A Bench to Bedside View of Uremic Toxins

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ABSTRACT

Reviewing the current picture of uremic toxicity reveals its complexity. Focusing on cardiovascular damage as a model of uremic effects resulting in substantial morbidity and mortality, most molecules with potential to affect the function of a variety of cell types within the vascular system are difficult to remove by dialysis. Examples are the larger middle molecular weight molecules and protein-bound molecules. Recent clinical studies suggest that enhancing the removal of these compounds is beneficial for survival. Future therapeutic options are discussed, including improved removal of toxins and the search for pharmacologic strategies blocking responsible pathophysiologic pathways.

During the development of the uremic syndrome, losses of kidney function are accompanied by deteriorating organ function attributable to the accumulation of uremic retention solutes. Compounds that exert an adverse biologic impact are called uremic toxins.[1] Recently, a complex picture has emerged of multiple compounds with different characteristics exerting divergent effects on organs.[1,2]

Does it make sense to seek out new uremic compounds or as yet undetected effects of known uremic toxins? In this review, we highlight how recent advances in our knowledge of uremic toxicity have led us to modify existing therapeutic concepts and discuss novel approaches that may be useful for future therapies of uremia.

In particular, we focus on the mechanisms potentially responsible for uremic cardiovascular damage, the major cause of morbidity and mortality in patients with chronic kidney disease (CKD).[3,4] Because the treatment of traditional risk factors for cardiovascular disease in the CKD population only partially reduces the proportion of cardiovascular deaths in comparison with the effects observed in the general population,[5] there is a need to elucidate these additional atherogenic mechanisms. This knowledge might, in turn, be applicable to the wider population, should the compounds that are pathophysiologically active in uremia also be elevated in people without CKD, as has already been observed for homocysteine and advanced glycation end products (AGE).

COMPLEXITY OF UREMIC TOXICITY AND UREMIC TOXIN RESEARCH

In 2003, the European Uremic Toxin Work Group (Eutox; http://EUTox.info) listed the 90 different uremic retention solutes known at that time.[2] Since then, at least 25 additional retention solutes have been identified,[6,7] creating a far more complex picture than was accepted a few years ago.

This highly diverse group of uremic retention solutes includes low molecular weight organic substances as well as peptides. As a result of differing hydrophobicity, low molecular weight organic compounds may either exist in free water-soluble forms or bind reversibly to serum proteins, thereby altering protein functions, such as reducing drug-binding capacity.[8] In CKD, peptides may be found in their native form or, as a consequence of exposure to the uremic milieu, become irreversibly altered through posttranslational modifications, resulting in changes in structure and function. Examples include the heterogeneous group of AGE, advanced oxidation protein products, and carbamoylated proteins, which occur when amino groups are modified by cyanate, which is spontaneously transformed from urea. The molecular weight of most of these peptides belongs to the higher "middle molecular" range (10 to 30 kD). Importantly, both protein-bound solutes and peptides are particularly difficult to re-

move by conventional dialysis treatments.

The identification and characterization of uremic retention solutes playing a main role in uremia-related complications is a prerequisite for the critical evaluation and systematic design of preventive and therapeutic interventions for patients with CKD. *In vitro* assays testing the biologic effects of individual solutes represent a straightforward tool to select rapidly candidates for further in-depth investigation; however, uniform approaches to the preparation of these compounds and the experimental techniques used are necessary to obtain reliable and comparable results. EUTox recently published basic protocols for the *in vitro* screening of uremic retention solutes, providing information about their availability, solubility, and the appropriate preparation of stock solutions.[9] The use of the correct concentrations of solutes is a precondition to obtaining relevant conclusions,[10,11] and it is recommended that the highest reported concentration in uremic plasma be used as a starting point, with evaluation of concentration dependence in cases in which a significant biologic effect is observed. The application of appropriate control conditions is necessary for the correct interpretation of the observed effects.

To evaluate the pathophysiologic impact of CKD, any biologic model system representative of the cellular dysfunction caused by uremia can be used, for example, by leukocytes for diminished immune defense or oxidative stress, endothelial cells for cardiovascular disease, smooth muscle cells for the progression of atherosclerosis, hepatocytes for disturbed metabolism, fibroblasts for fibrosis, and osteoblasts for renal osteodystrophy. Human cells should be used whenever possible, and if animal models are studied, a species for which the relevance to the human condition has already been proved should be chosen. In the following section, some recently described examples of uremic retention solutes are discussed with the potential to affect vascular damage.

