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Authors
Lau, Wei Ling
Vaziri, Nosratola D

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Review Article

Urea, a true uremic toxin: the empire strikes back

Wei Ling Lau* and Nosratola D. Vaziri*

*Division of Nephrology and Hypertension, University of California Irvine, Orange, CA, U.S.A.

Abstract

Blood levels of urea rise with progressive decline in kidney function. Older studies examining acute urea infusion suggested that urea was well-tolerated at levels 8–10× above normal values. More recent *in vitro* and *in vivo* work argue the opposite and demonstrate both direct and indirect toxicities of urea, which probably promote the premature aging phenotype that is pervasive in chronic kidney disease (CKD). Elevated urea at concentrations typically encountered in uremic patients induces disintegration of the gut epithelial barrier, leading to translocation of bacterial toxins into the bloodstream and systemic inflammation. Urea induces apoptosis of vascular smooth muscle cells as well as endothelial dysfunction, thus directly promoting cardiovascular disease. Further, urea stimulates oxidative stress and dysfunction in adipocytes, leading to insulin resistance. Finally, there are widespread indirect effects of elevated urea as a result of the carbamylation reaction, where isocyanic acid (a product of urea catabolism) alters the structure and function of proteins in the body. Carbamylation has been linked with renal fibrosis, atherosclerosis and anaemia. In summary, urea is a re-emerging Dark Force in CKD pathophysiology. Trials examining low protein diet to minimize accumulation of urea and other toxins suggest a clinical benefit in terms of slowing progression of CKD.

Key words: carbamylation, chronic kidney disease, inflammation, urea.

BACKGROUND: CONTROVERSY SURROUNDING UREA TOXICITY

Chronic kidney disease (CKD) is characterized by the accumulation of waste products that have the potential to dysregulate normal cellular functions, the so-called uremic toxins. These can be divided into small molecules (<500 Da) or middle molecules. Of the water-soluble small molecules, urea has the highest blood concentrations [1]. Uremic toxins contribute to accelerated cardiovascular disease in CKD via propagating non-traditional risk factors that include chronic inflammation, oxidative stress, protein-energy wasting, disordered mineral metabolism and deficiency of endogenous calcification inhibitors [2–4]. Urea is a 60 Da molecule that is the end-product of protein and nitrogen metabolism. It is a well-established surrogate marker of kidney function, protein intake and dialysis adequacy [5,6]; however, there has been much controversy about whether urea is truly pathogenic.

Early experiments examining urea infusions in animal models were done by Vauquelin and Segalas (1822), followed by Gigot-Suard (1870) and Treitz (1859) [7]. No toxicity was observed as low doses were used in animals with intact kidney function. In the late 1800s, detailed investigations by Herter showed that the mammalian kidney is able to excrete urea at a rate 12 times greater than that of the frog's Wolffian body (weight for weight), suggesting an evolutionary importance for efficient urea elimination [7]. In rabbits, dogs and monkeys following nephrectomy or ureter ligation, Herter noted that arrhythmias and muscle spasms would occur at blood urea levels of 0.3% (8–10× above normal blood content), with coma and subsequent cardiopulmonary arrest occurring at levels of 0.4–0.5% [7]. Other uremic retention products including middle molecules were unknown at the time, and were not assessed. Urea ingestion as high as 4 g/kg for 5 days has been reported to be harmless in piglets; however, dogs dosed orally with 5–30 g/kg urea develop weakness, gastrointestinal symptoms and eventual coma [8]. In the 1970s, Johnson et al. [9] added urea to the dialysate in three chronic haemodialysis patients and concluded that blood concentrations below 140 mg/dl are non-toxic. When serum urea was increased quickly above 170 mg/dl, there were mild symptoms such as headache and lethargy; moderate symptoms were observed at urea concentrations above 280 mg/dl [9] (consistent with the threshold of 10× above normal blood levels that was noted by Herter in animal studies [7]).

Clinical trials that address adequacy of dialysis in the end-stage renal disease (ESRD) population may be interpreted as providing indirect evidence for the absence of urea toxicity [10].

Abbreviations: 4D trial, die deutsche diabetes dialyse (German Diabetes and Dialysis Study); BCL2, B-cell lymphoma 2; CKD, chronic kidney disease; ESRD, end-stage renal disease; FHN trial, frequent haemodialysis network; HEMO trial, haemodialysis study group; LDL, low-density lipoprotein; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; O-GlcNAc, *O*-linked beta-*N*-acetylglucosamine; ROS, reactive oxygen species.

