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Review article
Pregledni članak

NONENZYMATIC POST-TRANSLATIONAL MODIFICATION DERIVED PRODUCTS: NEW BIOMARKERS OF PROTEIN AGING

NEENZIMSKE POSTTRANSLACIONE MODIFIKACIJE DERIVISANIH PRODUKATA: NOVI BIOMARKERI STARENJA PROTEINA

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Summary: During their biological life, proteins are exposed in a cumulative way to irreversible nonenzymatic post-translational modifications that are responsible for their molecular aging and generate specific by-products called »post-translational modification derived products« (PTMDPs). PTMDPs are involved in the pathogenesis of various diseases such as diabetes mellitus, renal insufficiency and atherosclerosis, and are potential biomarkers in clinical practice. Nonenzymatic glycation refers to the spontaneous binding of glucose and reducing sugars to free amino groups and is amplified by oxidative processes (referred to as »glycoxidation«). It generates many reactive by-products such as aldehydes and leads to the formation of »advanced glycation end products« (AGEs). AGEs accumulate *in vivo*, alter tissue organization and activate membrane receptors such as RAGE, which triggers inflammatory responses. Carbamylation is due to the binding of isocyanic acid, formed *in vivo* either by spontaneous dissociation of urea or by action of myeloperoxidase on thiocyanate, and generates homocitrulline from lysine groups. Carbamylation leads to alteration of the structural and biological properties of proteins, and favors inflammation and atherosclerosis. PTMDPs may be assayed by different methods, among others LC-MS/MS or immunoassays, constitute a promising field of investigation in basic research and are potential major biomarkers in laboratory medicine.

Keywords: post-translational modifications, glycation, carbamylation, aging, diabetes mellitus, chronic renal failure

Kratak sadržaj: Tokom svog biološkog života, proteini su na kumulativan način izloženi nepovratnim neenzimskim posttranslacionim modifikacijama odgovornim za njihovo molekulsko starenje usled kojih nastaju specifični sporedni produkti koji se nazivaju »posttranslacionim modifikacijama derivisanih produkata« (PTMDPs). PTMDPs učestvuju u patogenezi raznih bolesti, kao što su diabetes mellitus, renalna insuficijencija i ateroskleroza, i predstavljaju potencijalne biomarkere u kliničkoj praksi. Neenzimska glikacija odnosi se na spontano vezivanje glukoze i redukujućih šećera za slobodne amino grupe, dopunjeno oksidativnim procesima (što se naziva »glikoksidacija«). Tokom nje nastaju mnogi reaktivni sporedni produkti poput aldehida i ona dovodi do nastanka »naprednih krajnih proizvoda glikacije« (AGEs). AGEs se akumuliraju *in vivo*, menjaju organizaciju tkiva i aktiviraju membranske receptore kao što su RAGE, što pokreće inflamatorne odgovore. Karbamilacija je posledica vezivanja izocijanske kiseline, nastale *in vivo* spontanom disocijacijom uree ili delovanjem mijeloperoksidaze na tiocijanat, i stvara homocitrulin iz lizinskih grupa. Karbamilacija dovodi do promena u strukturnim i biološkim svojstvima proteina i potencira inflamaciju i aterosklerozu. PTMDPs se mogu odrediti pomoću različitih metoda, među kojima su LC-MS/MS i imunotestovi. PTMDPs predstavljaju oblast u osnovnom istraživanju koja donosi brojne mogućnosti i uz to su potencijalno važni biomarkeri u laboratorijskoj medicini.

Ključne reči: posttranslacione modifikacije, glikacija, karbamilacija, starenje, diabetes mellitus, hronično oboljenje bubrega

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Non-standard abbreviations: AGEs, advanced glycation end products; ALEs, advanced lipoxidation end products; AOPPs, advanced oxidation protein products; CDPs, carbamylation derived products; CML, N-(carboxymethyl)lysine; GC-MS, gas chromatography/mass spectrometry; 4-HNE, 4-hydroxynonenal; LC-MS/MS, liquid chromatography/tandem mass spectrometry; MALDI, matrix assisted laser desorption ionization; MDA, malondialdehyde; PTMDPs, nonenzymatic post-translational modifications derived products; RAGE, receptor for advanced glycation end products; RCCs, reactive carbonyl compounds.

