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Mass Spectrometric Analysis of Protein Tyrosine Nitration in Aging and Relevant Diseases

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Protein tyrosine nitration (PTN) participates in pathophysiological processes, such as aging and neurodegenerative diseases. Although mass spectrometry of nitrated peptides has become a powerful tool for the identification of nitrated peptides, the low stoichiometry of PTN hampers the successful analysis. Accumulation of 3-nitrotyrosine has been found to occur during the aging process; this was identified through mass spectrometry. There has been increasing evidences that PTN-induced changes in protein structure and function play important roles in diverse pathogenesis of aging related diseases, carcinogenesis, and immunomodulatory diseases. In this review, we focus on the recent progress in understanding of PTN with regard to age-related diseases using mass spectrometry.

INTRODUCTION

Protein tyrosine nitration (PTN) is a physiological event implicated in numerous biological processes modulated by nitric oxide (NO) occurring in a number of diseases (Lee et al., 2009a; Schopfer et al., 2003). PTN is characterized by series of oxidation processes, which is selective modification on the tyrosine residues exposed to intramolecular acidic or basic environment. Many recent studies have demonstrated that increased tyrosine nitration in different diseases, such as cancer, neurodegenerative diseases, and cardiovascular injury (Greenacre and Ischiropoulos, 2001; Kanski et al., 2005b) of numerous inflammatory responses (Kanski and Schoneich, 2005), age-related diseases such as age-related macular degeneration (AMD) (Murdaugh et al., 2010), cardiovascular disease, stroke, decreased immune responses (Ames, 1995; Beal, 1995), and even cancer (Kim et al., 2011). It has been reported that protein nitration contributes to cellular signaling mechanisms (Di Stasi et al., 1999; Squier and Bigelow, 2000) as PTN occurs in a specific and reversible way (Gow et al., 1996; Kamisaki et al., 1998). As a result of PTN, the structure and function of the modified proteins is affected. However, little is known about mechanisms and the target proteins for endogenous reactive nitrogen species (RNS) or reactive oxygen species (ROS). As the first step towards identification of the nitrated proteins in biological samples, proteomic technologies have been applied to characterize nitrated proteins from tissues and cell lines under disease settings. This review highlights the significance of PTN in the cellular pathophysiology of aging and relevant diseases.

REACTIVE NITROGEN SPECIES

Protein tyrosine nitration is a posttranslational modification in response to oxidative stress (Haddad et al., 1994; Ignarro, 1990; MacMillan-Crow et al., 1996; Masri, 2010) such as peroxynitrite anion ($ONOO^-$) and nitrogen dioxide ($^{\bullet}NO_2$). Radical and non-radical RNS refer to reactive molecules derived from nitric oxide (NO) and supeproxide including nitrosyl cation (NO^+), peroxynitrous acid (ONOOH), peroxynitrite ($ONOO^-$), and nitrylchloride (NO_2Cl). There are several ROS found in biological system. Protein nitration is the result of combinatorial reactions of RNS/ROS (Turko and Murad, 2002).

NO is a short-lived physiological messenger which is highly diffusible and lipophillic (Masri, 2010) in nature. NO regulates several significant physiological functions including vasodilation, respiration, cell migration, immune response and apoptosis (Muntane and la Mata, 2010). Nitric oxide is produced within cells by the actions nitric oxide synthases (NOS) (Drew and Leeuwenburgh, 2002). There are three distinct isoforms of NOS: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3) (Ignarro et al., 1987; Muntane and la Mata, 2010; Nathan, 1992). NO can carry out its function as an intracellular as well as extracellular messenger by diffusing over several cell diameters within a short time (Ames et al., 1993; Lancaster, 1994; Sohal and Orr, 1992). Increased NO production and subsequent ONOO- formation have been detected under hyperoxygenation states in which $O_2^{\cdot-}$ and $O_2^{\cdot-}$

-derived oxidants are known to be overproduced.

