Nitrotyrosine and Chlorotyrosine: Clinical Significance and Biological Functions in the Vascular System

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Submitted for publication August 11, 2005

The heme-containing enzyme myeloperoxidase (MPO) is both present and active in inflammatory conditions. This enzyme is potentiated by its formation of multiple inflammatory mediators. The two most common mediators are the modified tyrosines: nitrotyrosine and 3-chlorotyrosine. Along with other modified tyrosines, these molecules have been found to be elevated in atherosclerosis, lung disease, sepsis, vasculitis, and other inflammatory diseases. By treating some of these diseases, the levels of modified tyrosines have been shown to decrease. There have been a wide range of animal models designed to study the in vivo effects of these tyrosine molecules. In addition, there are also several reports in the literature of the in vitro actions of modified tyrosine molecules demonstrated by various cell-culture models. The purpose of this review is to evaluate the clinical significance and biological functions of these modified tyrosine molecules in atherosclerosis as well as a variety of other inflammatory conditions. It is timely information because of their association with diseases as well as lack of overview of their molecular actions. This review will focus on the formation, clinical significance, and animal and cell-culture models of these important molecules.

Key Words: nitrotyrosine; chlorotyrosine; myeloperoxidase; tyrosine; oxidative stress; vascular disease; atherosclerosis; inflammation; endothelial cell.

INTRODUCTION

Extensive evidence suggests that atherosclerosis is a chronic inflammatory process with a link to thrombogenicity. The pathogenesis of this process initially starts with oxidative damage induced by inflammatory cells on the arterial wall. Inflammatory cells such as monocytes, macrophages, and neutrophils release various oxidizing enzymes that are involved in this process [1]. One such enzyme is the heme protein myeloperoxidase (MPO). MPO is a glycosylated arginine-rich and basic protein, comprised of two subunits, a 59- to 64-kDa heavy subunit and a 14-kDa light subunit. MPO is expressed in neutrophils and monocytes, where it is secreted during activation of these cells and localizes in and around endothelial cells after leukocyte degranulation [2, 3]. Recently, MPO has been shown to play a role in atherogenesis. Individuals with MPO deficiency or mutations have a significantly reduced rate of cardiovascular disease as compared to the general population [4–6]. MPO is quite complex and has a number of different pathways. Two of its important metabolites are 3-chlorotyrosine and nitrotyrosine [7]. After undergoing MPO-mediated oxidation, the normally occurring tyrosine is converted into the non-physiological 3-chlorotyrosine, which serves as a marker for oxidative damage in arteries [8]. MPO-induced reactive nitrogen species can oxidize low-density lipoprotein (LDL), which is seen in atherosclerotic lesions [9].

Both 3-chlorotyrosine and nitrotyrosine have very important clinical significance as they both serve as markers for MPO activity. It is thought that these two molecules could cause oxidative damage contributing to atherosclerosis. These molecules could also cause tissue damage or dysfunction in many other inflammatory conditions. In addition, 3-chlorotyrosine is unique because it is heat stable and is not readily formed by artificial mechanisms, which make it an excellent marker for MPO-induced oxidation [10]. Nitrotyrosine is also a
biological marker for leukocyte-dependent formation of LDL nitrate [11–13]. Both molecules could be used to provide a "molecular fingerprint" for MPO-catalyzed oxidation.

The biological effects of free 3-chlorotyrosine have been studied by us and others. 3-Chlorotyrosine has been shown to cause endothelial dysfunction by decreasing endothelium-dependent vasorelaxation and endothelial nitric oxide synthase (eNOS) expression. In addition, 3-chlorotyrosine was also shown to cause an increase in free-radical production. 3-Chlorotyrosine residues bound to high-density lipoprotein (HDL) and LDL have both been linked to atherosclerosis [14, 15]. Patients with coronary artery disease were shown to have higher levels of 3-chlorotyrosine in HDL isolated from plasma when compared to normal serum controls [15, 16]. Recent evidence has also shown that 3-chlorotyrosine levels in LDL recovered from plaque of patients with an atherosclerotic aorta was 30 times higher when compared to the LDL obtained from the serum of normal donors [15]. This modified version of LDL is readily absorbed and processed by macrophages, resulting in cholesterol deposition and foam cell formation, both of which processes are essential in plaque formation [16]. Effects and actions of 3-chlorotyrosine and nitrotyrosine on vascular cells are different from those of other inflammatory cytokines and mediators. The importance of these molecules is vital in providing a measure of oxidation-induced atherosclerosis. In our review, we seek to explore the formation, mechanisms of action, and roles specific to 3-chlorotyrosine and nitrotyrosine. We have reviewed available animal and cell-culture models as well as clinical studies to better elucidate the roles of these active metabolites in oxidation-induced atherosclerosis.

