Pyridoxamine scavenges protein carbonyls and inhibits protein aggregation in oxidative stress-induced human HepG2 hepatocytes

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A B S T R A C T

Introduction of carbonyl groups into amino acid residues is a hallmark for oxidative damage to proteins by reactive oxygen species (ROS). Protein carbonylation can have deleterious effects on cell function and viability, since it is generally unrepairable by cells and can lead to protein dysfunction and to the production of potentially harmful protein aggregates. Meanwhile, pyridoxamine (PM) is known to scavenge various toxic carbonyl species derived from either glucose or lipid degradation through nucleophilic addition. PM is also demonstrated to catalyze non-enzymatic transamination reactions between amino and α-keto acids. Here, we found that PM scavenges protein carbonyls in oxidized BSA with concomitant introduction of carbonyl groups into amino acid residues, resulting either from oxidation of the amino acid side chains such as lysine, arginine, proline, and tryptophan, or from oxidation of the amino acid residues from the cleavage of peptide bonds by the α-amidation pathway or by oxidation of glutamyl residues. Protein carbonylation can provoke conformational alterations of the polypeptide chain, often leading to the loss of protein function. Furthermore, the carbonylation can elicit protein aggregation by promoting unfolding and formation of non-covalent as well as covalent bonds among proteins. Since protein carbonylation is an irreversible/unrepairable modification, despite recent reports arguing for a decarbonylation process, protein carbonyls are the most widely used biomarker for oxidative damage to proteins, and reflect cellular damage, aging, and several age-related disorders.

1. Introduction

The reactive oxygen species (ROS)-mediated oxidative modification of proteins and subsequent accumulation of modified proteins have been found in cells during aging, oxidative stress, and in various pathological states [1,2]. Among divergent modifications, introduction of carbonyl groups into amino acid residues is a hallmark for oxidative damage to proteins [1,2,3]. Direct oxidation of proteins by ROS yields reactive carbonyl derivatives resulting either from oxidation of the amino acid side chains such as lysine, arginine, proline, and tryptophan, or from oxidation of the amino acid residues from the cleavage of peptide bonds by the α-amidation pathway or by oxidation of glutamyl residues [2,4,5]. Protein carbonylation can provoke conformational alterations of the polypeptide chain, often leading to a loss of protein function. Furthermore, the carbonylation can elicit protein aggregation by promoting unfolding and formation of non-covalent as well as covalent bonds among proteins [6]. Since protein carbonylation is an irreversible/unrepairable modification, despite recent reports arguing for a decarbonylation process [3,7], protein carbonyls are the most widely used biomarker for oxidative damage to proteins, and reflect cellular damage, aging, and several age-related disorders [4,7].

Pyridoxamine 5′-phosphate (PMP) and pyridoxal 5′-phosphate (PLP) belong to the vitamin B6 family and function as co-enzymes for the transamination reaction. PLP/PMP-dependent aminotransferases catalyze transamination between primary amines and carbonyl compounds including keto acids, aldehydes, and ketones [8–10]. Intriguingly, it has been found that pyridoxamine (PM) and a variety of its analogues can non-enzymatically aminate a number of alkyl, aryl, and side chain functionalized α-keto acids to α-amino acids [11–13]. Meanwhile, PM scavenges various toxic carbonyl species derived from either glucose or lipid degradation through nucleophilic addition of its aminomethyl group, inhibiting the modification of proteins to advanced glycoxidation and lipoxidation end products (AGEs and ALEs) [14,15]. In animal model studies, PM also inhibited AGEs/ALEs formation and retarded the development of diabetic complications in streptozotocin-induced
diabetic rats [16].

In the present study, we found that PM non-enzymatically scavenges protein carbonyls accompanied by pyridoxal formation and recovers catalytic activity of oxidized lysozyme. Moreover, we demonstrated that treatment of H₂O₂-exposed HepG2 hepatocytes with PM significantly reduced levels of cellular carbonylated proteins and aggregated proteins, and also improved cell survival rate. Our findings suggest that PM might protect against oxidative stress-induced cell death by recovering impaired protein function and/or abrogating protein aggregation via scavenging protein carbonyls.

