

Protein Nitration in Cardiovascular Diseases

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Abstract—There is growing evidence that cardiovascular disease is associated with progressive changes in the production of free radicals and radical-derived reactive species. These intermediates react with all major cellular constituents and may serve

several physiological and pathophysiological functions. The nitration of protein tyrosine residues has been used as a footprint for in vivo production of radical and nonradical reactive species. Tyrosine nitration may alter protein function and metabolism and therefore, provides for further dysfunctional changes. This review focuses on an appearance of tyrosine nitrated proteins in cardiovascular tissues under different settings of cardiovascular disease. Sources of reactive species, putative mechanisms of protein

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nitration *in vivo*, as well as protein nitration under normal physiological conditions, are also described. The goal of this review is to attract more attention to identification of specific proteins, which undergo tyrosine nitration and to study a correlation between their altered function and pathology. Understanding

how protein nitration affects disease progression may offer a unique option for design of antioxidant therapy for the treatment of cardiovascular complications. At the same time, protein nitration can be a biological marker of efficiency of antioxidant therapy.

I. Introduction

Protein tyrosine nitration is a well established post-translational modification occurring in a number of diseases (Greenacre and Ischiropoulos, 2001). Tyrosine nitration may affect protein structure and function. A gain of function, as well as no effect on function have been reported for some nitrated proteins (Gole et al., 2000; Balafanova et al., 2002). However, the inhibition of function is a more common consequence of protein nitration (Ischiropoulos, 1998; Greenacre and Ischiropoulos, 2001). It has also been shown that nitration of a tyrosine residue may prevent the subsequent phosphorylation of that residue (Gow et al., 1996; Kong et al., 1996). Alternatively, nitration of tyrosine residues may simulate phosphorylation (MacMillan-Crow et al., 2000; Mallozzi et al., 2001) and results in the constitutively active proteins. Furthermore, tyrosine nitration may change the rate of proteolytic degradation of nitrated proteins and favor either its faster clearance or the accumulation of nitrated proteins in cells. Cumulatively, this suggests that protein nitration may be involved in a variety of functions, possibly including disease initiation and progression.

Many recent studies in cardiovascular research have demonstrated that there is an accumulation of nitrated proteins in different settings of cardiovascular disease. In this review, the following topics will be outlined: i) sources and mechanisms of protein nitration *in vivo*, ii) protein nitration in the cardiovascular system under physiological and pathological conditions, and iii) therapeutic implication of protein nitration.

II. Oxidative Pathways in Cardiovascular Disease

A. Reactive Nitrogen and Oxygen Species

Nitric oxide (NO) and superoxide (O_2^-) are probably the most relevant free radicals in biology. They are readily converted by enzymes or nonenzymic chemical reactions into reactive nonradical species, which can in turn give rise to new radicals. Radical and nonradical reactive nitrogen species (RNS¹) include NO , nitrogen dioxide (NO_2), nitrous acid (HNO_2), nitrosyl cation (NO^+), nitrosyl anion (NO^-), dinitrogen tetroxide (N_2O_4), dinitrogen trioxide (N_2O_3), peroxyxynitrite (ONOO^-), peroxyxynitrous acid (ONOOH), alkyl peroxy-

trites (ROONO), nitronium cation (NO_2^+) and nitryl chloride (NO_2Cl). Radical and nonradical reactive oxygen species (ROS) include O_2^- , hydroxyl radical (HO^\bullet), peroxy radical (RO_2^\bullet), alkoxy radical (RO^\bullet), hydroperoxyl radical (HO_2^\bullet), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1\text{O}_2$). Pathways of RNS and ROS production and clearance are overlapped and cross-regulate each other. Protein nitration is caused by a different combination of RNS and ROS, and we will use a term RNS/ROS throughout the whole review to underline the involvement both of them in general oxidative mechanisms and more specifically, in the nitration of the tyrosine residues within proteins.

There is emerging evidence that increased RNS/ROS production make a significant contribution to the progression of cardiovascular disease (Patel et al., 2000b; Cuzzocrea et al., 2001; Wattanapitayakul and Bauer, 2001; Droge, 2002), but the actual sources of these reactive species and mechanisms involved may not be identical in different settings.

B. Sources of NO

NO is produced from L-arginine by the enzyme nitric-oxide synthase (NOS). There are three isoforms of NOS: neuronal NOS (nNOS or NOS-I) originally identified in brain, inducible NOS (iNOS or NOS-II) originally identified in macrophages, and endothelial NOS (eNOS or NOS-III) originally identified in endothelial cells. Constitutive nNOS and eNOS require calcium and calmodulin as cofactors and generate low amounts of NO . Constitutively expressed mitochondrial NOS was recently reported (Ghafourifar et al., 2001). Its activity is also regulated by calcium. The iNOS that is expressed in macrophages, endothelial cells, fibroblasts, vascular smooth muscle cells and cardiac myocytes in response to inflammatory cytokines, does not require calcium and calmodulin as cofactors. Furthermore, it generates substantially larger amounts of NO for long periods of time (Moncada et al., 1991; Nathan, 1992; Cannon et al., 1998; Zweier et al., 2001). The expression of iNOS is regulated both at the level of transcription and at the level of iNOS mRNA stability. Catalytic activity of iNOS is regulated by the availability of the substrate, L-arginine, and of the cofactors, NADPH and tetrahydrobiopterin. Induction of iNOS expression is complemented by co-induction of cationic amino acid transporter proteins (increase the intracellular L-arginine level) and GTP cyclohydrolase (key enzyme of tetrahydrobiopterin synthesis).

¹ RNS, reactive nitrogen species; ROS, reactive oxygen species; NOS, nitric-oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; SOD, superoxide dismutase.

Increased NO production via induction of iNOS has been suggested as a major mechanism by which cytokines mediate cardiac contractile dysfunction and development of cardiovascular disease. Indeed, iNOS mRNA and iNOS protein expression were demonstrated in many different settings of cardiovascular disease (Schulz et al., 1995; Wildhirt et al., 1995; Cannon et al., 1998; Sawyer and Colucci, 1998; Zweier et al., 2001).

C. Sources of O_2^-

The major sources of intracellular O_2^- are uncoupling of mitochondrial electron transport and cytosolic oxidases, such as NADH/NADPH oxidase and xanthine oxidase. NOS may also generate O_2^- under specific conditions.

The uncoupling of mitochondrial electron transport is a classical mechanism of oxidant production with the developing consensus that O_2^- generation is controlled by the level of reduction of the respiratory chain (Turrens, 1997). Under hypoxic or ischemic conditions, the lack of oxygen supply disrupts the mitochondrial electron transport chain, resulting in many adverse events (Lemasters et al., 1997). Reoxygenation or reperfusion causes a massive production of ROS due to the resumption of oxygen supply to mitochondrial respiration (Lesnfsky et al., 1997). In addition to being a major source of ROS, mitochondria are also a target for their damaging effects. The phenomenon is that oxidative stress can lead to dysfunctional mitochondria, and dysfunctional mitochondria may self-amplify damage by generating further free radicals (Zorov et al., 2000).

Nonmitochondrial sources of O_2^- are also critical in cardiovascular disease (Wattanapitayakul and Bauer, 2001). The neutrophil NADPH oxidase may generate millimolar quantities of O_2^- and is involved in nonspecific host defense during infection. This complex protein is both constitutive and induced by pro-inflammatory stimuli. Different enzymatic forms of the NADPH oxidase resemble those of NOS and display diversity in regulation and the amount of free radical formed (Levonen et al., 2001). Vascular NADH/NADPH oxidase is activated by angiotensin II and significantly contributes to O_2^- production in the pathogenesis of angiotensin II-induced cardiovascular disease (Griendling et al., 1994; Wattanapitayakul et al., 2000).

