[Frontiers in Bioscience 8, d264-278, January 1, 2003]

NITRIC OXIDE (NO) – BIOGENERATION, REGULATION, AND RELEVENCE TO HUMAN DISEASES

Ka Bian and Ferid Murad

Department of Integrative Biology and Pharmacology, The Institute of Molecular Medicine, The University of Texas-Houston Medical School, 6431 Fannin, Houston, TX 77030

TABLE OF CONTENTS

1. ABSTRACT

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 as a signalling 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 150^{th} 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. 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. INTRODUCTION

NO research has enormously extended in the past 20 years and the role of NO in physiology and pathology has been extensively studied (1-3). This review focuses on the pathways of NO synthesis and metabolism in biological systems. The importance and relevance of interconversions between NO and other nitrogen oxides 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 regulatory pathways could lead to the development of new therapies for various pathological conditions which are characterized by un-balanced production of NO (4, 5).

3. THE GENERATION OF NO

3.1. Enzymatic synthesis of NO

3.1.1. Nitric Oxide Synthase

The first nitric oxide synthase (NOS) isoform to be purified was the neuronal or brain NOS or type I NOS (nNOS or NOS-1; 6). This was followed shortly thereafter by inducible NOS, also known as type II NOS (iNOS or NOS-2; 7, 8), and then by endothelial NOS or type III NOS (eNOS or NOS-3; 9-11). All three isoforms of the enzyme function as a homodimmer 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)

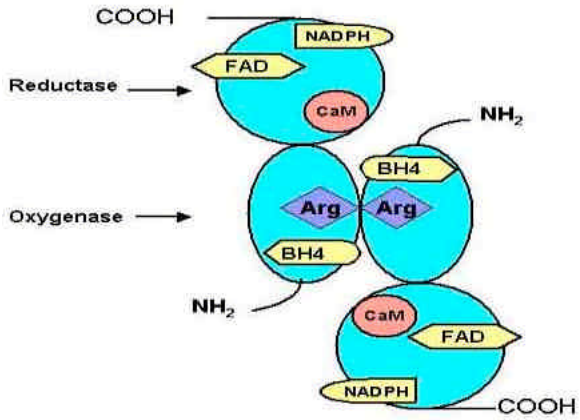

Figure 1. Functional structure of NOS homodimmer.

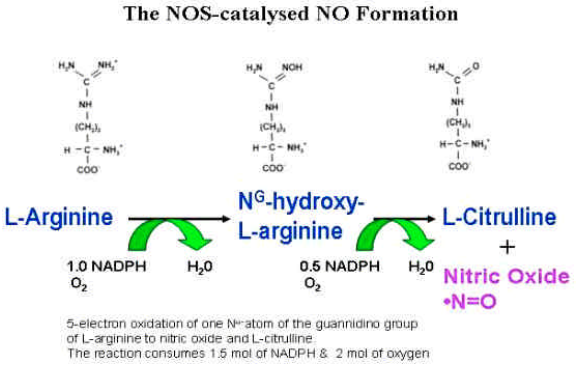

Figure 2. The NOS-catalysed nitric oxide formation.

domain (12, 13). The catalytically active isoforms exist as homodimers with tetrahydrobiopterin and heme serving to facilitate dimer formation (Figure 1; 14, 15). The carboxy terminal domain has considerable homology between the isoforms, and is homologous to cytochrome P450 (16). 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, as summarized in Figure 2. The NOS enzyme catalyzes the 5 electron oxidation of one N^{ω}-atom of the guanidino of L-arginine to NO and L-citrulline through the cofactors including: NADPH; flavin adenine dinucleotide (FAD); flavin mononucleotide (FMN); and tetrahydrobiopterin (H_4B)(17-19). 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 the enzyme mechanism.

3.1.2. NOS Function Regulation & NOS-2 Selective Inhibition

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 (20, 21). 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 (22). 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 (23-25). 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): **1)** Tissue injury and various inflammatory disorders (26, 27); **2)** Neuronal disease (28, 29); **3)** Auto-immune diseases (30, 31); **4)** Cancer or tumor cell proliferation (32-35); **5)** Angiogenesis and related pathological changes (36, 37); and **6)** Diabetes mellitus (38, 39).

Thus, selective inhibition of NOS-2 may have therapeutic potential for treatment of diseases mediated by the overproduction of NO (40). 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 (41, 42) and 2) guanidine inhibitor (aminoguanidine) which has some selectivity towards NOS-2 inhibition, but has low efficacy and causes severe side effects (42). The recent discovery made by us indicates that an alternative approach is possible (43). We have found NOS-2 expression can be selectively down regulated by *T. sprialis* infection (43). The features of this inhibition include: **a) Systemic**: local jejunal infection by *T. spiralis* induce 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; to paxillin, a housekeeper protein; or to cyclo-oxygenase-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 (44) that inhibition of NOS-2 expression by *T. sprialis* infection is dependent on the signaling pathway that includes the IL-4 receptor a subunit, receptor-associated kinases, Janus tyrosine kinase, and Stat6 in the suppression of NOS-2. It is surprising that activation of the IL4Ra/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 IL4Rα ligand could be involved in the IL-4Rα/Stat6 stimulating pathway which 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. 2. NOS-independent NO generation

3.2.1. Nitric oxide and the Nobel Prize

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 as a signalling molecule in the cardiovascular system. In the 1970s, Ferid Murad and his colleagues demonstrated that soluble guanylate cyclase was stimulated by nitrite-containing compounds, causing an increase in cGMP, which in turn brought about vascular relaxation. Murad (45, 46) first suggested that the activation of soluble guanylate cyclase may occur via the formation of NO. He was fascinated by the idea that a gas could regulate smooth muscle function and proposed that hormones and other endogenous factors may also act through NO. In 1980, Furchgott and his colleagues published an article (47) underlining the role of endothelial cells in the ACh induced relaxation of arterial smooth muscle and recognized that vasodilation by bradykinin, histamine, ATP was due to the same relaxing substance, which they named endothelial derived relaxing factor (EDRF). It was Ignarro (48-50) who went on to conclude that EDRF from the artery and vein is either NO or a chemically related radical species in the 1987 December issue of *Circulation Research*.

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, and was synthesised by Sombrero in 1847 (51). Another nitrate-containing compound, amyl nitrite, was discovered a few years later and was used by Guthrie in 1859. During his discovery of dynamite (trinitrotoluene [TNT]), Alfred Nobel had suffered from angina pectoris and was prescribed nitroglycerin for his chest pain in 1895 (51). He was certainly taken aback since he could not imagine how the nitro containing substance could be taken into the body. Almost a century later, organic nitrates and their gaseous metabolic end product, NO, were implicated in a vast array of biologically diverse activities.

3.2.2. Organic nitrates ($RONO_2$s)

Organic nitrates ($RONO_2$) are simple nitric and nitrous acid esters of alcohols. Clinically used $RONO_2$ compounds include GTN, pentaerythrityl tetranitrate (PETN), isosorbide dinitrate (ISDN), isosorbide dinitrate, and triethanolamine trinitrate biphosphate. The organic nitrates are susceptible to alkaline hydrolysis, but are generally stable in neutral or weakly acidic solution, which is one of the major differences comparing with the NONOates that will be discussed next. The medical usage as well as the biological effects of organic nitrates can be attributed to their intracellular conversion to nitrite ions and then to NO (52), which in turn activates guanylate cyclase and increases the cGMP content in cells (53, 54). Elevated cGMP ultimately leads to de-phosphorylation of the myosin light chain, resulting in smooth muscle relaxation. It has been suggested that multiple pathways contribute to NO formation from organic nitrates *in vivo*; however, the metabolic mechanism is poorly understood. It is not until recently, that researchers at Duke University Medical Center, have been able to make significant progress in the identification of the enzymatic mechanism of GTN bioactivation, eventhough the compound's discovery occurred over 150 years ago. Chen *et al* (55) purified a nitrate reductase known as mitochodrial aldehyde dehydrogenase (mtALDH) that specifically catalyzes GTN to form 1,2-glyceryl dinitrate and nitrite, which are eventually converted to NO. This finding demonstrates that the biotransformation of GTN occurs predominantly in mitochondria and indicates that attenuated biotransformation of GTN by mtALDH underlies the induction of nitrate tolerance. Although the study has not clarified every aspect of the pathway, patients taking organic nitrates esters for the treatment of acute ischemic syndromes and congestive heart failure will benefit from awareness of the contraindicated effect of certain classes of drugs that inhibit mtALDH activity, such as sulfonylurea hypoglycemics, chloral hydrate, and acetaminophen.

