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Carbamoylation of glomerular and tubular proteins in patients with kidney failure: a potential mechanism of ongoing renal damage[1]

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Summary

Background: Cyanate formed spontaneously from urea carbamoylates non-protonated amino groups of protein, irreversibly altering function, charge and structure. Carbamoylated proteins in renal tissue have not been examined hitherto.

Objectives: To identify homocitrulline (ε-amino-carbamoyl-lysine), a result of *in vivo* carbamoylation by urea-derived cyanate, from patients with renal disease or in newly transplanted kidneys by immunohistochemistry. To evaluate enzymatic activity of carbamoylated and non-carbamoylated matrix metalloproteinase-2 and correlate this with renal tissue carbamoylated *in vivo*.

Design: Anti-homocitrulline antibody is specific for homocitrulline and was used to identify carbamoylation of ε-amino-lysine in renal biopsies from patients with elevated BUN, with isolated proteinuria, and as controls, from normal donors at time of transplantation. Enzymatic activity of matrix metalloproteinase-2 carbamoylated *in vitro* was evaluated.

Results: Homocitrulline was present in glomerular basement membrane (8/10), mesangium (8/10), tubular epithelium and cytoplasm (7/10) and Bowman's capsule (1/10) in patients with elevated BUN. The discordant patterns of glomerular and tubular localization of homocitrulline versus immune complexes indicated that the carbamoylated proteins were not a component of immune deposits but were modified proteins in renal tissue. No homocitrulline was found in transplanted kidneys (14/15) or in proteinuric patients (2/2). Enzymatic activity of both human and rat matrix metalloproteinase-2 was strongly inhibited in a dose-dependent fashion when incubated with cyanate.

Conclusions: In situ carbamoylation in proteins occurred in kidneys of patients with renal dysfunction but not in normal newly transplanted kidneys. Decreased enzymatic activity of carbamoylated enzymes may alter specific renal regulatory mechanisms. Carbamoylated proteins with altered function and charge may represent a previously underestimated mechanism in renal pathophysiology.

Keywords: cyanate; urea; carbamoylation; matrix metalloproteinase-2

Introduction

Immunohistochemical staining in renal tissue identifies carbamoylation (the terms carbamoylation or carbamylation have been used by different authors but the terms have the same meaning). Post-translational modification of renal proteins due to urea-derived cyanate has not been previously reported. Renal tissues from patients with renal insufficiency were compared to tissues obtained from normal kidneys immediately after transplantation. Urea and cyanate increase as renal function decreases. Urea is the *in vivo* source of

Abbreviations

BUN	= blood urea nitrogen
ESRD	= end stage renal disease
FITC	= fluorescein isothiocyanate
GBM	= glomerular basement membrane
MMP-2	= matrix metalloproteinase-2
PBS	= phosphate buffered saline
PMN	= polymorphonuclear neutrophil

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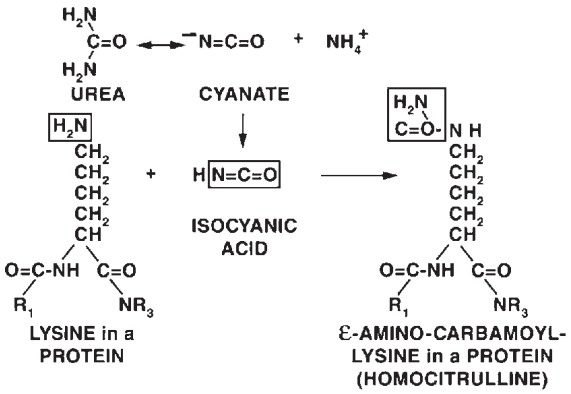


Figure 1

Formation of ε-amino-carbamoyl-lysine (homocitrulline) by urea-derived cyanate carbamoylating the non-protonated ε-amino group of lysine in a protein.

cyanate. Cyanate is in equilibrium with urea, therefore when a molecule of cyanate is removed by carbamoylation, a new molecule of cyanate is formed spontaneously. Among carbamoylated proteins found *in vivo* which have altered function are carbamoylated hemoglobin with a change in oxygen affinity [1] and carbamoylated low density lipoprotein (LDL) with an altered affinity for the LDL receptor [2]. Carbamoylation of enzymes, hormones, structural proteins, and transport proteins with altered function have been reported [3].