Xanthine oxidase, a metalloflavoprotein, is involved in the purine degradation pathway and generates O_2^- as a byproduct of its catalytic activity. Xanthine oxidase is an important source of O_2^- and has been implemented in the pathogenesis of injury following post-ischemic reperfusion. Its activity could be triggered by increased formation of the substrates, xanthine and hypoxanthine, due to ATP degradation during ischemia (Xia and Zweier, 1995). Chronic hypoxia or increased inflammatory cytokines can enhance xanthine oxidase activity and also cause its release into the plasma. It was shown, that the

elevated levels of circulating xanthine oxidase participate in endothelial dysfunction (Houston et al., 1999).

nNOS exhibits oxidase activity in the case of insufficient substrate or tetrahydrobiopterin supply (Heinzel et al., 1992; Pou et al., 1992). Cofactor-deficient nNOS cannot catalyze the five-electron oxidation of L-arginine to NO , but it can receive electrons from NADPH and donate them for one electron reduction of oxygen to O_2^- . Similar data were obtained with the inducible (Xia and Zweier, 1997) and endothelial (Vasquez-Vivar et al., 1998) NOS isoforms, demonstrating that enzymatic generation of O_2^- is a common feature of NOS. Implications of O_2^- generation from NOS in cardiovascular disease were reviewed (Vasquez-Vivar et al., 1999). However, recent studies (Xu, 2000a,b) suggested that NOS coenzyme and cofactors might cause O_2^- generation in the NOS-independent manner. These findings raise some question whether or not NOS actually does catalyze O_2^- formation.

D. Consequences of Oxidative Events

The net concentrations of NO at the tissue level may predict its protective or toxic effects. Many lines of evidence suggest that modulation of NO concentration will determine whether or not the roles played by RNS/ROS will be protective or detrimental to the cardiovascular system (Cannon et al., 1998; Ronson et al., 1999; McCarty, 2000; Patel et al., 2000a,b; Wattanapitayakul and Bauer, 2001; Zweier et al., 2001). Availability of NO is determined by the amounts produced and by the local chemical environment, which promotes either protection of NO by antioxidants or depletion of NO by O_2^- with RNS/ROS generation. Since protein tyrosine nitration is mainly detrimental to protein function, the major focus of this review will be on the adverse consequences of RNS/ROS generation.

Many studies conducted have illustrated that increased RNS/ROS production may be a unifying mechanism in cardiovascular disease progression. Adverse changes associated with RNS/ROS production have been found, essentially, at all levels of the cardiovascular system: including gene expression, signal transduction, energy metabolism, antioxidant defense and cell death (reviewed by Wattanapitayakul and Bauer, 2001). Molecular mechanisms of these oxidative events include post-translational modifications of proteins. Protein nitration is a prominent one, which attracts much attention (Ischiropoulos, 1998; Nakazawa et al., 2000; Greenacre and Ischiropoulos, 2001). However, it is not clear whether protein nitration and subsequent alteration of protein function contributes to progression of cardiovascular disease or simply reflects the presence of complications caused by oxidative stress.

III. Mechanisms of Protein Nitration in Vivo

Most of our knowledge regarding protein nitration is derived from in vitro experiments with albumin or free

tyrosine. The physiological relevance of these findings remains to be defined. Presumably, the nitration pathways *in vivo* are not mutually exclusive and may operate simultaneously. Given the complexity of biological systems, it is likely that the nitrating species responsible for protein nitration must be evaluated for every model of disease separately. Meanwhile, the mechanism(s) of *in vivo* nitration remains an area of active investigation and controversy (Beckman, 1996; Eiserich et al., 1996; Goldstein et al., 2000; Pfeiffer et al., 2000, 2001a,b; Reiter et al., 2000; Sawa et al., 2000; Zhang et al., 2001a). The most likely *in vivo* mechanisms for protein nitration are summarized in Fig. 1 and described below.

A. ONOO⁻-Dependent Tyrosine Nitration

Formation of ONOO⁻ by the diffusion-limited recombination of [•]NO with O₂⁻ is a reaction of considerable biological interest (Beckman et al., 1990; Radi et al., 2001). A second order rate constant of this reaction was independently determined as 4.3, 6.7, and 1.9 × 10⁹ M⁻¹ s⁻¹ (Huie and Padmaja, 1993; Goldstein and Czapski, 1995; Kissner et al., 1997). It has been suggested that ONOO⁻ can also be formed by the reaction of nitroxyl anion (NO⁻) with O₂ (Hogg et al., 1996). The latter reaction proceeds at a slower rate (5.7 × 10⁷ M⁻¹ s⁻¹) than that of [•]NO with O₂⁻. Perhaps, this reaction is physiologically relevant in some specific conditions since NO⁻ production from [•]NO could be catalyzed by cytochrome *c* (Sharpe and Cooper, 1998), and concentrations of O₂ *in vivo* are many orders of magnitude higher than concentrations of O₂⁻.

ONOO⁻ is a strong oxidant capable of modifying most biological molecules and compounds, including such amino acids as tyrosine, tryptophan, cysteine, and methionine (Radi et al., 1991; Alvarez et al., 1996, 1999). The detailed chemistry of the ONOO⁻-catalyzed reactions is beyond the scope of this review but can be found in other sources (Beckman and Koppenol, 1996; Koppenol, 1998; Squadrito and Pryor, 1998; Ducrocq et al., 1999; Radi et al., 2001). Nitration of free and protein-bound tyrosine to yield nitrotyrosine is a well established *in vitro* reaction of ONOO⁻. However, there has

been a debate over the physiological significance of these findings (Goldstein et al., 2000; Pfeiffer et al., 2000, 2001a,b). The major concern is that ONOO⁻ formation and ONOO⁻-catalyzed tyrosine nitration both require specific conditions that rarely occur in complex biological systems. For example, a requirement of precisely balanced rates of [•]NO and O₂⁻ production for ONOO⁻ formation (Pfeiffer and Mayer, 1998; Goldstein et al., 2000) or high concentrations of potentially ONOO⁻-specific scavengers in biological samples (Mayer et al., 1998). However, another study did not confirm the strict requirement of equimolar fluxes of [•]NO and O₂⁻ for oxidative pathways involving a direct reaction with ONOO⁻ (Jour'dheuil et al., 2001). In addition, direct reactions of ONOO⁻ with CO₂, transition metals, and superoxide dismutase (SOD) have been found to catalyze the nitration of tyrosine residues (Beckman et al., 1992; Ischiropoulos et al., 1992; Lymar et al., 1996). These reactions increase the rate of tyrosine nitration and may explain the ability of ONOO⁻ to nitrate proteins *in vivo* despite the presence of high concentrations of compounds, such as reduced glutathione, cysteine, or ascorbate, which act to inhibit radical formation and therefore prevent nitration.

A few more considerations could be helpful in discussing a role of ONOO⁻ in *in vivo* protein nitration. They include 1) tyrosine nitration in hydrophobic conditions and 2) tyrosine nitration in the absence of heme peroxidase.