3.2.3. S-nitrosothiols (RSNO)

As early as 1981, Ignarro's group demonstrated that the bioactivities of certain pharmacological nitrogen oxide (NOx) donors were attributed to reactions with cellular thiols (56), which is several years before making the observation that NO is actually synthesized endogenously in mammalian cells. There is now a large body of literature that implicates S-nitrosothiol (SNO) as an intermediate in nitric oxide–dependent and guanylyl cyclase–independent signaling processes. Reactive protein thiols are becoming regarded as major intracellular target of nitric oxide (57).

S-nitrosothiols (for detail see reviews: 58-62) are thio-esters of nitrite with the general structure R-S-N=O (RSNO); naturally occurring examples include S-nitrosocysteine, S-nitrosoglutathione and S-nitrosoalbumin, in which R is an amino acid, polypeptide and protein respectively. RSNOs are direct analogs of the nitrite esters of alcohols—the organic nitrites reviewed above. S-nitrosothiols can be synthesized from the reaction between thiols and nitrous acid (**Equation 1**) in extremely acidic condition (pH < 3).

$HNO_2 + RSH \longrightarrow RSNO + H_2O$ (Equation 1)

The decomposition of RSNOs is heat, UV light and metal ion (Cu^{2+}) dependent, which results in the formation of NO and the corresponding disulphide (RSSR; **Equation 2**). The reaction can be enhanced by the presence of ascorbate, thiols, high oxygen tension and pH > 3. In addition, the enzymic decomposition by γ-glutamyl transpeptidase, glutathione peroxidase and xanthine oxidase has also been demonstrated.

$2RSNO \longrightarrow RSSR + 2NO$ (Equation 2)

Although RSNOs such as S-nitroso-N-acetyl-penicillamine (SNAP), N-acetyl-S-nitrosopenicillaminyl-S-nitrosopenicillamine and S-nitrosoglutathione (GSNO) are commercially available, none of them has been used therapeutically due to the unpredictable rate of decomposition in the body. In contrast, an increasing number of proteins have been found to undergo S-nitrosylation *in vivo.* These S-nitrosothiol proteins have demonstrated an important role in many physiological as well as pathological processes. The study of S-nitrosohemoglobin is an example through which the significance of the process may be understood.

Hemoglobin (Hb) is one of the most well understood proteins in the human body. In addition to its well recognized function of O_2/CO_2 transportation, the allosteric transition of Hb has been shown to capture and deliver a third gas, NO (63). Hb exists in two alternative structures, known as R (for relaxed, high O_2 affinity) and T (for tense, low O_2 affinity). The allosteric transition of Hb (from R to T; see **Equation 3**) controls the reactivity of one pair of highly conserved cysteines (Cys93) that are located in a protected pocket within R conformation and are exposed in the T conformation. At high pO_2, endothelium-derived NO is captured by heme-iron in R-state Hb and the captured NO can then be transferred to Cys93 on R-state Hb, forming SNO-Hb (64). While reaching low pO_2 regions, allosteric conversion of SNO-Hb to the T-state promotes transnitrosation in which NO groups are transferred to acceptor thiols such as glutathione, or those in the red blood cells. The interaction of Hb with red blood cells membrane through the chloride/bicarbonate anion exchange protein AE1 is the best example for this proposed transition. AE1 contains two reactive cysteine residues that can be oxidatively crosslinked to the reactive β-chain cysteine in Hb through a disulphide bond. The study by Dr.Stamler's group indicated that transnitrosylation of a vicinal thiol in the cytoplasmic domain of AE1 is a strong candidate mechanism for the transfer of the NO group from the β-chain Cys93 of SNO-Hb to the red blood cells membrane 65, 66).

$CYSNO\text{-}Hb([Fe(II)O_2]_4) + e^- \leftrightarrow CYS\text{-}Hb([Fe(II)NO][Fe(II)]_3) + 4O_2$

R-state T-state (Equation 3)

S-nitrosation of cysteine residues in proteins also takes place in other cases. Albumin is an abundant circulating protein that may serve as an effectively reservoir of NO in its nitrosated form (67). The angiotensin-converting enzyme (ACE) inhibitor captopril is considered as a reduced thiol that has been reported acting as an NO donor after being nitrosated (68). Plasminogen activator (tPA) is another popular medicine with the ability to undergo nitrosation (69).

3.2.4. NONOates (diazeniumdiolates)

Anionic $[N(O)NO]^-$ groups (70) are chemically active and able to covalently attach to sulfur (forming $O_3S[N(O)NO]$), oxygen (forming $O[N(O)NO]$), and carbon (forming $CH_2([N(O)NO]^-)_2$). While the $[N(O)NO]^-$ group bound to nitrogen, NO is spontaneously released from the compounds (**Equation 4 and 5**).

$Et_2NH + 2NO + Brønsted\ base\ (B) \longrightarrow Et_2N[N(O)NO]^- + BH^+$

(Equation 4)

$X\text{-}[N(O)NO]^-$

$X\text{-}[N(O)NO]^- \longrightarrow X^- + 2NO$ (Equation 5)

* X = secondary amine group

The decomposition of the compounds containing $X\text{-}[N(O)NO]^-$ is pH-dependent; an acidic pH will extremely facilitate the proceeding which is different comparing with the organic nitrates ($RONO_2$) described previously. The decomposition theoretically follows first-order kinetics and is able to spontaneously generate up to two molecules of NO per [N(O)NO]- unit. The decomposition rate (also referred as half life of NO release) is the most important criteria for selecting proper NONOates for various purposes. Keefer (71) has proposed a unified nomenclature system in which X-[N(O)NO]- containing compounds are referred to as "*diazeniumdiolates*", rather than as "NONOates", "Drago complexes", "NOC" compounds, etc. The reason is that the "diazeniumdiolates" designation is derived from International Union of Pure and Applied Chemistry nomenclature rules and reflects the structure of the functional group in a universally recognizable way.

NO is involved in various physiological phenomena, such as vasorelaxation and neurotransmission. The major direction of NO augmentation for therapeutic usage should be NO signaling pathway oriented. Thus, the pathological conditions due to functional or quantitative NO insufficiency will be the targets for NO supplement therapy. Considering the first-order dissociation of NO, diazeniumdiolates (NONOates) make themselves well suited for a variety of biomedical applications, especially those requiring known rates of NO release. However, the biggest challenge for us to apply NO donors in medical practice is to design a better donor that can generate molecular NO at the right time and the right bodily location. An ideal polymeric-based NO donor could be considerable promise for biomedical applications in which local delivery of NO is desired.

3.2.5. Nitrite (nitrate)-derived NO

NO is produced in various quantities by NOS-dependent and –independent pathways in the body.

Although several mechanisms are responsible for NO scavenge, the final oxidative metabolite of NO are nitrite (NO_2^-) and nitrate (NO_3^-). On the other direction of the reaction, nitrate can be reduced to nitrite and then, nitrite can readily protonate under acidic conditions to form nitrous acid that can be transformed into NO by self-decomposition and reduction (see **Equation 6 and 7**; 72).

$$NO_2^- + H^+ \longrightarrow HNO_2 \quad (pKa \approx 3.3) \quad \text{(Equation 6)}$$

$$3\,HNO_2 \longrightarrow H_2O + 2NO + NO_3^-$$

$$2\,HNO_2 \longrightarrow H_2O + N_2O_3;$$

$$N_2O_3 \longrightarrow NO + NO_2 \quad \text{(Equation 7)}$$

This enzyme-independent NO formation has been shown to occur in humans under several physiological or pathological conditions. For instance, NO formation may occur from salivary nitrite, from nitrous acid in the stomach, from bacteriostasis in the gastrointestinal tract and in urine (73), and in the ischaemic rat heart due to local lower pH conditions (74). Using an amiNO series of nitric oxide sensor, we can observe the *in situ* NO generation by the addition of nitrite to acidified solution (pH = 2) in the presence of iodide ion (reducing agent) according to the **Equation 8**:

$$2NO_2^- + 4H^+ + 2I^- \longrightarrow 2NO + I_2 + 2H_2O \quad \text{(Equation 8)}$$

Consequently, the mole ratio of nitrite to NO is 1:1. Thus, the amount of NO generated equal the amount of nitrite added. To facilitate the equation 7 reaction, other reducing agents are also employed. It has been reported that NO formation is enhanced by ascorbic acid in the stomach and chewed lettuce in low pH condition. Lettuce contains various phenolic compounds and studies by Takahama *et al* (75) have indicated that phenolics such as quercetin can reduce nitrous acid to produce NO.