In vivo irreversible formation of homocitrulline (Fig. 1) occurs when urea-derived cyanate (isocyanic acid) reacts with the non-protonated ε-amino group of lysine at body pH and temperature [4, 5]. The formation of homocitrulline is cumulative over the life-span of the protein and results in the loss of net positive charges with functional changes in carbamoylated protein [3, 6, 7]. Homocitrulline was identified with anti-homocitrulline antibody [2, 8, 9, 10] that was specific for ε-amino–carbamoyl-lysine. N^{ε}-carboxymethyl lysine, an advanced glycosylation end product, also accumulates in patients with end-stage renal disease. In order to insure the specificity of the anti-homocitrulline antibody studies were performed to see if cross-reactivity occurred. The anti-homocitrulline antibody was found to be specific for homocitrulline and did not react with N^{ε}-carboxymethyl lysine either alone or in proteins [10].

In our previous studies, carbamoylation with formation of homocitrulline in hemoglobin occurred *in vivo* [8] and on cell surface proteins and cytoplasmic proteins of polymorphonuclear neutrophils (PMN) and some monocytes [11] in patients with end-stage renal disease (ESRD). *In vitro* carbamoylation of PMN inhibited the release of microbicidal superoxide [11]. The toxicity due to *in vivo* and *in vitro* carbamoylation of proteins and amino acids has been reviewed [3].

Matrix metalloproteinases, especially matrix metalloproteinase-2 (MMP-2), are important enzymes responsible for the turnover of extracellular matrix proteins. A decrease in MMP-2 activity induced by cyclosporine A resulted in significant total collagen accumulation in mesangial cells [12]. Therefore, inhibition of MMP-2 – that may also be the case with carbamoylation – may well promote the development of glomerulosclerosis.

Herein, we examined the effect of carbamoylation upon the function of MMP-2, an enzyme that plays an important role in the kidney [13, 14], and we examined renal tissue for the presence and location of *in vivo* carbamoylated proteins.

Materials and methods

Kidney biopsies: patients with renal insufficiency and elevated BUN

All biopsies were collected and handled in accordance with the needs of primary diagnostic evaluation. No material was collected which was not needed for diagnostic purposes. Renal biopsies were processed for light microscopy and electron microscopy according to standard procedures. A battery of immunofluorescent stains for immunoglobulins G, M, and A, kappa and lambda light chains, properdin, fibrin, and complement C3, C4, and C1q were also employed for non-transplanted kidneys. Tissues were archived as frozen material (–70 °C) until further processing. Materials were sectioned at 5 micron thickness, placed on glass slides, fixed with cold acetone, and frozen.

Table 1

Uremic patients: Clinical data.

Patient	sex	age (years)	BUN (mmol/l*)	creatinine (μmol/l**)	urinary protein (g/24 hr)	diagnosis
1	♂	22	43	1582	–	acute interstitial nephritis
2	♂	69	13	495	13.3	IgA nephropathy
3	♂	16	15	866	50	end-stage glomerulosclerosis
4	♀	59	23	946	10.2	membranoproliferative glomerulonephritis with crescents
5	♂	31	22	849	6.9	advanced diabetic nephropathy
6	♀	6	32	539	0.2	immune complex mediated, acute crescentic glomerulonephritis
7	♀	83	21	274	2.0	class IV lupus nephritis
8	♂	23	23	575	–	chronic interstitial nephritis with noncaseating granuloma
9	♀	67	24	460	–	membranoproliferative glomerulonephritis
10	♀	85	16	221	10.0	arterio- and arteriolar nephrosclerosis

* Reference range 2.9–8.2 mmol/l
** Reference range 53–115 μmol/l

Kidney biopsies: control material

Baseline biopsies (15) were obtained from normal kidney allografts at the time of transplantation and from 2 patients with a normal BUN and proteinuria.