2. Materials and methods

2.1. Preparation of oxidized BSA and lysozyme

Fatty acid free BSA and lysozyme chloride from egg white were obtained from Nacalai Tesque (Kyoto, Japan). BSA (1.1 mg/mL) and lysozyme (0.1 mg/mL) were incubated with 10 mM H₂O₂ in the presence of 100 μM FeSO₄ at 37 °C for 30 h in 0.1 M sodium phosphate buffer (PB, pH 7.4). After removal of insoluble protein aggregates by centrifugation, the oxidized protein was dialyzed for 24 h against three changes of PB containing 1 mM EDTA and two changes of PB. Protein concentration was determined using the BCA protein assay Kit (Nacalai Tesque).

2.2. Analysis of protein carbonyls by avidin blotting and spectrophotometric assay

Protein carbonyls were detected as previously described [17,18]. Briefly, protein carbonyls were labeled with biotin hydrazide (Sigma-Aldrich, St. Louis, MO, USA). The biotinylated proteins were reduced with DTT, and then subjected to SDS-PAGE. After the protein was transferred to nitrocellulose membrane, the protein carbonyls were detected with HRP-conjugated streptavidin (Beckman Coulter, Fullerton, CA, USA; 1:8000) and an ECL reagent, EzWestLumiOne (ATTO, Tokyo, Japan). β-Actin was also detected by immunoblotting using anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-rabbit IgG-HRP (Cell Signaling Technology, Danvers, MA, USA). Carbonyl content was quantified spectrophotometrically by the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) according to the published procedure [19].

2.3. Determination of pyridoxal by fluorescent HPLC

Pyridoxal was determined by fluorometric HPLC using a C-18 reversed phase column (LiChrospher RP-18, 125 × 4.0 mm, Merck, Darmstadt, Germany). The conditions were as follows: 2.2 mM 1-octane sulfonic acid in potassium dihydrogen phosphate and 19 mM 85% phosphoric acid and 4.0 mM triethylamine, adjusted to pH 2.75 (solvent A) and acetoniitrile (solvent B), isocratic elution with 7% solvent B, and a flow rate of 1.0 mL/min [20]. The eluate was monitored with excitation and emission wavelengths set at 296 and 385 nm, respectively.

2.4. Fluorescent HPLC analysis of carbonyl amino acids

After dialysis of protein samples, α-aminoacidic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) in proteins were labeled by reductive amination with NaN⁴BH₃ and p-aminoobenzic acid (ABA) to AAS-ABA and GGS-ABA. The detailed protocol was described in our previous reports [21,22]. After acid hydrolysis, AAS-ABA and GGS-ABA were determined by fluorometric HPLC using a C-18 reversed phase column (COSMOSIL SC18-AR-II, 250 × 4.6 mm, Nacalai Tesque). A discontinuous gradient was used with solvent A (50 mM sodium acetate, pH 5.0) and solvent B (acetonitrile) as follows: 0% B at 0 min, 8% B at 20 min at a flow rate of 1.5 mL/min. The eluate was monitored with excitation and emission wavelengths set at 280 and 350 nm, respectively.

2.5. Measurement of lysozyme activity

Lysozyme activity was measured by turbidimetric method with Micrococcus lysodeikticus (Nacalai Tesque) as substrate. The lysozyme solution (20 μL) was mixed with 180 μL of Micrococcus lysodeikticus suspension (1.0 mg/mL) in 100 mM sodium phosphate buffer (pH 7.0) in a 96-well plate. The turbidity was determined continuously at 570 nm using a microtiter plate reader. The initial rate of turbidity decrease was estimated from the linear portion of the earliest time points of the enzymatic reaction.

2.6. Cell culture and cell viability assay

The human hepatocyte cell line HepG2 (ATCC, Manassas, VA, USA) was cultured in DMEM (4.5 g/L glucose) supplemented with 10% FBS, 100 units/mL streptomycin, and 100 μg/mL penicillin at 37 °C in a humidified 5% CO₂ atmosphere. Prior to the experiments, the cells were incubated overnight with serum-free DMEM. Cell viability was measured by WST-8 assay using Cell Count Reagent SF (Nacalai Tesque) following the manufacturer’s instructions.

2.7. Preparation of soluble and insoluble protein fractions

Soluble and insoluble proteins were fractionated as previously described with some modifications [23]. Briefly, the cells were washed twice with ice-cold PBS and lysed in RIPA buffer supplemented with 1 × protease inhibitor cocktail (Nacalai Tesque) for 30 min on ice. Lysates were centrifuged at 16,000 ×g for 10 min at 4 °C to collect the soluble proteins present in the supernatant. After supernatant removal, the pellet was extracted in RIPA buffer three times, and then the insoluble protein pellet was solubilized in urea buffer (8 M urea, 2% CHAPS) supplemented with 1 × protease inhibitor cocktail and sonicated for 15 min.

