Kinetics of carbamylated haemoglobin in acute renal failure

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Abstract

Background. Carbamylation of proteins by isocyanic acid, the reactive form of cyanate derived from urea, is increased in uraemia and may contribute to uraemic toxicity. Kinetics of carbamylation that may reflect uraemic toxicity is not clearly defined in acute renal failure (ARF).

Methods. Twenty-eight patients with ARF and 13 with chronic renal failure (CRF) were included in the study in order to determine changes in carbamylated haemoglobin concentration (CarHb) in ARF. The usefulness of this parameter for differentiating ARF from CRF was also investigated. CarHb was measured by high-performance liquid chromatography after acid hydrolysis.

Results. Mean CarHb level (expressed as μg carbamyl valine per gram (CV/g) Hb) was significantly higher in ARF (54.3 ± 5.2) than in normal subjects (31.6 ± 1.3). On admission, CarHb level was correlated with duration of ARF prior to hospitalization in the intensive care unit ($r^2 = 0.723$, $P < 0.001$). CarHb was significantly higher at recovery in the subgroup of patients requiring haemodialysis than in the subgroup not requiring haemodialysis (82.4 ± 11.3 vs 46.7 ± 5.2, $P < 0.01$). Similarly dialysis patients lost more weight (8.6 ± 1.4 vs 2.7 ± 0.5 kg, $P < 0.005$) and had higher averaged blood urea levels in the 20 days prior to recovery (17.6 ± 1.9 vs 11.3 ± 1.8 mol/l, $P < 0.05$). After recovery, CarHb level decreased at a rate of 0.219 μg CV/g Hb per day in patients with reversible renal insufficiency. CarHb concentration was higher in patients with CRF. A cut-off CarHb value of 100 μg CV/g Hb had a sensitivity of 94% and a positive predictive value of 94% for differentiating ARF from CRF.

Conclusions. Kinetics of CarHb showed a near normal red blood cell life span in ARF. Changes in CarHb enabled, with a good sensitivity, the distinction to be made between patients who recovered from ARF and those with sustained renal impairment, whether due to prior CRF or resulting from parenchymal sequelae. Measurement of CarHb is valuable at clinical presentation of ARF in patients with an unknown medical history of renal disease.

Keywords: acute renal failure; carbamylation; chronic renal failure; haemodialysis; uraemia

Introduction

Carbamylation of haemoglobin (Hb) and other proteins occurs in uraemia. It results from the non-enzymatic post-translational modification of proteins by isocyanic acid, the reactive form of cyanate derived from the spontaneous dissociation of urea. For Hb, the most extensively studied protein in this setting, cyanic acid reacts with the terminal valine residues of both $\alpha$ and $\beta$ chains. Under physiological conditions, the level of isocyanate is ~1% that of urea [1]. Carbamylation may contribute to uraemic toxicity. It has been documented that carbamylated proteins are implicated in the atherogenesis of chronic renal failure (CRF) [2,3]. In cultured neuroblastoma cells, carbamylation of cellular proteins is associated with synthesis of the heat shock protein HSP 72 [4]. Carbamylation of lens proteins, such as actin, predisposes to cataract development [5]. In renal failure, near complete carbamylation of human serum albumin (HSA) resulted in a two-third reduction of the binding capacity of the protein for small anionic molecules [6].

Carbamylated haemoglobin (CarHb) is the most simple, useful and reproducible index for measuring the carbamylation reaction in vivo. Increased CarHb levels have been measured in patients with CRF or with acute renal failure (ARF). It has been postulated that CarHb level may reflect the intensity of uraemic toxicity as has been shown for glycated haemoglobin in the diabetic patient [7,8]. Consequently, CarHb could represent a suitable marker of dialysis adequacy in chronic haemodialysed patients [9,10]. Since the changes of CarHb with time have not been extensively investigated, we took the opportunity to study its
kinetics in patients with ARF and to evaluate its practical usefulness for differentiating ARF from CRF in the uraemic patient.