1. Tyrosine Nitration in Hydrophobic Conditions. In terms of the chemistry of tyrosine nitration, it seems likely that the local environment of the targeted tyrosine residue may play a key role in determining the final outcome of the reaction. Although much is known about the chemistry of tyrosine nitration in aqueous solution, detailed investigations of the chemistry of tyrosine nitration in the hydrophobic interior of membranes or hydrophobic regions of proteins have only recently begun (Goss et al., 1999; Zhang et al., 2001a). [•]NO and other oxides of nitrogen are hydrophobic gases. They have higher solubility in hydrophobic solvents. This suggests that the concentration of RNS may be higher in a

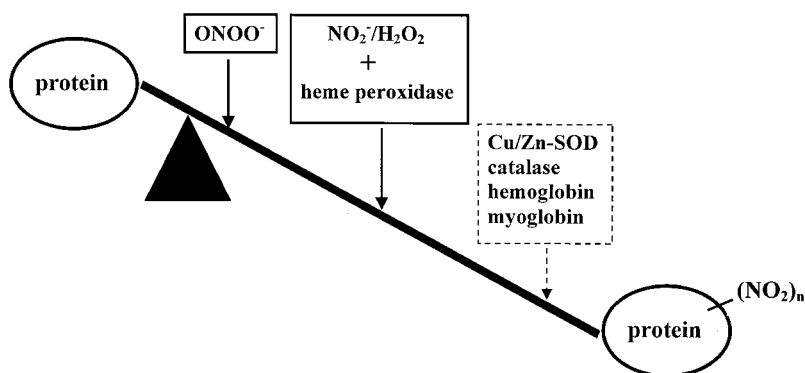


FIG 1. Protein nitration *in vivo*. ONOO⁻- and heme peroxidase-dependent protein nitrations are the most likely mechanisms. Other mechanisms, whose physiological relevance remains to be understood, include protein nitration catalyzed by some hemoproteins with pseudoperoxidase activity.

hydrophobic milieu. Even if the intrinsic rate constant of the RNS-mediated reaction within hydrophobic phase is the same as in the aqueous cytosol, the reaction is accelerated overall because of the increased reactant concentration and the lack of the hydrolysis reaction (Liu et al., 1998b). The ONOO⁻ can freely pass through lipid membranes, making ONOO⁻-mediated reactions in hydrophobic environment, such as cell membranes, organelles, lipoproteins, and sites buried in the protein tertiary structure, also of extreme relevance (Marla et al., 1997; Denicola et al., 1998; Boulos et al., 2000; Khairutdinov et al., 2000; Zhang et al., 2001a).

Another interesting observation is the stable tyrosyl free radical found in ribonucleotide reductase from different prokaryotes. This tyrosyl radical is essential for enzyme catalysis and located inside a rigid hydrophobic pocket (Ormo et al., 1995; Liu et al., 1998a). The remarkable stability of this radical (a half-life of several days at 25°C) is explained by the unique hydrophobic environment, which stabilizes the tyrosyl radical. The formation of the tyrosyl radical was suggested as a key element of tyrosine nitration. The possibility of stabilizing tyrosyl radical in the hydrophobic environment could be one more difference, which distinguishes tyrosine nitration in the hydrophobic environment from tyrosine nitration in the hydrophilic environment.

However, the argument that tyrosine nitration occurs in a hydrophobic environment is weakened by the fact that tyrosine residues mainly don't intend to be buried away from solvent. Although, there is not a universal method for measuring the relative affinities of amino acid residues for hydrophobic phases, the consensus of different approaches put the tyrosine residue in the middle of the hydrophobicity scale (Eisenberg, 1984). This limits the probability of the tyrosine residue appearing in the hydrophobic environment.

2. Tyrosine Nitration in the Absence of Heme Peroxidase. Both major mechanisms of protein tyrosine nitration, ONOO⁻- and heme peroxidase-dependent, probably overlap in vivo. However, specific conditions in vivo can favor one over the other. For example, it seems likely that mitochondria have no heme peroxidases. At the same time, the mitochondrial respiratory chain is a major source of O₂⁻. Considering the recent evidences for mitochondrial NOS (Giulivi et al., 1998; Ghafourifar et al., 2001), the intramitochondrial formation of ONOO⁻ is becoming apparent. Indeed, evidence for intramitochondrial ONOO⁻ formation was presented in recent publications (Ghafourifar et al., 1999; Valdez et al., 2000). Tyrosine nitration of mitochondrial proteins is also recognized (MacMillan-Crow et al., 1996, 2001; Park et al., 1999; Aulak et al., 2001; Riobo et al., 2001; Turko et al., 2001; Yamamoto et al., 2002).

Protein nitration was also observed under pathological conditions that were not associated with immune cell infiltration. For example, doxorubicin treatment caused cardiac accumulation of tyrosine nitrated proteins in

mice (Weinstein et al., 2000). At the same time, there was no histological evidence of neutrophil infiltration into cardiac tissue. These two observations together favor ONOO⁻ as a source of tyrosine nitrated proteins in the doxorubicin-treated mice.

B. Heme Peroxidase-Dependent Tyrosine Nitration

Besides ONOO⁻, it has become recognized that other reactions, such as nitrite-dependent heme peroxidase reactions also may give a rise to protein tyrosine nitration in vivo (Van der Vliet et al., 1997; Eiserich et al., 1998; Van Dalen et al., 2000; Pfeiffer et al., 2001b; Brennan et al., 2002). It has been shown that heme peroxidase enzymes (myeloperoxidases, eosinophil peroxidases, horseradish peroxidases) in the presence of nitrite and H₂O₂ can nitrate different proteins in heart homogenates (Sampson et al., 1998) or different pure proteins (Van der Vliet et al., 1997; Wu et al., 1999). This occurs through simultaneous oxidation of nitrite and tyrosine to nitrogen dioxide radical and tyrosyl radical, respectively. The subsequent reaction of these two radicals yields nitrotyrosine. Tyrosine nitration under these conditions was exclusively inhibited by catalase and azide (an myeloperoxidase inhibitor) but not by SOD. This suggests that the mechanism of tyrosine nitration is ONOO⁻-independent.

Protein tyrosine nitration could be achieved by the direct oxidation of nitrite by H₂O₂, but this reaction requires nonphysiological concentrations of H₂O₂. Alternatively, nitrate can be oxidized by myeloperoxidase-derived hypochlorous acid to form nitryl chloride, which is capable of nitrating protein tyrosine residues (Eiserich et al., 1996; Panasencko et al., 1997). However, other studies (Sampson et al., 1998; Ohshima et al., 1999) did not confirm physiological relevance of this reaction.

Nitrate balance studies consistently conclude that a greater amount of nitrite is excreted than can be accounted for by ingestion. Therefore, there are endogenous sources of nitrite production, namely NO and the products of NO metabolism (Oldreive and Rice-Evans, 2001). For example, in the vascular system, NO is rapidly oxidized to nitrate by reaction with oxyhemoglobin or methemoglobin (Radi, 1996). The reaction of ONOO⁻ with a wide variety of biomolecules results in the production of nitrite (Pryor and Squadrito, 1995). Since O₂⁻ is a precursor of H₂O₂, it appears that both major nitrating reactions in vivo share the same sources of substrates. However, their relative contribution to nitrotyrosine formation may vary depending on inflammatory models (Brennan et al., 2002).

The discrimination between these two mechanisms of tyrosine nitration could be mainly associated with infiltration of activated phagocytes, which contain high levels of heme peroxidases. Activated phagocytes, such as eosinophils and neutrophils or monocytes, play a central role in host defense mechanisms. However, the reactive

intermediates formed by these cells also can harm normal tissue and contribute to inflammatory injury. Myeloperoxidase and eosinophil peroxidase are the most abundant proteins in the activated phagocytes, and the state of phagocytic activation has been described as one of the early events in cardiovascular disease (Zahler et al., 1999; Frangogiannis et al., 2002). The infiltration of activated phagocytes during chronic settings of cardiovascular disease is not well established (Wattanapitayakul and Bauer, 2001). It is most likely that chronic settings favor the ONOO⁻-dependent mechanism of protein nitration over the heme peroxidase-dependent.