Correspondence: Nosratola D. Vaziri (email ndvaziri@uci.edu).

The ADEMEX (ADEquacy of PD in MEXico) study, a prospective randomized study in 965 peritoneal dialysis patients, compared a 2-year, higher intensity dialysis protocol with efficient removal of small molecular weight molecules (as assessed by a Kt/V of 2.0 and a creatinine clearance of 60 l/week) with a standard dialysis protocol. Patient survival was not different, suggesting that more intensive elimination of urea (and other water-soluble small molecular weight substances) was not beneficial [11]. Similarly, there was no mortality benefit in the HEMO study which focused on 1846 chronic haemodialysis patients randomly assigned to either high-intensity (Kt/V 1.45 and urea reduction ratio 75%) or standard-intensity (Kt/V 1.05 or urea reduction ratio 65%) haemodialysis treatment [12]. In secondary analysis, greater dialysis dose was found to have a mortality benefit in women which may reflect gender discrepancies when normalizing to volume (V); upon rescaling of dialysis dose to body surface area Daugirdas et al. [13] showed that women in the HEMO trial received substantially less dialysis due to having a lower anthropometric V per unit of surface area than men. Finally, in the frequent haemodialysis network (FHN) trial, haemodialysis intensity was increased by raising the number of dialysis sessions to 6 times/week in one group of 120 patients, as compared with standard 3 times/week in another group of 125 patients [14]. Despite a significantly different Kt/V between the two groups (2.57 in conventional compared with 3.6 in intensive group), there was no significant effect on cognition, serum albumin or requirement for erythropoiesis-stimulating agents. A significant benefit was found for the primary composite end point of death or 12-month change in left ventricular mass [14] and the survival benefit was sustained up to a median of 3.6 years out [15]; however, lower ultrafiltration requirements in the 6 days/week treatment group was a confounding factor. Post hoc analysis from the HEMO trial noted increased risk of all-cause and cardiovascular mortality at ultrafiltration rates over 10 ml/h·kg [16]. Thus, the survival benefit in the FHN trial could not be attributed to small molecule clearance alone. We caution that these 'negative' studies may have been unable to detect a clear benefit of urea reduction as end-organ damage may be too far advanced in the ESRD population, with patients often having been exposed to chronic urea elevation for several years. Haemodialysis therapy can itself exert adverse effects including activation of inflammatory pathways through blood exposure to the extracorporeal circuit as well as indiscriminate removal of useful small molecule nutrients. Finally, the ESRD milieu is extremely complex whereby variations in residual kidney function, middle molecule clearance, non-urea solute fluctuations, mineral/bone abnormalities and whole-body fluid balance all impact patient outcomes, leading some experts to denounce urea clearance (Kt/V) as an over-simplified measure of dialysis adequacy [17]. Indeed, efforts to investigate the potential benefits of urea-lowering may be most productive if focused on the pre-dialysis population, as with low protein diets discussed below.