Introduction

Many biochemical processes participate in the pathogenesis of aging and diseases. Among them, the »nonenzymatic post-translational modifications« alter proteins throughout their biological life in organisms. These chemical modifications may be considered hallmarks of protein molecular aging. The most studied reactions are glycation (or glycoxidation), carbonylation, and carbamylation (1). These cumulative modifications are usually characterized by the covalent binding of small metabolites to free reactive groups of proteins, followed by irreversible molecular rearrangements. The intensity of post-translational modifications depends on many factors, such as the concentration of the binding metabolite, which increases in various pathological situations, and the lifespan of the protein. In addition, these reactions are very intricate, and compete for a limited number of protein sites. They progressively occur during aging, and are amplified in various diseases such as diabetes mellitus, chronic renal failure or atherosclerosis.

Damaged proteins constitute a molecular substratum for many dysfunctions described in metabolic and age-related diseases. Accordingly, »nonenzymatic post-translational modification derived products« (PTMDPs) are considered useful potential biomarkers of these diseases (1, 2). The only biomarker currently assayed in routine practice is HbA_{1c}, which is considered the »gold standard« for assessing the efficiency of diabetic treatment. However, the implementation of mass spectrometry and proteomics in clinical laboratories could constitute a decisive step for the development of PTMDP evaluation in patients, provided standardization of assays is achieved.

This review summarizes the most prominent nonenzymatic post-translational modifications of proteins involved in molecular aging, and identifies the most suitable PTMDPs to be used as biomarkers in clinical practice.

Glycation, glycoxidation and carbamylation

Many nonenzymatic post-translational modifications occur concomitantly to trigger molecular aging of proteins (*Figure 1*), which is characterized by different features, e.g. accumulation of aged proteins (3), loss of biological functions, impairment of interactions (4, 5), generation of new antigenicity (6), interaction with new cellular receptors, mediating inappropriate biological effects (7).

The most common reaction is glycation, which refers to the binding of glucose or other reducing sugars to proteins. The initial formation of an instable Schiff base is followed by a molecular (»Amadori«) rearrangement to form stable ketoamines (»Amadori products«) which are further processed by a variety of

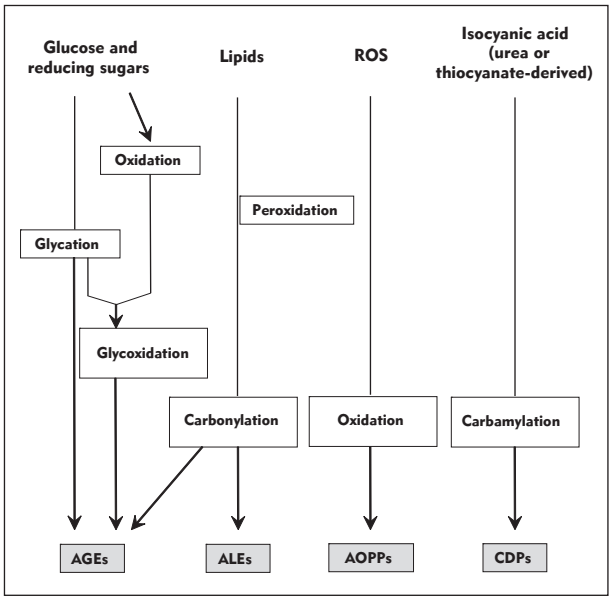

Figure 1 Formation of PTMDPs.

oxidative reactions (glycoxidation) and generate a wide group of complex compounds called »advanced glycation end products« (AGEs) (8, 9). Several AGEs, like pentosidine or N^{ε}(carboxymethyl)-lysine (CML), have been characterized, but many others remain unidentified, since glycation is triggered by many sugar-derived metabolites, and several AGEs are formed independently from the Amadori pathway, by direct binding of reactive carbonyl compounds (RCCs, e.g. glyoxal, methyglyoxal and 3-deoxyglucosone), generally on arginine residues (1). The structures of some characteristic AGEs are shown in *Figure 2A*.

AGEs accumulate in various tissues, where they interact with specific receptors, especially RAGE (»Receptor for advanced glycation end products«). The subsequent cell signalling, which triggers inflammatory processes, could play a significant role in the progression of various diseases (7, 10).

Oxidation also generates »advanced oxidation protein products« (AOPPs) (1, 11) such as methionine sulphoxide and 3-nitrotyrosine (12). RCCs generated by oxidative stress not only act as potent glycating agents but also support the carbonylation reaction, which leads to the formation of compounds which most derive from lipid peroxidation, called »advanced lipoxidation end products« (ALEs) (13).

Glycation, oxidation and carbonylation pathways are often entangled in living organisms (*Figure 1*), and are increased in diabetes mellitus and aging.