NITROTYROSINE

As one of the most important mediators of MPO, nitrotyrosine plays a key role in the process of oxidation seen early on in atherosclerosis and other inflammatory conditions including active lupus, rheumatoid arthritis, influenza, pancreatitis, cholecystitis, ulcerative colitis, and Crohn’s disease. Nitrotyrosine originates as tyrosine in both a free and protein-bound form. The protein-bound form that is involved in atherosclerosis is attached to LDL. This molecule is then nitrated to form the biologically active nitrotyrosine. The nitrate moiety is donated by the reactive nitrating intermediate peroxynitrite (ONOO⁻) [17]. Peroxynitrite in turn is formed from MPO-dependent oxidation of nitric oxide (NO). As recent studies have indicated, this MPO-mediated oxidation process occurs in a highly efficient manner in human serum [16]. Once modified, the nitrated form of LDL is then collected and consumed by macrophages via phagocytosis. The end product of this degradation is the deposition of cholesterol and foam cells that are vital in plaque development. Cigarette smoking has been shown to accelerate this nitrotyrosine-mediated oxidation [18].

One important aspect about the formation of nitrotyrosine that must be noted is that it is not solely generated by peroxynitrite [19–21]. MPO can also independently oxidize nitrite (NO₂⁻), a stable end product of NO metabolism, to form nitrogen dioxide (NO₂). NO₂ is also a reactive nitrogen species which in turn can generate tyrosine. A third pathway utilizes MPO-generated hypochlorous acid (HOCl⁻), which also oxidizes NO₂⁻ to nitryl chloride (NO₂Cl), which is also a reactive nitrogen species (Fig. 1).

Several recent investigations validate the clinical importance of nitrotyrosine. Multiple studies indicate that raised nitrotyrosine levels have been detected in atheromatous plaques when analyzed by immunohistochemistry [16, 22]. In addition, patients afflicted with diseases that have a high oxidative stress burden such as diabetes have been shown to have elevated plasma levels of nitrotyrosine [22]. Nitrotyrosine levels have been shown to be elevated in the serum of smokers [23]. Nitrotyrosine levels were also found to be higher in patients suffering from abdominal aortic aneurysms. During repair of their aneurysms, the nitrotyrosine levels became further elevated early on after reperfusion during open repair [24]. This further supports the theory of an inflammatory pathway for nitrotyrosine as it is apparently elevated with reperfusion injury. Interestingly, women from the same studies were shown to have lower levels of circulating nitrotyrosine, which may contribute to their lower vascular risk profile [24]. Finally, studies on patients undergoing hemodialysis have revealed that nitrotyrosine levels are elevated in these patients when compared to controls [25].

There have been several animal studies performed using nitrotyrosine linking it to inflammation via

FIG. 1. Potential pathways of nitrotyrosine and chlorotyrosine formation. MPO, myeloperoxidase; NO₂, nitrite; HOCl, hypochlorous acid; ONOO⁻, peroxynitrite; NO₃, nitrogen dioxide; NO₂Cl, nitryl chloride; Cl⁻, chloride; H₂O₂, hydrogen peroxide.
various models. Mice injected with *Klebsiella pneumonia* produced an inflammatory fluid rich in nitrotyrosine [26]. Rats genetically engineered to be spontaneously hypertensive have been shown to have an increased production of nitrotyrosine [27]. A study that links the association of nitrotyrosine with the oxidative stress of renal failure showed an increase in nitrotyrosine serum levels in uremic mice [28]. Rats that were injected intratracheally with lipopolysaccharide had elevated levels of nitrotyrosine in their bronchoalveolar lavage fluid [29].

Nitrotyrosine has also been tested in a variety of cell-culture experiments. Nitrotyrosine production has been tested in an assortment of different tumor cell lines including rat glioma and neuroblastoma [30]. Treating these cells with nitrotyrosine induced morphological changes, nuclear suffering, decreased viability, and growth inhibition [24]. Investigators have shown that the addition of peroxynitrite to astrocytes in the cell culture results in an increase in nitrotyrosine production [31].