Low protein diets in pre-dialysis CKD were first championed by the Italian nephrologists Giovannetti and Maggiore over 50 years ago [18], based on the rationale that decreased amino acid degradation and urea synthesis results in lowered urea accumulation [19,20]. Early observational studies appeared promising in slowing CKD progression, and were followed by randomized trials that were summarized in two major meta-analyses. In one meta-analysis, Fouque et al. compared a low protein diet of 0.3–0.6 g/kg/day with a usual diet (eight studies, $n = 1524$) over a follow-up period of 12–24 months in non-diabetic CKD patients [21]. Robertson et al. [22] compared diabetic CKD patients on 0.3–0.8 g/kg/day compared with 1–2 g/kg/day protein diet over a period of 4.5 months to 4 years (nine studies, $n = 585$). These publications highlighted a trend for slowing of CKD progression with dietary protein restriction, however it is important to note that compliance with low protein diet was poor across all studies. The largest randomized controlled trial to date is the MDRD (modification of diet in renal disease) study which measured GFR (glomerular filtration rate) via ^{125}I-iothalamate clearance, with subjects divided into Study A and Study B [23]. Study A compared 0.58 versus 1.3 g/kg/day protein diet in 585 subjects with GFR 25–55 ml/min/1.73 m^2 and found no difference in terms of CKD progression. Study B compared low protein diet 0.58 g/kg/day compared with very low protein diet of 0.28 g/kg/day supplemented by ketoacids in 255 patients with more advanced CKD (GFR 13–24 ml/min/1.73 m^2); the supplemented very low protein diet was associated with slower rate of GFR loss that almost reached statistical significance ($P = 0.07$) [23]. Adherence to prescribed protein intake was assessed via urinary urea excretion; blood urea levels were not reported as blood urea can be variable and is affected by a variety of factors aside from GFR and dietary protein (catabolic state, renal tubular handling, volume status, gastrointestinal bleeding [24]). Since then, other smaller randomized trials of low or very low protein diets supplemented with ketoacids or essential amino acids have noted various benefits, including delaying the need to initiate dialysis [25,26]. Protein restriction may have particular utility in the elderly population where dialysis initiation does not significantly prolong survival but instead is associated with sub-optimal quality of life and a high rate of complications. To this end, Brunori et al. randomized non-diabetic patients >70 years of age with CKD stage 5 (56 patients per group) to dialysis compared with a very low protein diet of 0.3 g/kg/day supplemented with keto-analogues, amino acids and vitamins. The diet intervention delayed dialysis initiation by a median of 10.7 months and was associated with a lower hospitalization rate, and similar survival [27]. The 2013 KDIGO (kidney disease improving global outcomes) guidelines suggest lowering protein intake to 0.8 g/kg/day in adults with estimated glomerular filtration rate (eGFR) <30 ml/min/1.73 m^2 (grade 2B evidence for pre-dialysis non-diabetic CKD patients, grade 2C evidence for diabetic CKD patients) [28]. An extensive review summarizing decades of experience with low-protein diets was recently published by a group of Italian nephrologists, where important variables including patient compliance, avoidance of malnutrition, and counselling on low phosphorus and low sodium intake are discussed [29].

THE RE-EMERGING DARK FORCE: CHRONIC UREA ACCUMULATION

From the above-mentioned studies involving urea infusions and haemodialysis survival, one could erroneously conclude that

high blood urea levels commonly encountered in CKD are well-tolerated and non-toxic. In this review, we discuss the emerging data that argue the opposite, including studies that demonstrate direct toxicity of urea on various tissues including the intestinal epithelium, vascular wall and adipocytes. Urea effects at these respective sites can promote systemic inflammation, vascular calcification and insulin resistance (Figure 1) [30–32]. Further, urea can exert indirect toxicity via carbamylation which alters the function of enzymes, hormones and other proteins (Figure 1) [33–35]. These collective downstream effects are a culmination of chronic exposure and would be missed in acute infusion studies; conversely, when evaluated in ESRD the disease process may be too far advanced for interventions to make a difference. Moreover, via activation of inflammatory pathways and catabolism through blood exposure to the extracorporeal system [36,37] as well as indiscriminate removal of useful small molecule nutrients, use of prolonged and/or frequent haemodialysis can cause adverse consequences and mask the beneficial effects of heightened urea removal. We need to discard the notion that urea is an innocuous biomarker of kidney function; rather, it is a bona fide uremic toxin with multiple pathophysiologic roles that promotes the accelerated aging phenotype seen in CKD [38]. Indeed, the overall encouraging data from low protein diet trials as described above supports a clinical advantage of restricting urea accumulation to limit end-organ effects. In the following sections, we review urea effects at the organ and cellular level (cardiovascular, gut and adipocyte) and discuss systemic implications. In the last section, we will address indirect urea toxicity whereby protein carbamylation has been associated with atherosclerosis, renal fibrosis and anaemia. It is important to note that although research over the past decade has advanced the field, interpretation of the clinical significance of urea toxicity has been and will remain a complex issue due to concurrence of other uremic toxins and patient comorbidities.