Biotransformation of NO and its related N-oxides undergoes a continuous succession of changes. In addition to endogenous L-arginine –derived NO synthesis that serves as a major source of nitrite/nitrate, a number of other factors could also influence the circulation and tissue levels of nitrite/nitrate: 1) Dietary intake: vegetables such as beets, celery, lettuce and other leafy vegetables are rich in nitrates. Nitrite/nitrate are also added in various food as preservatives. Both nitrite and nitrate are highly water soluble. Beside reduction of nitrite/nitrate to form NO in stomach mentioned above, the nitrate/H^+ cotransporter has been shown to play an important role in cell membrane nitrate intake (76). 2) Respiratory intake: NO reacts with oxygen in air to form nitrogen dioxide (NO_2). Both of NO_2 and NO are the major N-oxides (NO_X) in gaseous phase to which human are exposed. As described above, inhaled NO could react with hemoglobin, forming nitrosyl-hemoglobin from which nitrite and nitrate are generated. In the respiratory tract, NO_2 could further react with NO to form N_2O_3 that then hydrolyzes to form nitrite (77). In August 16 published Science magazine, Chuck *et al* (78) has reported a surprising discovery that oceans seawater is a huge source for alkyl nitrate such as methyl nitrate and ethyl nitrite. This observation further indicated the existence of organic nitrate in the interaction between human and its atmosphere.

While we discuss the metabolism of nitrite/nitrate, a brief overview of denitrification is helpful for further understanding the interconversion of inorganic nitrogen. Denitrification is the major biotic process in which nitrate is reduced to dinitrogen through a series of enzymic reactions (79). Nitrate reductase (NR), at least three classes of nitrate reductase (NAS, NAR and NAP) have been described (80): Bacterial assimilatory nitrate reductase (NAS) is located in the cytoplasmic compartment and participates in nitrogen assimilation. The bacterial membrane-bound nitrate reductase (NAR) is anchored to the cytoplasmic face of the membrane and is involved in anaerobic nitrate respiration. The periplasmic respiratory enzyme (NAP) is a two-subunit complex, located in the periplasmic compartment. All these enzymes are able to catalyse the reaction of nitrate to nitrite,as shown in **Eequation 9**.

$$NO_3^- + 2H^+ + 2e \longrightarrow NO_2^- + H_2O \quad \text{(Equation 9)}$$

Nitrite reductase (NIR), is a respiratory enzyme that can catalyse the one-electron reduction of nitrite to NO (**Equation 10**; 81):

$$NO_2^- + 2H^+ + e \longrightarrow NO + H_2O \quad \text{(Equation 10)}$$

Another inorganic nitrogen metablism enzyme is nitrous oxide reductase (N_2OS; 79) that catalyses the reduction of nitrous oxide (N_2O; produced by denitrification and other processes) to dinitrogen (N2) and H_2O (**Equation 11**).

$$N_2O + 2H^+ + 2e \longrightarrow N_2 + H_2O \quad \text{(Equation 11)}$$

Nitric oxide reductases (NORs) are found in bacteria and belong to the large enzyme family including cytochrome oxidases (82). At least two isoforms of NOR have been identified. One is a cytochrome bc-type complex (cNOR) that receives electrons from soluble redox protein donors. The another type (qNOR) lacks the cytochrome c component and uses quinol as the electron donor. The NOR catalyses the two electron reduction of NO to N_2O as described in **Equation 12**:

$$2NO + 2H^+ + 2e \longrightarrow N_2O + H_2O \quad \text{(Equation 12)}$$

It has been suggested (80) that all above enzymes may be ancestors of energy-conserving enzymes of the heme-copper oxidase superfamily. Thus, some aerobic microorganisms rely on the reduction of nitrogen oxides, especially nitrate, to gaseous products that enable them to grow in oxygen-limiting conditions and the ability to denitrify has been found in a wide variety of bacteria and even in some fungi.

Nevertheless, in the biology world, nitrate is the major source of nitrogen (N) for plants growth and

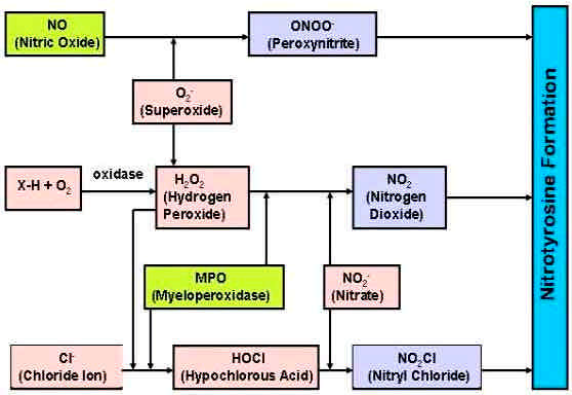

Figure 3. Biological pathways for NO2-Tyr formation.

development, providing cellular components and modulating gene expression. Although many studies on the effects of NO in plants focused only on the aspect of air pollutant, the present of NO signaling pathway in plants has been suggested by several groups and the evidence for the existence of NOS in higher plant cells has emerged since 1995 when Kuo (83) first reported that a anti-mammalian NOS antibody positively recognized protein from wheat germ. In 1996, the presence of NOS activities in plants has been reported (84), however, there is still lack of proteomic or genetic information regarding the plant nitric oxide synthase (85). As a powerful alternative pathway for NO production, non-enzymatic NO generation from nitrite has been studied extensively (see above for review). Nitrate reductase is capable of producing NO through one-electron reduction of nitrite using NAD(P)H as an electron donor. It has been reported that plant species, such as sunflower, spinach, sugar cane, corn, rape, spruce, and tobacco, emit NO gas under certain conditions (72). The significance of NO production in plants was described as an important player in several aspects, including symbiosis establishment, phytoalexin production, growth regulation, resistance to drought hardiness, and the activation of the plant defense system against pathogens (for detail see the reviews by 72, 85, 86).

4. NO OVERPRODUCTION & TYROSINE NITRATION

4.1. Life sciences where the ROS and the RNS meet

As the consequence of aerobic metabolism, the biological body is constantly generate reactive oxygen species (ROS) which include superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$) and nonradical hydrogen peroxide (H_2O_2). The discovery of nitric oxide further draw the attention towards reactive nitrogen species (RNS) which include $^{\bullet}NO$ (nitrogen monoxide) that can under going interconversion to form NO^+ (nitrosonium), and NO^- (nitroxyl anion) in certain cellular conditions[3,5,87,88]. 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 (5, 87, 88). Nitrite (NO_2^-) is another major oxidation product derived from NO, and can be oxidized by myeloperoxidase (MPO), lactoperoxidase (LPO), horseradish peroxidase to form a reactive nitrogen intermediate(s) such as nitrogen dioxide that is capable of nitrating tyrosine (Figure 3). MPO and other peroxidase are also able to use halides and pseudohalides as co-substrates to generate reactive intermediate hypochlorous acid, which further forms nitryl chloride that results in formation of NO2-Tyr (89-91). Thus, it is likely that multiple pathways participate in tyrosine nitration (Figure 3; 92, 93).

For a period of time, protein nitration was thought to be the evidence of the existence of peroxynitrite and hence protein nitration was the foot print of peroxynitite 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 (88, 94, 95). It has also been shown that tyrosine nitration can diminish a protein's effectiveness as a substrate for tyrosine kinases (96).

4.2. *In vivo* protein tyrosine nitration

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 (97-99) in more than 200 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 (4, 5, 92, 97).

4.2.1. MPO-catalyzed NO_2-Tyr formation

In 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 (100-104) have tried to establish *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 nitrogen dioxide and nitryl chloride are 1 electron oxidants that is not powerful enough to overcome endogenous antioxidants, while peroxynitrous acid can catalyze 2 electron oxidation and is not completely inhibited by these antioxidants (88). 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 *Trichinella spiralis*, a one host helminth (105, 106). Our studies have revealed that a severe inflammatory histological changes occurred in *T. spiralis*-infected jejunum that was associated with shortened villi, elongated crypts and a greatly thickened smooth muscle. These

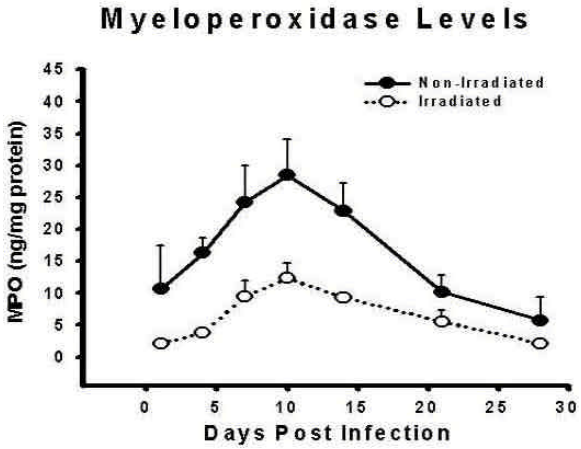

Figure 4. *Trichenelle spiralis* infection -induced elevation of MPO. Irradiation (open circle) shows the inhibition of MPO expression.

