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OXIDATIVE MODIFICATIONS AND CARBAMOYLATION OF AMINO ACIDS AND PROTEINS IN UREMIA

Carbamoylation of amino acids and proteins in uremia

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Carbamoylation of amino acids and proteins in uremia. Cyanate spontaneously transformed from urea increases as renal function decreased. Acting as a potential toxin, the active form of cyanate, isocyanic acid, carbamoylates amino acids, proteins, and other molecules, changing their structure, charge, and function. The resulting in vivo carbamoylation can modify the molecular activity of enzymes, cofactors, hormones, low-density lipoproteins, antibodies, receptors, and transport proteins. Antibodies specific for ε-amino-carbamoyl-lysine (homocitrulline) located carbamoylated proteins in situ in neutrophils, monocytes, and erythrocytes. Carbamoylated proteins were found in renal tissue from uremic patients but not in normal transplanted kidneys. The irreversible reaction with cyanate converts free amino acids (F-AAs) to carbamoyl-amino acids (C-AAs). The Carbamoylation Index (CI), C-AA/F-AA, quantifies the decrease of the F-AA pool for each essential amino acid. C-AAs contribute, in part, to malnutrition of uremia. C-AAs interfered with protein synthesis to lower ^{14}C hemoglobin synthesis in human reticulocytes and osteocalcin synthesis in rat osteosarcoma-derived tissue culture. Insulin-sensitive glucose uptake was decreased 33% in cultured rat adipocytes by α-amino-carbamoyl-asparagine. α-Amino carbamoylation occurs primarily in F-AA, while ε-amino carbamoylation of lysine in protein occurs continuously during the protein life span. Protein catabolism releases ε-amino-carbamoyl-lysine. Quantitation of α versus ε carbamoylation may yield a more sensitive measurement of protein intake versus protein catabolism, and could be useful in decisions concerning the time to initiate dialysis or subsequent changes in dialysis prescription. Carbamoylated molecules can block, enhance, or be excluded from metabolic pathways, thereby influencing the fate of noncarbamoylated molecules. Although not an "all-or-none" phenomenon, urea-derived cyanate and its actions are contributing causes of toxicity in uremia.

This review of carbamoylation presents data showing that urea-derived cyanate should be considered a uremic toxin (Fig. 1). Other uremic toxins and their roles in contributing to the uremic syndrome have been described recently [1]. Carbamoylation is the result of constant exposure and reaction with urea-derived cyanate, which increases in renal disease. Isocyanic acid reacts with amino acids, proteins, and other molecules in vivo, changing their molecular structure, charge, and function by carbamoylation [2]. Urea, $(H_2N)_2 - C = O$, spontaneously forms cyanate, $^{-}N = C = O$, and ammonia, $NH4^{+}$, at body pH and temperature [3]. Isocyanic acid, $HN = C = O$, the active form of cyanate, reacts irreversibly with the nonprotonated amino group of amino acids, forming α-amino-carbamoyl-amino acids (C-AAs) from free amino acids (F-AAs) [4, 5]. The irreversible carbamoylation forming ε-amino-carbamoyl-lysine (ε-N-C-lysine) occurs at multiple lysine sites within a protein with accumulation over the life span of the protein [4, 6]. When a molecule of cyanate is removed by carbamoylation, a new molecule of cyanate is formed because of the equilibrium between urea and cyanate. Reversible carbamoylation occurs at the hydroxyl (OH) group of tyrosine, serine, or threonine [6] and the sulfhydryl (SH) group of cysteine depending on the pH of the microenvironment [7].

IN VIVO CARBAMOYLATION OF PROTEINS IN UREMIC PATIENTS

The administration of cyanate as a treatment for sickle cell disease was discontinued after some patients developed cataracts [8] and peripheral neuropathy [9]. The continuous presence of cyanate in uremia differed from the cyanate doses in drug therapy. For this reason, we decided to examine tissue from patients with renal disease for in vivo carbamoylation.

In vivo carbamoylation of protein in the uremic patient with end-stage renal disease (ESRD) was determined using immunohistochemical reactions with antibody specific for the ε-N-C-lysine (homocitrulline) in protein in situ. Blood cells were examined from patients with a blood urea nitrogen (BUN) range of 32 to 102 mg/dL who were undergoing continuous ambulatory peritoneal dialysis (CAPD) for 2 to 135 months [10]. Carbamoylated lysine in protein was found in the cytoplasm of polymorphonuclear neutrophils and monocytes in monolayers of blood cells. Cell-surface proteins in living leukocytes contained ε-N-C-lysine. The effects of carbamoylation of leukocyte

Key words: cyanate, urea, carbamoyl-amino acids, uremic toxin.