C. Other Putative Mechanisms

Other mechanisms relevant to in vivo conditions have been described (McBride et al., 1999; Zhang et al., 2000; Grzelak et al., 2001; Kilinc et al., 2001; Ogino et al., 2001). These mechanisms of protein nitration vary slightly from those described above and are based on pseudoperoxidase activity of hemoproteins, such as copper/zinc superoxide dismutase (Cu/Zn-SOD), catalase, hemoglobin, and myoglobin. Perhaps, these reactions reflect the putative toxicity of hemoproteins as the potent oxidants capable of generating RNS/ROS and promoting oxidative damage.

Despite the obvious protective role of different SOD, the ability of SOD to produce strong oxidants can be damaging to cells. Initially, it was believed that H₂O₂ was metabolized by Cu/Zn-SOD to form hydroxyl radicals, which serve as a source of oxidative damage (Yim et al., 1993). Recent studies showed that tyrosine nitration could play a part in this damage. In the presence of bicarbonate (HCO₃⁻), Cu/Zn-SOD may act as a peroxidase in the physiological pH range (Sankarapandi and Zweier, 1999). HCO₃⁻ is abundantly present in biological systems and can dramatically alter the nitrating ability of RNS/ROS (Lymar et al., 1996). It was proposed that in the presence of nitrite/H₂O₂, HCO₃⁻ increases the peroxidase activity of Cu/Zn-SOD (Zhang et al., 2000). This causes generation of nitrogen dioxide and carbonate anion radicals with subsequent oxidation and nitration of tyrosine residues. These reactions may generate multiple tyrosine derivatives, including nitrotyrosine. Another study showed that Cu/Zn-SOD or Mn-SOD in the presence of [•]NO/H₂O₂ caused nitration of phenol and oxidation of dihydrorhodamine-1,2,3 to rhodamine-1,2,3 (McBride et al., 1999). The latter was interpreted as production of ONOO⁻. Collectively, these studies suggest an alternative mechanism, which may have in vivo implications to protein nitration.

Catalase is a heme peroxidase ubiquitously expressed throughout mammalian tissues that is involved in protecting cells from oxidative stress. Catalase can catalyze in vitro nitration of free tyrosine or tyrosine residues of bovine serum albumin in the presence of azide/H₂O₂ (Ogino et al., 2001). Oxidation of azide by the catalase/H₂O₂ system can generate azidyl radicals. Subsequent

reaction of the azidyl radicals with oxygen generates [•]NO. The involvement of these products in the catalyzed-dependent tyrosine nitration as well as its physiological relevance remains to be understood.

Hemoglobin, the main component of the erythrocyte, is a ONOO⁻ scavenger of physiological relevance (Minetti et al., 2000). However, hemoglobin exhibits different enzymatic activities (Giardina et al., 1995), including the pseudoperoxidase activity (Bao and Williamson, 1997; Alayash et al., 2001). Incubation of human hemoglobin with nitrite/H₂O₂ was found to induce self-nitration and nitration of bovine serum albumin (Grzelak et al., 2001). The hemoglobin-catalyzed nitration is not enhanced by HCO₃⁻ and is inhibited by cyanide. Presumably, hemoglobin may behave like peroxidase and perform tyrosine nitration by the mechanism analogous to that of peroxidase. This catalytic property of hemoglobin seems to be very important for protein nitration in circulating erythrocytes. However, there is controversy concerning the appearance of tyrosine nitrated proteins there, since a recent report showed no accumulation of nitrated proteins in the circulating erythrocytes (Kikugawa et al., 2000).

Another hemoprotein capable of catalyzing nitrotyrosine formation is the myoglobin (Kilinc et al., 2001). This reaction is nitrite/H₂O₂-dependent with a pH optimum of approximately 6.0. Most likely, it may occur under acidic pH and low oxygen tension produced during myocardial ischemia.

D. Selectivity of Protein Nitration

Apart from the mechanism of tyrosine nitration, the selectivity of protein nitration is also a subject of interest. It has been shown that the process of tyrosine nitration is residue-, protein-, and tissue-specific: not all tyrosine residues of a protein are nitrated and not all proteins are targets for nitration (Ischiropoulos, 1998; Souza et al., 1999). Certain proteins can be preferentially targeted for nitration. This selectivity may depend not only on the composition and structure of a given target, but also on its intracellular concentration, localization, and interaction with other molecules.

IV. Protein Nitration under Physiological Conditions

A. Oxidative Modification of Proteins and Redox Regulation

RNS/ROS exist in biological cells and tissues at low concentrations under normal physiological conditions and are involved in the redox regulation of many physiological functions (Droge, 2002). The balance between their rates of production and their rates of clearance determines their concentrations by various antioxidant compounds and enzymes. Redox regulation requires that this balance be changed, either by an increase in RNS/ROS production or a decrease in the activity of the

antioxidant system. There are several mechanisms for reestablishing the original redox state after such a temporary imbalance. Elevated RNS/ROS concentrations typically induce the expression of genes whose products exhibit antioxidative activity. Moreover, the rate of RNS/ROS synthesis is regulated by different feedback mechanisms, for example by direct inhibition of NOS by NO (Abu-Soud et al., 1995). The expression of iNOS is also regulated at the transcriptional and post-transcriptional level by signaling pathways that involve redox-responsive agents such as the transcriptional factor nuclear factor- κ B or mitogen-activated protein kinases (MacMicking et al., 1997).

Redox regulation under physiological conditions is often associated with oxidative derivatization of proteins. For example, certain signaling cascades involving protein tyrosine kinases can be enhanced by oxidative inhibition of protein tyrosine phosphatases (Hardwick and Sefton, 1995). All protein tyrosine phosphatases share a common sequence motif with a catalytically essential cysteine residue in the active center that can be inactivated by H_2O_2 (Denu and Tanner, 1998). H_2O_2 can also enhance the stimulation of the insulin receptor tyrosine kinase activity by insulin (Schmid et al., 1999). This redox effect is probably mediated by the oxidative derivatization of any of four cysteine residues in the tyrosine kinase domain of this membrane receptor.

B. Protein Nitration

Modification of cysteine residues described above represents a common mechanism of redox regulation. Nitration of tyrosine residues in proteins may also be important in redox regulation under physiological conditions. Nitration of tyrosine residues in proteins induces the change of tyrosine into a negatively charged hydrophilic nitrotyrosine moiety and causes a marked shift of the local pK_a of the hydroxyl group from 10.07 in tyrosine to 7.50 in nitrotyrosine. This is expected to change the function of a protein. A gain of function as well as no effect on function were reported for tyrosine nitrated proteins (Gole et al., 2000; Balafanova et al., 2002); however, the inhibition of function is a much more common consequence of protein tyrosine nitration (Ischiropoulos, 1998; Greenacre and Ischiropoulos, 2001). Nitration of a tyrosine residue may also prevent further phosphorylation of that residue (Gow et al., 1996; Kong et al., 1996). Alternatively, nitration of tyrosine residues may simulate phosphorylation (MacMillan-Crow et al., 2000; Mallozzi et al., 2001) and results in the constitutively active proteins. There is also evidence that tyrosine nitration may mimic regulatory cyclic adenylation of a specific tyrosine residue (Berlett et al., 1996, 1998).