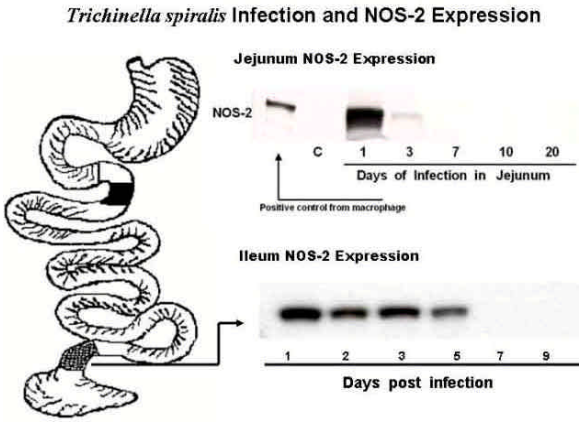

Figure 5. *Trichenelle spiralis* infection can set into motion systemic mechanism(s) that down regulate NOS-2 expression in both jejunum and ileum.

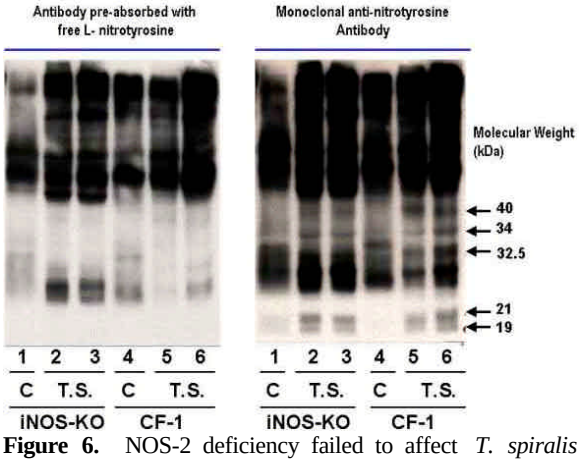

Figure 6. NOS-2 deficiency failed to affect *T. spiralis* infection (MPO)-induced protein tyrosine nitration. 5 distinct bands of NO2-Tyr were detected in the jejunum tissue after 7 days of parasite infection (T.S.). Comparing with the control (CF-1) mice, the comparable significant levels of tyrosine nitration can be found in NOS-2 KO mice. Detected NO2-Tyr bands were confirmed by anti-NO2-Tyr antibody that was pre-absorbed with free L-NO2-Tyr. Each indicated lane number represents an individual animal used in the experiment.

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 (Figure 4). However, a strikingly different induction of inducible NO synthase (NOS-2) was demonstrated in the inflammed 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 (Figure 5; 43, 106, 107). We have evaluated intestinal protein for NO2-Tyr formation at different time points after parasite infection. At 7 to 10 days post infection, 5 distinct bands of NO2-Tyr were detected in the jejunum (Figure 6). 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 *trichinella spiralis*-induced high MPO inflammation study. Despite the failure to 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 (Figure 6; 107). In summary, NO2-Tyr formation is observed following *trichinella 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 NO^{2+} or $ONOO^-$. While extremely brief half life of NO^{2+} (~1ns) made it unlikely to perform it's function in physiological condition (108), there is not enough direct evidence that support the formation of $ONOO^-$ by MPO (92). Thus, NO2-Tyr may be formed *in vivo* even with less NO circumstance, and effective suppression of MPO activity may be important in preventing NO2-Tyr formation.

4.2.2. Calcium Channel Protein Tyrosine Nitration

The first evidence that muscle contraction can be altered by nitration of key protein is suggested by the study of Schoneich's group (109). In skeletal muscle, sarcoplasmic-reticulum (SR) Ca^{2+}-ATPase isoforms 2 (SERCA2a) progressively accumulates a significant amount of NO2-Tyr with age. A correlation of the SR Ca^{2+}-ATPase activity and covalent protein modifications *in vitro* and *in vivo* suggests that tyrosine nitration may affect the Ca^{2+}-ATPase activity. By means of partial and complete proteolytic digestion of purified SERCA2a with trypsin or Staphylococcus aureus V8 protease, followed by Western-blot, amino acid and HPLC–electrospray-MS (ESI-MS) analysis, the study localized a large part of the age-dependent tyrosine nitration to the sequence Tyr294-Tyr295 in the M4–M8 transmembrane domain of the SERCA2a, close to sites essential for Ca^{2+} translocation.

Smooth muscle contractility is primarily regulated by alteration in intracellular free-Ca^{2+} concentration that is mainly due to the influx of extracellular- Ca^{2+} via Ca^{2+} channel or intracellular -Ca^{2+} release from the storages (110-112). Ca^{2+} -calmodulin-induced stimulation of myosin light chain kinase (MLCK) and subsequent phosphorylation of regulatory myosin light chain (MLC) eventually results in the contraction of the smooth muscle (112-114). What has become apparent from

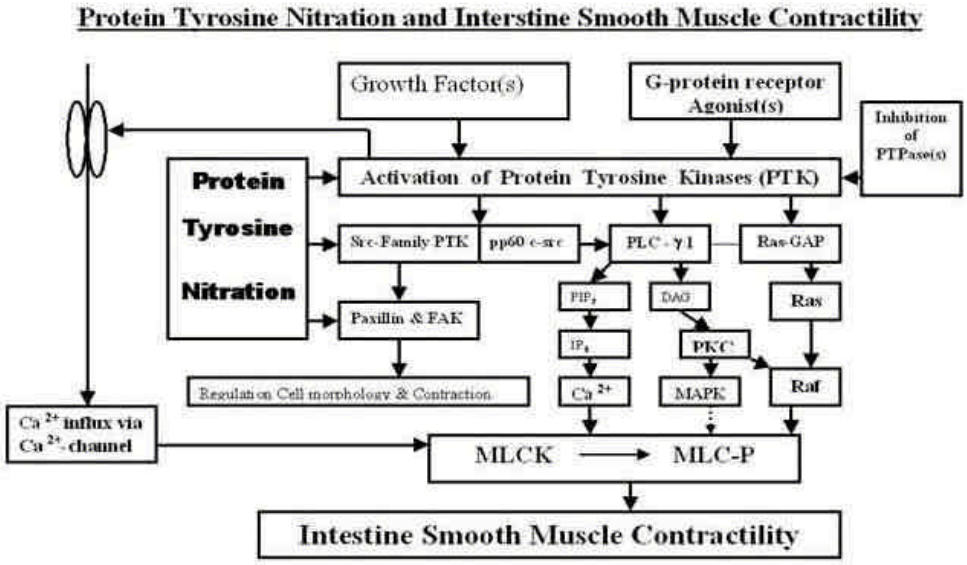

Figure 7. Role of protein tyrosine nitration in smooth muscle contractility.

work over the past decade, is that tyrosine kinase pathways are just as important for the control of rapid tissue response such as contraction, as they are for the regulation of delayed responses, such as gene transcription and cell division (reviewed by Di Salvo et. al. 115). Vascular smooth muscle function is altered in many conditions that are characterized by inflammation and/or sepsis, conditions that are not uncommon. Therefore, prevention and/or reversal of the vascular muscle dysfunction would likely have a positive impact on patient recovery. As we described in current review, nearly all inflammatory states are characterized by the generation of oxygen-derived free radicals as a common feature. Superoxide rapidly oxidizes NO to form $ONOO^-$ which not only terminates the physiological actions of NO, but also results in protein nitration. The experiments described here were designed to test the HYPOTHESIS that nitration of key proteins involved in smooth muscle contraction participates in the inflammation altered circulation function. A rapidly growing body of evidence suggests that enhanced protein tyrosine phosphorylation is associated with smooth muscle contraction (Figure 7). Emphasis of our projects was placed on the presence and possible effect of nitration of tyrosines contained in relevant tyrosine kinases and/or substrates that are linked to the regulation of smooth muscle contractility.

As mentioned above, activation of MPO is a key route for NO2-Tyr formation. MPO is a heme enzyme of neutrophils that uses hydrogen peroxide and nitrite to generate nitrogen dioxide and hypochlorous acid which in turn catalyzes the nitration of tyrosine. Using our *trichinella spiralis* infection model, we were able to evaluate the NO2-Tyr formation in intestinal smooth muscle tissue. At 7-10 days post helminth infection, homogenates of inflamed jejunum were immunoprecipitated with anti-NO2-Tyr and anti-Ca^{2+}-channel α1C antibodies. The precipitates then were detected with anti-NO2-Tyr antibodies using Western blotting techniques (Figure 8: left panel). Results from this experiment indicated that the L-type Ca^{2+}-channel protein was nitrated during inflammation (116).