**CHLOROTYROSINE**

MPO utilizes a variety of co-substrates with H$_2$O$_2$. During one of these reactions shown in Fig. 1, MPO catalyzes the formation of hypochlorous acid (HOCl$^-$) from the Cl$^-$ ion and H$_2$O$_2$. HOCl in turn is a potent chlorinating oxidant. It has several known targets. One of these targets is the molecule tyrosine in both its free and its bound forms. When reacted with HOCl$, tyrosine is converted into the 3-chlorotyrosine molecule. Although there is a variety of other chlorinated oxidation products formed from MPO, none are ideal to serve the role as a marker. Most of these molecules lose their halide group and spontaneously decompose. 3-Chlorotyrosine, on the other hand, is a stable molecule that makes it ideal for use as a specific marker for MPO activity both in vivo and in vitro [10].

3-Chlorotyrosine production has been studied in clinical studies in various pathological conditions. In patients suffering from asthma, 3-chlorotyrosine concentration was elevated in the urine when compared to normal controls [32]. In another investigation, a review of tracheal aspirates of preterm infants with pulmonary dysfunction revealed elevated levels of 3-chlorotyrosine [33]. Sputum samples of patients with cystic fibrosis analyzed by nuclear magnetic resonance imaging revealed elevated levels of 3-chlorotyrosine [34]. With regards to atherosclerosis, 3-chlorotyrosine levels recovered from human atherosclerotic plaque were 30 times elevated when compared to normal serum controls [10]. Patients with chronic renal failure have ongoing oxidative stress and often are plagued with vascular disease. Hemodialysis in these patients activates neutrophils. In these patients, serum concentrations of 3-chlorotyrosine were also shown to be markedly elevated when compared to those from age- and sex-matched controls [35]. Finally, a recent article revealed that urine collected from patients who had inflammatory vasculitis had elevated levels of 3-chlorotyrosine when compared to normal controls [36].

To examine the production of 3-chlorotyrosine in vivo, several animal models have been designed. Mice that underwent cecal ligation and puncture evoked a large inflammatory response. The inflammatory fluid generated from these animals was examined and found to contain large amounts of 3-chlorotyrosine [26]. In another model, mice were injected with *K. pneumonia* and an inflammatory exudate was created. This exudate was also found to be abundant in 3-chlorotyrosine [26]. In yet another model, peritoneal fluid from septic MPO-deficient mice were noted to have much lower levels of 3-chlorotyrosine when compared to wild-type mice under similar septic conditions [37].

The in vitro effects of 3-chlorotyrosine have been studied using various cell lines. The stability of the molecule permits it to be easily studied as a marker for inflammation, neutrophil, and MPO activity. Smooth muscle cells treated with the reactive oxidation intermediate HOCl$^-$/H$_2$O$_2$ showed elevated levels of 3-chlorotyrosine production [38]. A similar result has also been shown with cultured neutrophils [39]. Other studies have confirmed this process by the addition of the antioxidant Vitamin C, which reverses this process in smooth muscle cells [38]. In other studies, human neutrophils that have phagocytosed the bacteria *Escherichia coli* were evaluated and demonstrated high levels of 3-chlorotyrosine by mass spectrophotometry [39]. In a bacterial study, *Staphylococcus aureus* treated with HOCl$^-$ converted its tyrosine residues to 3-chlorotyrosine [40].

**OTHER MODIFICATIONS OF TYROSINE**

Although nitrotyrosine and 3-chlorotyrosine appear to be the most active tyrosine metabolites, there are several other oxidation products of tyrosine including dityrosine, trityrosine, and 3-bromotyrosine [10]. Dityrosine is one such tyrosine metabolite. Although not uniquely generated from MPO, several studies have demonstrated MPO-dependent formation of dityrosine. The pathway of this molecule formation has been confirmed using electron spin resonance spectroscopy [41]. Trityrosine is formed when MPO catalyzes the formation of the tyrosyl radical from 1-tyrosine. This tyrosyl radical then undergoes immediate dimerization to form the molecule trityrosine [42]. Another modification of tyrosine that is often overlooked is 3-bromotyrosine. This molecule is formed when the enzyme MPO produces the reactive oxygen species HOCl, which then reacts with Br$^-$ to produce several brominating intermediates, which ultimately result in the production of 3-bromotyrosine. As with all tyrosine metabolites generated from MPO, these molecules promote protein cross-linking and initiation of lipid
peroxidation. These features are characteristic in lipoproteins and lipids recovered from human atheromas [37].