MASS SPECTROMETRIC ANALYSIS OF PROTEIN TYROSINE NITRATION

Recently, mass spectrometry (MS)-based profiling methods have been employed to identify protein tyrosine nitration and understand their roles (Kanski and Schoneich, 2005), (Bigelow and Qian, 2008). While the availability of antibodies for nitrotyrosine (Beckmann et al., 1994), (Girault et al., 2001) has made it possible to characterize protein tyrosine nitration, the low stoichiometry of nitrated tyrosine residues and the lack of specificity of the antibodies have hampered identification of the modification. Therefore, MS-based identification of nitrated proteomes is critical to understand their potential roles in various pathophysiological processes. To selectively isolate nitrated peptides, various affinity purification methods were previously developed including chemical derivation strategies such as biotinylation, S-acetylthioacetate formation (Zhang et al., 2007), schiff base strategy (Lee et al., 2009b) and fluorinated carbon tagging (Kim et al., 2011). These methods have contributed to the research of PTN.

PROTEIN NITRATION IN AGING AND AGE RELATED DISEASES

Aging itself is a combination of changes in our body and the impact of what we do with our bodies. This happens on multiple levels:

Accumulated Damage

Toxins, the UV radiation from sunlight, harmful foods, pollution and other toxins all take their toll on our bodies. Over time, these toxins can lead to tissue damage and the body "falls behind" in maintaining and repairing the cells, tissues and organs.

Metabolic Aging

After age 45, the average individual loses around 10% of their muscle mass per decade (Janssen and Ross, 2005). This equates to losing about one-third to one-half a pound of muscle each year. Physical activity plays a role in both body composition and metabolism during the aging process. Research shows that most individuals gradually reduce their level of physical activity as they age, which further reduces their number of calories needed to maintain weight. As we go through our day, our cells are turning food into energy, which produces byproducts that can be harmful. This process of metabolizing and creating energy results in damage to our body over time.

These days some significant progress has been made to understand and explain the effects of oxidants (reactive oxygen and reactive nitrogen species) on the oxidative stress on lipids, proteins, and DNA and how this contributes to the aging process (Ames et al., 1993; Leeuwenburgh et al., 1998; Sohal and Orr, 1992). Till now very little is known about the process of aging that makes it remained a mystery. It is documented that increased nitration is often contribute to the development of age-related diseases. Excess peroxynitrite may affect modulation of mitochondrial respiration that can act as platform for development of prevalent neurodegenerative diseases (Poderoso, 2009). Proteomic analysis by ESI-MS/MS had illustrated that flotillin-1 and α-tubulin are nitrated in rat as a consequence of aging (Dremina et al., 2005). Age dependent accumulation of 3-nitrotyrosine (3-NT) on skeletal muscle glycogen phosphorylase b (Ph-b) is reported in experimental rat model (Fugere et al., 2006).

Age-Related Macular Degeneration (AMD/ARMD)

Age-Related Macular Degeneration (AMD) is progressive condition that results in the gradual deterioration of the macula, the portion of the retina provides the ability to see fine detail, and loss of vision from the center of the field of vision. AMD is the leading cause of vision impairment, resulting in functional limitations and legal blindness in the elderly population usually over the age of 50 (Mitchell et al., 1995). AMD develops when the retina's blood supply diminishes. The macula's high concentration of cones, the cells responsible for color and fine detail vision, makes it especially vulnerable to damage and its cells begin to die. The death of the cells results in diminished vision. AMD may affect one eye at first, though nearly always affects both eyes as it progresses.

There are two forms of AMD, atrophic (commonly known as dry) and neovascular (commonly known as wet). All AMD begins as the atrophic form, in which the nourishing outer layer of the retina withers, or atrophies. Approximately 90 percent of AMD remains in this form and progresses slowly. In the remaining 10 percent, new blood vessels begin to grow erratically within the choroid, the blood-rich membrane that nourishes the retina. These blood vessels are thin and fragile, and bleed easily. The resulting hemorrhages cause the retina to swell, distorting the macula and accelerating the loss of cells.

The pathophysiology of AMD cannot be explained simply. This may include genetic predispositions, accumulation of lipofuscin and drusen, local inflammation and neovascularization (Murdaugh et al., 2010). Several research groups screen the genomes from different groups of AMD patients and discovered a commonly inherited variant (Y402H) of the complement factor H (CFH) gene that significantly increases the risk of AMD (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). Findings of these research groups link up the genetics and inflammation to the AMD. Some recent research progresses evidence that Components of Drusen, Inflammation and oxidative stress involved in the development of AMD (Hollyfield et al., 2008; Laine et al., 2007; Skerka et al., 2007; Wang et al., 2008). Large fluxes of nitric oxide (NO) are released through the activation of inducible nitric oxide synthase during inflammation (Carreras et al., 1994). In AMD patients significantly higher plasma NO levels than control subjects are reported (Evereklioglu et al., 2003). Bruch's membrane lies between the choroidal capillary bed and