Protein tyrosine nitration has been detected in numerous tissues under apparently normal physiological conditions (Greenacre and Ischiropoulos, 2001). In the cardiovascular system, basal protein nitration was

found in all major types of cells, such as myocytes, endothelial cells, fibroblasts, and vascular smooth muscle cells (Davidge et al., 1998; Frustaci et al., 2000; Kajstura et al., 2001). Basal protein nitration was also found in plasma (Khan et al., 1998; Marfella et al., 2001). Some of these nitrated proteins were identified. Myofibrillar creatine kinase (Mihm et al., 2001a), prostacyclin synthase in coronary arteries (Zou et al., 1999), and heart succinyl-CoA:3-oxoacid CoA-transferase (Turko et al., 2001) were demonstrated to be nitrated under normal physiological conditions. Several structural proteins, such as myosin heavy chain, α -actinin, and desmin were also found nitrated in control atrial myocytes (Mihm et al., 2001b). These data are consistent with the emerging perspective that low levels of tyrosine nitration may be a physiological regulator of a signaling pathway.

C. Feedback Regulation

It is widely accepted that nitration of tyrosine residues *in vivo* is derived from enzymatically produced NO. This implies that tyrosine nitration is a critical component of NO biochemistry and could function as a negative feedback modulator of NO production. The recent study on the murine lung epithelial cells (Robinson et al., 2001) demonstrated that ONOO⁻ treatment causes accumulation of nitrotyrosine in iNOS and inhibits NO production. ONOO⁻-dependent inhibition of iNOS may be a mechanism of attenuating iNOS activity at inflammatory sites *in vivo*. ONOO⁻ can also inhibit the activity of xanthine oxidase and O₂⁻ production (Lee et al., 2000). Down-regulation of xanthine oxidase activity may serve as the feedback to limit further ONOO⁻ formation. Presumably, many other proteins associated with the RNS/ROS functions can be regulated by tyrosine nitration.

D. Rationale for "Denitrase"

Protein nitration occurs under normal physiological conditions and affects the function of many proteins. To be a regulatory mechanism, protein nitration requires reversibility. Indeed, putative denitrase activity was demonstrated in several publications (Gow et al., 1996; Kamisaki et al., 1998; Kuo et al., 1999, 2002). This activity was monitored by the decreased intensity of nitrotyrosine immunoreactive bands in Western blots and increased nitrate levels in reaction mixtures. However, neither an enzyme catalyzing this reaction nor a product of this reaction was identified. The reversibility of protein tyrosine nitration remains to be elucidated. Meanwhile, the biological rationale for this type of enzymatic activity is summarized below.

In the presence of NAD(P)H and a corresponding reductase the nitrotyrosine could be enzymatically reduced to the nitro anion radical (Krainev et al., 1998). The nitro anion radical is then oxidized by molecular oxygen to yield O₂⁻ and regenerate nitrotyrosine. Thus, once formed *in vivo*, nitrotyrosine may cause depletion of

NAD(P)H and promote repetitive redox cycling with excessive O_2^- generation.

Instead of repair, the proteolytic degradation of tyrosine nitrated proteins may discharge nitrated tyrosine residues. Indeed, the accelerated degradation of mildly oxidized proteins is a normal cellular function. However, extensively oxidized proteins are poor substrates for proteases and may accumulate in cells (Davies, 2001). Furthermore, nitration of tyrosine can change chymotrypsin-like proteolytic selectivity. For example, chymotrypsin was found to be capable of cleavage next to nitrated tyrosine residues but at a considerably slower rate than next to unmodified tyrosine residues (Souza et al., 2000).

Proteolytic degradation of tyrosine nitrated proteins actually results in the appearance of free nitrotyrosine. Free nitrotyrosine in vivo could also be derived from direct nitration of free tyrosine. The levels of free nitrotyrosine vary in different tissues, (Greenacre and Ischiropoulos, 2001) but were detected everywhere. It was demonstrated that systematic administration of free nitrotyrosine markedly attenuates the subsequent hemodynamic responses to α 1- and β -adrenoceptor agonists in anesthetized rats (Kooy and Lewis, 1996). Inhibition of the hemodynamic action of angiotensin II by free nitrotyrosine may be involved in the pathogenesis of inflammatory conditions, such as atherosclerosis, ischemia-reperfusion, and sepsis, where tyrosine nitration is favored (Kooy and Lewis, 1996). It was also shown that physiological concentrations of free nitrotyrosine can induce vascular and endothelial dysfunction of rat thoracic aorta segments in vitro (Mihm et al., 2000).

Review of possible adverse functions of protein-bound or free nitrotyrosine assumes an apparent need for repair of this modification and warrants further research on putative denitrases.

V. Protein Nitration in Cardiovascular Disease

A. Cardiovascular Inflammation

The induction of iNOS in response to pro-inflammatory cytokines or endotoxin (bacterial lipopolysaccharide) has been implicated in cardiovascular dysfunction. The production of large amounts of 1NO during inflammatory challenge leads to the formation of RNS/ROS capable of oxidizing many biological molecules including protein tyrosine nitration. Human autopsy specimens obtained from patients with a diagnosis of sepsis demonstrated intense nitrotyrosine immunoreactivity in the endocardium, myocardium, and coronary vascular endothelium and smooth muscle (Kooy et al., 1997). Following endotoxin or interleukin- 1β treatment, tyrosine nitrated proteins were found in myocardium (Oyama et al., 1998; Cheng et al., 1999), aorta (Szabo et al., 1995), plasma (Kamisaki et al., 1997), and cultured cardiomyocytes (Combes et al., 2001). Immunohistochemical studies showed a co-induction of iNOS, cyclooxygenase

and protein tyrosine nitration in endocardial endothelium and coronary arteriole endothelium in rabbits after endotoxin administration (Mebazaa et al., 2001). The treatment with NOS inhibitors prevented tyrosine nitration. Cytokine-induced myocardial dysfunction was also associated with overproduction of O_2^- (Cheng et al., 1999). These data indicate that changed equilibrium between 1NO and O_2^- is involved in the pathogenesis of the cardiovascular inflammation. Furthermore, the time course studies of several pro- and anti-oxidant variables throughout endotoxemia indicate that endotoxin-induced myocardial dysfunction is caused by the sum of complex interactions between various oxygen- and nitrogen-derived radicals (Iqbal et al., 2002). Altered protein functions caused by tyrosine nitration could be a portion of this pathogenesis.

B. Autoimmune Myocarditis

Acute viral myocarditis is a potentially lethal disease in humans. Autoimmune myocarditis, an experimental model for human postviral heart disease, could be induced in laboratory animals by injection of cardiac myosin. It was shown that autoimmune heart disease is accompanied by iNOS expression and accumulation of tyrosine nitrated proteins in inflammatory macrophages as well as in cardiomyocytes (Bachmaier et al., 1997; Ishiyama et al., 1997; Shin et al., 1998). Focal myocarditis was sufficient to induce nitrotyrosine formation throughout the whole heart muscle (Bachmaier et al., 1997). Aminoguanidine, the iNOS inhibitor, prevented myocardial destruction, inflammatory cell infiltration and decreased immunostaining for tyrosine nitrated proteins.

