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Original article

Myeloperoxidase-mediated protein lysine oxidation generates 2-aminoadipic acid and lysine nitrile in vivo

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ABSTRACT

Recent studies reveal 2-aminoadipic acid (2-AAA) is both elevated in subjects at risk for diabetes and mechanistically linked to glucose homeostasis. Prior studies also suggest enrichment of protein-bound 2-AAA as an oxidative post-translational modification of lysyl residues in tissues associated with degenerative diseases of aging. While in vitro studies suggest redox active transition metals or myeloperoxidase (MPO) generated hypochlorous acid (HOCl) may produce protein-bound 2-AAA, the mechanism(s) responsible for generation of 2-AAA during inflammatory diseases are unknown. In initial studies we observed that traditional acid- or base-catalyzed protein hydrolysis methods previously employed to measure tissue 2-AAA can artificially generate protein-bound 2-AAA from an alternative potential lysine oxidative product, lysine nitrile (LysCN). Using a validated protease-based digestion method coupled with stable isotope dilution LC/MS/MS, we now report protein bound 2-AAA and LysCN are both formed by hypochlorous acid (HOCl) and the MPO/H_2O_2/Cl^- system of leukocytes. At low molar ratio of oxidant to target protein N^{ε}-lysine moiety, 2-AAA is formed via an initial N^{ε}-monochloramine intermediate, which ultimately produces the more stable 2-AAA end-product via sequential generation of transient imine and semialdehyde intermediates. At higher oxidant to target protein N^{ε}-lysine amine ratios, protein-bound LysCN is formed via initial generation of a lysine N^{ε}-dichloramine intermediate. In studies employing MPO knockout mice and an acute inflammation model, we show that both free and protein-bound 2-AAA, and in lower yield, protein-bound LysCN, are formed by MPO in vivo during inflammation. Finally, both 2-AAA and to lesser extent LysCN are shown to be enriched in human aortic atherosclerotic plaque, a tissue known to harbor multiple MPO-catalyzed protein oxidation products. Collectively, these results show that MPO-mediated oxidation of protein lysyl residues serves as a mechanism for producing 2-AAA and LysCN in vivo. These studies further support involvement of MPO-catalyzed oxidative processes in both the development of atherosclerosis and diabetes risk.

1. Introduction

2-Aminoadipic acid (2-AAA) is a low abundance amino acid previously suggested as an intermediate in some minor pathways of lysine catabolism in humans [1,2]. Interest in this relatively uncommon amino acid has increased recently, however, due to its suggested enrichment both in disease-associated tissues and plasma in subjects at risk for development of diabetes. For example, using an untargeted metabolomics approach, Gerszten and colleagues recently reported that plasma levels of 2-AAA herald increased future risk of developing diabetes [3]. Moreover, physiological levels of 2-AAA were shown to promote pancreatic beta cell insulin secretion and lower plasma glucose levels in murine models, further suggesting potential involvement of 2-AAA as a modulator of glucose homeostasis [3]. In earlier studies it was suggested that protein lysyl residue oxidation by myeloperoxidase (MPO) – generated halogenating oxidants [4–10] may serve as a possible mechanism for generation of 2-AAA as a post translational modification of protein and lipoprotein lysyl residues [11]. Subsequent

Abbreviations: *m/z*, mass to charge; MPO, myeloperoxidase; 2-AAA, 2-aminoadipic acid; LysCN, lysine nitrile; Ac-Lys, N^{α}-acetyl-lysine; Ac-2AAA, N^{α}-acetyl-2-aminoadipic acid; Ac-LysCN, N^{α}-acetyl-lysine nitrile; PMN, polymorphonuclear leukocytes; PMA, Phorbol 12-myristate 13-acetate; TG, thioglycollate; ZM, zymosan