AFFECTED CELL SYSTEMS

Leukocytes

For many years, studies have revealed dual effects of uremic retention solutes on leukocyte function: Blunting upon stimulation, which has been linked to infection, and basal activation linked to microinflammation, malnutrition, and atherosclerosis.[1,2,12–15] The major leukocyte subtypes affected by uremic conditions are polymorphonuclear cells, specifically neutrophils and mononuclear cells of the monocyte/macrophage type.[2] It is predominantly the latter cell type that is activated by uremic retention solutes, enhancing vascular damage.

Guanidino compounds are small water-soluble uremic retention solutes that have been implicated in neurotoxicity.[16] Until recently, no potential for cardiovascular damage had been attributed to the guanidines except for asymmetric dimethylarginine, which inhibits inducible nitric oxide synthase (iNOS), an endothelial protective enzyme[17,18]; however, guanidino compounds have now been shown to stimulate leukocytes, with methylguanidine and guanidino acetic acid significantly enhancing the LPS-stimulated production of TNF-α by normal monocytes.[19]

AGE accumulate in the plasma of uremic patients and induce an increase in leukocyte oxidative stress.[14] Until recently, the biologic effect of AGE had been studied mainly with artificially prepared AGE, which might not be representative of AGE compounds really present in uremia, such as fructoselysine, N-ε-carboxymethyllysine, pyrraline, or pentosidine.[2] Glorieux *et al.* studied the proinflammatory effect of several AGE compounds that are retained in uremia, Arg I (arginine modified with glyoxal), carboxyethyllysine, and carboxymethyllysine, demonstrating increased production of free radicals by monocytic cells.[20] It is interesting that one of the studied AGE (Arg II) had no effect at all on leukocytes, showing that the behavior of a number of compounds belonging to a specific group cannot automatically be extrapolated to all solutes of this group.

Because it has been established that p-cresol in humans exists predominantly as the conjugate p-cresylsulfate (pCS), which is a protein-bound substance,[21] the effect of pCS on leukocyte oxidative burst activity has been compared with that of the parent compound, p-cresol.[22] Whereas p-cresol suppresses leukocyte activity, p-cresylsulfate enhances baseline leukocyte activity.[22,23] This highlights the important point that conjugates do not necessarily have the same effects as the parent compound.

Homocysteine, another protein-bound uremic toxin, activates NF-κB in macrophages, which is associated with a significant increase in intracellular superoxide anion levels,[24] an effect abolished by folic acid. Phenylacetic acid, also a protein-bound retention solute, inhibits iNOS expression in a dosage-dependent manner.[25] Inhibition of either endogenous NOS or iNOS may reinforce vascular damage. Furthermore, phenylacetic acid inhibits Ca^{2+}-ATPase activity, increasing intracellular Ca^{2+} concentrations.[26]

Napoleone *et al.*[27] demonstrated that leptin, a protein-bound peptide that accumulates in uremia, induces tissue factor expression by mononuclear cells. Tissue factor is a pivotal agonist in the clotting cascade and contributes to atherosclerosis by playing a key role in thrombosis and inflammation. When either a leptin antibody or leptin receptor antibody was added in these experiments, before leptin exposure, the observed effect was inhibited.

Endothelium

Endothelial dysfunction plays an important role in the development of atherosclerotic vascular disease.[28] Besides the classical causes of endothelial dysfunction, such as hypertension, diabetes, and dyslipidemia, CKD *per se* also plays a role. Patients with CKD have alterations in endothelial properties with increases in both plasminogen activator inhibitor-1 and von Willebrand factor, whereas tissue plasminogen activator decreases, suggesting a procoagulant state at the endothelial surface.[29] Regulation of vascular tone is also impaired with de-

creased endothelium-dependent vasodilation[30] associated with the inhibition of endothelial NOS by uremic solutes such as asymmetric dimethylarginine,[17] AGE,[31] and homocysteine.[32] CKD also induces oxidant stress and inflammation in endothelial cells and production of reactive oxygen species in cultured endothelial cells by the protein-bound uremic toxin indoxyl sulfate.[33] TNF synthesis is also enhanced by AGE.[31]