BREAKDOWN OF THE INTESTINAL BARRIER AND DISORDERED GUT MICROBIOME

The gastrointestinal tract has gained recognition as a major source of chronic inflammation in CKD. Elevated urea affects gut permeability and inflammation via [1] breakdown of the tight junction barrier, and [2] modifying the microbial flora. The early studies of urea infusion in the 1800s noted transudation of bloodstream urea into the intestinal lumen as evidenced by congestion of the gut mucus membrane [7]. Two inter-connected mechanisms have been described by which elevated urea leads to degradation of the intestinal epithelial barrier. Firstly, urea diffuses from the blood into the gut lumen and is metabolized by gut bacterial urease to ammonia [$CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2NH_3$]; the latter is hydrolysed into caustic ammonium hydroxide [$NH_3 + H_2O \rightarrow NH_4OH$] which is capable of dissolving proteins [39,40]. Although breath ammonia is elevated in ESRD patients [41,42] due to microbial conversion of urea in the gastrointestinal tract and the oral cavity [43,44], abnormal systemic ammonia levels have not been reported in CKD. *In vitro*, confluent cultured human colonocytes exposed to media containing urea at clinically relevant concentrations 42 or 74 mg/dl show a concentration-dependent fall in trans-epithelial electrical resistance and loss of tight junction proteins [40]. When urease was added to the culture media to simulate the presence of microbial flora, loss of tight junction proteins was amplified and there was detachment of the monolayer [40].

Once urea-induced breakdown of the enterocyte barrier is initiated, this triggers a second mechanism whereby influx of leucocytes and local inflammation [45,46] induces retraction and endocytosis of the transcellular tight junction proteins (claudins and occludin) [47,48]. The net result is a 'leaky gut' with paracellular movement of not only bacterial fragments but also microbe-derived luminal toxins (discussed below) into the bloodstream, thus promoting chronic systemic inflammation.

Gut bacterial DNA fragments have been detected in the blood of both pre-dialysis CKD and chronic haemodialysis patients [30,49]. Work by de Almeida Duarte et al. [50] demonstrated penetration of bacteria across the intestinal wall and their detection in the mesenteric lymph nodes in uremic rats. Levels of circulating endotoxin, which is derived from the cell wall of Gram-negative bacteria, increase with severity of CKD stage and are most elevated in chronic haemodialysis and peritoneal dialysis patients [51,52]. Haemodialysis patients may be particularly susceptible to increased endotoxin translocation from the gut due to systemic circulatory stress and intradialytic regional ischaemia, and exogenous sources of endotoxin are a concern if water purity is compromised. Of note, blood endotoxin levels were not significantly different between haemodialysis and peritoneal dialysis patients in the study by McIntyre and colleagues [52]. In a cohort of 306 haemodialysis patients, blood endotoxin levels correlated with severity of systemic inflammation in the absence of clinically detectable infection [31]. The systemic inflammation in CKD has been linked with progression of renal failure, cardiovascular morbidity and death [53].

Elevated urea also negatively affects the intestinal flora, which then promotes generation of gut-derived uremic toxins. The bacteria that make up the gut microbiome have an important symbiotic relationship with the host, providing energy-rich metabolites and vitamins to enterocytes [54]. Plant-derived polysaccharides or resistant starches transit intact to the colon where they are degraded by *Bacteroides* and fermented to release hydrogen, carbon dioxide, alcohol and short-chain fatty acids (acetate, butyrate, propionate and D-lactate) [54]. The energy-rich short-chain fatty acids are a central nutrient source for enterocytes [54]. In the CKD milieu, influx of urea and other toxins as well as pH alterations due to local production of NH_4OH exerts a selection pressure in the gut lumen, resulting in the expansion of bacterial families that express urease, uricase and indole and *p*-cresol-forming enzymes [55]. Conversely, there are decreased numbers of bacteria that are able to produce the short-chain fatty acid butyrate [55]. In phylogenic microarray analysis of microbial DNA isolated from the stool samples of 24 ESRD patients compared with 12 healthy controls, there were significant differences in the abundance of over 200 bacterial operational taxonomic units belonging to 23 bacterial families [56].