Clinical studies

The blood urea nitrogen (BUN) and creatinine were measured with a COBAS Roche Biochemical Analyzer (Roche Diagnostic Systems, Inc., Montclair, N. J.) or a Hitachi 747 Chemistry Analyzer (Boehringer Mannheim, Indianapolis, IN). Diagnosis of renal disease was based on clinical, biochemical and pathologic data.

Immunohistochemical identification with anti-homocitrulline antibody

Fixed sections were washed twice with phosphate buffered saline (PBS), pH 7.2, 10 minutes for each wash, covered with 10% normal goat serum for 5 minutes to block nonspecific reactions with the secondary antibody, and then washed. Thereafter, each slide was washed 3 times with PBS for 3 minutes each wash. Next, the tissue was covered with the primary anti-homocitrulline antibody prepared in this laboratory in guinea pigs using homologous guinea pig carbamoylated low density lipoprotein [2, 8, 9] which was directed specifically against ε-amino-carbamoyl-lysine and reacted equivalently with other proteins containing ε-amino-carbamoyl-lysine [9]. Then slides were kept in a moist chamber at 24 °C for 20 hours. The primary antibody was removed by washing. The tissue was covered with the secondary antibody, goat anti-guinea pig IgG-FITC, H- and L-chain specific (Southern Biotechnology Associates, Inc., Birmingham, AL), 1:10 in PBS. After 1 hour in a moist chamber at 24 °C, the secondary antibody was removed by washing. The slides were coverslipped using Immu-Mount (Shandon, Pittsburgh, PA). Tissues were examined using an epifluorescence microscope with a standard FITC filter set and the fluorescence labeling of homocitrulline was photographed. The primary antibody bound to homocitrulline antigen for 20 hours at 6 °C was negative for immunohistochemical tissue staining. Goat anti-guinea pig IgG-FITC antibody alone was also negative. No tissue autofluorescence was seen.

Exposure of human MMP-2 and of rat mesangial cell MMP-2 to cyanate *in vitro*

Purified human MMP-2 (20 ng/assay; Anawa Trading SA, Wangen, Switzerland) and rat mesangial cell culture supernatant (20 µl/assay) containing MMP-2 were separately exposed to various concentrations of potassium cyanate (Fluka, Buchs, Switzerland), such as 0 mM, 25 mM, 50 mM, 75 mM, and 100 mM, at pH 7.4. Incubations were performed for 1 hour at room temperature. Immediately thereafter, samples were subjected to a continuously recording fluorometric assay, as described by us and others [15, 16]. The assay used is based on the measurements of the continuous hydrolysis of the quenched fluorescent substrate, (7-methoxycoumarin-4-yl)Acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH_2 (MCA; Bachem, Bubendorf, Switzerland: λ_{ex} 328 nm, λ_{em} 393 nm). Assays were performed in triplicate, the enzymatic activity was measured by linear regression, with a slope obtained from serial measurements over a period of 5 hours, and the per cent inhibition was calculated. Furthermore, samples containing human MMP-2 were subjected to Western blot analysis as described previously [17] using an anti-homocitrulline antibody [8, 10].

Statistics

To compare biopsies of patients with renal failure with normal kidney material, we used the Fisher's exact test.

Study approval was obtained from the Institutional Review Board of the University of Tennessee Health Science Center.

Results

Patients with renal insufficiency

The clinical data and the patient diagnoses are found in Table 1. The BUN ranged from 13 to 43 mmol/l and creatinine ranged from 221 to 1582 µmol/l. Homocitrulline *in situ* is reported in Table 2. The renal sites of immunoglobulins and complement are reported in Table 3.

Homocitrulline in the protein of the glomerular basement membrane in 8 of 10 kidneys was identified by linear fluorescence. The glomerulus of patient 4 (Fig. 2 A) is typical of that seen in the 8 patients showing homocitrulline in the glomerular basement membrane (Table 2) in the absence of immune deposition (Table 3). In one case with

Table 2

Uremic patients: renal site of homocitrulline.