A new insight into endothelial dysfunction is also provided by the observation of circulating endothelial microparticles. These are intact vesicles derived from cell membranes that arise from two processes, cell membrane activation and apoptosis.[34] Microparticles can originate from endothelial cells and also from other cells, such as platelets, monocytes, granulocytes, and erythrocytes. Microparticles are involved in the regulation of coagulation and apoptosis, and pathologic conditions associated with microparticles have been described. A defect in microparticle generation is responsible for Scott syndrome, a bleeding disorder,[34] whereas increased microparticle formation is observed in cardiovascular disease, diabetes, and both undialyzed and hemodialyzed patients with CKD.[35,36] The generation of endothelial microparticles is elicited *in vitro* by the presence of indoxyl sulfate.[35] Patients who had CKD and were treated with high-efficiency hemodiafiltration during 4 mo showed a decrease in the number of endothelial microparticles when compared with patients who were treated with conventional high-flux hemodialysis.[37]

A remarkable characteristic of the endothelium is its capacity for continuous regeneration and repair.[38] This involves two mechanisms: The classically described proliferation of adjacent endothelial cells and the more recently described homing of circulating endothelial progenitor cells (EPC).[38] These latter cells may be mobilized from bone marrow in response to cytokines or ischemia or derive from circulating leukocytes.[39] In CKD, endothelial repair mechanisms are altered,

representing a possible threat to vascular integrity. Some uremic toxins such as indoxyl sulfate reduce endothelial proliferation,[40] and serum from uremic patients decreases the ability of EPC to migrate.[41] In addition, patients with CKD generally have a decrease in the number of circulating EPC,[42] although contrary observations have been described, possibly as a result of inflammation or ischemia.[41]

Other Effects

Besides leukocytes and endothelial cells, platelets also play a central role in vascular damage by inducing hemostasis and arterial thrombosis.[43] Platelets interact with coagulation factors, in particular thrombin, a potent platelet-activating agonist,[44] and during thrombin-induced aggregation, almost the entire content of platelet granules is released.[45,46]

Platelets from patients with renal failure have increased intracellular concentration of the diadenosine polyphosphates. Diadenosine pentaphosphate (Ap_5A) and diadenosine hexaphosphate (Ap_6A) act as strong growth factors for vascular smooth muscle cells (VSMC) *via* P2Y receptors.[47] Because enhanced VSMC growth is a hallmark of atherosclerosis in renal failure, the increased amount of diadenosine polyphosphates in platelets may play an important role in causing increased cardiovascular damage. Furthermore, diadenosine polyphosphates are strong vasoconstrictors with direct effects on vascular tone mediated by P2X receptors.[48,49] Thus, diadenosine polyphosphates may be one as yet unidentified cause of hypertension in renal failure.

In addition to platelets, renal tissue is a source of diadenosine polyphosphates, and renal tubular cells release Ap_5A and Ap_6A.[50] Because of the close proximity of tubules and peritubular vessels in the kidney, these diadenosine polyphosphates may act in a paracrine manner to promote vascular disease by inducing VSMC proliferation. Diadenosine polyphosphates are predominantly protein bound and characterized by a middle molecular weight, factors that hamper their removal from

the plasma by conventional hemodialysis.[51]

Another evolving area in uremia research is the role of structural variants of angiotensin, with a novel angiotensin peptide, angiotensin-A (Ang-A), recently identified in human plasma.[52] The affinity of Ang-A to the AT_1 receptor is nearly equal to that of Ang II; however, its vasoconstrictive effect is lower. Thus, Ang-A is a less potent and only partial AT_1 agonist. It is interesting that the affinity of Ang-A to the AT_2 receptor is higher than that of Ang II. Whether the impact of Ang-A at the AT_2 receptor also translates into an increase in intrinsic activity will require the development of a suitable model to study AT_2-mediated signaling events.