C. Heart Failure

The failing heart displays a disruption of fundamental regulatory processes. Among them is a balance between generation of 1NO and ROS (Saavedra et al., 2002). Altered cross talk between 1NO and oxidative stress may cause protein nitration. Indeed, extensive cardiac protein nitration was demonstrated in multiple settings of cardiac failure (Ferdinandy et al., 2000; Cesselli et al., 2001; Feng et al., 2001; Mihm et al., 2001a). iNOS is expressed in the myocardium after myocardial infarction and in heart failure. Studies on the iNOS(-/-) mutant and wild-type mice demonstrated that iNOS expression after myocardial infarction causes myocardial dysfunction and results in higher mortality in wild-type compared with iNOS(-/-) mutant mice. At the same time, myocardial infarction significantly increased the levels of myocardial and plasma nitrotyrosine (Feng et al., 2001). Accumulation of tyrosine nitrated proteins was also coupled with apoptotic cell death in the paced dog heart. Myocyte, endothelial cell, and fibroblast apoptosis was detected before the impairment of cardiac function became apparent. Cell death increased with the

duration of pacing and followed progressively increased nitrotyrosine formation (Cesselli et al., 2001).

Statins, hydroxymethylglutaryl coenzyme A reductase inhibitors, attenuate angiotensin II-induced cellular signaling. Cerivastatin improved left ventricular remodeling after myocardial infarction and decreased the nitrotyrosine protein level in rats (Bauersachs et al., 2001).

Not much is known about specific proteins, which undergo nitration and which altered function may contribute to myocardial dysfunction. Mihm et al. (2001a) demonstrated that myofibrillar creatine kinase is highly sensitive to nitration in cardiac failure in vivo. The myofibrillar isoform of creatine kinase is an important controller of myocyte contractility. The myocyte contraction depends upon complex and tightly regulated high-energy phosphate production and utilization. The energetics of myocyte contraction are severely altered in myocardial dysfunction. Increased oxidative stress has been implicated in the pathology of multiple cardiac disease states. Nitration of critical tyrosine residues in the active site of creatine kinase and impairment of its catalytic activity could be a link between increased oxidative stress and myocardial dysfunction (Mihm et al., 2001b, 2002).

D. Ischemia-Reperfusion Injury

Reperfusion of ischemic myocardium is the definitive treatment to attenuate myocardial injury. Unfortunately, reperfusion itself causes additional tissue damage mediated by several factors including inflammatory response and consequently altered production of RNS/ROS (Wang and Zweier, 1996; Liu et al., 1997; Yasmin et al., 1997; Zweier et al., 2001). RNS/ROS generation can cause oxidative modifications of proteins. This could be a critical factor in post-ischemic myocardial injury.

Although the molecular mechanisms of injury remain to be elucidated, many studies showed that repetitive episodes of ischemia-reperfusion caused an increased formation of nitrotyrosine in cardiac tissue. Furthermore, various competitive inhibitors of the NOS enzyme have been shown to reduce the level of cellular protein nitration and to reduce reperfusion injury in various settings (Wang and Zweier, 1996; Liu et al., 1997; Yasmin et al., 1997; Mori et al., 1998; Hayashi et al., 2001; Zhang et al., 2001b; Zweier et al., 2001; Baker et al., 2002). Preconditioning of isolated rat hearts before subsequent ischemia-reperfusion also reduced formation of free nitrotyrosine measured in the perfusate (Csonka et al., 2001). All these studies support a role of protein tyrosine nitration in the genesis of post-ischemic myocardial injury. However, little is known about specific protein targets for nitration.

A recent publication (Zou and Bachschmid, 1999) implicates prostacyclin synthase. Prostacyclin synthase, an enzyme with antithrombotic, antiproliferative, and dilatatory functions in the normal vasculature, was found

to be nitrated and inactivated in isolated bovine coronary arteries following hypoxia-reoxygenation. The administration of NOS inhibitors or SOD prevented nitration and inactivation of enzyme and abolished coronary vasospasm induced by hypoxia-reoxygenation (Zou and Bachschmid, 1999). The current conclusion is that nitration and inactivation of prostacyclin synthase results in accumulation of unmetabolized prostaglandin H₂, which causes the observed vasospasm.

E. Cardiac Allograft Rejection

Cardiac transplantation is an effective therapy for end-stage heart failure. However, cardiac allograft rejection remains a problem and is the leading cause of death in cardiac transplant recipients after the first year. It is broadly accepted that the immunological and inflammatory reactions in the myocardium are the major component of the pathological changes observed during cardiac allograft rejection, but the molecular mechanisms, which ultimately cause rejection, are not completely understood. There is a large body of evidence that the death of cardiac myocytes is the hallmark of cardiac allograft rejection and that NO produced by macrophages infiltrating the myocardium or by the cardiac myocyte itself is potentially cytotoxic to heart muscle cells (Szabolcs et al., 1996; Cannon et al., 1998).

During cardiac allograft rejection, there is significant release of cytokines as a part of the immune response to foreign antigens present in the cells of transplanted heart. Cytokines cause expression of iNOS, which generates large amounts of NO for long periods of time. iNOS mRNA, iNOS enzyme activity, and immunostaining for iNOS protein were increased in macrophages, endothelial cells, vascular smooth muscle cells, and cardiac myocytes in rejected cardiac allografts (Szabolcs et al., 1996, 1998; Sakurai et al., 1999; Akizuki et al., 2000; Wildhirt et al., 2001). All these studies also demonstrated the accumulation of tyrosine nitrated proteins, suggesting that tyrosine nitration may play a role in the rejection process. Experiments with iNOS inhibitors, O₂⁻ scavengers, and iNOS knockout mice have proved an association of cardiac myocyte death with iNOS expression and nitration of myocyte proteins (Szabolcs et al., 1998, 2001; Sakurai et al., 1999; Akizuki et al., 2000; Wildhirt et al., 2001).

F. Transplant Coronary Artery Disease

Transplant coronary artery disease is a major cause of late mortality after cardiac transplantation in humans. Studies on tissue sections from patients with transplant coronary artery disease revealed iNOS expression in neointimal macrophages and smooth muscle cells. Normal coronary arteries had no evidence of iNOS expression. Similar to the setting of acute and chronic cardiac allograft rejection, iNOS expression in human arteries with transplant coronary artery disease was associated with extensive nitration of protein tyrosines (Ravalli et

al., 1998). Studies on atherosclerotic lesions from patients with transplant coronary artery disease revealed colocalization of two enzymes involved in the inflammatory response, iNOS and cyclooxygenase-2. Protein nitrotyrosine was found in the same distribution as that of iNOS and was colocalized with cyclooxygenase-2 in macrophages (Baker et al., 1999). These findings indicate that protein tyrosine nitration might be involved in the process leading to the development of transplant coronary artery disease.

G. Hypertension

Various experimental models of hypertension, including genetic and induced by angiotensin II or by aortic banding, have implied that this pathophysiological state is associated with endothelial dysfunction, increased O_2^- production, and an apparent decrease in the production of 'NO. Molecular mechanisms involved in the development of endothelial dysfunction as well as the effect of hypertension on iNOS expression and activity remain to be fully elucidated. Analysis of O_2^- production, iNOS expression, and protein tyrosine nitration in aortas from aortic-banded rats revealed that an enhanced O_2^- production alone is not sufficient to produce endothelial dysfunction. Substantial changes were observed only when iNOS expression and O_2^- production were increased and were associated with the enhanced protein tyrosine nitration (Bouloumie et al., 1997). Abdominal aortic coarctation above the renal arteries leads to severe hypertension proximal to the site of stenosis. Western blot analysis with anti-nitrotyrosine antibody revealed marked increase in nitrotyrosine abundance in the heart and the aorta segment proximal to the stenotic site in aortic-banded rats (Barton et al., 2001). The enhanced protein tyrosine nitration after the exposure to ONOO⁻ also was found in aortas from hypertensive rats compared with normotensive Wistar-Kyoto rats (Cassasi et al., 2001).