Patient	glomerulus			tubules	
	basement membrane	mesangium	Bowman's capsule	perinuclear	cytoplasm
1	+ (linear)	+	–	–	–
2	+ (linear)	+	–	+	–
3	–	–	–	–	–
4	+ (linear)	+	–	+	+
5	+	+	–	+	+
6	–	+	+	–	+
7	+ (linear)	–	–	–	+
8	+ (linear)	+	–	–	–
9	+ (linear)	+	–	+	–
10	+ (linear)	+	–	+	+

+ = Positive – = Negative

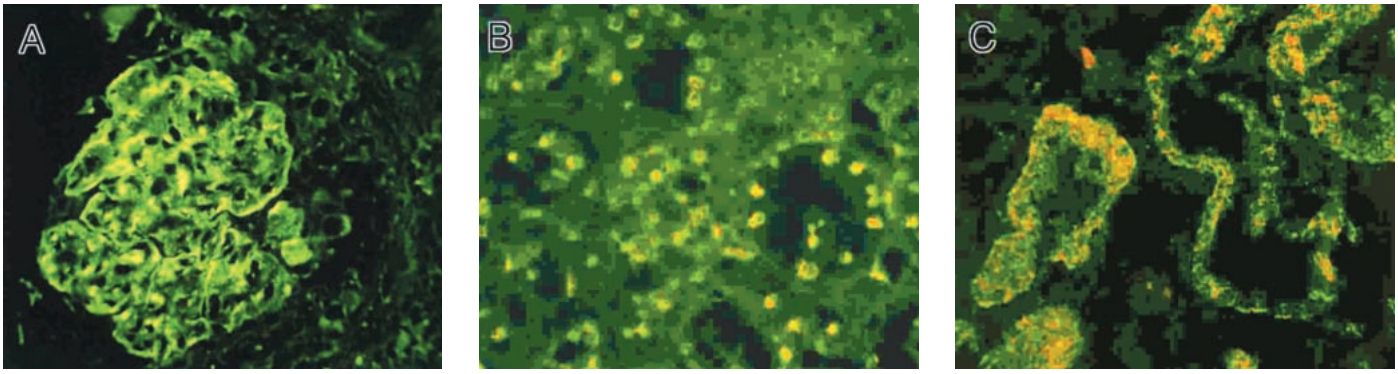


Figure 2

A. Patient 4. Membranoproliferative glomerulonephritis with crescents. Linear fluorescence of the glomerular basement membrane identifiying the presence of homocitrulline in protein. Fluorescence identifies the presence of homocitrulline in protein in the mesangial cells and extracellular matrix (original magnification 400×).
B. Patient 2. IgA nephropathy. Perinuclear fluorescence in the cells of the tubules identifying the presence of homocitrulline in protein. Linear fluorescence of the glomerular basement membrane similar to Figure 2A was also found in this patient (original magnification 400×).
C. Patient 10. Arterio- and arteriolar nephrosclerosis. Fluorescence of the tubular epithelium identifying the presence of homocitrulline in protein (original magnification 400×).

Table 3

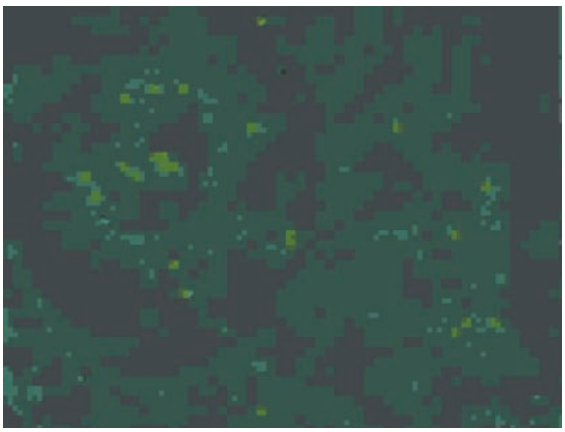
Uremic patients: Renal site of immunogobulins and complement.