There are several investigations that report on the clinical significance of these tyrosine residues. In the extreme oxidative stress environment seen in children with Kwashiorkor, urinary levels of dityrosine were markedly elevated when compared to normal, well-nourished children [43]. In another study, patients with cystic fibrosis in whom there is an increased amount of oxidative stress were examined for levels of dityrosine in the sputum. The dityrosine levels in these patients were elevated when compared to normal individuals [44]. Dityrosine production is elevated in atherosclerosis owing to the increased oxidation of LDL. Human carotid plaques were evaluated and found to have high levels of dityrosine when compared to controls [45]. In another study, a review of patients with a history of hypercholesterolemia was done where serum levels of dityrosine was evaluated. Increased levels of dityrosine was noted when compared to controls. As a follow-up to this study, the same patients were then treated with the antioxidant statin drug atorvastatin for 12 weeks; interestingly a decrease in the amount of dityrosine was noted [46]. The disorders collectively referred to as vasculitis are characterized by a high level of inflammatory stress. Patients with known vasculitis disorders were evaluated and found to have elevated levels of 3-bromotyrosine along with 3-chlorotyrosine [36]. Several pulmonary inflammatory conditions have also been shown to have increased levels of 3-bromotyrosine. Urinary levels of 3-bromotyrosine were elevated in asthmatic patients [32]. Patients with cystic fibrosis were also found to have elevated levels of 3-bromotyrosine in addition to the other tyrosine metabolites as mentioned previously [34]. Finally, as with the other tyrosines, elevated levels of 3-bromotyrosine were demonstrated in human atheromas [47].

To better study these newer tyrosine residues in vivo, several animal studies have been devised. Exercise is known to cause an increase in oxidation. Livers from rats were analyzed after subjecting them to the physical stress of a treadmill. In these rat livers, the production of dityrosine along with nitrotyrosine was significantly increased. In addition, these rats were then treated with the antioxidant eriocitrin, which decreased oxidized tyrosine production [48]. The oxidation model of atherosclerosis has also been demonstrated in vivo with oxidative tyrosine residues. Rabbits fed a cholesterol-rich diet were confirmed to have atherosclerosis. The livers and kidney of these animals were then evaluated for dityrosine and nitrotyrosine levels and found to be elevated. This effect was reversed again with the addition of an antioxidant. The aging process is a natural oxidative stress [49]. Urine from rats aged for a 2-year period were studied and found to have increased levels of dityrosine when compared to younger rats [50].

As with 3-chlorotyrosine, mice subjected to septic conditions due to fecal contamination of the peritoneal cavity were found to have elevated levels of 3-bromotyrosine [26]. To prove that 3-bromotyrosine is produced from MPO, septic mice that were genetically engineered to be deficient in MPO were evaluated for 3-bromotyrosine in peritoneal fluid collections. In these animals, the levels of 3-bromotyrosine was significantly decreased when compared to wild-type mice, a similar result as that seen with 3-chlorotyrosine [37].

The functions of dityrosine, trityrosine, and 3-bromotyrosine have been investigated using a variety of cell-culture methods. Parkinson’s disease is a neurodegenerative process where the exact etiology is still unclear. There is recent evidence indicating that the oxidation of the intracellular protein alpha-synuclein may be involved in the initiation of disease. Human dopaminergic cell lines that are known to over-express alpha-synuclein were treated with various reactive oxygen species generators. The results indicate that there was a significant increase in dityrosine production [51]. The formation of 3-bromotyrosine was studied by harvesting eosinophils from porcine blood. Using high-pressure liquid chromatography techniques, the production of 3-bromotyrosine from eosinophil peroxidase and MPO was observed and quantified [52]. To study the generation of trityrosine, porcine eosinophils were isolated and tested for trityrosine production. Using mass spectrometry, eosinophil-peroxidase-dependent production of trityrosine was demonstrated [53].