2.8. ProteoStat staining assay

A ProteoStat® aggresome detection kit (Enzo life sciences Inc., Plymouth Meeting, PA, USA) was used to characterize aggregated proteins in HepG2 hepatocytes. The cells were stained with the ProteoStat dye and DAPI (Nacalai Tesque) according to the manufacturer’s protocol. Then, the cells were imaged using a fluorescence microscope, BZ-9000 (Keyence, Osaka, Japan).

3. Results

3.1. Scavenging of protein carbonyls by PM

We initially established that PM concentration- and time-dependently converts phenylpyruvic acid to phenylalanine under physiological conditions at pH 7.4 and 37 °C (Supplementary Fig. S1). To evaluate whether or not PM directly scavenges protein carbonyls, we attempted to detect carbonyl groups in PM-treated oxidized BSA using biotin hydrazide as a molecular probe [17,18]. After labeling protein carbonyls with biotin hydrazide, the cells were stained with the ProteoStat dye and DAPI (Nacalai Tesque) according to the manufacturer’s protocol. Then, the cells were imaged using a fluorescence microscope, BZ-9000 (Keyence, Osaka, Japan).
of BSA to hydroxyl radicals, followed by dialysis to remove Fenton’s reagent, and incubated OxBSA with 0–100 mM PM for 0–15 h. As seen in Fig. 1B and C, treatment of OxBSA with PM resulted in concentration- and time-dependent decreases in carbonyl group contents. Moreover, we quantified total protein carbonyl groups were detected by avidin blotting.

PM scavenges protein carbonyls in OxBSA. (A) BSA (0.1 mg/mL) was incubated with 0–100 mM H$_2$O$_2$ in the presence of 100 mM Fe$^{2+}$ in PB (pH 7.4) at 37 °C for 15 h. After being labeled with biotin hydrazide, the protein carbonyl groups were detected by avidin blotting. (B) OxBSA (0.1 mg/mL) was incubated with 0–100 mM PM in PB at 37 °C for 15 h. The biotin-labeled protein carbonyl groups were detected by avidin blotting. (C) OxBSA (0.1 mg/mL) was incubated with 0–100 mM PM in PB at 37 °C for 0–15 h. The biotin-labeled protein carbonyl groups were detected by avidin blotting. (D) OxBSA (0.1 mg/mL) was incubated with or without 100 mM PM in PB at 37 °C for 15 h. Protein carbonyls were derivatized with DNPH and determined by spectrophotometric assay. Data are presented as means ± SEM (n = 6). (E) PM (100 μM) was incubated with or without native BSA (0.1 mg/mL) or OxBSA (0.1 mg/mL) in PB at 37 °C for 15 h. Pyridoxal was determined by fluorometric HPLC. Data are presented as means ± SEM (n = 4). (F) Chemical structures of GGS and AAS. (G and H) OxBSA (0.1 mg/mL) was incubated with or without 100 mM PM in PB at 37 °C for 15 h. After fluorescent derivatization and acid hydrolysis, GGS (G) and AAS (H) were determined by fluorometric HPLC. Data are presented as means ± SEM (n = 3). *P < 0.05, **P < 0.001 compared as indicated (ANOVA, Tukey’s test).

We also observed significant production of pyridoxal during incubation of PM with BSA and oxBSA but not during incubation of PM alone (Fig. 1E), implying that PM might scavenge protein carbonyls via transamination. To further characterize the protein carbonyl scavenging action of PM, we determined levels of GGS and AAS (Fig. 1F), the most predominant forms of the carbonyl amino acids [22,24], in proteins. AAS is the oxidation product of the lysine residue after exposure to oxygen free radicals, whereas GGS...
originated from the oxidation of arginine and proline. After derivatization with the fluorescent reagent ABA, the PM-treated and untreated BSAs were hydrolyzed, and analyzed by fluorometric HPLC (Supplementary Fig. S2). As shown in Fig. 1G and H, we demonstrated that the incubation of OxBSA with 100 μM PM for 15 h caused significant declines in GGS and AAS by about 75% and 70% of untreated OxBSA, respectively.