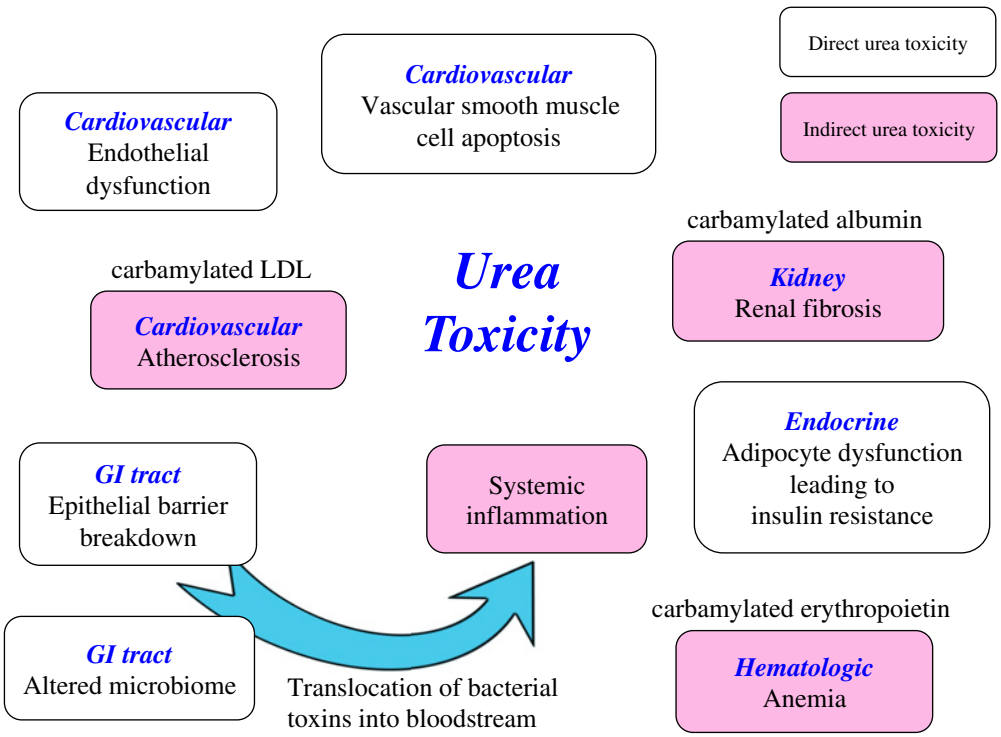

Figure 1 **Direct (white boxes) and indirect (shaded boxes) effects of urea toxicity in various organ systems**
Cardiovascular: Elevated urea induces vascular smooth muscle cell apoptosis which can promote vascular calcification. Urea also induces ROS production by endothelial cells which leads to endothelial dysfunction. Carbamylation is the modification of a protein's function via reaction with isocyanic acid, a breakdown product of urea. Carbamylation of LDL decreases its recognition by the LDL receptor but increases uptake by macrophage scavenger receptor class A, thus driving atherosclerosis. *Kidney:* Carbamylated albumin has been shown to drive interstitial fibrosis. *GI (gastrointestinal) tract:* Diffusion of urea into the gut lumen drives breakdown of epithelial tight junctions by production of caustic ammonium hydroxide, and by inducing local inflammation. Urea and other metabolic toxins, as well as a low fibre diet in CKD, alters the gut microbiome and favours expansion of bacterial families that produce uremic toxins such as indoxyl sulfate. Bacterial fragments and uremic toxins translocate through the leaky gut wall into the bloodstream, driving systemic inflammation. *Hematologic:* Carbamylation of erythropoietin decreases its ability to stimulate red blood cell production, contributing to anaemia in CKD. *Endocrine:* Urea induces ROS production in adipocytes and leads to insulin resistance.

Further, CKD patients are often advised to adhere to diets that are low in fermentable plant fibre (low potassium diet) and poor in symbiont-rich cheese/yogurt (low phosphorus diet). This change in food substrate also affects bacterial composition, jeopardizing microbial nutrient production. Uremic toxins produced by the altered microbiome include indoxyl sulfate and *p*-cresyl sulfate; these diffuse into the bloodstream across the injured 'leaky gut' as described above and drive systemic inflammation and cardiovascular morbidity [53,57,58].

Aronov et al. [59] confirmed the colonic origin of several known uremic toxins (including *p*-cresyl sulfate and indoxyl sulfate) and many as-yet unidentified products in the plasma of patients with ESRD, by comparing data obtained from ESRD patients who had undergone colonic resection with data from ESRD patients with intact colon and normal control individuals. More than 30 mass spectroscopy-detected solutes were present in the plasma from ESRD patients with colons but were either absent or present in significantly lower concentration in those without colons. Nearly all of these compounds were significantly lower in control individuals, suggesting that they represented uremic solutes.