Plasma Ang-A is increased in renal failure. The Ang-A/Ang II plasma ratio of healthy individuals is <0.2, but in renal failure, this ratio increases to up to 0.7. This may indicate increased activity of decarboxylase in mononuclear cells, decreased enzymatic degradation, or impaired renal removal. Increases in the half-life of other low molecular weight peptides have also been described in renal failure. Currently, conventional enzyme immunoassays do not distinguish between Ang II and Ang-A, because these assays quantify the sum of Ang II and Ang-A.[52]

Summary

As research continues, more and more uremic toxins are uncovered with the potential to have significant impacts on a variety of cell types and functions within the vascular system. The aforementioned uremic toxins can be added to the list published in 2001, summarizing the compounds known at that time to have the potential to affect vascular quality (Table 1).[2] These recent data confirm that most pathophysiologically relevant compounds are molecules that are "difficult to remove by dialysis," such as the larger "middle molecules," protein-bound molecules, and molecules such as guanidines, which show a kinetic behavior that differs markedly from our current marker urea.[53,54]

Table 1. Compounds with the potential to provoke vascular damage[a]

Middle molecules[b]
  AGE
  Ang-A
  dinucleotide polyphosphates
    Ap_5A
    Ap_6A
  leptin
  TNF-α
Protein-bound molecules[b]
  AGE[c]
  dinucleotide polyphosphates
    Ap_5A
    Ap_6A
  homocysteine
  indoxyl sulfate
  leptin
  pCS
  phenylacetic acid
  TNF-α
Small water-soluble compounds
  ADMA[d]
  guanidino acetic acid[d]
  methylguanidine[d]

[a]ADMA, asymmetric dimethylarginine.
[b]Protein-bound molecules and middle molecules can be defined as molecules that are "difficult to remove by standard dialysis strategies." Middle molecules might at the same time be protein bound; in that case, these are mentioned under the heading of protein-bound molecules as well as under that of middle molecules.
[c]AGE exist in their free form or are incorporated into amino acids, peptides, and proteins by irreversible linking; this is a different way of protein binding as compared with the relatively reversible links for most other protein-bound compounds.
[d]Although these guanidino compounds are small water-soluble solutes, they nevertheless have a different kinetic behavior compared with the prototypic water-soluble compound urea.

TRANSLATION INTO THERAPEUTICS

Current Situation

Most *in vitro* knowledge of uremic toxins implicated in vascular damage has pointed to a critical role for solutes that are difficult to remove by dialysis (Table 1). This knowledge has stimulated the development of randomized, controlled trials that have suggested superior cardiovascular outcomes for large-pore dialyzer membranes in secondary[55–58] or primary analyses (Membrane Permeability Outcome [MPO] study; data presented at the 2007 meeting of the European Renal Association–Renal Dialysis and Transplantation Association in Barcelona and at the 2007 American Society of Nephrology in San Francisco).[59,60] Whether further enhancing the convective removal of solutes will improve outcomes, as suggested by the relationship between β_2-microglobulin and survival[61] and additional observational studies,[62,63] will need to be confirmed by controlled trials.

The finding of a potential role for the guanidines in vascular damage is interesting because of their extended volume of distribution[53,54]; this results in poor clearance from the extravascular compartment during hemodialysis with substantial rebound occurring at the end of dialysis.[53] This could be countered by increasing dialysis time and/or frequency.[64,65] Problems of intercompartmental transfer might represent a major limitation to the removal of other molecules as well, such as β_2-microglobulin.[66] As many toxins have been shown to be generated by inflammation,[2,67] it seems prudent that dialysis conditions should be minimally proinflammatory, by avoiding dialysate impurities,[68] central venous catheters,[69] and membrane bioincompatibility.[70]

The Future

Two principal therapeutic options exist to improve further the treatment of uremia: The first is to enhance the removal of uremic toxins and the second is to develop pharmacologic approaches to interfere with their toxic effects. Although the maximal removal capacity of currently available diffusive and especially convective strategies has probably not yet been achieved, the question arises as to how much additional improvement is achievable. With regard to convection, technical refinements are still possible, but these must be friendly to patients and users. Importantly, nonspecific strategies to increase the removal of uremic toxins might also eliminate essential solutes that are beneficial (*e.g.*, trace metals) or medications, and these unwanted effects will need to be assessed and compensated for in the future. With both conventional diffusive and convective therapies, increasing treatment time and/or frequency[64,65,71] or the molecular weight cutoff of membranes[72] might offer another option to improve uremic toxin removal without the need for new technologies.