Patient	glomerulus basement membrane	mesangium
1	–	IgM, C3, C4, Clq
2	–	IgM, IgA, κ, λ, C3
3	IgM, C3, Clq, Properdin	–
4	–	IgG, IgM, C3, C4, Properdin
5	–	IgM, C3, Properdin
6	–	C3
7	–	IgG, IgM, IgA C3, C4, Clq
8	–	IgM, C3
9	–	IgG, C3
10	–	–

Tubules (perinuclear and cytoplasm): No immunogobulin or complement found, except in patient 6 where C3 was found

Figure 3

Transplanted kidney, reperfusion biopsy. Carbamoylated protein was not evident. This photomicrograph is typical of renal tissue seen in individuals with a normal BUN (original magnification 400×).



sclerosed glomeruli, a more diffuse staining was seen. Typical also is the homocitrulline found in the extracellular matrix and mesangial cells (Fig. 2 A). Homocitrulline due to carbamoylated protein seen *in situ* in mesangial cells was demonstrated in 8 of 10 kidneys (Table 2).

In the tubular cells, homocitrulline was located in the perinuclear area (Fig. 2 B) and/or in the cytoplasm (Fig. 2 C) in 7 kidneys. Not all tubules in the same tissue section had carbamoylated cellular proteins. No immunoglobulins or complement were found in the tubules of these patients. Homocitrulline was present in the renal tubules of patients with primary glomerular disease, i.e. IgA nephropathy (Fig. 2 B) and membranoproliferative glomerulonephritis (Table 2), and in arterio- and arteriolar nephrosclerosis (Fig. 2 C) all without immune deposition.

Casts of fluorescent protein were located within some tubules. Fluorescent carbamoylated protein was seen within Bowman's capsule in the same site where material was identified as a crystalline deposit by electron microscopy (patient 6).

Control studies: biopsies from transplanted kidneys and proteinuric patients with normal BUN levels

No homocitrulline was found in biopsies from proteinuric patients with BUN levels of 8.2 mmol/l or less, or in biopsies of transplanted kidneys from 14 of 15 patients. This was significantly different from those with renal failure ($p < 0.001$). Figure 3 shows a typical example of a reperfused transplanted kidney where no homocitrulline was found. In 1 of the 15 transplanted kidneys a slight fluorescent labeling was seen in the cytoplasm of tubular cells.

Cyanate inhibited the in vitro activity of MMP-2

The purified human MMP-2 and MMP-2 synthesized by rat mesangial cells in tissue culture was examined for the effect of cyanate upon enzymatic activity. The activity of purified human MMP-2 served as the control without cyanate. The presence of 25 mM, 50 mM, 75 mM and 100 mM cyanate inhibited the proteolytic enzymatic activity of MMP-2 in a dose-dependent manner. Inhibition was 3%, 26%, 65% and 97% compared to the control without cyanate (Fig. 4). The pres-

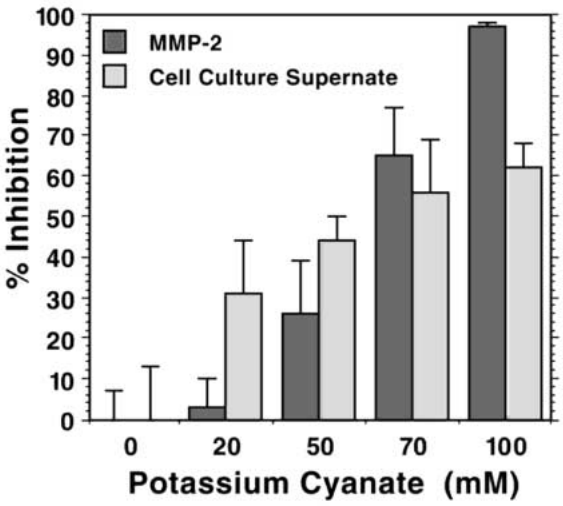


Figure 4

Inhibition of both purified human MMP-2 or MMP-2 in supernatant from rat mesangial cell culture incubated with increasing concentrations of cyanate. Results represent the mean of 3 assays and 0% inhibition is defined as MMP-2 activity in the absence of cyanate.

ence of carbamoylated MMP-2 was confirmed by Western blot analysis using anti-homocitrulline antibody (data not shown). Carbamoylation is a rapid process occurring within minutes. Therefore, the incubation time of 1 hour was largely sufficient for carbamoylation to occur, thereby showing the cyanate dose-dependent inhibition of MMP-2.

