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Carbamylated albumin is a potent inhibitor of polymorphonuclear neutrophil respiratory burst

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Abstract **Carbamylation is a post-translational modification of proteins characterized by the binding of cyanate to amino groups, increased in renal failure. Pathophysiological consequences of carbamylation and adverse effects of carbamylated proteins on cell functions are poorly understood. We studied the influence of carbamylated albumin on polymorphonuclear neutrophil (PMN) O_2^- production. Carbamylated albumin significantly decreased O_2^- production in PMNs stimulated by type I collagen, but not by phorbol 12-myristate 13-acetate or tumor necrosis factor-α. This effect was related to inhibition of p^{125}FAK phosphorylation. Such an alteration of neutrophil oxidative functions might explain characteristic complications of renal failure, such as increased occurrence of inflammation or infections.**

Keywords: Carbamylation; Albumin; Chronic renal failure; Polymorphonuclear neutrophils; Respiratory burst

1. Introduction

Cell functions are deeply modulated by specific interactions with circulating or tissue proteins. For example, polymorphonuclear neutrophils (PMNs) are activated by contact with extracellular matrix (ECM) proteins such as type I collagen, resulting in stimulation of respiratory burst and secretion of specific enzymes [1]. However, these interactions may be impaired by the numerous post-translational modifications which alter proteins in living organisms. Post-translational modifications are due to the non-enzymatic binding of low molecular weight substances (e.g. oses, lipids or their by-products) to proteins, generating alterations of structural and functional properties [2].

A relevant *in vivo* post-translational modification is carbamylation, which is characterized by the non-enzymatic binding of urea-derived cyanate to free amino groups of proteins, especially to ε-NH_2 group of lysine residues, thus generating homocitrulline residues [3].

Carbamylation of proteins is amplified in patients with chronic renal failure (CRF) because of increased serum urea concentration [4–6]. For example, it has been shown that albumin, the most abundant serum protein, was carbamylated during CRF [7]. However, no studies have been devoted to the potential involvement of carbamylated albumin in pathophysiological processes. Especially, interactions of carbamylated albumin with PMNs or other inflammatory cells, which play an important role in inflammatory processes and participate in host defense against infections, are still unknown. Previous works of our laboratory have shown that carbamylated type I collagen was able to inhibit PMN oxidative functions through a mechanism involving $\alpha_L\beta_2$ integrin and subsequent p^{125}FAK phosphorylation [8]. In this study, we hypothesized that carbamylated albumin was also able to interfere with PMN oxidative functions, and addressed the question of the potential role of circulating carbamylated proteins in the dysregulation of PMN functions in CRF.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise mentioned.

2.2. Preparation of type I collagen

Type I collagen was prepared from Sprague–Dawley rat tail tendons by acetic acid extraction and dissolved at 2 mg/mL in sterile 18 mM acetic acid at 4 °C [9].

2.3. Preparation of PMNs

Whole blood was obtained on informed consent from healthy subjects of the laboratory staff. PMNs were isolated using a single-step centrifugation procedure through a metrizoate-polyprep gradient (Nycomed, Oslo, Norway) at 750 g for 35 min at room temperature [9]. The PMN-rich layer was separated, washed once in Dulbecco's solution (NaCl 137 mM, KCl 2.7 mM, HEPES 30 mM, glucose 10 mM, $CaCl_2$, 2 H_2O 1.3 mM, $MgCl_2$, 2 H_2O 1 mM, pH 7.4) and centrifuged at $600 \times g$ for 15 min at room temperature. Contaminating erythrocytes were eliminated by hypotonic lysis in 15 mM ammonium chloride.

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Abbreviations: CRF, chronic renal failure; FAK, focal adhesion kinase; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophil; TNF-α, tumor necrosis factor-α

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2.4. Albumin carbamylation

Human albumin (Baxter, Maurepas, France) was carbamylated by incubation with 0.1 M KCNO in a 150 mM phosphate buffer, pH 7.4, at 37 °C for 1, 2 or 6 h. Free KCNO was removed by extensive dialysis against distilled water. Control series were prepared in the presence of 0.1 M KCl according to the same protocol. Electrophoresis of control and carbamylated albumin was performed on a 10% (m/v) polyacrylamide gel without SDS. Amino acid analysis was carried out by cation exchange chromatography after acid hydrolysis using a Hitachi 8800 analyzer (ScienceTec, Les Ullis, France).