Angiotensin II is a natural regulator of blood pressure and a well recognized participant in many cardiovascular diseases (Stroth and Unger, 1999). It was shown that increased gene expression of several subunits of NADH/NADPH oxidase and subsequent generation of oxygen-derived free radicals (particularly O_2^-) are involved in the vascular response to angiotensin II (Pagano et al., 1998), and this may be an important component of angiotensin II-mediated cardiovascular disease (Rajagopalan et al., 1996; Laursen et al., 1997). Although the role of angiotensin II in cardiovascular disease is established, the molecular mechanisms by which it participates have not been elucidated. Recent studies have shown that oxidant stress response to angiotensin II includes extensive tyrosine nitration of proteins in the vascular endothelium (Wattanapitayakul et al., 2000; Wang et al., 2001). This protein nitration correlates with the extent of endothelial dysfunction observed and is probably associated with increased production of ONOO⁻ at the early stage

of angiotensin II action. Angiotensin II is a peptide, and it could be a target for tyrosine nitration caused by ONOO⁻. Studies on in vitro tyrosine nitration of angiotensin II demonstrated that nitration of the tyrosine residue totally inhibits vasoconstrictive properties of angiotensin II in vivo (Ducrocq et al., 1998).

Studies on rat model of lead-induced hypertension point to enhanced ROS-mediated inactivation of 'NO with sequential increase of abundance of tyrosine nitrated proteins in many tissues, including heart (Vaziri et al., 1999). Concomitant administration of vitamin E ameliorated hypertension and tissue levels of nitrotyrosine. The beneficial effects of vitamin E support the role of increased RNS/ROS activity in the pathogenesis of hypertension.

H. Atherosclerosis

The pro- and anti-atherogenic role of 'NO is broadly reviewed (Patel et al., 2000a,b). One explanation for pro-atherogenic role is the modification of proteins and lipids caused by RNS/ROS derived from altered 'NO metabolism. A series of studies demonstrated protein nitration in human atherosclerotic tissue (Beckman et al., 1994; Buttery et al., 1996; Leeuwenburgh et al., 1997; Luoma et al., 1998; Cromheeke et al., 1999; Depre et al., 1999; Hunter et al., 1999). Protein tyrosine nitration was associated with iNOS expression and detected in iNOS-positive macrophage-rich lesions at different stages of atherosclerosis (Luoma et al., 1998; Cromheeke et al., 1999). Furthermore, iNOS and nitrotyrosine immunoreactivity were detected in complex heterogeneous cellular plaques, in relatively acellular fibrous plaques, and in myointimal plaques (Hunter et al., 1999). The presence of iNOS and nitrotyrosine in plaque also correlated with plaque instability in patients (Depre et al., 1999; Hunter et al., 1999). Presumably, tyrosine nitrated proteins with altered function may promote atherogenesis, counteracting the well established anti-atherogenic effects of 'NO.

Specific protein targets for nitration in atherosclerosis remain to be identified. A recent study (Zou et al., 1999) on bovine atherosclerotic arteries revealed tyrosine nitration of prostacyclin synthase. This study focused on the early stages of atherosclerosis, when arteries display focal thickening without signs of necrosis or rupture of plaques. It is likely that earlier nitration and inactivation of prostacyclin synthase and subsequent accumulation of pro-thrombotic prostaglandin H_2 may predispose further platelet aggregation and thrombus formation.

I. Diabetes

Diabetes causes early development of cardiovascular complications (Grundy et al., 1999). There is emerging evidence that RNS/ROS make a significant contribution to the progression of diabetes and its complications (Honing et al., 1998; Rosen et al., 2001). Several recent publications focused on diabetes-associated protein ni-

tration (Frustaci et al., 2000; Ceriello et al., 2001, 2002a,b; Kajstura et al., 2001; Marfella et al., 2001; Turko et al., 2001). It was shown that the apoptosis of myocytes, endothelial cells, and fibroblasts in heart biopsies taken from diabetic patients is associated with intracellular levels of nitrotyrosine (Frustaci et al., 2000). A positive correlation between accumulation of nitrotyrosine and myocyte apoptosis in the diabetic heart was also demonstrated (Kajstura et al., 2001).

Furthermore, perfusion of isolated rat hearts in conditions of high glucose concentration was accompanied by the formation of nitrotyrosine and evident cardiac cell apoptosis (Ceriello et al., 2002a). These support the concept of oxidative stress as a mediator of the vascular damage caused by hyperglycemia. These also consider protein tyrosine nitration as a marker of oxidative damage in diabetes. Marfella et al. (2001) showed that acute hyperglycemia in normal subjects causes an oxidative stress as evidenced by the raised circulating protein nitrotyrosine levels during the hyperglycemic clamp. Ceriello et al. (2001) demonstrated that nitrotyrosine plasma levels were correlated with plasma glucose concentrations in type II diabetic patients. They also demonstrated that postprandial hyperglycemia is accompanied by nitrotyrosine generation (Ceriello et al., 2002b). These observations may have important implications for the pathogenesis of vascular dysfunction in diabetes, if the pathway(s) for the increase of protein-bound nitrotyrosine levels will be established.

Recently, we found that the mitochondrial protein succinyl-CoA:3-oxoacid CoA transferase undergoes tyrosine nitration in the rat heart following streptozotocin administration (Turko et al., 2001). To our knowledge, this is the first study to identify the increase of tyrosine nitration of a specific protein in diabetes. Succinyl-CoA:3-oxoacid CoA transferase is located in the mitochondrial matrix and catalyzes the formation of acetoacetyl-CoA from acetoacetate (Laffel, 1999). This is the rate-determining step of ketone body conversion into acetyl-CoA, which subsequently enters the citric acid cycle. Diabetes is associated with a variety of abnormalities in myocardial energy metabolism (Sato et al., 1995). Accumulating evidence has implicated changes in myocardial energy substrate use as a contributing factor to diabetes-associated cardiomyopathies (Stanley et al., 1997). Our finding that succinyl-CoA:3-oxoacid CoA transferase undergoes tyrosine nitration and exhibits lower catalytic activity in the diabetic heart (Turko et al., 2001) is consistent with the postulated shift in the source of acetyl-CoA for the citric acid cycle in diabetic hearts (Stanley et al., 1997).

Endothelial dysfunction is a critical initial factor in the development of diabetic vascular disease (Laight et al., 2000). Exposure of human aortic endothelial cells to high glucose (30 or 44 mM) results in tyrosine nitration and inactivation of prostacyclin synthase (Zou et al., 2002). This can change thromboxane/prostaglandin H₂

receptor stimulation and explain an increased endothelial apoptosis in diabetes.

J. Cigarette Smoking

Cigarette smoking, as well as secondhand smoke, is considered a risk factor for cardiovascular disease, but the mechanism of the adverse effect of smoking is not fully understood. Cigarette smoke contains abundant free radicals including [•]NO. A shared feature among cardiovascular disease risk factors is the generation of increased RNS/ROS. Hence, cigarette smoke may induce some of its damaging effects by free radical mechanisms. It was shown that exposure to cigarette smoke extracts, prepared by bubbling the gas phase of smoke into phosphate-buffered saline, converts free tyrosine to nitrotyrosine (Yamaguchi et al., 2000). Exposure of plasma to gas-phase cigarette smoke causes depletion of antioxidants, induces lipid peroxidation, and is capable of converting tyrosine to nitrotyrosine in proteins (Eiserich et al., 1995). Human plasma proteins, such as fibrinogen, transferrin, plasminogen, and ceruloplasmin, were found to have tyrosine nitrated residues in active smokers (Pignatelli et al., 2001). Studies on the platelets from chronic smokers demonstrated intra-platelet nitrotyrosine formation, which was associated with increased platelet aggregation and with lower intra-platelet levels of reduced glutathione and ascorbate (Takajo et al., 2001). Oral administration of ascorbate to smokers restored these parameters compared with the nonsmokers group. The data suggest that cigarette smoke may cause damage of biomolecules, including tyrosine nitration of proteins, and that endogenous antioxidants can attenuate some of these adverse effects.