MMP-2 present in cell culture supernatant of rat mesangial cells was inhibited in a similar fashion, 31%, 44%, 56% and 62%, using identical cyanate concentrations of 25 mM, 50 mM, 75 mM and 100 mM cyanate, when compared to the control without cyanate (Fig. 4). This demonstrated the presence of enzymatically active MMP-2 in the supernatant of rat mesangial cells in culture. Both human MMP-2 and rat MMP-2 were carbamoylated by cyanate, resulting in inhibition. Inhibited proteolytic activity is an important cause for change in function.

Discussion

There are many proposed mechanisms favoring the progression of renal insufficiency [18]. In many chronic renal diseases, a sufficiently strong initial insult to the kidneys is followed by a relentless and irreversible decline in renal function. Therefore, there exists a need for the identification of factors supporting or enhancing the process of progressive decrease in glomerular filtration rate. This investigation provides the first evidence of *in situ* carbamoylation of renal proteins and carbamoylation-induced decrease in MMP-2 activity, and thus may provide another potential mechanism for the progression of renal insufficiency.

Urea-derived cyanate is a reactive molecule that alters the charge and structure of amino acids and proteins (Fig. 1) as well as their function [3]. Evidence of the adverse effects of *in vivo* carbamoylation was found when cyanate administered as a therapeutic agent in sickle cell anemia caused peripheral neuropathy [19] and cataracts [20]. In 1960, decreased activity of the enzyme ribonuclease in urea solution was found to be the result of carbamoylation of lysine residues forming homocitrulline by urea-derived cyanate [21]. Since that time, carbamoylation of many enzymes and hormones resulting in altered functional activity has been reported [22–28]. We demonstrated that cyanate, in a time- and dose-dependent manner, when incubated with PMNs, strongly inhibited the ability of carbamoylated PMNs to release microbicidal superoxide [11]. The enzymatic activity of both purified human MMP-2 and rat mesangial MMP-2 in the supernatant of rat mesangial cell culture was inhibited by cyanate in a dose-dependent manner *in vitro*. The inhibition of proteolytic activity of matrix-degrading enzymes has a profound effect upon catabolism of extracellular matrix and remodeling of injured tissue [29]. This investigation shows that, in uremia, there is *in vivo* carbamoylation of proteins in the kidney. We found homocitrulline in extracellular matrix proteins which results in a carbamoylated protein with changes of charge and structure. We have also shown that *in vitro* carbamoylation inhibits the proteolytic activity of matrix-degrading enzymes, such as MMP-2. Inhibited proteolytic activity is an important change in function. As nephropathies progress and urea levels increase, MMP-2 activity may decrease below a critical level required for physiologic matrix metabolism, as a result of carbamoylation. As a consequence, there may be a progressive increase of extracellular matrix in the mesangium, a process that results in glomerulosclerosis [14, 29, 30]. In the observed repair of renal tissue, the ongoing degradation of macromolecules by proteolysis is followed by rearrangement and deposition of new components, all of which may be altered by carbamoylation. A cycle associated with glomerulosclerosis [15–18] may occur where there is a decrease of carbamoylated MMP-2 proteolytic activity, and a progressive increase of extracellular matrix in the mesangium. Also, the *in vivo* carbamoylation of extracellular matrix proteins may render these proteins less susceptible for degradation by MMP-2. It is conceivable that inhibition of MMP-2 activity by carbamoylation supports the development of glomerulosclerosis as the final stage of many renal diseases. As discussed herein, the biological relevance of inhibition of MMP-2 proteolytic enzymatic activity by carbamoylation *in vitro* has clinical implications and carbamoylation of MMP-2 *in vivo* with MMP-2 inhibition by urea-derived cyanate is the subject of future investigation.