As advances have been made in our understanding of the importance of the gut microbiome in CKD, there has been growing interest in the use of prebiotics (non-digestible food ingredients that can stimulate growth and/or activity of beneficial gut bacteria) and probiotics (living organisms ingested via food or supplements that can improve the health of the host). Our group recently reported that the prebiotic amylose maize resistant starch, which reaches the colon undigested and is metabolized by bacteria to short-chain fatty acids, improved creatinine clearance and reduced kidney fibrosis in CKD rats [60]. A follow-up study revealed marked improvements in serum, urine and cecal fluid metabolomics in conjunction with decreased gut microbial dysbiosis [61]. A meta-analysis of controlled feeding trials found that fibre supplementation significantly decreased serum urea levels in a pooled cohort of 143 participants but there was significant interstudy heterogeneity and urea lowering was not a dose–response effect [62]. All trials were of crossover design and most

were of short duration (median follow-up was 4.5 weeks), and the majority of trials (86%) were of low study quality as assessed by the Heyland Methodological Quality Score (<8). Probiotics have also been tested with the goal to produce a less pathogenic microflora so as to reduce production of uremic toxins. In two 1996 reports involving small cohorts of haemodialysis patients, treatment with Lactobacillus preparations decreased blood levels of uremic toxins [63,64] and improved nutritional status [63]. A multi-national crossover trial in patients with CKD stage 3 and 4 noted significant decrease in blood urea levels and improved quality of life scores after treatment with a proprietary formulation of *S. thermophilus*, *L. acidophilus* and *B. longum* over 6 months [65]. A recent trial examined the combination of pro- and pre-biotic therapy over 6 weeks in pre-dialysis CKD patients (Synbiotics Easing Renal Failure by Improving Gut Microbiology, SYNERGY) and noted decrease in serum *p*-cresyl sulfate and microbiome alterations [66]. Studies of longer duration are needed to assess hard clinical outcomes of pre- and pro-biotics in the CKD population.

VASCULAR WALL TOXICITY

The traditional risk factors of diabetes mellitus, smoking, dyslipidaemia and hypertension do not fully explain the accelerated rate of cardiovascular disease in the CKD population. Non-traditional uremic risk factors for vascular dysfunction and calcification include chronic oxidative stress and inflammation, hyperphosphatemia, parathyroid hormone and vitamin D imbalances, anaemia and deficiency of endogenous calcification inhibitors [4,67–70]. Recent studies suggest direct urea toxicity on vascular smooth muscle cells and endothelial cells. Exposure of human aortic smooth muscle cells to 20 mM (56 mg/dl) urea in culture medium induces expression of BAD [B-cell lymphoma 2 (BCL2)-associated death promoter], a pro-apoptotic member of the BCL2 family [71]. Co-exposure of cells to urea and 7-ketocholesterol, a cholesterol oxidation product known to have potent pro-apoptotic activity, resulted in further increase in pathologic vacuolization and apoptosis [71]. Thus, urea-mediated sensitization of cells to the pro-apoptotic effect of oxidative stress, exerted by oxidized cholesterol for instance, may contribute to the increased apoptosis observed in the arterial wall of CKD patients [72]. Apoptosis in turn is mechanistically linked to vascular medial calcification with its downstream consequences of arterial stiffness, hypertension and heart failure [4].

Endothelial dysfunction is a strong predictor of subsequent cardiovascular events [73], and presence of endothelial dysfunction is evident in early stages of CKD [74]. Patients with renal insufficiency had impaired endothelium-dependent vasodilation, higher levels of diene conjugates and lipid hydroperoxide, and lower total antioxidative activity compared with healthy controls [74]. High urea concentrations have been shown to be damaging to the endothelial cells of the tunica intima. D'Apolito et al. [75] reported that incubation of human aortic endothelial cells with 20 mM urea resulted in increased mitochondrial reactive oxygen species (ROS) production and activation of pro-inflammatory pathways through increased protein kinase C and hexosamine activity, NFkB induction, and accumulation of intracellular advanced glycation end products. The vascular wall effects of urea remain to be confirmed in human trials.

INSULIN RESISTANCE

Cultured 3T3-L1 adipocytes exposed to 20 mM urea for 48 h release ROS, resulting in O-GlcNAc (*O*-linked beta-*N*-acetylglucosamine) modification of several downstream insulin signalling effectors with decreased insulin-stimulated IRS-1 (insulin receptor substrate-1), and a reduction in glucose transport by 76.4% [32]. Mannitol was used as an osmotic control and had no effect on insulin-stimulated glucose transport. A dose-dependent relationship with impaired insulin signalling was observed between urea concentrations of 10–40 mM [32]. Similarly, CKD mice showed increased systemic ROS levels in tandem with elevated insulin resistance-associated adipokines. The investigators went on to show that urea infusion in normal animals was able to induce insulin resistance with >2.5-fold increase in plasma levels of insulin resistance-associated adipokines, without side effects of haemolysis or osmotic diuresis [32]. Insulin resistance in both CKD and normal mice were prevented by antioxidant superoxide dismutase (SOD)/catalase mimetic treatment; however potential confounders such as urea-induced intestinal inflammation and hepatic recycling of the infused urea were not addressed. Overall, these experiments suggest that urea-induced insulin resistance may contribute to the high rates of impaired glucose homoeostasis observed in the CKD population [76,77] but the clinical significance remains to be confirmed via human trials.

