Uremic Toxin–Targeting as a Therapeutic Strategy for Preventing Cardiorenal Syndrome

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Chronic kidney disease (CKD) is a global health problem. CKD patients are at high risk of developing cardiovascular disease (CVD), including coronary artery disease, heart failure and stroke. Several factors invoke a vicious cycle of CKD and CVD, which is referred to as “cardiorenal syndrome”. Among these factors, the compounds retained through loss of renal excretion play a pathological role in causing atherosclerosis and CVD. These compounds have been broadly classified as uremic toxins because of their accumulation with declining renal function and cytotoxicity. The major uremic toxins contributing to CVD are asymmetric dimethylarginine (ADMA), advanced glycation endproducts (AGE), and trimethyl amine N-oxide (TMAO). ADMA is linked to CVD through regulation of nitric oxide (NO), a major vasodilator. Advanced glycation endproducts (AGE) are a heterogeneous group of molecules formed by a non-enzymatic reaction between reducing sugars and amino acids, lipids, and DNAs. It has been demonstrated that in the diabetic milieu hyperglycemia is mainly associated with AGE formation. Recently, reduced renal excretion and increased production of AGE were shown to promote AGE accumulation in several organs of CKD patients. Therefore, AGE is also a recognized uremic toxin. Not only is it translocated from the blood stream to visceral organs, excess AGE causes crosslinking of matrix molecules disrupting matrix–matrix and matrix–cell interactions. AGEs are also capable of binding to the receptor for AGE (RAGE), which, in turn, produces intracellular reactive oxygen species (ROS) and stimulates several intracellular signaling molecules, leading to the production of inflammatory and profibrotic cytokines.

Indoxyl sulfate and p-cresyl sulfate are uremic toxins derived from the breakdown of amino acids by the microbiota, which accumulate and correlate with enhanced cardiovascular mortality in CKD patients. In addition to...
ADMA, AGE, and TMAO in Cardiorenal Disease

Figure 1. Schematic of mutual relationships between uremic toxins and cardiorenal syndrome. AGE, advanced glycation endproduct; ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; ROS, reactive oxygen species; TMAO, trimethyl amine N-oxide.

ADMA

Biology of ADMA
ADMA is an analog of L-arginine, a substrate for NO. ADMA reduces NO synthesis by antagonizing L-arginine and competes for the uptake of L-arginine into cells through transporters, leading to subsequent damage to endothelial cells (ECs). ADMA is shown to directly reduce phosphorylation of endothelial NO synthase (eNOS); administration of exogenous ADMA exacerbated vascular damage in eNOS-deficient mice. ADMA also regulates ROS production and activates the RAS, both of which are major regulators of cardiovascular and renal function. Therefore, ADMA is an attractive therapeutic target for the prevention of cardiorenal syndrome.

Various types of cells can synthesize methylarginines, including ADMA, which are catalyzed by protein–arginine methyltransferase (PRMT). Next, free monomethyl- and dimethyl-arginine molecules are released from cells after proteolysis of methylated proteins and can be detected in tissues or blood. More than 80% of ADMA undergoes hydrolysis, converting to L-citrulline and amine by dimethylarginine dimethylaminohydrolase (DDAH) (Figure 2).

Based on the metabolic pathway of ADMA, possible mechanisms of ADMA accumulation in CKD patients are: (1) increased methylation stress on arginine, (2) increased release of methylated arginine because of protein degradation (increased protein catabolism), (3) decreased degradation by DDAH, or (4) reduced renal excretion of ADMA. Of these, it is speculated that DDAH may strongly correlate with accumulation of ADMA because a large proportion of ADMA undergoes DDAH-mediated degradation. DDAH is known to be a redox-sensitive enzyme. In long-term cultured vascular ECs, DDAH activity and expression decreased in a time-dependent manner and, by contrast, ADMA concentration was upregulated over time, both of which were rescued by treatment with antioxidants, suggesting that ROS seem to be the main contributor to regulating the ADMA–DDAH axis.
However, their efficacy of lowering ADMA in humans is not well established. In rodent models, overexpression of DDAH prevented PC loss and ischemia-associated renal fibrosis in 5/6 subtotal nephrectomy (Nx). These data indicate that ADMA is not only a biomarker, but plays a fundamental and pathological role in the progression of CKD. Metformin and pioglitazone reduce the circulating ADMA levels and increase NO levels through upregulation of DDAH in rodent models. However, their efficacy of lowering ADMA in humans is still controversial, so further studies are required.