Partly as a result of the use of liver supportive therapies, several sophisticated techniques have recently been developed to enhance the removal of protein-bound molecules and/or larger compounds through convective strategies, adsorption from whole blood, or combinations of adsorption and convection/diffusion. The manipulation of convection is based on large-pore filtration, which purposely leaks large solutes and even albumin. The albumin loss may range up to 50 g per treatment, which must then be replaced, together with other plasma components, as proposed for selective plasma exchange therapy.[73]

Direct adsorption from blood by hemoperfusion with bead columns[74] has probably not yet reached its full potential. One interesting possibility is the targeted elimination of selected molecules responsible for uremic complications, and the technology required to do this is currently available, as shown for β_2-microglobulin[75]; however, a classification of uremic solutes according to their importance is needed to permit a clinically and economically justified choice of target molecules.

Adsorption when combined with convective therapies, such as large-pore filtration with subsequent adsorption of filtrate and its reinfusion, has shown some utility.[76,77] Similarly, adsorption can be combined with diffusion when used for dialysis against dialysate containing lipophilic elements[78] or albumin.[79] Adsorption of spent peritoneal dialysate, with reinfusion into the peritoneal cavity, may be another diffusive/adsorptive approach that has the advantage of eliminating biocompatibility reactions with blood constituents.

In addition to improving removal, a second option is to neutralize the toxic effects of uremic retention solutes by drug administration. A number of such measures are already in practice, mostly based on empirical experience or from evidence collected in the general popula-

Table 2. Currently applied pharmacologic strategies to prevent uremic complications[a]

Strategy	Effect
Aspirin	Decrease procoagulant and proinflammatory effects
AST-120	Adsorb indoxyl sulfate
Antihypertensives	Decrease hypertension and preserve kidney function
ACEi, ARB, and/or renin inhibitors	Decrease hypertension
	Preserve kidney and heart function
	Inhibition of AGE generation
β blockers	Decrease hypertension
	Neutralize catecholamines
Diuretics	Decrease hypertension
	Combat fluid overload
Statins	Combat dyslipidemia
Phosphate binders	
calcium containing	Decrease P, PTH
non–calcium containing	Decrease Ca × P, P, PTH
ESA	Combat anemia
Folic acid	Decrease homocysteine
Resins (e.g., kayexalate)	Decrease potassium

[a]ACEi, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; Ca × P, calcium-phosphate product; ESA, erythropoiesis-stimulating agents; P, phosphate; PTH, parathyroid hormone.

tion (Table 2). This approach has the advantage of having an impact not only on approximately 0.1% of the global population with stage 5 CKD but also on the approximately 10% of the population with stages 3 and 4 CKD, who are also affected by the major consequences of uremia, such as cardiovascular disease.[3] Another option might be the modification of the intestinal flora to affect the

Table 3. Potential targets for the neutralization of uremic effects[a]

Activity of receptors
Changes in intracellular calcium level
  influx of intracellular calcium
  release of calcium from intracellular stores
  activity of Ca^{2+}-ATPase
Activation/inhibition of MAPK
  ERK1/2
  JNK
  P38
Activation/inhibition of transcription factors
  NF-κB
  AP-1
Activation/inhibition of PKC
Generation of ROS
  inhibition of production
  neutralization
Effects at the transcriptional level (mRNA)
Effects at the posttranscriptional level

[a]AP-1, activator protein-1; extracellular signal–regulated kinase 1/2; JNK, C-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; ROS, reactive oxygen species.

generation of uremic toxins or their precursors.[80] To design more targeted approaches, uremic solutes and their pathophysiologic effects need to be better characterized and classified; several pathways that could be explored are listed in Table 3.

CONCLUSIONS

The current picture of uremic toxicity is complex because of the groups of compounds that are retained and pathways affected. Molecules that are difficult to remove by dialysis, such as the larger middle molecular weight molecules and protein-bound molecules, play significant roles in uremic toxicity, and recent clinical studies suggest that enhancing the removal of these compounds has a beneficial effect on survival. Future therapeutic options include improved or novel removal of toxins and/or the search for pharmacologic inhibitors of the relevant pathophysiologic pathways and the opportunity to improve the quality of life for patients with kidney failure.

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