3.2. Reactivation of oxidized lysozyme by PM

ROS-mediated protein carbonylation alters the conformation of the polypeptide chain due to the increased hydrophobicity, eventually leading to protein inactivation and aggregation [6,25]. Indeed, as shown in Fig. 2A, the exposure of lysozyme to Fenton reagent significantly reduced its bacteriolytic activity, and coincidently evoked the formation of protein aggregates (Supplementary Fig. S3). Based on our results, we speculated that PM might reactivate oxidized proteins by scavenging protein carbonyls. Hence, PM-treated oxidized lysozyme was evaluated by determining its bacteriolytic activity and protein carbonyl content. Oxidized lysozyme was prepared by exposure of lysozyme to hydroxyl radicals, followed by removal of insoluble protein aggregates and Fenton’s reagent, and then incubated with 0–500 μM PM for 24 h. As shown in Fig. 2B and C, PM effectively recovered oxidized lysozyme activity, and concomitantly scavenged protein carbonyls in a concentration-dependent manner. These results suggest that PM treatment might exert cytoprotective action against oxidative stress-injured cells through scavenging of protein carbonyls.

3.3. Decline in protein carbonyls and aggregates in H2O2-exposed HepG2 cells by PM treatment

Oxidative stress-related carbonylation of cellular proteins triggers the accumulation of high-molecular-weight aggregates in cells [26]. Increased protein aggregation eventually elicits cell death due to aggregate-associated cytotoxicity [27]. To assess the cytotoxicity and actions of PM, we used HepG2 hepatocytes because liver is exposed to high levels of endogenous and exogenous ROS, and in liver diseases, ROS are involved in cell death and liver injury [28,29]. The exposure of HepG2 cells to H2O2 resulted in the generation of multiple carbonylated cellular proteins (Fig. 3A), and coincidently provoked significant cell death in a dose-dependent manner (Supplementary Fig. S4). To determine whether cellular protein carbonyls can be reduced by PM, we treated H2O2-exposed HepG2 cells with or without PM for 15 h. Consequently, we found that PM treatment effectively decreased the levels of cellular carbonylated proteins (Fig. 3B), and also improved cell survival rate (Fig. 3C).

To ascertain the inhibitory potency of PM against cellular protein aggregation, we stained the cells with ProteoStat dye, a fluorescent molecular rotor dye that specifically interacts with denatured proteins within aggregates [30,31]. As observed by fluorescence microscopy (Fig. 4A), the exposure of HepG2 cells to H2O2 markedly gave rise to the accumulation of aggregated proteins in the cytoplasm, whereas the PM treatment for 15 h significantly diminished aggregate formation in the H2O2-exposed cells. Protein aggregates are known to remain insoluble in strong detergent buffers but can be solubilized in urea [32]. To further verify whether PM prevents the accumulation of carbonylated protein-related aggregates, we analyzed the detergent soluble and insoluble proteome by SDS-PAGE/avidin blotting. The exposure of HepG2 cells to H2O2 caused a significant accumulation of highly carbonylated insoluble proteins (Supplementary Fig. S5). As shown in Fig. 4B and C, we demonstrated that the treatment of H2O2-exposed HepG2 cells with 100 μM PM for 15 h significantly decreased levels of carbonylated insoluble proteins. These findings suggest that PM might protect against oxidative stress-induced cellular dysfunction by abrogating protein aggregation via scavenging of protein carbonyls.