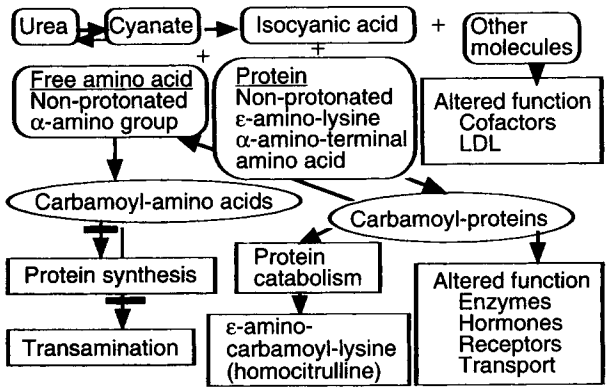

Fig. 1. The alteration of metabolic events by cyanate. (Modified with permission from *Wien Klin Wochenschr*, from Figure 1 in [2]).

protein were examined since a change in protein structure and function could be a factor related to infection in uremic patients. Neutrophils from normal subjects were incubated with cyanate in vitro. Cyanate, in a dose- and time-dependent manner, decreased the production of superoxide to a level that interfered with microbicidal activity [10]. Hemoglobin in erythrocytes also contained ϵ-N-C-lysine [10, 11] in addition to α-N-C-valine [2].

Carbamoylation of renal proteins in situ could also occur. Therefore, we examined renal tissue using the immunohistochemical reactions with antibody specific for ϵ-N-C-lysine [10–12]. Renal tissue was obtained from patients with different degrees of renal insufficiency with BUN ranging from 36 to 121 mg/dL. Control tissue obtained from healthy reperfused transplanted kidneys and in proteinuric patients with a normal BUN did not show the presence of carbamoylated protein. However, in the patients with elevated urea levels, ϵ-N-C-lysine was found in the glomerular basement membrane, in mesangium, in tubular perinuclear membrane, and in the cytoplasm of some tubular cells [2]. The tubular carbamoylation may be in part from carbamoylated molecules in transport. However, the carbamoylation of mesangial protein and extracellular matrix proteins, including enzymes, could lead to a decreased catabolism, which may lead to glomerulosclerosis and fibrosis. The mesangial enzyme matrix metalloproteinase-2 (MMP-2) has been shown to regulate glomerular cell proliferation and differentiation [13]. Examination of the effect of cyanate on MMP-2 from rat mesangial cells in tissue culture and human MMP-2 showed a dose-dependent inhibition of enzyme activity, which may be related to the changes found in restructuring the mesangium in the presence of uremia.

CARBAMOYLATION ALTERS ENZYME ACTIVITY

Renewed interest in the chemical reactivity of cyanate occurred in 1960 when the modification of protein by cyanate in urea solution caused the carbamoylation of 4.7 of 10 ϵ-amino groups of lysine in ribonuclease with a decrease in enzymatic activity of 65% [14]. Enzymes functioning in the catabolic pathways of amino acids were examined. Threonine dehydratase is one of a group of enzymes that depends on the coenzyme pyridoxal 5′ phosphate (PLP) forming a bond with the ϵ-amino group of lysine for enzymatic activity [15]. When the ϵ-amino group in the enzyme is carbamoylated, the reaction with PLP cannot take place, thereby blocking the catabolic pathway of threonine. Other PLP-dependent enzymes are alanine and aspartate amino transferases [16–18]. The transfer of amino groups between amino acids and α-keto-acids is inhibited when these enzymes are carbamoylated, thereby interfering with amino acid metabolism. The essential amino acid isoleucine requires a PLP-dependent amino transferase for its catabolic pathway. In addition, carbamoylation of PLP itself has been reported to interfere with threonine dehydratase [15]. Chymotrypsin carbamoylated at the hydroxyl group of serine had decreased enzymatic activity showing that the hydroxyl group reaction with cyanate was important [19].

Enzymes in glucose metabolism such as glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase have decreased activity when carbamoylated [20]. The altered functions of many other carbamoylated proteins are included in a review of urea and carbamoylation [20]. Molecular site-directed mutagenesis that alters protein function through replacement of lysine residues could lead to identification of additional molecules that are adversely affected by carbamoylation.

