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Original article

Carbamylation is a competitor of glycation for protein modification *in vivo*

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ABSTRACT

Aim. – Chronic kidney disease (CKD) and diabetes mellitus are two diseases that accelerate protein molecular ageing through carbamylation and glycation reactions, characterized by the binding of urea-derived isocyanic acid and of sugars on proteins, respectively. These two reactions target the same protein amino groups and, thus, compete with each other. Such competition may arise especially in diabetic patients with nephropathy. This study aimed to evaluate their potential competitive effects *in vitro* and under conditions reproducing CKD and/or diabetes *in vivo*.
Methods. – Albumin was incubated *in vitro* with glucose, urea or cyanate. Carbamylation *in vivo* was enhanced in normal and diabetic (*db/db*) mice by either subtotal nephrectomy or cyanate consumption. Homocitrulline, carbamylated haemoglobin and furosine were measured by LC–MS/MS, fructosamine by colorimetric assay and HbA_{1c} by immunological assay.
Results. – Reciprocal inhibition between carbamylation and glycation was observed during albumin incubations *in vitro*. Besides, 5 weeks after induction of CKD *in vivo*, plasma homocitrulline concentrations were similar in both diabetic and non-diabetic mice, whereas fructosamine and HbA_{1c} were decreased (−23% and −42%, respectively) in diabetic mice with CKD compared with only diabetic ones. Fructosamine and HbA_{1c} were also decreased in cyanate-spiked water-drinking mice compared with plain water-drinking diabetic mice.
Conclusion. – Carbamylation competes with glycation *in vivo*, especially under conditions of high glycation. Thus, the classic markers of glycaemic control should be interpreted with caution in diabetic patients with CKD because of this competitive effect.

Introduction

Several chronic diseases, including chronic kidney disease (CKD) and diabetes mellitus, are characterized by increased intensity of non-enzymatic post-translational modifications (NEPTMs) that alter the structural and functional properties of proteins through the formation of bioactive post-translational modification-derived products (PTMDPs) [1]. The most prominent reaction is glycation, elicited by the initial binding of glucose and other sugars to amino groups of proteins, followed by Amadori rearrangement and its subsequent reactions (for example, oxidation, cleavages, molecular rearrangements, formation of cross-links). Glycation leads first to the formation of stable Amadori products, such as glycated haemoglobin (HbA_{1c}) and serum fructosamine, and then to a group of complex compounds called "advanced glycation end-products" (AGEs; for example, N[6]-carboxymethyllysine and pentosidine) [2].

Because of the role of oxidation in the formation of AGEs, the entire process is referred to as "glycoxidation" by many authors.

Abbreviations: AGEs, Advanced glycation end products; CDPs, Carbamylation-derived product; CKD, Chronic kidney disease; cLDL, Carbamylated low-density lipoprotein; HCit, Homocitrulline; NEPTMs, Non-enzymatic post-translational modifications; PTMDPs, Post-translational modification-derived products.

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However, AGEs may also be formed by non-oxidative processes after direct binding of carbonyl compounds produced from sugar-derived metabolites, such as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The classic carbonyl compounds produced through this process are glyoxal and methylglyoxal. These compounds interact preferentially with lysine and arginine residues, generating hydroimidazolone AGEs as byproducts (glyoxal-hydroimidazolone-1 [G-H1], methylglyoxal-hydroimidazolone-1 [MG-H1] and argpyrimidine).

Glycation and glycoxidation rates are increased in patients with diabetes because of hyperglycaemia, and in patients with CKD because of sustained oxidative stress and decreased clearance of AGEs. Glycation-derived products arouse interest as biomarkers (HbA_{1c} is routinely used for evaluating glycaemic control) [3] and as pathogenic agents, as AGEs have deleterious effects within tissues through interactions with membrane receptors, especially RAGEs (receptor of AGEs), which trigger inflammatory cell responses accounting for AGE toxicity [4,5].

However, other NEPTMs are involved in the pathophysiology of chronic diseases. In CKD, elevated urea concentrations reinforce the intensity of another NEPTM, carbamylation, which refers to the non-enzymatic binding of isocyanic acid, mainly formed by urea dissociation, to protein amino groups. The most characteristic carbamylation-derived product (CDP), homocitrulline (HCit), derives from isocyanic acid binding to the ε-amino group of lysine residues [6]. Like glycation, carbamylation is able to alter protein properties and their interactions with cells [7]. Clinically, the carbamylation rate of plasma proteins has been associated with increased cardiovascular mortality [8,9] and morbidity [10–12].