In another experiment (Figure 8: right panel), a segment of jejunum was taken from a control animal and strips of longitudinal muscle were removed and placed in tissue baths such that isometric contractions could be recorded. Treatment of a muscle strip with 50 nM MPO for 60 mins resulted in a marked delay in the onset and a decrease in the velocity of the phasic phase of a carbachol-induced contraction. However, the amplitude of the tonic phase of the contraction was not attenuated (115). Because the phasic phase of smooth muscle contraction has been shown to be due mainly to the influx of Ca^{2+} via voltage-gated Ca^{2+}-channels, the response to potassium was determined. The potassium-induced contraction also was markedly inhibited. These preliminary results suggest that changes in muscle contractility perhaps due to nitration of voltage-dependent Ca^{2+} channels which occurs in the presence of elevated MPO activity. Application of the results of our experiments will add significant knowledge to the cardiovascular field and contribute to our understanding of the regulation of contraction of smooth muscle under normal and inflammatory conditions.

4.3. Mechanisms underlie peroxidases-induced protein tyrosine nitration

MPO is a tetrameric, basic (PI > 10) heme protein with the molecular weight of ~150 kDa that is comprised of two identical disulfide-linked protomers, each of which possesses a protoporphyrin-containing 59-64 kDa heavy subunit and a 14 kDa light subunit (117). MPO plays a fundamental role in the killing of invading bacteria, viruses, and fungi by forming strong intermediates (Figure 3) which can result in protein oxidation and nitration. While MPO reacts with H_2O_2, a dismutation product of neutrophils-generated $O_2^{\bullet-}$, in the present of physiological

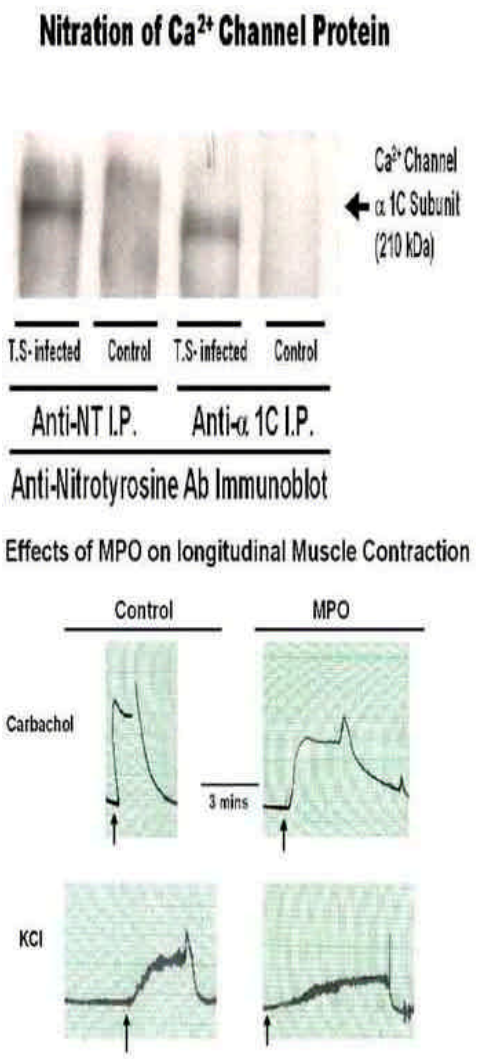

Figure 8. Effect of MPO on Ca^{2+} channel protein nitration and smooth muscle contraction. Left panel: Homogenates of 10 days *T. spiralis*-infected jejunum were immunoprecipitated with anti-NO2-Tyr and anti-Ca^{2+} -channel a1C antibodies. The precipitates then were cross treated with anti-Ca^{2+}-channel a1C and NO2-Tyr antibodies using Western blotting techniques. Results from both experiments indicated that the L-type Ca^{2+}-channel protein was nitrated during inflammation. Right panel: The strips of longitudinal muscle were removed from jejunum and placed in tissue baths and isometric contractions were recorded. Treatment of a muscle strip with 50 nM MPO for 60 mins resulted in a marked delay in the onset and a decrease in the velocity of the phasic phase of a carbachol-induced contraction. The potassium-induced contraction also was markedly inhibited.

concentration of Cl^-, a radical intermediate hypochlorous acid (HOCl) is formed (89, 118) (**Equation 13**):

$$H_2O_2 + Cl^- + MPO \longrightarrow H_2O + HOCl \quad \text{(Equation 13)}$$

HOCl is generally regarded as a primary microbicide and can further react with NO_2^- to form transitory nitryl chloride (NO_2Cl) that is capable of nitrating tyrosine residue. However, the rate constant for the HOCl- NO_2^- reaction under physiological conditions is relatively low due to the existence of numerous reductants that are more highly reactive toward HOCl. As we have described in the Figure 3, MPO can oxidize nitrite to a species that is capable of causing tyrosine nitration. A postulated reaction scheme[91] is summarized in **Equation 14, 15 and 16**.

$$\begin{aligned} &MPO + H_2O_2 \longrightarrow \text{Compound I} + H_2O \\ &\text{Compound I} + NO_2^- \longrightarrow \text{Compound II} + NO_2^\bullet \\ &\text{Compound II} + NO_2^- \longrightarrow MPO + NO_2^\bullet \end{aligned} \quad \text{(Equation 14-16)}$$

In addition to MPO, the family of human peroxidases includes eosinophil peroxidase, uterine peroxidase, lactoperoxidase, salivary peroxidase, thyroid peroxidase and prostaglandin H1/2 synthases. As indicated, peroxidases are enzymes that utilize hydrogen peroxide to oxidize substrates. Furthermore, several peroxidases have been demonstrated to cause protein tyrosine nitration. An interesting feature of those proteins is that they are heme-containing protein. It has been long recognized that heme is a key molecule in the responses to environmental stress, including oxygen. The heme molecule promotes most biological oxidation processes involved in oxygen transport, mitochondrial respiration, drug metabolism, steroid biosynthesis, cellular antioxidant defenses, and signal transduction processes (119, 120). Thus, heme is an oxidant in several model systems and the pro-oxidant effects of free heme and hemoprotein may be attributed to the formation of hypervalent states of the heme iron (121, 122). Therefore, we decided to test the hypothesis that free heme as well as iron play important role in NO2-Tyr formation. We used bovine serum albumin (BSA) as substrate to test the effect of tyrosine nitration by either hemin or iron. Nitration of BSA was confirmed by Western blotting with an antibody against NO2-Tyr. As demonstrated in Figure 9 and 10, BSA could be strongly nitrated by either himin or Fe^{2+}.

While iron is essential for a number of cellular activities, an excess of cellular iron becomes toxic that involves many organs leading to a variety of serious diseases such as liver disease, heart disease, diabetes mellitus, hormonal abnormalities, dysfunctional immune system, etc. The tissue damage associated with iron overload is believed to result primarily from free radical reactions mediated by iron since iron is an effective catalyst in free radical reactions. The detection of protein nitration by iron may offer another explanation for the pathological changes induced by iron overload and which could be managed or prevented by inhibition of NO2-Tyr formation.

There has been an enormous interest in NO since its discovery. As a simple but well known conclusion, NO is a double edge sword mediator in that it can exert beneficial or detrimental effect depending on the physiopathological context. Therapy targeting the action of NO has a long history such as the organic nitrates, while

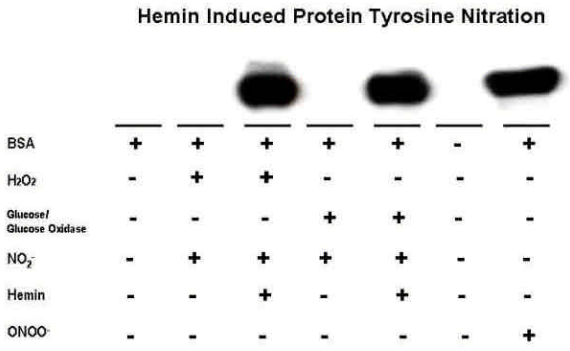

Figure 9. Result of Western blot with anti-NO2-Tyr antibody shows tyrosine residue in BSA could be nitrated under the condition of heme-NO_2-H_2O_2 treatment system.

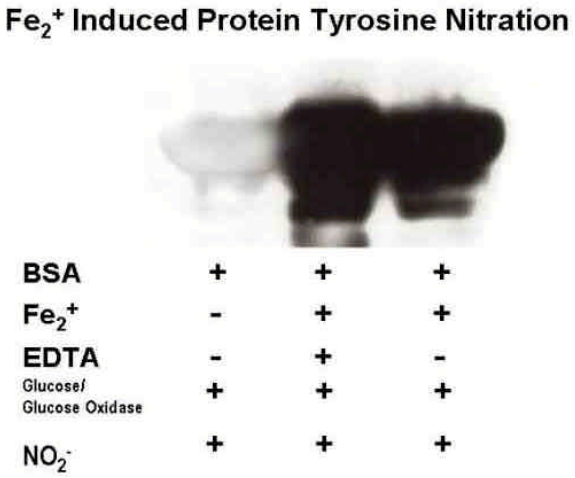

Figure 10. Western blot with anti-NO2-Tyr antibody indicates the role of iron in BSA tyrosine nitration.

still is very young due to the enormous effort in understanding the biology of NO.