CONCLUSIONS AND PROSPECTIVE VIEW

MPO is a heme-containing enzyme that is unique to neutrophils and monocytes. When activated, these cells release MPO in response to inflammatory stimuli. The majority of MPO is released by neutrophils. In addition, eosinophils have a related enzyme known as eosinophil peroxidase which also has similar functions. Both of these enzymes exert their activity by catalyzing reactions resulting in the formation of a wide variety of oxidants. These oxidants in turn further propagate to produce multiple modified tyrosine molecules. The two most well studied of these molecules are nitrotyrosine and 3-chlorotyrosine. These tyrosine moieties are commonly seen in a wide variety of inflammatory conditions (Table 1). There is some controversy as to the exact origin of these molecules. Specifically, there are some MPO-independent pathways that have also been shown to produce modified tyrosine molecules. However, in general, they serve as markers of MPO activity and inflammation.

Although nitrotyrosine and chlorotyrosine are elevated in many inflammatory conditions, their roles in vascular pathology and underlying molecular mechanisms are largely known. Atherosclerosis is currently known as an inflammatory condition. Inflammation and
oxidative processes are key components of atherosclerosis. Evidence indicates that modified tyrosine residues may play a key role in this process starting from fatty streak formation and leading to plaque rupture and thrombosis. Modified tyrosine residues on LDL are consumed by macrophages and decomposed to form fatty streaks along the arterial wall, which initiates the process of plaque formation. It has been observed but not understood why women have a lower incidence of hypercholesterolemia and coronary artery disease. Recently, with increased smoking among women, this gap has closed. By studying the levels of modified tyrosines, we may be able to further understand this discrepancy.

Modified tyrosine residues are also seen in a variety of other inflammatory conditions. Several models of lung inflammation and injury have been studied where elevated levels of modified tyrosines are seen. The elevated levels of 3-chlorotyrosine in asthmatics confirm that this disease is inflammatory in origin. Levels of dityrosine could be potentially used as markers for the degree of cystic fibrosis in the lung. Hypertension and diabetes are not routinely thought of as inflammatory conditions. However, with proof that elevated levels of modified tyrosine residues are present in these patients, there is potential to characterize these conditions as inflammatory in origin and to also quantify the degree of inflammation by comparing levels of modified tyrosines. Sepsis is a lethal inflammatory condition where an inflammation switch is turned on in the body. By using our knowledge of modified tyrosine levels in this disease, we may be able to further understand and treat this complex condition. A well-known but often poorly understood inflammatory condition is vasculitis. This condition can also be further understood and followed by assessing the levels of modified tyrosines in these patients.

With the aging of the population, the prevalence of inflammatory conditions such as atherosclerosis will only increase. It is estimated that in the US alone there are over a 100 million people suffering from hypercholesterolemia and atherosclerosis. As researchers and clinicians, we are obligated to find new ways to measure and treat this disease. One possible method would be by further studying the relationship between modified tyrosine residues and atherosclerosis. An example of this can be seen with the statins. Statin drugs are well known antioxidants [54], which are routinely used to treat hypercholesterolemia. Decreased levels of modified tyrosines have been shown as a result of statin therapy [55]. This could potentially be expanded to be performed with a wide variety of other drugs like clopidogrel and aspirin. In addition, MPO activities could be inhibited by several compounds including p-hydroxy-benzoic acid hydrazide, potassium cyanide, sodium azide, 3-amino-1,2,4-triazole, salicylhydroxamic acid, benzohydroxamic acid, ceruloplasmin, and dapsone [56–58]. Theoretically, these MPO inhibitors could reduce nitrotyrosine and chlorotyrosine formation in the inflammatory conditions, thereby reducing tissue injury due to oxidative stress. Thus, MPO inhibitors, hypochlorite scavengers such as taurine or methionine, as well as many antioxidants may have potential applications in treating or preventing the progression of atherosclerosis. However, such experiments or clinical trials are very limited. Further investigations are warranted. Furthermore, the potential use of modified tyrosine research is not limited to just atherosclerosis. As indicated in this review, there are a wide variety of other inflammatory conditions that are poorly understood and have the potential to be further investigated.

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ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants R01 HL065916, R01 HL072716, R01 EB-002436, and R01 HL083471 (C.C.); R01 DE15543 and R21 AT003094 (Q.Y.); R01 HL75824 (A.B.L.); and K08 HL076545 (P.H.L.).

REFERENCES