Patients and methods

Patients

Forty-one patients were included in the study. They were gathered into two groups: 28 had ARF and 13 had CRF. The following criteria were selected for inclusion in the group with ARF: (i) absence of previous proven CRF defined before the episode of ARF by clinical history and a baseline serum creatinine concentration < 133 μmol/l (1.5 mg/dl); (ii) absence of blood transfusion before admission; (iii) a serum creatinine concentration > 350 μmol/l (4.0 mg/dl) at inclusion. In 25 patients, ARF was due to acute tubular necrosis (ATN) and in three to obstructive nephropathy. In all cases, precise timing of clinical events was taken in order to estimate CarHb burden. ATN was related to infection in seven patients (two nosocomial Staphylococcus aureus septicaemia, three Gram-negative-related bacteraemia, one leptospirosis and one Hantaan virus infection). ARF was observed after myocardial infarction in five patients (infarction occurred after surgery in three cases). Drug induced renal injury was documented in four patients (one, methotrexate; one, cisplatin; one, radiocontrast medium; one, intravenous immunoglobulins). The other proven causes of ARF were two rhabdomyolysis, one lymphoma-associated hyperuricaemic ARF, 1 tubulointerstitial nephritis with uvetis and one postpartum ARF. Finally three patients developed typical ATN in a context of sepsis without isolation of a specific pathogen. Obstructive nephropathy was related to prostatic adenoma (two cases) or ectopic bladder catheterism (one case). During the acute phase of the disease, five patients received more than two blood units, five received two blood units and 18 were not transfused. Patients with CRF were programmed for haemodialysis and carefully monitored. They were included at initiation of iterative haemodialysis. CRF was due to nephroangiosclerosis in four patients, glomerulonephritis in three, diabetes nephropathy in two and renovascular disease with hypertension in one. Tubulointerstitial nephritis was documented in two cases and aetiology was unknown in one case. None had any episode of acute worsening of renal failure in the 6 months preceding inclusion. Their creatinine clearance value was between 5.5 and 9.5 ml/min. Fifteen healthy volunteers were used as normal controls.

Clinical biochemistry

Blood samples were taken from all the patients on admission and thereafter until recovery, which was defined by a serum creatinine concentration < 200 μmol/l (2.26 mg/dl). CarHb was measured using the high-performance liquid chromatography (HPLC) technique proposed by Kwan [11], with slight modifications. In short, red blood cells were separated and washed with isotonic sodium chloride. Washed blood cells were stored at −20 °C until analysis. A 0.5-ml sample was hydrolysed by adding 1 ml of concentrated HCl and heated at 110 °C for 2 h. The hydrolysate was cooled in dry ice. NaOH 10 M (2 ml) was added to obtain a pH up to 4, before mixing with 100 μl of the internal standard solution. N-Carbamyl-D, L-valine (CV) (32 mg/l) and N-carbamyl-D, L-norvaline (96 mg/l) (Sigma Aldrich, St Quentin Fallavier, France) were used as both standard and internal standard. The solution was extracted with 5 ml of ethyl acetate, after vortexing for 1 min, and centrifuged at 3000 g for 15 min. Supernatant (4.5 ml) was mixed with 2 ml of 1 M NaHCO3 and centrifuged at 3000 g for 10 min. The solvent was evaporated to dryness under nitrogen stream. The residue was dissolved in 0.5 ml of mobile phase. The latter consisted of a mixture of purified water (Milli Q; Millipore, St Quentin en Yvelines, France) containing 20 ml of HPLC grade acetonitrile (2%) and 1 ml of concentrated acetate (pH 4). The injected volume sample was 20 μl, the pump speed was 0.45 ml/min and the detection wavelength 203 nm. Intra- and interassay variation coefficients were <5%. In addition, using the same procedure, the mean recovery of CV added to washed red blood cells was 99%. Regular automated techniques were used to measure routinely haemoglobin, blood urea, serum creatinine and electrolytes.

Results

Baseline characteristics of patients with ARF or with CRF as compared with healthy controls

<table>
<thead>
<tr>
<th>Table 1: Baseline characteristics of patients with ARF or with CRF as compared with healthy controls</th>
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<tbody>
<tr>
<td>Healthy controls</td>
</tr>
<tr>
<td>Mean age (years)</td>
</tr>
<tr>
<td>Male-Female</td>
</tr>
<tr>
<td>Blood urea (mmol/l)</td>
</tr>
<tr>
<td>Plasma creatinine</td>
</tr>
<tr>
<td>CarHb (μmol/l)</td>
</tr>
</tbody>
</table>

*P<0.0001; **P<0.001; ***P<0.005.
Kinetic of carbamylated haemoglobin in ARF

Fig. 1. Regression curve analysis between blood urea concentration and duration of ARF (left) or CarHb concentration (right). No significant relationship was documented.

Fig. 2. Regression curve analysis between CarHb level and duration of ARF on the day of admission. CarHb concentration was tightly correlated with the duration of ARF.

Fig. 3. Kinetics of blood urea and CarHb concentration during the follow-up. CarHb level was increased on the day of recovery despite normalization of blood urea concentration.

Changes in CarHb concentration varied during the time-course but did not parallel those of blood urea seen in two representative patients with ARF, as shown in Figure 5. One of the patients had sustained ARF due to acute tubulo interstitial nephritis and their serum creatinine level returned to 164 μmol/l, 67 days after the apparent onset of ARF; CarHb and blood urea decreased in parallel. The other patient had ARF due to sepsis; his serum creatinine concentration returned to 96 μmol/l after 42 days. However, CarHb concentration remained higher than normal at 69.6 ± 6.1 mg CV/g Hb.