5. ACKNOWLEDGEMENTS

The financial support of this research was provided by the John S. Dunn, Harold and Leila Mathers, and Robert A. Welch Foundations; as well as The University of Texas, and National Institute of Health.

6. REFERENCES

1. Murad, F., Nitric oxide signaling: would you believe that a simple free radical could be a second messenger, autacoid, paracrine substance, neurotransmitter, and hormone? *Recent Prog Horm Res*, 53, 43-59; discussion 59-60. (1998)

2. Murad, F., Cellular signaling with nitric oxide and cyclic GMP. *Braz J Med Biol Res*, 32, 1317-27. (1999)

3. Murad, F., Discovery of some of the biological effects of nitric oxide and its role in cell signaling. *Biosci Rep*, 19, 133-54. (1999)

4. Bian, K., K. Davis, J. Kuret, L. Binder, and F. Murad, Nitrotyrosine formation with endotoxin-induced kidney injury detected by immunohistochemistry. *Am J Physiol*, 277, F33-40. (1999)

5. Bian, K. and F. Murad, Diversity of endotoxin-induced nitrotyrosine formation in macrophage-endothelium-rich organs. *Free Radic Biol Med*, 31, 421-9. (2001)

6. Bredt, D. S., P. M. Hwang, C. E. Glatt, C. Lowenstein, R. R. Reed, and S. H. Snyder, Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature*, 351, 714-8. (1991)

7. Nathan, C. and Q. W. Xie, Nitric oxide synthases: roles, tolls, and controls. *Cell*, 78, 915-8. (1994)

8. Nathan, C., Inducible nitric oxide synthase: regulation subserves function. *Curr Top Microbiol Immunol*, 196, 1-4. (1995)

9. Janssens, S. P., A. Shimouchi, T. Quertermous, D. B. Bloch, and K. D. Bloch, Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. *J Biol Chem*, 267, 14519-22. (1992)

10. Marsden, P. A., K. T. Schappert, H. S. Chen, M. Flowers, C. L. Sundell, J. N. Wilcox, S. Lamas, and T. Michel, Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett*, 307, 287-93. (1992)

11. Sessa, W. C., J. K. Harrison, C. M. Barber, D. Zeng, M. E. Durieux, D. D. D'Angelo, K. R. Lynch, and M. J. Peach, Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J Biol Chem*, 267, 15274-6. (1992)

12. Liu, Q. and S. S. Gross, Binding sites of nitric oxide synthases. *Methods Enzymol*, 268, 311-24. (1996)

13. Xia, H. and D. S. Bredt, Cloned and expressed nitric oxide synthase proteins. *Methods Enzymol*, 268, 427-36. (1996)

14. Marletta, M. A., Another activation switch for endothelial nitric oxide synthase: why does it have to be so complicated? *Trends Biochem Sci*, 26, 519-21. (2001)

15. Bredt, D. S., Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radic Res*, 31, 577-96. (1999)

16. White, K. A. and M. A. Marletta, Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry*, 31, 6627-31. (1992)

17. Kwon, N. S., C. F. Nathan, C. Gilker, O. W. Griffith, D. E. Matthews, and D. J. Stuehr, L-citrulline production from L-arginine by macrophage nitric oxide synthase. The ureido oxygen derives from dioxygen. *J Biol Chem*, 265, 13442-5. (1990)

18. Bush, P. A., N. E. Gonzalez, J. M. Griscavage, and L. J. Ignarro, Nitric oxide synthase from cerebellum catalyzes the formation of equimolar quantities of nitric oxide and citrulline from L-arginine. *Biochem Biophys Res Commun*, 185, 960-6. (1992)

19. Ignarro, L. J., G. M. Buga, L. H. Wei, P. M. Bauer, G. Wu, and P. del Soldato, Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc Natl Acad Sci U S A*, 98, 4202-8. (2001)

20. Klatt, P., B. Heinzel, M. John, M. Kastner, E. Bohme, and B. Mayer, Ca^{2+}/calmodulin-dependent cytochrome c reductase activity of brain nitric oxide synthase. *J Biol Chem*, 267, 11374-8. (1992)

21. Mayer, B., M. John, and E. Bohme, Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. *FEBS Lett*, 277, 215-9. (1990)

22. Ignarro, L. J., G. Cirino, A. Casini, and C. Napoli, Nitric oxide as a signaling molecule in the vascular system: an overview. *J Cardiovasc Pharmacol*, 34, 879-86. (1999)

23. Weisbrodt, N. W., M. Lai, and Z. Gottesfeld, Ileal nitric oxide formation is altered in the young rat in response to endotoxin: effects of exposure to alcohol in utero. *Alcohol*, 17, 247-51. (1999)

24. Gottesfeld, Z., M. Maier, D. Mailman, M. Lai, and N. W. Weisbrodt, Splenic sympathetic response to endotoxin is blunted in the fetal alcohol-exposed rat: role of nitric oxide. *Alcohol*, 16, 19-24. (1998)

25. Xie, Q. W. and C. Nathan, Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon-gamma and bacterial lipopolysaccharide. *Trans Assoc Am Physicians*, 106, 1-12. (1993)

26. Calatayud, S., D. Barrachina, and J. V. Esplugues, Nitric oxide: relation to integrity, injury, and healing of the gastric mucosa. *Microsc Res Tech*, 53, 325-35. (2001)

27. Abramson, S. B., A. R. Amin, R. M. Clancy, and M. Attur, The role of nitric oxide in tissue destruction. *Best Pract Res Clin Rheumatol*, 15, 831-45. (2001)

28. Dawson, V. L. and T. M. Dawson, Nitric oxide in neurodegeneration. *Prog Brain Res*, 118, 215-29. (1998)

29. Heneka, M. T. and D. L. Feinstein, Expression and function of inducible nitric oxide synthase in neurons. *J Neuroimmunol*, 114, 8-18. (2001)

30. Amin, A. R., M. Attur, and S. B. Abramson, Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis. *Curr Opin Rheumatol*, 11, 202-9. (1999)

31. Liu, J. S., M. L. Zhao, C. F. Brosnan, and S. C. Lee, Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am J Pathol*, 158, 2057-66. (2001)

32. Kong, G., E. K. Kim, W. S. Kim, Y. W. Lee, J. K. Lee, S. W. Paik, J. C. Rhee, K. W. Choi, and K. T. Lee, Inducible nitric oxide synthase (iNOS) immunoreactivity and its relationship to cell proliferation, apoptosis, angiogenesis, clinicopathologic characteristics, and patient survival in pancreatic cancer. *Int J Pancreatol*, 29, 133-40. (2001)

33. Kondo, S., S. Toyokuni, T. Tsuruyama, M. Ozeki, T. Tachibana, M. Echizenya, H. Hiai, H. Onodera, and M. Imamura, Peroxynitrite-mediated stress is associated with proliferation of human metastatic colorectal carcinoma in the liver. *Cancer Lett*, 179, 87-93. (2002)

34. Kagoura, M., C. Matsui, M. Toyoda, and M. Morohashi, Immunohistochemical study of inducible nitric oxide synthase in skin cancers. *J Cutan Pathol*, 28, 476-81. (2001)

35. Aaltoma, S. H., P. K. Lipponen, and V. M. Kosma, Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer. *Anticancer Res*, 21, 3101-6. (2001)

36. Wilson, K. T., Angiogenic markers, neovascularization and malignant deformation of Barrett's esophagus. *Dis Esophagus*, 15, 16-21. (2002)

37. Franchi, A., O. Gallo, M. Paglierani, I. Sardi, L. Magnelli, E. Masini, and M. Santucci, Inducible nitric oxide synthase expression in laryngeal neoplasia: correlation with angiogenesis. *Head Neck*, 24, 16-23. (2002)

38. Morris, B. J., A. Markus, C. L. Glenn, D. J. Adams, S. Colagiuri, and L. Wang, Association of a functional inducible nitric oxide synthase promoter variant with complications in type 2 diabetes. *J Mol Med*, 80, 96-104. (2002)

39. Johannesen, J., A. Pie, F. Pociot, O. P. Kristiansen, A. E. Karlsen, and J. Nerup, Linkage of the human inducible nitric oxide synthase gene to type 1 diabetes. *J Clin Endocrinol Metab*, 86, 2792-6. (2001)

40. Bryk, R. and D. J. Wolff, Pharmacological modulation of nitric oxide synthesis by mechanism-based inactivators and related inhibitors. *Pharmacol Ther*, 84, 157-78. (1999)

41. Inoue, H., I. Arai, S. Shibata, and S. Watanabe, NG-nitro-L-arginine methyl ester attenuates the maintenance

and expression of methamphetamine-induced behavioral sensitization and enhancement of striatal dopamine release. *J Pharmacol Exp Ther*, 277, 1424-30. (1996)