The effect of homocitrulline found in the glomerular basement membrane (GBM) upon the

structure and regulatory function in the renal glomerulus is currently unknown. The GBM provides a structural support against the high intraglomerular hydrostatic pressure and functions as a negatively charged permselective filtration barrier [13]. Molecules are filtered according to molecular size (increasing retention with increasing size) and by ionic charge. It is possible that carbamoylation may affect the filtration parameters when either the GBM or the molecules being filtered are carbamoylated. In both situations, the carbamoylated GBM and the carbamoylated molecules presenting for filtration have an altered structure. In carbamoylated proteins, the positively charged amino group becomes a neutral group. Because of this modification, the carbamoylated protein has a greater net negative charge which is evident in the electrophoretic mobility of carbamoylated protein compared to the native protein [31].

The variability of the presence of homocitrulline in tubules (Table 2, Figures 2 B, 2 C) may be due to carbamoylation of renal cellular structural and cytoplasmic proteins in contrast with transport of carbamoylated proteins. Homocitrulline concentration in protein depends upon urea concentration, duration of elevated urea levels, and the turnover time of the protein. Carbamoylation of the amino-terminal valine in hemoglobin (valine hydantoin) may be used to differentiate acute from chronic renal failure [32–35] and may be a marker for adequacy of therapy. Continuous exposure to urea-derived cyanate in low levels results in carbamoylation of low-turnover proteins. For this reason, carbamoylation of protein can occur at normal BUN levels in healthy individuals. Low levels of valine hydantoin were reported in healthy subjects and diabetics without chronic renal failure [36].

The role of homocitrulline in the perinuclear membrane is currently unknown but it may be possible that changes in protein structure by carbamoylation could alter the processes of molecular transport. Because the formation of homocitrulline is irreversible, the presence of carbamoylated protein indicates a protein with a long lifespan that has lysine residues in a microenvironment favorable for carbamoylation. The flow of molecules between the cytoplasm and nucleus of the cell is regulated by the perinuclear membrane, a continuation of the endoplasmic reticulum [37].

Homocitrulline was not seen in the perinuclear membrane or cytoplasm of tubules in healthy reperfused transplanted kidneys, nor in the proteinuric patients with a normal BUN.

Carbamoylated proteins, such as extracellular matrix proteins and MMP-2, GBM proteins, proteins in endoplasmic reticulum and cytoplasmic proteins, are present in patients with kidney failure, have different structures and may have different functional properties. Therefore, protein carbamoylation may represent a previously underestimated mechanism for the relentless progression of renal dysfunction. Additional insight into the function of carbamoylated proteins and the availability of a new immunohistochemical method for their detection may lead to new approaches for study of renal disease or its therapy.

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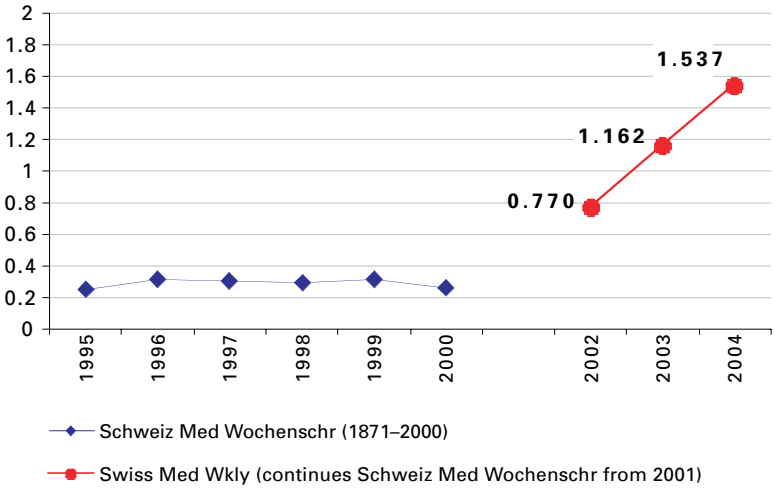

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