INDIRECT TOXICITY: PROTEIN CARBAMYLATION IN CKD

Carbamylation is a spontaneous post-translational protein modification that occurs through exposure to cyanate, which is the deamination byproduct of urea. Carbamylation involves the irreversible modifications of primary amines and reversible modifications of thiols, hydroxyls, phenols and imidazole groups [78,79] via addition of a 'carbamoyl' moiety ($2CONH_2$) to a functional group [80]. Under physiologic conditions, urea slowly dissociates into cyanate and its tautomer isocyanate. Cyanate is non-reactive but is rapidly converted to isocyanic acid which is a reactive electrophile with high affinity for nucleophilic groups such as primary amines (Figure 2) [80]. Isocyanic acid has a physiologic concentration approximately 45 nmol/l in humans that can reach 140 nmol/l in patients with advanced CKD [81]. The carbamylation of lysine by isocyanic acid generates a new residue called homocitrulline which can be used as a marker of carbamylation [82].

Carbamylation of proteins modifies their charge thus affecting their structure and function. It is a part of normal protein molecular aging in mammals, and has been associated with formation of cataracts [83] and decreased activity of the hormones

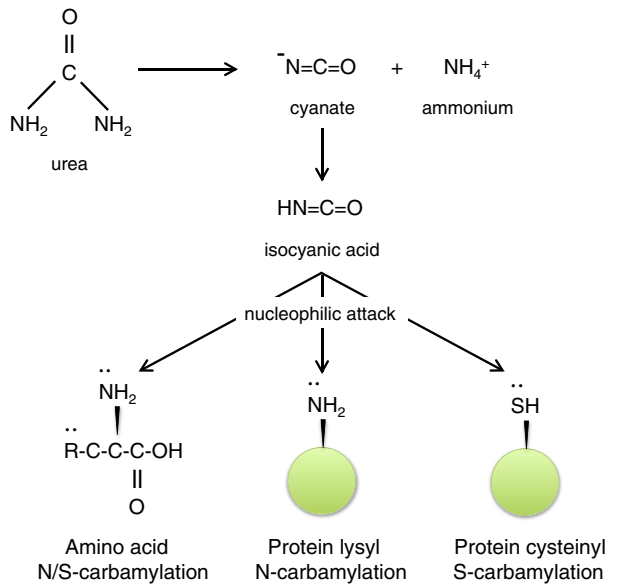

Figure 2 Chemical pathways of carbamylation in uraemia that lead to irreversible modifications of amino acids, and protein lysyl and cysteinyl side chains. Under physiologic conditions, urea slowly dissociates into cyanate which is rapidly converted to isocyanic acid, a reactive electrophile with high affinity for nucleophilic groups.

erythropoietin [84] and insulin [85]. Gorisse et al. [86] compared accumulation of carbamylated collagen and elastin in the skin during the life span of 3 mammalian species. Interestingly, when compared with non-CKD humans and bovines, mice were noted to have the highest rate of accumulation of carbamylated proteins in the skin; mice had the physiologically highest serum urea concentration (8–11 mmol/l compared with 2–7 mmol/l in humans) and the shortest life span among the three species studied [86].

In studies involving 1000 non-CKD individuals from Genebank, a clinical repository of subjects undergoing left heart catheterization, plasma levels of protein-bound homocitrulline (reflecting total plasma protein carbamylation) independently predicted increased risk of coronary artery disease, future myocardial infarction, stroke and death [33]. Carbamylation of plasma proteins has also been linked with increased mortality in the ESRD population. Among a cohort of 347 ESRD patients, plasma protein-bound homocitrulline levels was linked with increased death risk [34]. Similarly, plasma carbamylated albumin was associated with increased mortality risk in 187 patients from the ArMORR (accelerated mortality on renal replacement) study and in 1161 diabetic ESRD patients from the 4D cohort [87,88]. Levels of carbamylated proteins reflect average urea level over an extended period, and thus may be a better indicator of dialysis adequacy than prevailing measurements [80]. Indeed, Berg et al. [87] showed that carbamylated serum albumin (carbamylated on Lys-549) correlated with time-averaged blood urea concentrations and was a stronger predictor of 1-year mortality in incident haemodialysis patients than Kt/V or urea reduction ratio.