2.5. Measurement of superoxide anion (O_2^-) production

O_2^- production was measured by the superoxide dismutase inhibitable reduction of ferricytochrome c reaction [10]. 10^6 PMNs were suspended in Dulbecco's solution in presence of 0.1 μM cytochrome c. O_2^- production was induced by adding: (i) type I collagen (200 μg/mL), (ii) phorbol 12-myristate 13-acetate (PMA) (100 ng/mL), or (iii) tumor necrosis factor-α (TNF-α) (100 ng/mL), in the presence or absence of control or carbamylated albumin (200 μg/mL). The reaction was stopped by addition of 50 U superoxide dismutase, and absorbance variation at 550 nm was measured as an index of the amount of liberated O_2^-. Test tubes supplemented with 50 U superoxide dismutase were used as blanks assessing the specificity of the reaction. The amount of O_2^- produced was calculated by using an ε value of 15.5 $mM^{-1}.cm^{-1}$ and expressed in nmol of O_2^- per 10^6 cells.

2.6. Analysis of p^{125}FAK phosphorylation

PMNs (10^7 cells/ml in Dulbecco's solution) were first preincubated for 30 min at 20 °C in the presence of 2.5 mM phenylmethylsulfonyl fluoride and 5 mM sodium orthovanadate. The temperature was raised to 37 °C for 5 min prior to the stimulation by 200 μg/mL type I collagen in the presence or absence of control or carbamylated albumin (200 μg/ml). The incubation was stopped after 2 min by centrifugation at 1000 × *g* for 5 s at 4 °C. The supernatants were discarded and cells lysed by addition of 150 μl of boiled sample buffer (0.125 M Tris, 4% (m/v) SDS, 10% (v/v) glycerol, 0.01% (m/v) bromophenol blue, pH 6.8). 50 μl of cell lysates were submitted to electrophoresis. Total and phosphorylated p^{125}FAK were evaluated by Western-blotting using rabbit polyclonal antibodies raised against total p^{125}FAK (Biosource, Camarillo, CA, USA) and [Y^{397}]phosphorylated-p^{125}FAK (Biosource), respectively [8].

2.7. Statistical calculations

All experiments were performed in triplicate and the results expressed as means ± S.D. Significance of differences was calculated using the Student's *t*-test.

3. Results

3.1. Effect of carbamylation on amino acid composition and electrophoretic properties of albumin

Albumin was carbamylated by incubation with 0.1 M KCNO for 1, 2 and 6 h at 37 °C and amino acid composition evaluated by cation exchange chromatography. Carbamylation was evidenced by the formation of homocitrulline residues from lysine residues, in an incubation time-dependent manner (Table 1). For example, albumin incubated for 6 h with 0.1 M KCNO contained 80 residues of lysine per 1000 amino acid residues vs. 102 in native albumin, whereas homocitrulline contents were respectively 19 residues vs. 0 per 1000. No other significant change was noticed (data not shown).

When submitted to electrophoresis, carbamylated albumin displayed a faster mobility as compared to control albumin. The difference in electrophoretic mobility increased with carbamylation time, i.e. with the number of modified lysine residues (Fig. 1). These characteristics evidenced the binding of cyanate to lysine residues, and were further used as internal quality control parameters in our experiments. In standard experiments, preparations of albumin carbamylated for 6 h were used.

3.2. Effect of carbamylated albumin on PMN activation

In preliminary experiments performed as previously described [8], we have checked that carbamylated albumin neither exerted a toxic effect on PMNs nor modified PMNs adhesion on type I collagen (data not shown). Subsequently, the effect of carbamylated albumin on O_2^- production by PMNs was tested in the presence of three stimulators of PMN activation : type I collagen, PMA and TNF-α. When PMNs were stimulated by type I collagen, O_2^- production was decreased by more than 70% by carbamylated albumin (200 μg/mL), whereas control albumin had no significant effect at the same concentration (Fig. 2A). By contrast, PMNs stimulated by PMA or TNF-α showed no alteration of O_2^- production, neither by control nor by carbamylated albumin at 200 μg/mL (Fig. 2B and C).

