} -ATPase activity. In light of the possibility that a high concentration of heme may facilitate protein nitration, we compared NO_2 -Tyr formation in tissue homogenates from various organs. In the presence of heme, H_2O_2 and NO_2^- , considerable protein nitration was observed in homogenates of heart and skeleton muscle but not in the brain, liver, and kidney. Increasing the exogenous heme concentration, however, promoted nitration in the brain, liver, and kidney. To further elucidate the role of endogenous heme in NO_2 -Tyr formation, we compared protein nitration in homogenates of heart under conditions of with or without exogenous heme. A significant protein nitration could be induced by application of NO_2^- and H_2O_2 alone into the heart homogenate, and the addition of exogenous heme did not further increase the level of NO_2 -Tyr formation. Thus, hemoprotein-rich tissues such as cardiac muscle are vulnerable to protein nitration in pathological conditions characterized by the overproduction of H_2O_2 and NO_2^- or nitric oxide. Further elucidation of the behavior of the heme (iron) on reaction with the $\text{NO}_2^-/\text{H}_2\text{O}_2$ system is essential to the development of therapeutic methods that will focus on the prevention of oxidation and nitration.

The common feature of all putative mechanisms of protein nitration is the direct or indirect dependence on H_2O_2 (Fig. 2). H_2O_2 is a small, diffusible, and ubiquitous molecule that can be synthesized and destroyed rapidly in response to various stimuli. Apart from its involvement in tyrosine nitration, the H_2O_2 molecule is an intracellular messenger that regulates different signaling pathways (59, 60) through stimulation of tyrosine phosphorylation. Thus, the intracellular concentration of H_2O_2 is an important factor in modulation of cardiovascular function. We summarized a large number of

Fig. 2. The common feature of all putative mechanisms of protein nitration is the direct or indirect dependence on H_2O_2 . At the same time, the H_2O_2 molecule is an intracellular messenger. The “Ying and Yang” dual sides of H_2O_2 are illustrated in this Fig.

studies so far and illustrated the “Ying and Yang” dual sides of H_2O_2 in the scheme shown in Fig. 2. A signaling role for H_2O_2 is now established, but many questions remain to be answered. The role of H_2O_2 in different sub-cellular compartments is unclear: The specific H_2O_2 signaling induced by different stimuli still remains to be explored, and the interaction between H_2O_2 signalings and its physiologic or pathological consequences are attractive but need more work. The unanswered question is whether some tyrosines are undergoing nitration while excess endogenous H_2O_2 is produced. If so, it needs to be determined whether the formation of $\text{NO}_2\text{-Tyr}$ interferes with the H_2O_2 -stimulated signaling pathway. What are the possible pathophysiological implications of the heme(Fe)/ $\text{H}_2\text{O}_2/\text{NO}_2^-$ nitration system in cardiovascular disease is another question that should be addressed.

Acknowledgments

The financial support of this research was provided by the John S. Dunn, Harold and Leila Mathers, and Robert A. Welch Foundations (AU1437), as well as The University of Texas and The Shanghai University of TCM. We also acknowledge the supports for E-Institutes for Nitric Oxide and Inflammatory Medicine (E-04010) and Key Project (05ZZ11) from Shanghai Municipal Education Commission, Key Project of Shanghai Committee of Science & Technology (05JC14056).

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