4. Discussion

Protein carbonylation is believed to have deleterious effects on both cell function and viability, since it is generally unrepairable by cells and can lead to protein dysfunction and to the production of potentially harmful protein aggregates. Oxidized proteins are normally recognized and degraded by intracellular proteases, such as the proteasome complex. Nevertheless, ROS can react with these proteolytic complexes inducing a decline in their functionality [33],
Primary carbonylation can occur via direct attack at the polypeptide backbone and the amino acid side chains by hydroxyl radicals, which can be produced by the Fenton reaction and ionizing radiation [5,34]. Alternatively, carbonyl groups are also introduced in proteins by adduction of carbonyl-containing peroxidized lipids (4-hydroxynonenal, malondialdehyde, acrolein, etc.) or carbohydrates (i.e. glycation) [2,27]. There is now increasing evidence that protein carbonylation is selective in inactivating particular proteins such as annexin A1, Pin1, and SOD1 [1,2,34,35]. Elevated levels of protein carbonyls in tissue proteins have been detected during aging and diverse pathological states such as Alzheimer’s disease, amyotrophic lateral sclerosis, diabetes, and Parkinson’s disease, thus suggesting their potential causative role in normal aging and pathogenesis [1,2,4]. In this study, we demonstrated that PM non-enzymatically scavenges carbonyl groups including AAS and GGS residues in the oxidized BSA with concomitant formation of pyridoxal (Fig. 1). Although the precise molecular mechanism by which PM scavenges chemically diverse protein carbonyls remains to be elucidated, it is clear that the first step of the reaction is, at least, the addition of the primary amino group of PM to the carbonyl group in the oxidized protein, yielding a carbinolamine that is dehydrated to a Schiff base adduct (Supplementary Fig. S6). Considering the basis of the transamination reaction [8,9], the resulting Schiff base adduct might convert into a pyridoxaldimine adduct through the trans-Schiff base reaction. Then, the adduct might release pyridoxal through hydrolysis, thereby resulting in the amination of oxidized proteins.

Furthermore, it should be noted that PM treatment effectively recovered oxidized lysozyme activity with an accompanying decline in carbonyl group contents (Fig. 2). Our data suggest that scavenging of carbonyl groups in oxidized lysozyme by PM, might, at least in part, be attributed to reactivation of lysozyme activity. We also observed that PM treatment after exposure of H2O2 to HepG2 cells reduced the levels of cellular carbonylated proteins (Fig. 3B) and prevented the accumulation of carbonylated protein-related aggregates (Fig. 4). Once formed, such aggregates are
extremely resistant to proteolysis and can act as inhibitory compounds towards both the proteasome and lysosome degradation pathways [33]. Consequently, the aggregates can cause cytotoxicity via necrosis or apoptosis, and have been associated with a large number of age-related disorders, including Parkinson’s disease, Alzheimer’s disease, and cancer [26,27]. Hence, prevention of protein carbonylation and removal of carbonylated proteins are crucial for cell survival under oxidative stress. Indeed, we proved that PM treatment of H_2O_2-exposed HepG2 cells significantly increased cell survival rate (Fig. 3C).

Recently, PM has emerged as a promising pharmacological agent for protecting against the progressive tissue damage that occurs in diabetic nephropathy, diabetic retinopathy, hyperlipidemia, and other diseases [14]. This development is ascribed to the initial discovery that PM is a potent inhibitor of AGEs and ALEs formation, which has been closely related to the pathogenesis of diabetic complications and ageing [14,15]. Although the reported PM levels in rat plasma range from 0.001 to 0.02 μM, upon pharmacological supplementation in rats, plasma PM levels significantly rise, in some cases reaching values ~100 μM [14]. On the other hand, in toxicity studies on animals and humans, PM showed extremely low toxicity in oral administration with no reported adverse effects [14]. Our results demonstrated that treatment of H_2O_2-exposed HepG2 hepatocytes with 100 μM PM effectively decreased levels of cellular carbonylated proteins and significantly increased cell survival rates (Fig. 3). It is therefore conceivable that PM may act as an efficient scavenger of protein carbonyls in vivo, especially as it has been demonstrated that physiologically relevant concentrations of

Fig. 4. PM decreases the accumulation of aggregated proteins in H_2O_2-exposed HepG2 hepatocytes. (A) The cells were treated with 2 mM H_2O_2 for 5 h, followed by washing twice with serum-free DMEM. The cells were further incubated with or without 100 μM PM in serum-free DMEM for 15 h. After incubation, the cells were stained with ProteoStat dye and DAPI, and imaged using fluorescence microscopy. (B and C) The cells were treated with 2 mM H_2O_2 for 5 h, followed by washing twice with serum-free DMEM. The cells were further incubated with 0–100 μM PM in serum-free DMEM for 15 h. After derivatization with biotin hydrazide, the detergent soluble and insoluble proteins were analyzed by CBB staining (B) and avidin blotting (C).
PM are sufficient to cause cytoprotective effects against H₂O₂-exposed HepG2 hepatocytes.

**Conflict of interest**

None.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at [http://dx.doi.org/10.1016/j.bbrc.2017.03.147](http://dx.doi.org/10.1016/j.bbrc.2017.03.147).

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