Carbamylated albumin-mediated inhibition of PMN O_2^- production triggered by type I collagen was dose-dependent. No effect of carbamylated albumin was found at 2 μg/mL, whereas the inhibitory effect increased with higher concentrations, reaching about 12% (NS) at 20 μg/mL and 73% ($P < 0.01$) at 200 μg/mL (Fig. 3A).

This effect also depended on albumin carbamylation rate, since inhibition of O_2^- production by PMNs was more pronounced in presence of 200 μg/ml 6 h-carbamylated albumin

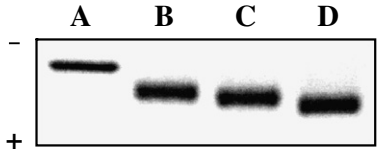

Fig. 1. Influence of carbamylation on albumin electrophoretic mobility. 2 μg of control (A) or carbamylated albumin incubated with KCNO for respectively 1 h (B), 2 h (C) and 6 h (D) were submitted to 10% (m/v) polyacrylamide gel electrophoresis.

Table 1
Homocitrulline content of carbamylated albumin

	Control albumin	Carbamylated albumin		
Incubation time	–	1 h	2 h	6 h
Homocitrulline	0	4.5 ± 0.3	8.1 ± 0.5	19.0 ± 1.0
Lysine	101.6 ± 1.0	95.4 ± 0.5	91.7 ± 0.7	80.3 ± 0.3

Albumin was incubated for 1, 2 or 6 h with 0.1 M KCNO (carbamylated) or 6 h with 0.1 M KCl (control) in phosphate buffer at 37 °C. Amino acid analysis was performed by cation exchange chromatography. Values are means ± S.D. ($n = 3$) and expressed as number of amino acid residues per 1000.

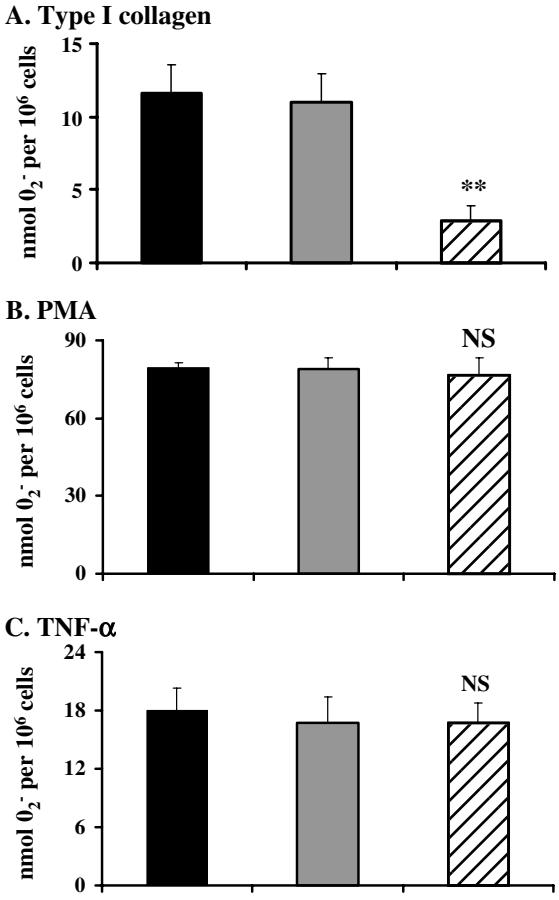

Fig. 2. Effect of carbamylated albumin on O_2^- production by PMNs stimulated by different effectors. PMNs were stimulated by 200 μg/mL type I collagen (A), 100 ng/mL PMA (B), or 100 ng/mL TNF-α (C), in Dulbecco's solution without albumin (closed bar), or containing either control (grey bars) or carbamylated (hatched bars) albumin (200 μg/mL). O_2^- production was assessed by the superoxide dismutase inhibitable reduction of cytochrome c reaction. Values are means ± S.D. ($n = 3$). Statistical differences vs. series with control albumin: NS: not significant, ** $P < 0.01$.