TABLE 1 | Nitroproteomic investigation from physiological tissues of aging

Condition	Tissue	Species	Separation Method	Detection Method	# of Nitro proteins	# of Nitro peptides	Reference
AGING	Skeletal muscle	Rat	2DE	MALDI-TOF MS LS-ESI MS/MS	8	-	(Kanski et al., 2003)
	Heart homogenate	Rat	2DE	LC-ESI MS/MS	13	1	(Kanski et al., 2005a)
	Heart homogenate		IP/1DE		9	-	
	Heart mitochondria				35		
	Skeletal muscle	Rat	IEF/1DE	LC-ESI MS/MS	11	12	(Kanski et al., 2005b)
	Brain	Rat	1DE	LC-ESI MS/MS	2	-	(Dremina et al., 2005)
	Muscle	Rat	1DE	LC-ESI MS/MS	1	3	(Sharov et al., 2006)
	Muscle	Rat	1DE	MALDI-TOF MS	5	5	(Fugere et al., 2006)
	Brain (Cerebellum)	Rat	2DE	LC-ESI MS/MS	16	-	(Gokulrangan et al., 2007)
			IEF/1DE		22	-	
			IEF		4	4	
	Heart	Rat	IEF	LC-ESI MS/MS	10	10	(Hong et al., 2007)
	Brain	Dog	2DE	MALDI-TOF MS	-	-	(Opii et al., 2008)

retinal pigment epithelial (RPE) cells. The exchange of various materials between the underlying choriocapillaris and overlying RPE occurs through Bruch's membrane (Sellner, 1986). Several researches have suggested that age-related damage to Bruch's membrane allows the accumulation of abnormal extracellular deposits, called drusen, between the basal lamina of the RPE and the inner collagen layer of Bruch's membrane (Crabb et al., 2002; Newsome et al., 1987) which is believed to elicit a local inflammatory response (Anderson et al., 2002; Yasukawa et al., 2007). An impaired RPE function was observed in non-enzymatic nitration of RPE basement membrane in tissue culture and also tyrosine nitration has been shown to occur in photoreceptor cells (Miyagi et al., 2002). 3-nitrotyrosine is a specific hallmark for inflammation-induced oxidative damage to proteins. In addition to proteins, Bruch's membrane also contains lipofuscin which is a mixture of autofluorescent material that accumulates in the RPE cells and is reported to photochemically generate a series of reactive oxygen species, including singlet oxygen, hydrogen peroxide, and superoxide anions (Rozanowska et al., 1998) that can stimulate oxidative stress in RPE. One of the major organic soluble chromophores in lipofuscin is A2E (2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E, 3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-- (2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]—pyridinium). Increasing accumulation of 3-nitrotyrosine and nitro-A2E in human Bruch's membrane with advancing patient age were identified which can demonstrate the inflammation mediated and non-enzymatic nitration in the progression of AMD.

Additionally, we summarize nitroproteomic studies with aging related tissues using mass spectrometry and their references in table 1 in order to help more understanding of aging related diseases in terms of PTN.

PROTEIN NITRATION AND CARCINOGENESIS

Carcinogenesis or oncogenesis or tumorigenesis can literally be defined as a process by which normal cells are transformed into cancer cells. This deadly process is characterized by a progression of changes on cellular and genetic level that ultimately reprogram a cell to undergo uncontrolled cell division and formation of a malignant mass.

Although several researches have been conducted to elucidate the mechanism of NO in tumor biology, it is still a mystery to the scientists. The growth of tumor is regulated by interactions of endothelial cells of the tumor vasculature, tumor-infiltrating immune cells such as T lymphocytes and macrophages, and the tumor cells themselves (Sutherland et al., 1988). Almost all of these cellular components have been shown to generate NO in vitro which has been proposed to be an important mediator of tumor growth (Jenkins et al., 1994; Lamas et al., 1992; Wink et al., 1998). NO has bifunctional role in carcinogenesis leading to either promotion or inhibition of tumor growth (Masri, 2010) . Both infectious and non-infectious injuries and irritation can initiate inflammatory response that can lead to a subsequent respiratory burst, an increased uptake of oxygen that leads to the release of free radicals from leukocytes, including activated macrophages. This process can damage surrounding cells and consequently can drive carcinogenesis by altering targets and pathways that are crucial to normal tissue homeostasis (Coussens and Werb, 2002). Chronic inflammation contributes to about one in four of all cancer cases worldwide. RNS induce oxidative and nitrosative stress results in DNA damage and inhibition of DNA repair enzymes through direct or indirect