It is also known that the ADMA concentration is a biomarker for left ventricular hypertrophy, cardiovascular events, coronary artery calcification, and death in patients with CKD. However, to date, few studies have identified the direct mechanism of the ADMA-modulated interaction between CKD and CVD. One possible mechanism is the intracellular, but not circulating, ADMA–DDAH system. Dowsett et al demonstrated that endothelium-specific deletion of DDAH profoundly impaired the angiogenic response without altering the circulating ADMA concentration. Overexpression of DDAH in cardiomyocytes preserves cardiac function after acute myocardial infarction through inhibition of ROS and caspase activities. More recently, Yokoro et al proposed ADMA-modulated renal anemia as another possible mechanism of ADMA mediating the substantial crosstalk between CKD and CVD. They found that erythropoietin receptor and erythrophorere, an iron metabolism regulator in response to erythropoietin, leading to reduced erythrocyte differentiation and hemoglobin synthesis. This deleterious effect of ADMA on renal anemia very likely facilitates the cardiorenal syndrome.

**Pathology of ADMA in CVD With CKD**

In patients with CKD, plasma ADMA levels are significantly increased compared with patients in the early phase of kidney dysfunction. ADMA seems to be involved in increased albuminuria associated with CKD. An in vitro study using isolated glomeruli showed that ADMA enhanced the permeability of albumin through the basement membrane. Adenovirus-mediated overexpression of DDAH in mice showed a reduction in ADMA concentration and urine albumin excretion in both 5/6 subtotal nephrectomy (Nx), a remnant kidney model with advanced CKD, and diabetic models. Those studies suggested increased ADMA levels can lead to albuminuria through direct regulation of glomerular filtration.

Oxygen is usually supplied to the tubulointerstitium through peritubular capillaries (PC) that branch from the efferent renal arteriole. Thus, a loss in PCs combined with interstitial fibrosis will decrease the oxygen diffusion rate and evoke interstitial hypoxia during advanced stages of CKD. Matsumoto et al found a positive correlation between ADMA and the degree of PC loss, but overexpression of DDAH prevented PC loss and ischemia-associated renal fibrosis in 5/6 subtotal Nx. A recent study also revealed that ROS-dependent degradation of DDAH occurred after ischemic reperfusion injury, which accelerated the loss of PCs in parallel with ADMA accumulation in the plasma and kidney tissue. These data indicate that ADMA is not only a biomarker, but plays a fundamental and pathological role in the progression of CKD. Metformin and pioglitazone reduce the circulating ADMA levels and increase NO levels through upregulation of DDAH in rodent models. However, their efficacy of lowering ADMA in humans is still controversial, so further studies are required.

**AGE–RAGE Axis in CKD**

**Biology of AGE and RAGE**

Reducing sugars, such as glucose, react non-enzymatically with an α-amino group or lysine residue at the N-terminal of amino acids to form a Schiff base. After Amadori compound, a stable deoxyketose addition compound, is formed, various reactions occur to generate dicarbonyl compounds as intermetabolites and then finally AGE, such as dehydration, condensation, oxidation, reduction, and formation of intermolecular crosslinking. Proteins in the body, especially those with a slow turnover rate, such as collagens, are highly exposed to reducing sugars for a long period. Therefore, the lysine and arginine residues of these proteins are likely AGE modified, which induces protein polymerization, reduces solubility, and lowers susceptibility to proteases; thus, AGES are rarely degraded. Crosslinking of AGES in visceral organs leads to irreversible organ damage.