CARBAMOYLATION ALTERS HORMONE ACTIVITY

Carbamoylation can affect hormone action. Carbamoylated insulin showed a decrease of one-third of its biological activity. In addition, carbamoylation at a different site in insulin caused immunologic reactivity to decrease 20% [21]. Carbamoylation of the hydroxyl group of tyrosine in oxytocin caused a change in activity. The dicarbamoyl-oxytocin, amino-carbamoyl-hydroxyl-carbamoyl-oxytocin, was not active and acted as an inhibitor of oxytocin [22]. Amino-carbamoyl-oxytocin showed slight activity and did not inhibit oxytocin [22]. Purified erythropoietin carbamoylated to form ϵ-N-C-lysine was inactive in bone marrow tissue culture [23].

CARBAMOYLATION OF LOW-DENSITY LIPOPROTEINS

Carbamoyl-low-density lipoprotein (C-LDL) is autoimmunogenic in guinea pigs producing specific antibodies against ϵ-N-C-lysine [12]. C-LDL prevented the binding

of LDL to cell surface receptors in human fibroblasts and prevented the displacement of unmodified LDL from the receptor [24]. C-LDL had a decreased clearance from plasma when 20% of the lysines were converted to ϵ-N-C-lysine [25]. LDL isolated from uremic patients had a slower clearance in rabbits than did LDL from normal subjects [26]. C-LDL carbamoylated in uremia is an example of another molecular change that alters the binding, transport, and excretion of LDL.

CARBAMOYL-AMINO ACIDS INTERFERE WITH PROTEIN SYNTHESIS

In uremia, the continual exposure of amino acids to cyanate results in F-AA being converted to C-AA. Theoretically, the irreversibly modified α-amino group cannot form a peptide bond with the carboxyl group of another amino acid in protein synthesis. To explore this issue, we examined synthesis of hemoglobin and synthesis of osteocalcin. Incorporation of ^{14}C-amino acids into human hemoglobin in reticulocytes was compared in the absence or presence of α-N-C-leucine, α-N-C-tyrosine, and α-N-C-aspartic acid. The radiolabeled hemoglobin was decreased by 23% in the presence of C-AA, and the slope of incorporation in a 30-minute incubation was 170 cpm/mL/min without C-AA and only 60 cpm/mL/min in the presence of C-AA [2].

The synthesis of osteocalcin (bone γ-carboxyglutamic acid protein) in the presence of α-N-C-leucine, α-N-C-tyrosine, α-N-C-glutamic acid, and α-N-C-aspartic acid (0.22 to 0.29 μmol/mL) was decreased 27% in 24 hours compared with cultures without C-AA. The mol ratio of C-AA to F-AA was 1 mol of α-N-C-tyrosine to 1.8 mol of tyrosine and 1 mol of α-N-C-leucine to 2.8 mol of leucine in the media. Therefore, the concentration of the F-AA was not a limiting factor for protein synthesis. The synthesis of osteocalcin was measured by radioimmunoassay in rat osteosarcoma derived tissue stimulated by 1,25-dihydroxy vitamin D_3. The concentration of α-N-C-leucine and α-N-C-tyrosine used in this study was similar to the range found in plasma of 30 patients with ESRD on CAPD [2]. C-AAs are contributing factors in decreased protein synthesis, muscle wasting, and anemia in uremia.

INSULIN-SENSITIVE GLUCOSE UPTAKE AND C-AA

After testing 15 C-AA for a role in induction of insulin resistance in cultured rat adipocytes, only α-N-C-asparagine reduced insulin-sensitive glucose uptake in a dose-dependent manner to a maximal decrease of 33%. The half maximally effective concentration was 148 μmol/L, which is in the range of α-N-C-asparagine found in uremic patients. The binding of ^{125}I-insulin to its receptor was not affected, nor was hexokinase or glucose phosphorylation affected. The F-AA asparagine also had no effect. These results indicated that the insulin-sensitive glucose transport system GLUT4 was affected by α-N-C-asparagine [2]. Thus, a heretofore unknown aspect of insulin resistance in uremia was shown to be the result of the interaction of a C-AA and a transport protein.

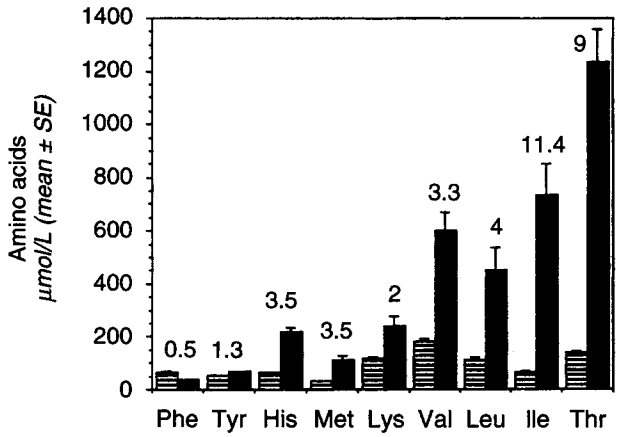

Fig. 2. Carbamoyl-amino acids formed from free amino acids shown as the Carbamoylation Index. Symbols are: (▤) free-amino acids (F-AA); (■) α-amino-carbamoyl-amino acids.