42. Salerno, L., V. Sorrenti, C. Di Giacomo, G. Romeo, and M. A. Siracusa, Progress in the development of selective nitric oxide synthase (NOS) inhibitors. *Curr Pharm Des*, 8, 177-200. (2002)

43. Bian, K., Y. Harari, M. Zhong, M. Lai, G. Castro, N. Weisbrodt, and F. Murad, Down-regulation of inducible nitric-oxide synthase (NOS-2) during parasite-induced gut inflammation: a path to identify a selective NOS-2 inhibitor. *Mol Pharmacol*, 59, 939-47. (2001)

44. Bian K, Zong. M., Harari Y, Weisbrodt N, Murad F., Down regulation of NOS-2 by an IL-4Ra-dependent, T cell-independent pathway during parasite-induced gut inflammation. *Gastroenterology*, 122, A143. (2002)

45. Arnold, W. P., C. K. Mittal, S. Katsuki, and F. Murad, Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci U S A*, 74, 3203-7. (1977)

46. Katsuki, S., W. Arnold, C. Mittal, and F. Murad, Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cyclic Nucleotide Res*, 3, 23-35. (1977)

47. Furchgott, R. F. and J. V. Zawadzki, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, 288, 373-6. (1980)

48. Ignarro, L. J., R. E. Byrns, G. M. Buga, and K. S. Wood, Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ Res*, 61, 866-79. (1987)

49. Ignarro, L. J., G. M. Buga, K. S. Wood, R. E. Byrns, and G. Chaudhuri, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A*, 84, 9265-9. (1987)

50. Ignarro, L. J. and K. S. Wood, Activation of purified soluble guanylate cyclase by arachidonic acid requires absence of enzyme-bound heme. *Biochim Biophys Acta*, 928, 160-70. (1987)

51. Marsh, N. and A. Marsh, A short history of nitroglycerine and nitric oxide in pharmacology and physiology. *Clin Exp Pharmacol Physiol*, 27, 313-9. (2000)

52. Ignarro, L. J., H. Lippton, J. C. Edwards, W. H. Baricos, A. L. Hyman, P. J. Kadowitz, and C. A. Gruetter, Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J Pharmacol Exp Ther*, 218, 739-49. (1981)

53. Bennett, B. M., H. Schroder, L. D. Hayward, S. A. Waldman, and F. Murad, Effect of in vitro organic nitrate tolerance on relaxation, cyclic GMP accumulation, and guanylate cyclase activation by glyceryl trinitrate and the enantiomers of isoidide dinitrate. *Circ Res*, 63, 693-701. (1988)

54. Papapetropoulos, A., C. Y. Go, F. Murad, and J. D. Catravas, Mechanisms of tolerance to sodium nitroprusside in rat cultured aortic smooth muscle cells. *Br J Pharmacol*, 117, 147-55. (1996)

55. Chen, Z., J. Zhang, and J. S. Stamler, Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci U S A*, 99, 8306-11. (2002)

56. Ignarro, L. J., P. J. Kadowitz, and W. H. Baricos, Evidence that regulation of hepatic guanylate cyclase activity involves interactions between catalytic site -SH groups and both substrate and activator. *Arch Biochem Biophys*, 208, 75-86. (1981)

57. Gow, A. J., Q. Chen, D. T. Hess, B. J. Day, H. Ischiropoulos, and J. S. Stamler, Basal and stimulated protein S-nitrosylation in multiple cell types and tissues. *J Biol Chem*, 277, 9637-40. (2002)

58. Richardson, G. and N. Benjamin, Potential therapeutic uses for S-nitrosothiols. *Clin Sci (Lond)*, 102, 99-105. (2002)

59. Al-Sa'doni, H. and A. Ferro, S-Nitrosothiols: a class of nitric oxide-donor drugs. *Clin Sci (Lond)*, 98, 507-20. (2000)

60. Gaston, B., Nitric oxide and thiol groups. *Biochim Biophys Acta*, 1411, 323-33. (1999)

61. Hogg, N., Biological chemistry and clinical potential of S-nitrosothiols. *Free Radic Biol Med*, 28, 1478-86. (2000)

62. Hogg, N., The biochemistry and physiology of S-nitrosothiols. *Annu Rev Pharmacol Toxicol*, 42, 585-600. (2002)

63. Patel, R. P., Biochemical aspects of the reaction of hemoglobin and NO: implications for Hb-based blood substitutes. *Free Radic Biol Med*, 28, 1518-25. (2000)

64. McMahon, T. J., R. E. Moon, B. P. Luschinger, M. S. Carraway, A. E. Stone, B. W. Stolp, A. J. Gow, J. R. Pawloski, P. Watke, D. J. Singel, C. A. Piantadosi, and J. S. Stamler, Nitric oxide in the human respiratory cycle. *Nat Med*, 8, 711-7. (2002)

65. Pawloski, J. R., D. T. Hess, and J. S. Stamler, Export by red blood cells of nitric oxide bioactivity. *Nature*, 409, 622-6. (2001)

66. Joshi, M. S., T. B. Ferguson, Jr., T. H. Han, D. R. Hyduke, J. C. Liao, T. Rassaf, N. Bryan, M. Feelisch, and J. R. Lancaster, Jr., Nitric oxide is consumed, rather than

conserved, by reaction with oxyhemoglobin under physiological conditions. *Proc Natl Acad Sci U S A*, 99, 10341-6. (2002)

67. Jourd'heuil, D., K. Hallen, M. Feelisch, and M. B. Grisham, Dynamic state of S-nitrosothiols in human plasma and whole blood. *Free Radic Biol Med*, 28, 409-17. (2000)

68. Jia, L. and R. C. Blantz, The effects of S-nitrosocaptopril on renal filtration and blood pressure in rats. *Eur J Pharmacol*, 354, 33-41. (1998)

69. Stamler, J. S., D. I. Simon, O. Jaraki, J. A. Osborne, S. Francis, M. Mullins, D. Singel, and J. Loscalzo, S-nitrosylation of tissue-type plasminogen activator confers vasodilatory and antiplatelet properties on the enzyme. *Proc Natl Acad Sci U S A*, 89, 8087-91. (1992)

70. Morley, D. and L. K. Keefer, Nitric oxide/nucleophile complexes: a unique class of nitric oxide-based vasodilators. *J Cardiovasc Pharmacol*, 22 Suppl 7, S3-9. (1993)

71. Fitzhugh, A. L. and L. K. Keefer, Diazeniumdiolates: pro- and antioxidant applications of the "NONOates". *Free Radic Biol Med*, 28, 1463-9. (2000)

72. Yamasaki, H., Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition in vivo. *Philos Trans R Soc Lond B Biol Sci*, 355, 1477-88. (2000)

73. Lundberg, J. O., M. Herulf, M. Olesen, J. Bohr, C. Tysk, N. P. Wiklund, E. Morcos, P. M. Hellstrom, E. Weitzberg, and G. Jarnerot, Increased nitric oxide production in collagenous and lymphocytic colitis. *Eur J Clin Invest*, 27, 869-71. (1997)

74. Zweier, J. L., P. Wang, A. Samouilov, and P. Kuppusamy, Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med*, 1, 804-9. (1995)

75. Takahama, U., T. Oniki, and S. Hirota, Oxidation of quercetin by salivary components. Quercetin-dependent reduction of salivary nitrite under acidic conditions producing nitric oxide. *J Agric Food Chem*, 50, 4317-22. (2002)

76. Zhou, J. J., L. J. Trueman, K. J. Boorer, F. L. Theodoulou, B. G. Forde, and A. J. Miller, A high affinity fungal nitrate carrier with two transport mechanisms. *J Biol Chem*, 275, 39894-9. (2000)

77. Ellis, G., I. Adatia, M. Yazdanpanah, and S. K. Makela, Nitrite and nitrate analyses: a clinical biochemistry perspective. *Clin Biochem*, 31, 195-220. (1998)

78. Chuck, A. L., S. M. Turner, and P. S. Liss, Direct evidence for a marine source of c1 and c2 alkyl nitrates. *Science*, 297, 1151-4. (2002)

79. Zumft, W. G., Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev*, 61, 533-616. (1997)

80. Richardson, D. J. and N. J. Watmough, Inorganic nitrogen metabolism in bacteria. *Curr Opin Chem Biol*, 3, 207-19. (1999)

81. Cheesman, M. R., S. J. Ferguson, J. W. Moir, D. J. Richardson, W. G. Zumft, and A. J. Thomson, Two enzymes with a common function but different heme ligands in the forms as isolated. Optical and magnetic properties of the heme groups in the oxidized forms of nitrite reductase, cytochrome cd1, from Pseudomonas stutzeri and Thiosphaera pantotropha. *Biochemistry*, 36, 16267-76. (1997)