Another important nonenzymatic post-translational modification is carbamylation, which refers to the binding of isocyanic acid to amino groups. The reaction with ε-NH_2 lysine residues elicits the formation of homocitrulline (*Figure 2B*), the most characteristic carbamylation-derived product (CDP). Isocyanic

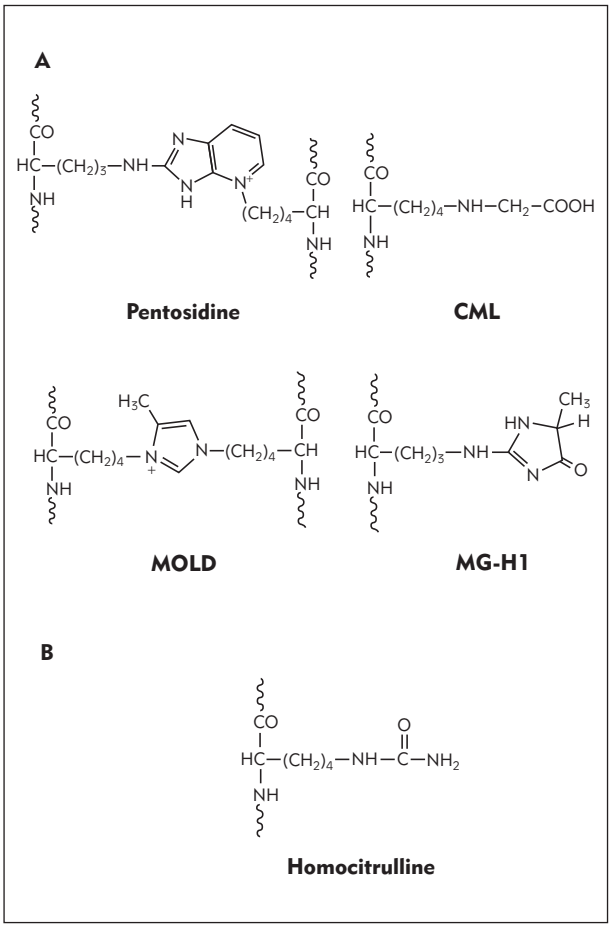

Figure 2 Structure of some typical PTMDPs (A: AGEs; B: CDP).

acid is generated either from the spontaneous dissociation of urea (14) or from the transformation of thiocyanate by myeloperoxidase (15). This reaction is increased during chronic renal failure and atherosclerosis.

PTMDPs are associated with the development of long-term complications of age-related or metabolic diseases (1, 7, 8, 13–18). In the organism, PTMDPs are either found bound to proteins or under peptidic or free forms, since damaged proteins may undergo limited proteolysis, and release modified peptides or amino acids in blood and urine (19).

The exact mechanisms responsible for the progression of protein molecular aging are difficult to identify since many of these reactions compete for a limited number of sites, generally ε-NH_2 of lysine residues. For that reason, each reaction cannot be considered separately. For example, many post-translational modifications of proteins are involved in atherosclerosis. Oxidation, glycation, carbonylation and carbamylation of low density lipoproteins (LDLs), and possibly high-density lipoproteins (HDLs), seem to be simultaneously involved (13, 15, 20–23).

Measurement of PTMDPs in biological fluids

PTMDPs exhibit a large variety in structure, stability and reactivity. Their measurement requires specific and highly sensitive methods.

The most commonly evaluated glycation products are glycated hemoglobin (HbA_{1c}) and fructosamines, which are typical Amadori products. HbA_{1c}, the most abundant glycated hemoglobin, results from the binding of glucose on the N-terminal valine residues of globin β chains. HbA_{1c} is considered the gold standard of diabetic survey and provides a retrospective reflection of the quality of glycemic control during the past 6–8 weeks (24).

For a long period, there has been a lack of assay standardization. In the 2000s, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has designated HbA_{1c} as the reference measurand, and has developed a reference method for HbA_{1c} measurement. This method quantifies the glycated N-terminal hexapeptide of Hb β chain (obtained by Glu-C endoproteinase digestion) by HPLC separation coupled to electrospray ionization mass spectrometry or to capillary electrophoresis (25). All HbA_{1c} assay methods must be traceable to this internationally accepted reference method (26).

HbA_{1c} assay cannot be used in some clinical circumstances such as anemia, blood transfusion or in the presence of some Hb variants (27). In this case, the measurement of plasma fructosamine or glycated albumin may constitute a valuable alternative solution, but provide information on the quality of diabetes control in the 2–3 weeks before sampling only (28, 29). Other specific glycated proteins like apolipoproteins may be measured by immunoassays (30) and could constitute interesting biomarkers in diabetes mellitus and atherosclerosis, but their use in routine practice is limited.