Urea diffuses easily due to its small molecular size and is distributed in total body water. Given its ubiquity in all tissue compartments, the potential resulting effects of urea exposure are far-reaching. The prevalence of protein carbamylation throughout a variety of organ systems was recently demonstrated in mice with and without CKD [89]. The investigators used liquid chromatography–tandem mass spectrometry to measure homocitrulline in a variety of tissues including the aorta, kidney, bone, skin, liver and heart. Low-turnover extracellular matrix proteins such as collagen demonstrated highest carbamylation content. Compared with non-CKD control mice, there was a 2-fold increase in carbamylation burden in 75% nephrectomized mice at 20 weeks in all the aforementioned tissues [89].

Carbamylation has been implicated in several facets of CKD pathophysiology.

- Renal fibrosis can be partly driven by carbamylation: kidney mesangial cells cultured with carbamylated fetal bovine serum proteins develop a pro-fibrosis phenotype with increased synthesis of collagen I and IV [90]. In an amphibian model (axolotl or Mexican salamander), intraperitoneal injection of carbamylated albumin induces renal peritubular fibrosis via induction of NFkB, transforming growth factor-beta (TGF-β), epidermal growth factor (EGF) and endothelin-1 by tubular cells [91].
- Atherosclerosis is increased in the presence of carbamylated low-density lipoprotein (LDL) which induces vascular endothelial cell apoptosis; carbamylation of LDL also decreases its recognition by the LDL receptor but increases uptake by macrophage scavenger receptor class A [33,92,93]. Carbamylated LDL also increases the adhesion of monocytes to endothelial cells through enhanced vascular cell adhesion protein-1, CD106 (VCAM-1) and intercellular adhesion molecule-1, CD54 (ICAM-1) expression [94], and induces proliferation of vascular smooth muscle cells [93]. Apostolov and colleagues found that serum carbamylated LDL from ESRD patients was elevated more than 3-fold compared with healthy controls [95], and later showed that oral urea administration in ApoE-deficient CKD mice increased plasma carbamylated LDL by 8-fold and accelerated atherosclerosis [35].
- In terms of anaemia of CKD, as mentioned above carbamylation has been linked with altered erythropoietin function [84]; a prospective cohort study of 158 haemodialysis patients found that levels of carbamylated serum albumin predicted resistance to erythropoiesis-stimulating agents and higher mortality risk [96].

Finally, recent observational studies have noted that increased protein carbamylation in ESRD patients is associated with amino acid deficiencies [87]. ESRD patients are at risk for depletion of amino acids due to protein-energy wasting and increased catabolic state on haemodialysis [37,97]. It has been proposed that since cyanate has a greater affinity for α-amino groups on free amino acids than for lysine side chains on proteins, free amino acids compete for cyanate binding and thus are protective by acting as natural scavengers for carbamylation [80]. A clinical trial is ongoing to investigate amino acid therapy for reduction in carbamylation in haemodialysis patients, as a first step toward

exploring the use of this therapy to reduce uremic complications (ClinicalTrials.gov identifier NCT01612429).

CONCLUSIONS

Research over the past decade has significantly advanced our understanding of urea toxicity at the cellular and systemic levels, as urea concentration increases with CKD progression. Taken together, the accumulating evidence suggests a negative impact of elevated urea on patient outcomes. Consequently, urea *per se* probably participates in the pathogenesis of cardiovascular disease, CKD progression, insulin resistance, intestinal disease and anaemia, and contributes to an overall accelerated aging phenotype. However, direct proof of the impact of elevated urea is currently lacking and will be difficult to ascertain, given its obligatory co-existence with inflammation and retention of other uremic toxins. Urea concentrations start to rise in early stages of CKD, and urea-induced damage is far advanced by the time patients reach ESRD and dialysis initiation, potentially limiting the benefits of urea-lowering interventions in the ESRD population. Clinical trials using low protein diets in pre-dialysis CKD to curb accumulation of urea and other toxic by-products of protein catabolism suggest benefits in terms of slowing progression of kidney failure, if patient compliance and avoidance of malnutrition are adequately addressed.

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