82. Watmough, N. J., G. Butland, M. R. Cheesman, J. W. Moir, D. J. Richardson, and S. Spiro, Nitric oxide in bacteria: synthesis and consumption. *Biochim Biophys Acta*, 1411, 456-74. (1999)

83. Kuo, W., Kuo, TW., Jones, DL., Baptiste, J Jr., Nitric oxide synthaseimmunoreactivity in baker's yeast, lobster and wheat germ. *Biochemical Archives*, 11, 73-78. (1995)

84. Ninnemann, H. and J. Maier, Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photoconidiation of Neurospora crassa. *Photochem Photobiol*, 64, 393-8. (1996)

85. Beligni, M. V. and L. Lamattina, Nitric oxide: a non-traditional regulator of plant growth. *Trends Plant Sci*, 6, 508-9. (2001)

86. Beligni, M. V., A. Fath, P. C. Bethke, L. Lamattina, and R. L. Jones, Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiol*, 129, 1642-50. (2002)

87. Davis, K. L., E. Martin, I. V. Turko, and F. Murad, Novel effects of nitric oxide. *Annu Rev Pharmacol Toxicol*, 41, 203-36. (2001)

88. Hanafy, K. A., J. S. Krumenacker, and F. Murad, NO, nitrotyrosine, and cyclic GMP in signal transduction. *Med Sci Monit*, 7, 801-19. (2001)

89. Eiserich, J. P., M. Hristova, C. E. Cross, A. D. Jones, B. A. Freeman, B. Halliwell, and A. van der Vliet, Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature*, 391, 393-7. (1998)

90. Wu, W., Y. Chen, and S. L. Hazen, Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. *J Biol Chem*, 274, 25933-44. (1999)

91. van der Vliet, A., J. P. Eiserich, B. Halliwell, and C. E. Cross, Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem*, 272, 7617-25. (1997)

92. Souza, J. M., E. Daikhin, M. Yudkoff, C. S. Raman, and H. Ischiropoulos, Factors determining the selectivity of

protein tyrosine nitration. *Arch Biochem Biophys*, 371, 169-78. (1999)

93. Brennan, M. L., W. Wu, X. Fu, Z. Shen, W. Song, H. Frost, C. Vadseth, L. Narine, E. Lenkiewicz, M. T. Borchers, A. J. Lusis, J. J. Lee, N. A. Lee, H. M. Abu-Soud, H. Ischiropoulos, and S. L. Hazen, A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem*, 277, 17415-27. (2002)

94. Turko, I. V., S. Marcondes, and F. Murad, Diabetes-associated nitration of tyrosine and inactivation of succinyl-CoA:3-oxoacid CoA-transferase. *Am J Physiol Heart Circ Physiol*, 281, H2289-94. (2001)

95. Marcondes, S., I. V. Turko, and F. Murad, Nitration of succinyl-CoA:3-oxoacid CoA-transferase in rats after endotoxin administration. *Proc Natl Acad Sci U S A*, 98, 7146-51. (2001)

96. Gow, A. J., D. Duran, S. Malcolm, and H. Ischiropoulos, Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett*, 385, 63-6. (1996)

97. Ischiropoulos, H., Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys*, 356, 1-11. (1998)

98. Raina, A. K., G. Perry, A. Nunomura, L. M. Sayre, and M. A. Smith, Histochemical and immunocytochemical approaches to the study of oxidative stress. *Clin Chem Lab Med*, 38, 93-7. (2000)

99. Nakazawa, H., N. Fukuyama, S. Takizawa, C. Tsuji, M. Yoshitake, and H. Ishida, Nitrotyrosine formation and its role in various pathological conditions. *Free Radic Res*, 33, 771-84. (2000)

100. Beckman, J. S. and W. H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*, 271, C1424-37. (1996)

101. Estevez, A. G., N. Spear, S. M. Manuel, R. Radi, C. E. Henderson, L. Barbeito, and J. S. Beckman, Nitric oxide and superoxide contribute to motor neuron apoptosis induced by trophic factor deprivation. *J Neurosci*, 18, 923-31. (1998)

102. Halliwell, B., K. Zhao, and M. Whiteman, Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. *Free Radic Res*, 31, 651-69. (1999)

103. Hayashi, Y., Y. Sawa, S. Ohtake, N. Fukuyama, H. Nakazawa, and H. Matsuda, Peroxynitrite formation from human myocardium after ischemia-reperfusion during open heart operation. *Ann Thorac Surg*, 72, 571-6. (2001)

104. Galinanes, M. and B. M. Matata, Protein nitration is predominantly mediated by a peroxynitrite-dependent pathway in cultured human leukocytes. *Biochem J*, Pt. (2002)

105. Bian, K., Harari, Y., Lai, M., Phan, T., Mailman, R., Weisbrodt, N., Murad, F., Intestinal nitrotyrosine formation in rats. *Gastroenterology*, 116, A844. (1999)

106. Bian, K., Harari, Y., Zhong, M., Lai, M., Weisbrodt, N., Murad, F., Intestine protein nitration induced by myeloperoxidase. *Gastroenterology*, 120. (2001)

107. Bian, K., Harari, Y., Zhong, M., Weisbrodt, N., Murad, F., Tissie inflammation and tyrosine nitration: role of nitric oxide and myeloperoxidase in nitrotyrosine formation. *FASEB Journal*, 15, A199. (2001)

108. Lymar, S. V. and J. K. Hurst, Carbon dioxide: physiological catalyst for peroxynitrite-mediated cellular damage or cellular protectant? *Chem Res Toxicol*, 9, 845-50. (1996)

109. Viner, R. I., D. A. Ferrington, T. D. Williams, D. J. Bigelow, and C. Schoneich, Protein modification during biological aging: selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase in skeletal muscle. *Biochem J*, 340 (Pt 3), 657-69. (1999)

110. Bian, K. and N. Toda, Reoxygenation and calcium-induced cerebroarterial contractions as affected by vasodilator agents. *J Cereb Blood Flow Metab*, 8, 808-15. (1988)

111. Bian, K. and K. Hermsmeyer, Ca^{2+} channel actions of the non-dihydropyridine Ca^{2+} channel antagonist Ro 40-5967 in vascular muscle cells cultured from dog coronary and saphenous arteries. *Naunyn Schmiedebergs Arch Pharmacol*, 348, 191-6. (1993)

112. Bian, K. and R. D. Bukoski, Modulation of resistance artery force generation by extracellular Ca^{2+}. *Am J Physiol*, 269, H230-8. (1995)

113. Bian, K. and R. D. Bukoski, Myofilament calcium sensitivity of normotensive and hypertensive resistance arteries. *Hypertension*, 25, 110-6. (1995)

114. Bian, K., K. Ishibashi, and R. D. Bukoski, 1,25(OH)2D3 modulates intracellular Ca^{2+} and force generation in resistance arteries. *Am J Physiol*, 270, H230-7. (1996)

115. Di Salvo, J., S. R. Nelson, and N. Kaplan, Protein tyrosine phosphorylation in smooth muscle: a potential coupling mechanism between receptor activation and intracellular calcium. *Proc Soc Exp Biol Med*, 214, 285-301. (1997)

116. Bian K, W. N., Murad F., Ca^{2+}-channel protein nitration and intestinal smooth muscle contractility. *Gastroenterology*, 120, A200. (2001)

117. Podrez, E. A., D. Schmitt, H. F. Hoff, and S. L. Hazen, Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. *J Clin Invest*, 103, 1547-60. (1999)

118. Podrez, E. A., H. M. Abu-Soud, and S. L. Hazen, Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic Biol Med*, 28, 1717-25. (2000)

119. Paoli, M., J. Marles-Wright, and A. Smith, Structure-function relationships in heme-proteins. *DNA Cell Biol*, 21, 271-80. (2002)

120. Smith, A., Homeostasis of heme in health and disease: current aspects of the structural biology of heme-protein interactions and of gene regulation. *DNA Cell Biol*, 21, 245-9. (2002)

121. Ryter, S., E. Kvam, and R. M. Tyrrell, Heme oxygenase activity determination by high-performance liquid chromatography. *Methods Enzymol*, 300, 322-36. (1999)

122. Ryter, S. W. and R. M. Tyrrell, The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic Biol Med*, 28, 289-309. (2000)

Key Words: Nitric Oxide; Nitric Oxide Synthase; Organic Nitrate; Nitric Oxide Donor; S-Nitrosothiols; Nitrotyrosine; Peroxidase; Hemin; Ca^{2+} Channel, Review

Send correspondence to: Dr Ka Bian, Department of Integrative Biology, Physiology, and Pharmacology, The University of Texas-Houston Medical School, 6431 Fannin, Houston, TX 77030, Tel: 713-500-7488, Fax: 713-500-0790, E-mail: ka.bian@uth.tmc.edu