A recent study implicated tyrosine nitration of mitochondrial MnSOD in hearts from mice exposed to cigarette smoke (Knight-Lozano et al., 2002). Exposure to cigarette smoke also caused increased mitochondrial DNA damage. These data together support the concept that intramitochondrial RNS/ROS levels increase with cardiovascular disease risk factor, cigarette smoking. Chronic exposure to cigarette smoke could ultimately result in mitochondrial dysfunction, an important early event in cardiovascular disease caused by oxidative stress (Knight-Lozano et al., 2002).

K. Aging

Cardiovascular disease increases in frequency with age, even in the absence of established risk factors. The underlying molecular mechanisms associated with age-related cardiovascular disease have not been elucidated, but might involve impaired [•]NO activity (McCann et al., 1998). For example, the endothelium-dependent relaxation declines with increasing age (Tschudi et al., 1996). Another recent study (Van der Loo et al., 2000) demonstrated that there is an accumulation of tyrosine nitrated proteins in the aortas of old rats compared with aortas of young rats. One of the nitrated proteins iden-

tified was Mn-SOD. Nitration of Mn-SOD leads to significant reduction of its activity (Yamakura et al., 1998; MacMillan-Crow et al., 1999). Mn-SOD is the major antioxidant enzyme in the mitochondria, and even partial inhibition can have adverse consequences. It is also likely, that the degree of Mn-SOD nitration may be a molecular marker of vascular aging (Van der Loo et al., 2000).

One more example of age-related nitration of a specific protein is reported for sarcoplasmic reticular Ca-ATPase isolated from the skeletal muscle (Schoneich et al., 1999; Viner et al., 1999). Inactivation of Ca-ATPase and decreased ATP utilization during aging may represent an adaptive response that functions to down-regulate energy metabolism and the associated generation of RNS/ROS (Squier, 2001).

The studies mentioned above indicate that protein nitration can have both detrimental and protective effects in aging. However, another study (Leeuwenburgh et al., 1998) indicated no significant increase in levels of nitrotyrosine in heart, skeletal muscle, and liver from young and old female rats.

VI. Therapeutic Implications

Major therapies for cardiovascular disease include drugs that affect vascular tone, cardiac contractility, fluid status, or lipid levels (Wattanapitayakul and Bauer, 2001). However, in light of the critical roles of RNS/ROS in cardiovascular disease, it seems reasonable to expect that antioxidant therapy may also have value. Perspectives of antioxidant therapy for cardiovascular disease were recently reviewed (Cuzzocrea et al., 2001; Snoeckx et al., 2001; Wattanapitayakul and Bauer, 2001). They mainly include different interventions to reduce RNS/ROS generation, such as antioxidant food supplements, ONOO⁻ decomposition catalysts, SOD-mimics, and modulations of expression of antioxidant enzymes, including molecular chaperones.

The susceptibility to oxidative stress is a function of the overall balance between factors that exert oxidative stress and those that exhibit antioxidant capability. Oxidative damage can, therefore, be described as a consequence of excessive oxidative stress and/or insufficient antioxidant potential. Under normal physiological conditions, [•]NO, O₂^{•-}, ONOO⁻, and other RNS/ROS are part

of the delicately balanced redox regulation. Once it was altered by cardiovascular disease, the development of procedures for reestablishing the original balance may be a central issue of research on cardiovascular disease. However, flooding the system with antioxidants or the overexpression of antioxidative enzymes may be just as harmful as excessive exposure to RNS/ROS (Droge, 2002). The major problem is the identification of the narrow line that separates advantageous and detrimental effects of RNS/ROS. Protein nitration is a type of oxidative damage that occurs in cardiovascular disease. Many studies summarized in this review found a positive correlation between protein nitration and cardiovascular disease progression and concluded that protein nitration can be a biological marker of disease progression. At the same time, protein nitration can also be a biological marker of effectiveness of antioxidant therapy.

Such an example is brought up by studies on statin therapy. Statins, cholesterol-lowering agents, can also improve the stability of the mRNA for eNOS and enhance the generation of [•]NO (Lefer et al., 2001). It was demonstrated in an experimental model of myocardial infarction, that cerivastatin treatment improved left ventricular remodeling, whereas decreased the level of tyrosine nitrated proteins (Bauersachs et al., 2001). Another study showed that simvastatin treatment reduced the atherosclerotic area in the thoracic aorta of rabbits fed a 0.5% cholesterol diet and, at the same time, decreased nitrotyrosine staining (Thakur et al., 2001). It is recognized that eNOS-derived [•]NO is a potent inhibitor of leukocyte recruitment at sites of inflammation. It seems likely that eNOS-derived [•]NO does not cause protein nitration, and protein nitration could still be a marker of the efficiency of statin therapy.

Another question is whether altered functions of tyrosine nitrated proteins contribute to disease development. Basically, the answer to this question requires identification of a specific tyrosine nitrated protein and a positive correlation between altered function of this protein and development of complications. Several studies (Zou and Bachschmid, 1999; Zou et al., 1999; Van der Loo et al., 2000; Knight-Lozano et al., 2002; Mihm et al., 2001a, 2002) fit these requirements and propose that protein nitration is a link between increased oxidative

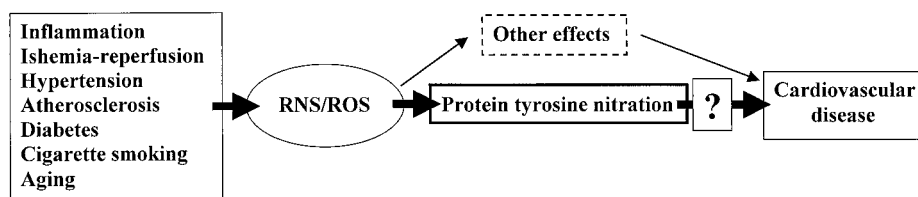


FIG 2. Does protein nitration contribute to cardiovascular disease? Increased RNS/ROS production may be a unifying mechanism in cardiovascular disease progression. RNS/ROS production causes protein nitration, whose specific role in cardiovascular disease warrants further studies. Multiple effects of RNS/ROS, other than protein nitration, include other protein modifications, modification of molecules other than proteins, and transcriptional regulation.

stress and cardiovascular complications. Collectively, this suggests that the search for antioxidants that prevent protein nitration may offer a unique therapeutic option for the treatment of cardiovascular complications.

VII. Future Directions

The emerging experimental data summarized in this review show protein nitration as an internal feature of cardiovascular disease (Fig. 2). The question is whether protein nitration occurs at an early stage of cardiovascular disease, contributing to the development of complications, or whether it is merely a consequence of the oxidative tissue damage, reflecting the presence of complications. The question is important and warrants future studies on detailed profiling of tyrosine nitrated proteins in different settings of cardiovascular disease. Additionally, function and metabolism of tyrosine nitrated proteins remain an area of research in need of rigorous study.

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