Many AGES, such as pyrralin and crossline, are generated by a glycation-dependent pathway; hence, hyperglycemia is closely associated with the generation of AGES. However, in recent years, oxidative stress has emerged as an alternative route for AGE production. N(ε)-carboxymethyl lysine (CML), a typical AGE, is synthesized via oxidative cleavage of Amadori compounds or through auto-oxidation of glucose. CML accumulation has been observed in the glomeruli of not only diabetic patients but also in individuals with hypertension or lupus nephritis. Taguchi et al demonstrated that aldosterone, a major regulatory hormone of sodium reabsorption, directly promotes the production of ONOO−, a type of oxidative stress, which accelerates intracellular CML generation in cultured murine podocytes.

**Pathology of AGE and RAGE in CVD With CKD**

Presently, the possible mechanisms explaining AGE accumulation in CKD are as follows: (1) reduction in AGE elimination as renal clearance decreases, (2) increased oxidative stress (induced by other uremic toxins) upregulates
in intracellular AGE production, or (3) uremic toxin-induced deterioration of insulin resistance in skeletal muscle accelerates glycation.\textsuperscript{32} The cytotoxicity of AGEs can be exerted by direct AGE modification of intracellular proteins, nucleic acids, and lipids. Further, AGE induces cell injury by interacting with its receptor (RAGE). RAGE is a single-transmembrane receptor that belongs to the immunoglobulin superfamily. It is expressed in vascular ECs, glomerular epithelial cells, tubular epithelial cells, and mesangial cells in the kidney and in cardiomyocytes, vascular cells, and fibroblasts in the heart.\textsuperscript{33} The engagement of AGE with RAGE enhances the generation of intracellular oxidative stress, activates intracellular signaling, such as nuclear factor-\(\kappa\)B (NF-\(\kappa\)B), Mitogen-activated protein kinase, and protein kinase C via activation of Diaphanous-1, Ras-related C3 botulinum toxin substrate-1, and the cell division cycle.\textsuperscript{34} The binding of ligands to RAGE also enhances NF-\(\kappa\)B and subsequent transcriptional activity, which, in turn, increase RAGE expression, inducing further activation of NF-\(\kappa\)B\textsuperscript{35} and thus creating a positive feedback loop (Figure 3).

AGE deposition and upregulation of RAGE are already observed in conditions that lead to CKD,\textsuperscript{36,37} such as hypertension and diabetes, and the upregulation of AGE with CKD further activates this pathway. The activation of the AGE–RAGE axis that already exists in the early stages of CKD is not only linked to the onset of CVD, but also inhibits the recovery process, thus throwing a one–two punch. AGEs may contribute to developing low-turnover bone disease in CKD patients via downregulation of osteoblasts and reduced secretion of parathyroid hormone.\textsuperscript{38} AGEs are also involved in vascular calcification: an independent predictor of cardiovascular death, which is a serious concern.\textsuperscript{39} Further, the AGE–RAGE axis is aberrantly activated in atherosclerotic plaques in human and murine models of atherosclerosis.\textsuperscript{32} Soro-Paavonen et al showed that genetic deletion of RAGE reduced hyperglycemia-induced atheromatous plaque with reduction of oxidative stress and several NADPH subunits.\textsuperscript{41} In addition, the cardiac AGE–RAGE axis is linked to HF and CAD.\textsuperscript{42} Hypoxia/reoxygenation injury upregulates RAGE in cardiomyocytes, together with an increase in AGES.\textsuperscript{43} Genetic deletion of RAGE led to a reduction in the infarction area, fewer apoptotic cells, and preserved cardiac contractility in rodent models of myocardial infarction.\textsuperscript{44}

Clinical research has shown that CML deposition in the vasculature is closely associated with systolic blood pressure\textsuperscript{45} and stiffness in the arteries.\textsuperscript{46} Clinical research has shown that CML deposition in the vasculature is closely associated with systolic blood pressure and stiffness in the arteries.\textsuperscript{46}

Taken together, these findings suggest inhibition of this axis is an attractive target for therapeutic intervention to inhibit progression to the cardiorenal syndrome.