Advanced glycation end products may be measured by various methods. An easy way to evaluate AGEs is to quantify their fluorescence at specific wavelengths. However, non-fluorescent AGEs are not measured, and these techniques are prone to various interferences, especially from fluorescent AOPPs. This principle has been developed for designing a non-invasive device able to evaluate skin autofluorescence. It turns out that this parameter correlates with plasma concentrations of AGEs and to the clinical complications of diabetes mellitus, renal failure or atherosclerosis (31).

Specific HPLC methods allow the quantification of many AGEs, such as pentosidine, CML, or argpyrimidine (1). Immunogenic properties of AGEs have also been used to develop »home-made« ELISA methods, for example for CML (32), which are however subject to analytical problems and are not standardized, which does not allow interlaboratory comparisons.

The most recent approaches use mass spectrometry. Especially, liquid chromatography/mass spectrometry (LC-MS) applications have been preferred, since the development of tandem mass spectrometric detectors has significantly increased the sensitivity of the techniques. LC-MS/MS methods have been developed for the quantitative evaluation of many AGEs including CML, pentosidine, hydroimidazolones, bis(lysyl)imidazolonium AGEs and monolysyl AGEs (33). However, major difficulties of standardization remain, particularly due to the lack of availability of convenient standards.

The measurement of reactive intermediate compounds (RCCs such as 4-HNE, MDA or 3-deoxyglucosone) is difficult because of their high reactivity and instability. They may be assayed by various poorly specific reactions, such as the thiobarbituric acid reactive substances (TBARS) assay (34). RCCs may also be specifically evaluated by GC-MS or HPLC after chemical stabilization (13, 35).

AOPPs may be assayed by a simple but non-specific colorimetric method, which grossly evaluates dityrosine content (36) or more specifically by LC-MS/MS (33, 37).

Several carbamylated proteins or CDPs are promising biomarkers in various diseases, especially renal failure and atherosclerosis. For instance, carbamylated hemoglobin (cHb) may be evaluated by measuring globin β chain N-terminal carbamyl-valine residue by HPLC/UV quantification of the valine hydantoin derivative obtained after acid hydrolysis (38). Carbamylated hemoglobin is considered a valuable marker of chronic renal failure (39), and interferes with HbA_{1c} measurement, with a variable intensity according to the methods used (40). Carbamylated lipoproteins seem to be promising markers in atherosclerosis (22, 23). Homocitrulline measurement by LC-MS/MS allows a specific and sensitive assessment of the rate of protein carbamylation (15).

The future of new biomarkers

PTMDPs are numerous, their formation is complex, and their metabolisms are entangled. The choice of relevant new markers will be based on various items:

Analytical relevance

PTMDPs may be bound to proteins, bound to peptides or free, so that the measured form has to be clearly determined. Some of them may be exogenous (41). Preanalytical conditions, such as the nature of the biological fluids used and the sampling conditions have to be defined (42). End products seem more suitable than intermediates, which are highly reactive and labile.

Field methods must meet criteria of specificity and sensitivity, but also of practicality and robustness. ELISA methods allow the determination of specific modified proteins, whereas LC-MS/MS methods, that are recognized for their better sensitivity and specificity when compared to classical HPLC methods (43), allow to measure specific AGEs (33).

Also, proteomics (MALDI-MS) and spectroscopic methods could be cutting edge technologies used for PTMDPs evaluation (44, 45). As PTMDPs generate specific spectral »fingerprints«, Raman spectroscopy has been recently proposed for the non-invasive evaluation of AGE-induced modifications (46).

Clinical relevance

Some well identified PTMDPs (e.g. pentosidine, CML or homocitrulline), that have already been evaluated in published clinical studies, appear to be of significant interest as markers.

A major concern is the complexity of interpreting the results in a given clinical context, since these compounds represent the outcome of different simultaneous and competing chemical reactions. The combination of several markers using algorithms may be a relevant approach to provide a »score« of the molecular aging of proteins, and would strengthen the concept of »metabolic memory« of the organism (47).

Novel markers

The enzymes involved in the mechanisms of protein repair (e.g. glyoxalases, or fructosamine-3-kinase, which catalyzes the removal of fructosamines from glycated proteins (48)), seem promising tools. Relevant genetic markers are under investigation as well, such as glyoxalase I and RAGE gene polymorphisms, which have been shown to be associated with diabetic complications and vascular damage (49). Their individual use in selected populations, however, still needs to be clarified.

Conclusion

Many reactions contribute to the development of long-term complications of metabolic or chronic diseases, generating potentially interesting biomarkers. Their use in routine practice implies the transfer of sensitive technologies such as LC-MS/MS into clinical chemistry laboratories, provided the standardization of the methods and the validation of guidelines for interpretation in specific pathological situations is achieved.

Conflict of interest statement

The author stated that there are no conflicts of interest regarding the publication of this article.

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