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studies by both our group and Monnier and colleagues suggested not only MPO, but also metal catalyzed oxidative processes may participate in 2-AAA formation [11–14]. Indeed, protein bound 2-AAA has been reported to be enriched at sites known to harbor enhanced levels of protein oxidation products, including high density lipoprotein recovered from human atherosclerotic lesions, and insoluble collagen from aged skin, particularly from subjects with diabetes or renal disease [11–14]. Interestingly, while biosynthetic pathways for generating 2-AAA are not observed in mammals, 2-AAA is a reported intermediate in the synthesis of lysine in certain fungi, as well as in the biosynthesis of penicillin in β-lactam-producing fungi [15,16]. It has also been reported as a metabolite produced from bacteria of the genus Thermus [17]. Despite the association between 2-AAA levels and the risk for developing diabetes [3], and its potential links to both vascular disease and degenerative diseases of aging [11–14], neither direct demonstration of oxidative pathway(s) that may participate in 2-AAA formation in vivo in mammals, nor detailed investigation of the biochemical pathway and structural intermediates involved in 2-AAA formation from protein lysyl residues, have been reported.

Wilson and colleagues recently reported that the major product of protein lysyl residue oxidation by reagent hypochlorous acid (HOCl) is not 2-AAA, but instead the unusual adduct lysine nitrile (LysCN, 2-amino-5-cyanopentanoic acid) [18]. While these purely in vitro studies used a high HOCl relative to target (protein lysyl residue) ratio, they reported up to 80% yield of LysCN when a maximal HOCl to target ratio is used (~100 to 600-fold excess) [18]. Nitriles in general can be acid labile [19], and acid hydrolysis was used in all (including our own) prior reported studies detecting protein-bound 2-AAA enrichment in tissues [11–14]. These provocative results suggesting LysCN as a potential HOCl generated oxidation product of protein lysyl residues therefore raised concerns to us of whether protein-bound 2-AAA is even a post translational oxidation product that exists in vivo, and whether 2-AAA is formed by the MPO/H_2O_2/Cl^- system in vivo.

In this paper we report on our pursuit to elucidate the products and reaction mechanisms of protein lysyl residue oxidation by HOCl [6,20–25], MPO-generated chlorinating oxidants [4,5,7–10], and activated leukocytes both in vitro and in animal models employing wild type (WT) and MPO knockout (MPO-KO) mice. The studies presented herein establish that 2-AAA is a major, and LysCN a minor, post translational oxidation product of lysyl residues formed at sites of inflammation. Our studies further demonstrate substantial enrichment of both 2-AAA and LysCN within human aortic atherosclerotic plaque, a site previously shown to harbor enriched content of both MPO and alternative MPO-generated oxidation products [5,10,26–29], and elucidate the reaction pathway responsible for MPO-generated chlorinating oxidants in forming these lysine oxidation products.

2. Materials and methods

2.1. Materials

N^{α}-Boc-L-Lysine and N^{α}-acetyl-L-Lysine used as surrogates for protein lysyl residues were purchased from Chem-Impex International, Inc. (Wood Dale, Illinois). D_3-2-aminoadipic acid was purchased from C/D/N isotopes Inc. (Pointe-Claire, Quebec). $^{13}C_6$, $^{15}N_2$-Lysine was purchased from Cambridge isotope laboratories, Inc. (Andover, Massachusetts). Sodium hypochlorite (NaOCl), H_2O_2, ammonium hydroxide (NH_4OH), trifluoroacetic acid and organic solvents were obtained from Fisher Scientific Co. Pronase was purchased from Roche life science (Indianapolis, Indiana). SAX SPE cartridges were obtained from Jordi lab (Mansfield, Massachusetts). Axis-Shield Polymorphprep was purchased from Cosmo Bio USA (Carlsbad, California). All other materials were obtained from Sigma-Aldrich, unless otherwise indicated.

2.2. Ethical considerations

All animal model studies were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic. All study protocols and informed consent for human subjects were approved by the Cleveland Clinic Institutional Review Board. Informed consents were obtained for isolation of human MPO, neutrophils and all other subject samples.