In order to demonstrate the therapeutic potential of inhibition of the AGE–RAGE axis, we created a DNA–aptamer directed against glyceraldehyde–AGE (AGE–DNA–aptamer) using the systematic evolution of ligands by Exponential enrichment (SELEX) method. We have previously demonstrated that an AGE–DNA–aptamer attenuated streptozotocin-induced diabetic nephropathy with a reduction in renal AGE deposition.\textsuperscript{46} More recently, we generated a DNA–aptamer directed against RAGE and confirmed that the RAGE–DNA–aptamer also prevented progression of kidney injury in both diabetic\textsuperscript{47} and hypertension-prone mice.\textsuperscript{48} The RAGE–aptamer is highly distributed to multiple organs, including the heart, kidney, and aorta.\textsuperscript{47} These findings suggest that AGE– and RAGE–aptamers prevent many of the pathological effects of the cardiorenal syndrome. Considering that the DNA–aptamer has been shown to be safe in humans,\textsuperscript{48} and rarely induces adverse effects, these compounds have a high clinical potential for preventing cardiorenal syndrome.

TMAO

Biology of TMAO in CKD

More than 100 metabolites have been reported as uremic toxins. Of these, indoxyl sulfate, p-cresyl sulfate, and TMAO, produced in the intestine by gut flora, correlate very highly with the incidence of cardiovascular comorbidities of CKD, suggesting the presence of a gut–renal axis for cardiorenal syndrome.\textsuperscript{49}

TMA is generated in the gut from betaine, L-carnitine and its metabolite \(\gamma\)-butyrobetaine, choline and other choline-containing compounds that are present in the diet. The major dietary contributions come from red meat, eggs, dairy products and salt-water fish, which are rich in choline, lecithin, and carnitine.\textsuperscript{50} These precursors are converted into TMAO by various enzymes within the gastrointestinal tract. Absorbed TMA is delivered to the liver where Flavin-dependent monooxygenase (FMO) isofoms 1 and 3 convert it to TMAO,\textsuperscript{51} which is then distributed not only to the liver, but the brain, muscle, kidney, and intestine.\textsuperscript{52} Approximately 50% of TMAO excreted via urine is attributed to secretion via tubular epithelial cells; the other 50% after glomerular filtration.

Pathology of TMAO in CVD With CKD

After reports of the pathological effects of TMAO on arteriosclerosis,\textsuperscript{53} gut microbiota-related uremic toxins have
gained attention in recent years. Increase in the TMAO level is associated with poor prognosis in chronic HF patients, even after normalizing for traditional cardiovascular risk factors. To date, it has been demonstrated that a high concentration of serum TMAO is independently linked to high prevalence of CVD, CKD progression, metabolic syndrome, and type2 diabetes.

In CKD patients, TMAO is upregulated because of maladaptive gut microbiota with a concomitant inability to excrete it. Xu et al also demonstrated that serum TMAO was elevated in mice with fecal microbiota transplants from CKD patients compared with those from healthy individuals. Further, CKD patients display abnormal upper gastrointestinal tract digestive function, thus allowing undigested dietary proteins to reach the large intestine and increasing the concentration of the precursors to uremic compounds, leading to the increase in uremic toxins, including TMAO.

Possible mechanisms to explain how TMAO invokes cardiovascular events are: (1) promoting an imbalance in cholesterol uptake, (2) accelerating proatherogenic foam cell migration to the arterial wall, (3) directly interrupting platelet activation and enhancing platelet responsiveness and thrombosis, and (4) probable activation of the RAS. Further, intake of high levels of TMAO induced upregulation of kidney injury molecule-1 (KIM-1), a tubular damage marker that positively correlates with serum TMAO levels, and promoted renal fibrosis in rodent model, suggesting that TMAO itself contributes to progression of CKD. It is also known that the serum concentration of TMAO increases with poor excretion of TMAO, and this effect could be reversed by renal transplantation, suggesting that, if CKD was present, the TMAO level would be mainly regulated by residual kidney function. It can be assumed that loss of renal excretion of TMAO evokes further increase in TMAO with progression of CKD, having a huge effect on CVD and atherosclerosis. Thus, TMAO is at the center of the vicious cycle between CKD and CVD.