(−80%, $P < 0.01$) than in presence of 2 h- or 1 h- carbamylated albumin (−38%, $P < 0.01$ and −17%, $P < 0.05$, respectively) (Fig. 3B).

No intrinsic scavenger effect of carbamylated albumin was found in a cell-free system (xanthine oxidase/hypoxanthine system) of O_2^- production (data not shown).

3.3. Effect of carbamylated albumin on p^{125}FAK phosphorylation

To investigate whether p^{125}FAK phosphorylation was involved in the effect of carbamylated albumin on PMNs stimulated by type I collagen, cell lysates were analyzed by Western-blotting with a specific antibody directed against [Y^{397}]phosphorylated-p^{125}FAK.

PMNs stimulated by type I collagen exhibited an increased tyrosine phosphorylation of p^{125}FAK when compared to unstimulated PMNs. p^{125}FAK phosphorylation was significantly decreased in presence of carbamylated albumin (200 μg/mL), whereas no inhibition was noticed with control albumin (Fig. 4).

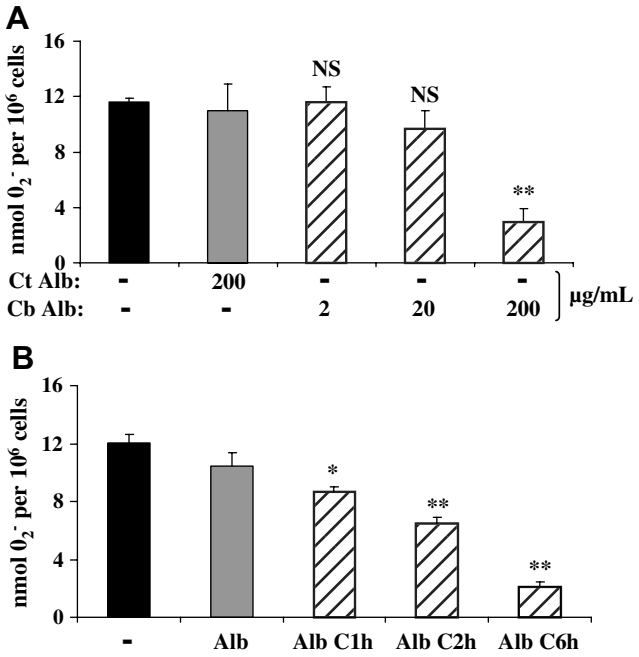

Fig. 3. Dose- and carbamylation rate-dependence of carbamylated albumin-induced inhibition of PMN O_2^- production. O_2^- production by PMNs was induced by type I collagen (200 μg/mL) in presence of control albumin (Ct Alb) and various concentrations (0, 2, 20 and 200 μg/mL) of 6 h-carbamylated albumin (Cb Alb) (A), or in presence of albumin (200 μg/mL) with different carbamylation rates (1 h (C1h), 2 h (C2h) or 6 h (C6h)) (B). O_2^- production was assessed by the superoxide dismutase inhibitable reduction of cytochrome *c* reaction. Values are means ± S.D. ($n = 3$). Statistical differences vs. series with control albumin: NS: not significant, *: $P < 0.05$, **: $P < 0.01$.

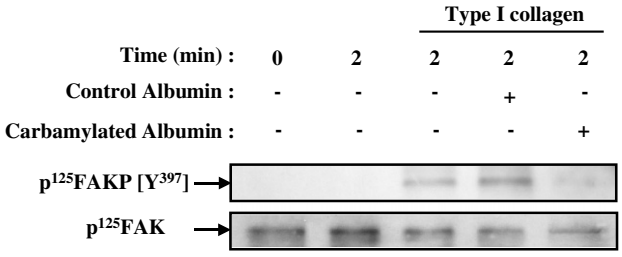

Fig. 4. Effect of carbamylated albumin on p^{125} FAK phosphorylation. PMNs were stimulated by type I collagen (200 μg/mL) in presence or absence of control or carbamylated albumin (200 μg/mL) for 2 min at 37 °C. Total and phosphorylated p^{125}FAK in cell lysates were analyzed by Western-blotting using rabbit polyclonal antibodies raised against total p^{125}FAK and [Y^{397}]phosphorylated-p^{125}FAK, respectively.