FATE OF FREE AMINO ACIDS IN UREMIA

Amino acids have been measured in plasma, urine, and dialysate in patients with ESRD maintained by hemodialysis (HD) or CAPD. The early studies of amino acids [27, 28] in uremia did not measure C-AA because the α-amino-group was derivatized in vivo by carbamoylation. In C-AA, there was no free α-amino group available to be derivatized in vitro in the method used to measure amino acids. Therefore, the values for amino acids in the uremic patient did not measure all of the amino acids that were present. In agreement with others, we have shown that the F-AA in plasma of healthy subjects differed from those in uremic patients where essential amino acids, especially tyrosine and the branched chain amino acids, valine, leucine, and isoleucine, were significantly lower. Patients with ESRD on CAPD were followed in a longitudinal study measuring F-AA and C-AA in plasma [29]. A ratio (C-AA/F-AA; called the Carbamoylation Index, CI) was calculated as μmol/L (mean ± SE). The CI showed that for one molecule of F-AA, there was a specific number of F-AA molecules removed from the F-AA pool by carbamoylation. Molecules of carbamoylated essential amino acids calculated as the CI are seen in Figure 2. For example, with a CI of 1.3, the anabolic availability of the essential amino acid tyrosine is decreased by one half [30]. The increase in C-AA may be explained by a block in the catabolic pathway of the F-AA because of a carbamoylated en-

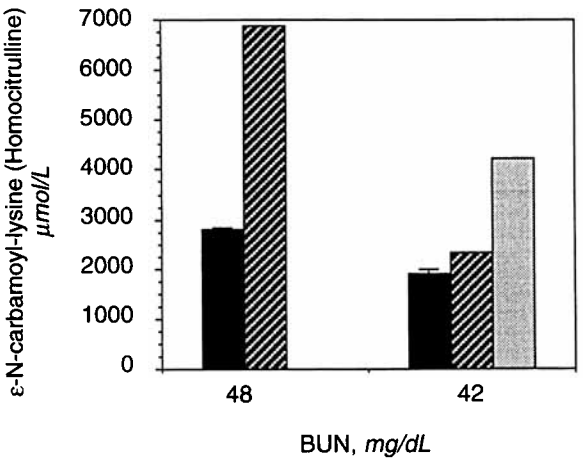

Fig. 3. Protein catabolism measured by ε-amino-carbamoyl-lysine (homocitrulline). Peritoneal clearance partially compensates for loss of residual renal function. Symbols are: (■) 6 hours plasma (mean ± SE); (▨) 24 hours dialysate; (■) 24 hours urine.

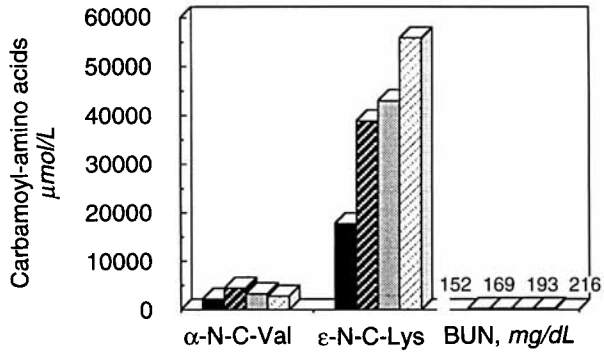

Fig. 4. In an ESRD patient who was not a candidate for dialysis, the α-amino-carbamoyl-valine initially increases as the BUN increases and then decreases with decreased protein intake. ε-Amino-carbamoyl-lysine measures protein catabolism and reflects increase in urea with time. Symbols are: (■) 3 months before death; (▨) 2.5 months before death; (■) 0.5 months before death; (▨) 2 days before death.

zyme and/or a carbamoylated cofactor such as PLP [15]. This is the case for threonine dehydratase and for transamination of isoleucine, where both the enzymes are PLP dependent. The decrease in the anabolically active F-AA pool by carbamoylation forming C-AA contributes in part to the malnutrition found in uremic patients.