2.3. General procedures

In vitro oxidation reactions were performed in 50 mM phosphate buffer (PB), pH = 7.0 supplemented with 100 μM diethylene triamine pentaacetic acid (DTPA). The concentrations of NaOCl and H_2O_2 were determined spectrophotometrically (ε292 (NaOCl) = 350 M^{-1} cm^{-1} [30] and (ε240 (H_2O_2) = 39.4 M^{-1} cm^{-1} [31]). Human MPO was isolated, characterized and quantified as described [7]. Human neutrophils were isolated by ficoll-hypaque buoyant density centrifugation as described previously [32]. Phorbol myristate acetate was prepared in dimethyl sulfoxide (DMSO). Cell experiments were performed in Hanks' balanced salt solution, pH 7.2 (no calcium, magnesium, or phenol red) (Thermo Fisher) with 100 μM DTPA.

2.4. Clinical specimens

Human aortic tissues were recovered at the time of vascular or heart transplantation surgery. All tissues were rinsed in ice-cold normal saline immediately, submerged in buffer (65 mM sodium phosphate, pH 7.4, 100 μM DTPA, 100 μM butylated hydroxytoluene), snap frozen with liquid N_2 and stored at −80 °C under argon until used for analysis.

2.5. Synthesis of lysine nitrile, N^{α}-acetyl-2-aminoadipic acid, N^{α}-acetyl-lysine nitrile

Lysine nitrile (2-amino-5-cyanopentanoic acid) synthesis was carried out according to the method of Yamazaki [33] with modifications. Generally, NaOCl (4.5 mmol) was added to the stirring solution of N^{α}-Boc-L-Lysine (500 mg, 2 mmol) in ethanol (10 mL). The mixture was stirred at room temperature for 24 h. Afterwards, the reaction mixture was poured into H_2O (50 mL) and then was extracted with dichloromethane (CH_2Cl_2, 3 × 30 mL). The solution was concentrated by rotary evaporation and the residue purified on a silica gel column and eluted with CH_2Cl_2. The Boc blocking group was removed with a solution of 50% trifluoroacetic acid in CH_2Cl_2. The resultant lysine nitrile was analyzed by high resolution mass spectrometry (MS) and showed the anticipated elemental composition and fragmentation pattern. The theoretical exact mass to charge ratio (m/z) for LysCN $[MH]^+$ ($C_6H_{22}N_2O_2$) is 143.0821 au, the detected m/z was $[MH]^+$ = 143.0820 au, Δppm = 0.4 au.

N^{α}-acetyl-2-aminoadipic acid (Ac-2AAA) was synthesized following the method of Ravindranath [34]. Due to the low solubility of 2-AAA in water, triethylamine (TEA) (6 mmol) was added to H_2O (2 mL) to solubilize 2-AAA (320 mg, 2 mmol). Acetic anhydride (4 mmol) was then added and the mixture was sonicated for 30 min in an FS60 sonic water-bath (Fisher Scientific). This process was repeated after adding another aliquot of acetic anhydride (4 mmol). After sonication, the Ac-2AAA was purified by reverse phase HPLC. The product was confirmed by high resolution MS analysis and showed the anticipated elemental composition and fragmentation pattern (m/z $[M\text{-}H]^-$ = 202.0715 au and the theoretical exact mass ($[M\text{-}H]^-$) of Ac-2AAA = 202.0715 au, Δppm = 0.0 au).

N^{α}-acetyl-lysine nitrile (Ac-LysCN) was synthesized by adding NaOCl (5 mM) to N^{α}-Ac-Lys (2 mM) in 50 mM sodium phosphate, pH = 7.0. The solution was heated at 40 °C for 72 h. Ac-LysCN was isolated from the solution by reverse phase HPLC and dried under reduced pressure in a vacuum centrifuge. The product was analyzed by