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remained elevated but blood urea returned to a normal level.

CarHb burden was higher in the subgroup of patients requiring dialysis, as documented by a significantly higher CarHb value at recovery of renal function (Table 2). Duration of ARF was similar in haemodialysed patients and non-dialysed patients (46.2 ± 5.8 vs 40.5 ± 8.4 days). Haemodialysed patients were characterized by an impressive body weight loss (8.6 ± 1.4 vs 2.7 ± 0.5 kg) and a higher than average blood urea level in the 20 days prior to recovery (17.6 ± 1.9 vs 11.3 ± 1.8 mmol/l), which indicated a higher protein catabolic rate.

A significant positive correlation was documented between blood urea level and CarHb at recovery (Table 3). This relationship persisted after exclusion of patients who received more than two blood units ($r^2 = 0.377$, $P = 0.002$) or after exclusion of all tranfused patients ($r^2 = 0.225$, $P = 0.055$). A better correlation indicated that CarHb concentration decreased at a rate of 0.22 µg CV/g Hb per day.

Fig. 4. Change of CarHb level after recovery from ARF. Regression analysis indicated that CarHb concentration decreased at a rate of 0.22 µg CV/g Hb per day.

In patients with CRF at the start of dialysis, mean blood urea concentration was 41.2 ± 2.3 mmol/l and CarHb level 129.0 ± 8.1 µg CV/g Hb. This latter value was significantly higher than in patients with ARF, in spite of a similar high blood urea concentration (Table 4). To assess the clinical usefulness of CarHb measurement for differentiating ARF from CRF, we

Table 2. Characteristics of ARF in dialysed and non-dialysed patients

<table>
<thead>
<tr>
<th></th>
<th>Dialysed ARF patients ($n = 18$)</th>
<th>Non-dialysed ARF patients ($n = 10$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood urea at entrance (mmol/l)</td>
<td>34.8 ± 3.5</td>
<td>21.8 ± 2.1*</td>
</tr>
<tr>
<td>Plasma creatinine at entrance (µmol/l)</td>
<td>610 ± 54</td>
<td>543 ± 65</td>
</tr>
<tr>
<td>CarHb at entrance (µg CV/g Hb)</td>
<td>55.6 ± 6.2</td>
<td>51.9 ± 5.8</td>
</tr>
<tr>
<td>Weight loss during ARF (kg)</td>
<td>8.6 ± 1.4</td>
<td>2.7 ± 0.5***</td>
</tr>
<tr>
<td>Blood urea at recovery (mmol/l)</td>
<td>11.2 ± 1.3</td>
<td>7.9 ± 1.3</td>
</tr>
<tr>
<td>Mean blood urea during the 20 days before recovery (mmol/l)</td>
<td>17.6 ± 1.9</td>
<td>11.3 ± 1.8*</td>
</tr>
<tr>
<td>CarHb at recovery (µg CV/g Hb)</td>
<td>82.4 ± 11.3</td>
<td>46.7 ± 5.2**</td>
</tr>
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</table>

*P < 0.05; **P < 0.01; ***P < 0.005.

Table 3. Determination coefficient between CarHb and blood urea in patients with ARF during the recovery phase

<table>
<thead>
<tr>
<th>Time-related event</th>
<th>$n$</th>
<th>$r^2$ value</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>At admission</td>
<td>28</td>
<td>0.119</td>
<td>0.07</td>
</tr>
<tr>
<td>During follow up</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>urea level at DR*</td>
<td>28</td>
<td>0.350</td>
<td>0.001</td>
</tr>
<tr>
<td>urea level 10 days before DR*</td>
<td>28</td>
<td>0.471</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>urea level 20 days before DR*</td>
<td>28</td>
<td>0.457</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At late control</td>
<td>13</td>
<td>0.531</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

DR, day of recovery. *Significant after exclusion of transfused patients.
calculated the sensitivity, specificity and positive predictive value of CarHb concentration at various cut-off values in both groups of haemodialysed patients. A cut-off value of 100 μg CV/g Hb resulted in sensitivity of 94%, specificity of 92% and a positive predictive value of 94% for differentiating ARF from CRF. With a lower cut-off of 80 μg CV/g Hb, sensitivity decreased to 83% while specificity and the positive predictive value remained unchanged.