4. Discussion

Various post-translational modifications occur throughout protein lifespan *in vivo*. In patients with chronic renal failure, carbamylation of proteins occurs at an abnormal rate. This phenomenon is the direct consequence of blood urea increase, since this molecule spontaneously dissociates to cyanate, which in turn binds to NH_2 groups of proteins, particularly to ε-NH_2 groups of lysine residues to form homocitrulline residues [3]. Such a reaction has also been described after dissociation of carbamyl phosphate into cyanate [11,12]. Increased amounts of carbamylated proteins in blood and tissues have been well

documented in CRF [4,6,13], especially in the case of haemoglobin [5]. Many studies have evaluated the impact of carbamylation on structure and/or function of proteins, enzymes or hormones, generating most of the time a loss of activity or of mechanical properties [3,14,15]. Surprisingly, considerably less information is available about carbamylation of albumin, the major circulating protein, even though the increase of albumin carbamylation rate has been clearly demonstrated in uremic patients [5]. Moreover, only few data are available about the adverse effects of carbamylated proteins on cell interactions. Indeed, only one study has demonstrated that carbamylated serum proteins could activate mesangial cells to a profibrogenic phenotype [16]. Nevertheless, we have shown that carbamylated type I collagen, a major component of extracellular matrix, was able to selectively regulate metabolic functions of inflammatory cells (*i.e.* monocytes and PMNs) [8,17]. Taking into account the dysregulation of leukocyte functions in CRF [18], we have hypothesized that carbamylated albumin could influence PMN oxidative functions, as carbamylated collagen did.

Albumin was carbamylated *in vitro* by incubation at 37 °C with 0.1 M KCNO for 1–6 h. The carbamylation rate was in agreement with that obtained for other carbamylated proteins [16]. Moreover, the alteration of electrophoretic mobility with incubation time is consistent with previous results [7], and confirmed the modification of protein charge by carbamylation.

Besides the effect of carbamylation on albumin composition and electrophoretic properties, we have shown that carbamylated albumin actually modulated PMN functions, even at low concentration (200 μg/mL), corresponding to a modification of 0.01% of lysine residues in serum albumin. This result is in agreement with carbamylation rates described by Balion et al. in uremic patients [5]. Carbamylated albumin *per se* had no direct effect on PMN O_2^- production, but significantly inhibited O_2^- release when PMNs were stimulated by type I collagen. Interestingly, the inhibition of PMN O_2^- production triggered by carbamylated albumin was carbamylation rate-dependent, and proved specific of type I collagen activation among the stimulating agents studied: no inhibition was observed when PMNs were stimulated by PMA or TNF-α. Previous works of our laboratory have shown that type I collagen was able to induce O_2^- production by human PMNs through an interaction mediated by $\alpha_L\beta_2$ integrin [9], thus inducing subsequent p^{125}FAK phosphorylation [8]. Our results suggest that carbamylated albumin alters type I collagen interaction with $\alpha_L\beta_2$ integrin, since we showed that p^{125}FAK phosphorylation on tyrosine 397, triggered by type I collagen, was strongly inhibited in presence of carbamylated albumin. However, the inhibitory effect of carbamylated albumin on PMNs activation is independent from any effects on adhesion, as in the case of carbamylated type I collagen [8].

In conclusion, our results suggest that carbamylation represents an important post-translational modification of proteins *in vivo*, besides glycoxidation, which occurs in CRF, diabetes mellitus and ageing [2,19]. We have shown that carbamylated albumin was a potent inhibitor of human PMN respiratory burst. Our findings suggest that PMNs, which are key-cells involved in host defense system through the respiratory burst, may have a lower capacity to respond to specific stimulating agents and to generate an inflammatory response in presence of carbamylated albumin. These results could partly explain the high occurrence of inflammatory and infectious complications in patients with CRF, responsible for higher morbidity and mortality.

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