C-AA IN ESRD MANAGED BY HEMODIALYSIS

In ESRD patients on HD, the reduction rate of C-AA during dialysis varied according to the C-AA (μmol/L, mean ± SE) and ranged from 25 to 65%. The appearance of C-AA in plasma from post-HD to pre-HD was calculated as μmol/L/hour (mean ± SE) and varied according to the C-AA. The rate of carbamoylation is dependent on the pKa of the amino group and the urea concentration. Further studies are necessary to investigate the removal of C-AA in the ongoing efforts to modify HD strategies.

DIABETES AND ESRD

Two different modifications of the ε-amino group of lysine and their methods of identification require clarification. The immunohistochemical identification of ε-N-C-lysine, a result of carbamoylation, should not be confused with immunohistochemical identification of N^{ϵ}-(carboxymethyl)lysine (CML), a glycoxidation product [31] reported as an advanced glycation end product (AGE) in the diabetes-renal disease literature. The anti–ε-N-C-lysine antibody used in studies reported herein is specific for ε-N-C-lysine and does not recognize CML. The reverse-phase high-performance liquid chromatographic (RP-HPLC) method used to identify and quantitate C-AA as reported herein reports only ε-N-C-lysine, not CML. We have demonstrated that these two molecules in a mixture are separated by different retention times, and each is recovered at greater than 90%.

Theoretically, in patients with both ESRD and diabetes, lysine residues that are glycosylated are not available for carbamoylation. To test this hypothesis, ε-N-C-lysine in patients with ESRD alone was compared with patients with ESRD and non–insulin-dependent diabetes mellitus (NIDDM) matched by BUN. ε-N-C-lysine was significantly higher ($P < 0.001$) in patients with ESRD alone than in ESRD NIDDM patients. ε-N-C-lysine was 5433 ± 485 μmol/L in ESRD and 3692 ± 221 μmol/L (mean ± SE) in NIDDM ESRD patients. Both glycosylation and carbamoylation contribute to protein modification and to the pathology that occurs in patients with diabetes and ESRD.

ε-N-C-lysine (homocitrulline) in ESRD measures continuous cyanate modification of proteins and is related to time-averaged urea concentration.

The concentration of ε-N-C-lysine (homocitrulline) in plasma, urine, and dialysate in ESRD patients on CAPD measures total ongoing protein catabolism. ε-N-C-lysine levels in plasma and peritoneal dialysate are compared with those found in plasma, dialysate, and urine in Figure 3. It is clear that peritoneal clearance partially compensates for the loss of residual renal function.

The longitudinal study of α-N-C-lysine and ε-N-C-lysine in postprandial plasma ($N = 84$) from patients with ESRD treated by CAPD was measured by RP-HPLC. During this time, the mean BUN values ranged from 58 to 66 mg/dL. The mean Kt/V of 2 did not change with time. Carbamoylation values over a 15-year period included predialysis values and adjustment to CAPD (first year) and loss of residual renal function, and thereafter varied according to intake or catabolism.

In a patient with ESRD who was not a candidate for dialysis, a comparison of α-N-C-valine and ε-N-C-lysine is seen in Figure 4. The initial increase in α-N-C-valine is an indication of the rising BUN. As uremia worsens, the α-N-C-valine level falls secondary to markedly decreased protein intake. The ε-N-C-lysine shows the catabolic rate of protein breakdown as it is compared with urea concentration over time. The increase in ε-N-C-lysine represents an increase in protein catabolism and higher urea concentrations. The comparison of α-N-C-valine and ε-N-C-lysine is valid, since valine and lysine are each 7% of the average protein.

The toxicity of cyanate should be considered in future decisions concerning new modes of ESRD therapy. This may include different forms of amino acid supplementation, new dialysis modalities that remove cyanate to a greater extent, or the use of an as yet undetermined "cyanate trap" along with current dialysis therapy.

CONCLUSION

Cyanate causes a cascade of molecular changes that contribute to the abnormalities seen in uremia (Fig. 1) and affects amino acids, enzymes, coenzymes, antibodies, hormones, and receptors by changing structure, charge, and function. Carbamoylated molecules can block, enhance, or be excluded from metabolic pathways and affect binding and trafficking and the fate of noncarbamoylated molecules. C-AAs with irreversible carbamoylation of the α-amino group contribute to decreased protein synthesis and a decreased F-AA pool. ε-Amino carbamoylation occurs at multiple lysine sites, is cumulative over the life span of the protein, and reflects the turnover rate of the protein and the time-averaged urea concentration. The concentration of ε-N-C-lysine in plasma, urine, and dialysate is a direct measure of protein catabolism. The removal of urea, cyanate, and carbamoylated molecules appears to alleviate partially the metabolic and functional changes found in uremia. Toxicity from carbamoylation is not an "all-or-none" phenomenon, but actions of cyanate contribute to toxicity seen in uremia.

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