Many studies have described the role of carbamylated proteins in the development of chronic diseases. For instance, carbamylated plasma proteins stimulate cultured mesangial cell synthesis of collagen I and IV, favouring fibrosis [13]. Carbamylation of lipoproteins promotes atherosclerosis. In animals, administration of carbamylated low-density lipoprotein (cLDL) induces their endothelial and subendothelial accumulation [14]. Although little is known of the receptor for carbamylated proteins, it has been shown that cLDL links with LOX-1 receptor on endothelial cells [15] and causes their dysfunction through nitric oxide (NO) synthase uncoupling [16]. In addition, it has recently been demonstrated that carbamylation happens physiologically during chronological ageing and is directly related to life expectancy [17].

In humans, NEPTMs cannot be considered on their own, as their ultimate effects on protein and tissue functions constitute the combined outcome of the simultaneous occurrence of all these various and complex non-regulated processes, which happen physiologically at a basal level, especially upon ageing [17,18], and are amplified in pathological conditions [19,20]. However, while the total number of theoretically available sites susceptible to modification by NEPTMs within proteins is considerable, only a small proportion are reactive sites and for a number of reasons (including steric hindrance, pKa), which means that, in many proteins, the same sites are common targets for both these reactions. For example, the major site of haemoglobin modification is the N-terminal valine residue of β chains, modified in HbA_{1c} as well as in carbamylated haemoglobin [21].

In complex situations like diabetic nephropathy, where both glycation and carbamylation intensities are increased, competition among NEPTMs is to be expected. This could result in a variety of possible modification rates, leading to different effects on circulating proteins as well as at tissue and cell levels. However, to date, no investigations have focused on this topic. Thus, the present study has looked at the competition between glycation and carbamylation of blood proteins, using incubations *in vitro* and mouse models of diabetes and CKD *in vivo*.

Methods

Chemicals and reagents

HCit was purchased from MP Biochemicals Europe (Illkirch, France), and d_8-lysine and d_7-citrulline, used as internal standards, from CDN Isotopes (Quebec, Canada). Synthetic peptides used for carbamylated haemoglobin quantification were obtained from Proteogenix (Schiltigheim, France). Mass spectrometry (MS)-grade acetonitrile was obtained from VWR International (Strasbourg, France), hydrochloric acid from Merck (Darmstadt, Germany) and other reagents of analytical grade from Sigma-Aldrich (St. Louis, MO, USA).

Competition in vitro

Human serum albumin (Baxter, Guyancourt, France) was solubilized (1 g/L) in a 300 mM phosphate buffer (pH 7.4) containing 0.05% (m/v) sodium azide, supplemented with urea (20 mM), glucose (20 mM) or sodium cyanate (0.2 mM or 20 mM), depending on the experimental conditions. Incubation media were filtered (0.45-μm filter) and incubated for 3 weeks at 37 °C under sterile conditions. After incubation, samples were dialyzed for 48 h at 4 °C against 0.15 M of NaCl to remove glycating (glucose) and/or carbamylating (urea and cyanate) agents.

Animal models

Nine-week-old male diabetic *db/db* (C57Bl/KsJ-*db/db*) mice and their matching controls (*db/+*) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Animals were fed *ad libitum*, and housed in plastic cages, with sawdust-covered floors, in a room kept at a constant ambient temperature and a 12-h light–dark cycle. All animal procedures were conducted in accordance with French government policies (*Services vétérinaires de la santé et de la production animale, Ministère de l'agriculture*; Veterinary services of health and animal production, Ministry of agriculture), and following guidelines for the care and use of laboratory animals; the study protocol was approved by our institution's animal care committee (*Comité d'éthique en expérimentation animale de Reims Champagne Ardenne*, *registration 56*).

Subtotal nephrectomy model

All *db/db* and *db/+* mice were randomly assigned to either a CKD group (CKD–diabetic group, CKD group) or a control group (diabetic group, control group). A two-step subtotal nephrectomy procedure was used to induce CKD: surgery was performed under isoflurane anaesthesia. Half of the left kidney's poles was removed by kidney pole incision, with bleeding controlled by electrocoagulation. One week later, the right kidney was removed under the same anaesthetic conditions, achieving a 75% reduction of total renal mass and so leading to chronic kidney failure. Control animals underwent sham operations. Five weeks after the surgery, mice were sacrificed, using xylazine (Rompun 2%; Bayer, Leverkusen, Germany) and ketamine (Clorketam 1000; Vétoquinol SA, Lure, France) anaesthesia at 60 μg/g and 120 μg/g body weight, respectively; blood was collected in heparin-containing tubes by cardiac puncture. Plasma and red blood cells were stored at −80 °C until analysis.

Cyanate consumption model

The *db/db* (diabetic) and *db/+* (control) mice were randomly assigned to four groups that received either plain water (wa-control group and wa-diabetic group) or water spiked with 1 mM of sodium cyanate (cy-control group and cy-diabetic group), with renewal of cy-containing water twice a week. After 6 weeks, the

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