mechanisms. Post-translational modifications of proteins and the induction of mutations in cancer-related genes which is mediated by nitration, nitrosation, acetylation, phosphorylation or poly-ADP-ribosylation are some major events that might increase the cancer risk (Muntane and la Mata, 2010). High levels of NO can develop carcinogenic nitrosamines or can directly modifying DNA or DNA repair proteins are proved to be genotoxic (Wink et al., 1991). In carcinogenesis, mediated by NO, several alterations in apoptosis, DNA repair and cell-cycle checkpoints are prominent (Jaiswal et al., 2001; Melino et al., 1997; Pervin et al., 2001). Several research results highlighted that substantial modification of key biological target(s) including DNA repair proteins and transcription factor known to be inhibited by NO.

PROTEIN NITRATION AND IMMUNOMODULATION

Immunomodulation refers to the action undertaken by the medication on autoregulating processes that steer the immunological defense system that can lead to autoimmune diseases. It was documented that the persistence of nitrated proteins and peptides may activate the immune system and result in the production of immunoglobulins that specifically recognize 3-nitrotyrosine in human plasma (Ischiropoulos, 2009; Thomson et al., 2007). It has also been reported in the study of mouse and rabbit models that nitrated proteins and peptides readily elicit production of antibodies (Heijnen et al., 2006; Xu et al., 2006). This argument can be supplemented by the work of Herzog et al (62). According to this research, tyrosine nitrated peptides from hen egg-white lysozyme elicited production of monoclonal antibodies in transgenic mice that express hen egg-white lysozyme (Herzog et al., 2005). Gendelman and co-workers demonstrated that specific immune response to nitrated α-synuclein produced a dynamic immuno-inflammatory response in mice that led to the degeneration of dopamine-producing neurons (Giasson et al., 2000). Infection of with Listeria monocytogenes in mice motivated the generation of antigen-presenting cells (APC's) that contain nitrated peptides, demonstrating that APC's generated nitrated peptides in vivo, that may significantly augment the immunogenic response to the infectious agent as well. Another functional feature of tyrosine nitration in the immune system is that the binding of peptide–major histocompatibility complex dimers to CD8 (cluster of differentiation 8) positive T cells is disrupted by the myeloid-derived suppressor cells via nitration of tyrosine residues in the T cell receptor–CD8 complex (Nagaraj et al., 2007). This finding can summarize the tyrosine nitration as a provider of novel mechanism of T-cell tolerance in cancer and operator as an immune system modulator. So it can be demonstrated that nitration of proteins may elevate generation of antibodies to autologous proteins and immunological responses may profoundly influence in autoimmune and inflammatory diseases.

CONCLUSION

Nitration is an early-stage event that may mechanize onset and progression of several diseases. Several experimental data suggest that pathogenesis of major, chronic age-related diseases are associated with activation of redox-sensitive transcription factors. Protein tyrosine nitration appears to be a pathologically important posttranslational modification in various disease models. Commonly used techniques for characterization of protein tyrosine nitration are tandem mass spectrometry and immunostaining following 2-D PAGE. Due to extremely low stoichiometry of nitrated tyrosine residues of proteins in biological samples, it is hard to identify nitrated tyrosine residues without suitable enrichment process. The identification of nitrated proteins in disease models using a enrichment process is necessary for understanding of the pathophysiological roles of protein tyrosine nitration in the target diseases.

ABBREVIATIONS

3-NT	3-nitrotyrosine
AMD	age-related macular degeneration
CD8	cluster of differentiation 8
ESI-MS	electrospray ionization- mass spectrometry
MS	mass spectrometry
NO	nitric oxide
NOS	nitric oxide synthases
eNOS or NOS-3	endothelial NOS
iNOS or NOS-2	inducible NOS
nNOS or NOS-1	neuronal NOS
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPE	retinal pigment epithelial cell
PTN	protein tyrosine nitration

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