**Discussion**

High CarHb levels are always documented in patients with uraemia whether they have an acute or a chronic syndrome [12,13]. Because carbamylation is a non-enzymatic reaction, the resulting level of carbamylated proteins, i.e. haemoglobin, is expected to be closely related to blood urea level. In the group of patients with ARF, correlation between these two parameters was weak at admission pointing out the role of length of exposure to high urea levels in CarHb generation. The higher the duration of exposure of reactive proteins to high urea concentration, the higher the amount of CarHb obtained. This relationship was documented by the significant correlation obtained between the first CarHb concentration and the apparent duration of ARF (Figure 2). The weak correlation between CarHb and blood urea concentration observed might be due to the obviously different turnover of the two molecules. This result was at variance with those of Stim et al. [13] but was confirmed by Vaden et al. [14]. Using an experimental model in dogs, Vaden found that CarHb concentration did not correlate with serum urea nitrogen or creatinine concentrations. The influence of time was not emphasized in this study as it is in ours. In prerenal failure, Frazao et al. [15] found that a significant elevation of CarHb concentration (defined as being >2 SD above the mean) may be observed after 4 days of transient renal failure. Balian et al. [16] failed to document any clear relationship between CarHb or short-life carbamylated plasma protein concentration and predialysis urea level in a 6 month longitudinal study of seven stable, thrice weekly haemodialysed patients.

Increased protein catabolism is a hallmark of ARF. It is governed by the severity of the underlying disease and by complications such as severe infection or metabolic acidosis [17,18]. Consequently urea generation rate differs between patients. Blood urea concentration is also dependent on its diffusion volume, closely related to hydration. In patients requiring dialysis as compared with those who did not need dialysis, CarHb levels were similar at admission but higher at recovery. A careful control of body fluids and a higher increase of blood urea due to oliguria might explain these differences at recovery. The highest CarHb burden will slowly decrease due to the long life span of red blood cells. Obvious increased protein catabolism was observed in these patients since a significantly higher body weight loss was documented during the acute phase of the disease. Metabolic acidosis is a frequent finding in ARF and may by itself contribute to protein catabolism. Acidosis was not correlated with the carbamylation process, but in most of the patients, acidosis was partially corrected by buffer administration at the time of CarHb measurement. The pH dependence of carbamylation of both α and β subunits has been demonstrated *in vitro* [19]. Increasing pH shifts the equilibrium $\text{CNO}^- + \text{H}^+ = \text{HCNO}$ to the left resulting in reduced cyanic acid concentration. In CRF, metabolic acidosis has been identified as a weak but independent factor of carbamylation in multiple regression analysis [13].

Another factor of CarHb generation is the amount of substrate reactive to cyanate. *In vitro*, a linear relationship has been documented which then reaches a plateau, reflecting a saturable process [13]. *In vivo*, it seems probable that saturation of reactive sites is quickly reached. It has been documented that haemoglobin, at least in CRF, is also modified by several chemical reactions that may compete for the same substrates. Advanced glycation end products (AGE) are formed during non-enzymatic glycation and oxidation reactions in normoglycaemic uraemic patients [20]. In such reactions (carbonyl stress), carbonyl compounds derived from autoxydation of carbohydrates and lipids are reacting with protein amino groups. Furthermore, oxidants may react with amino acid residues such as tyrosine, leading to the formation of protein cross-linking products, which are now designated as advanced oxidation protein products (AOPP); this latter reaction has been well described with albumin [21]. A clear relationship has been demonstrated between AOPP and AGE [21].

In order to avoid a mixed population of reactive blood cells we considered the 10 patients who were transfused during the first days of hospitalization. Their exclusion from the study did not change the significance of the results of the whole group of patients with ARF. Therefore we can assume that the carbamylation process also modified transfused red blood cells.

The clearance of CarHb was 0.219 μg CV/g Hb per day, once the plasma urea level was <10 mmol/l, indicating that normalization of CarHb takes some 180 days. This result suggests that the life span of red blood cells containing CarHb is similar to that of normal red blood cells. This result might be confirmed,
since the slope of the regression curve was lower than expected due to the fact that blood urea concentration remained higher than normal, even in the follow-up 60 days after apparent clinical recovery.

The clinical presentation of CRF is frequently similar to that of ARF. The distinction between ARF and CRF may be difficult and requires numerous biological investigations. Among them, CarHb measurement is of interest. Our results clearly indicate that a CarHb value < 80 μg CV/g Hb is in favour of ARF, therefore CarHb evaluation may be helpful for nephrology referral and as well as for evaluating sequelae of ARF. Using a CarHb cut-off value of 100 μg CV/g Hb, the sensitivity and specificity of CarHb concentration for differentiating ARF from CRF in Vaden’s study were 95.6 and 84.2%, respectively [14]. These values are very close to those from our study of the human.

In summary, duration of exposure to even slightly increased blood urea levels is the main determinant of carbamylation in ARF. CarHb level may better predict ARF duration than blood urea level. Due to the slow turnover of red blood cells that harbour CarHb, kinetics of CarHb adds more precise predictive value to the diagnosis and the prognosis of ARF than the normal blood urea determination.

References


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