Mutual Relationship Among Uremic Toxins

AGE and ADMA

Bucala et al first demonstrated in 1991 the capability of AGES to quench NO in ECs. Since then, several studies have been conducted to prove the relationship between AGES and NO. eNOS activity was significantly attenuated in cultured vascular ECs when treated with serum from CKD patients, suggesting a role of uremic toxins. Ando et al demonstrated a positive correlation between plasma levels of ADMA and serum AGES, with an inverse correlation with endothelial function as assessed by flow-mediated vasodilatation (FMD) in the branchial arteries. They additionally found that AGES increased ROS generation in ECs in a dose-dependent manner through interaction with the RAGE. AGES decreased gene expression levels of DDAH-II, an isoform of DDAH present in vascular cells, which resulted in an increase in ADMA, and both were completely blocked by an antioxidant, N-acetylcysteine. AGES were shown to regulate PRMT and DDAH expression, leading to increased ADMA concentration, by modulating ROS in cultured proximal tubular cells and mesangial cells. In addition, the serum ADMA level in patients with septic shock is elevated when compared with healthy individuals, which also correlates with serum AGE levels (P=0.002) after multivariate stepwise regression analyses. On the basis of these findings, AGES appear to be closely related with ADMA generation in various cell types through modulation of the ADMA metabolic pathway, and the AGE–ADMA combination will likely have a greater effect on NO production than either compound alone.

TMAO and ADMA

A high burden of TMAO induces H\textsubscript{2}O\textsubscript{2} production and decreased eNOS expression in the thoracic aorta of mice. Coincubation with TMAO accelerates production of ROS, reduces NO generation, and promotes the senescence phenotype in human umbilical vein ECs and cultured endothelial progenitor cells. Considering that ROS mediates intracellular ADMA production through alteration of PMRT or DDAH, TMAO-elicited ROS presumably regulates intracellular ADMA, causing endothelial dysfunction and subsequent CVD. The TMAO level positively correlates with serum ADMA levels in clinical research as well. Taken together, TMAO is likely involved in ADMA production, but, further studies are necessary to elucidate the crosstalk between TMAO and ADMA.

AGE–RAGE Axis and TMAO

As mentioned earlier, red meat, eggs, and fish are the main dietary sources of betaine and L-carnitine, precursors of TMAO. Interestingly, when cooked these dietary products contain huge amounts of AGE-bound proteins, which also contribute to increased AGE concentrations. Excess intake of these products poses a high risk for elevated levels of both TMAO and AGES. Consequently, a low-protein diet is expected to decrease the production of uremic toxins and AGEs, and may be considered as a therapy for cardiorenal syndrome. Because a clinical study showed that protein restriction therapy within 0.55 g/kg/day induced malnutrition in only 3 (0.7%) of 423 patients with advanced CKD, restriction therapy within 0.55 g/kg/day induced malnutrition in only 3 (0.7%) of 423 patients with advanced CKD, 0.6–0.8 g/kg/day induced malnutrition in only 3 (0.7%) of 423 patients with advanced CKD, and may be considered as a therapy for cardiorenal syndrome. Because a clinical study showed that protein restriction therapy within 0.55 g/kg/day induced malnutrition in 0.6–0.8 g/kg/day induced malnutrition in only 3 (0.7%) of 423 patients with advanced CKD, 0.6–0.8 g/kg/day induced malnutrition in only 3 (0.7%) of 423 patients with advanced CKD. Thus, AST-120 is also a potent therapeutic agent for cardiorenal syndrome.

The accumulation of uremic toxins is one of the main characteristics of advanced CKD and is responsible for
CVD, the leading cause of death in patients with CKD. Recent studies have demonstrated that uremic toxins begin accumulating in the body from the early stage of CKD. Some of these uremic toxins, such as AGEs, are not degraded, causing long-term tissue and organ damage. As described here, direct cardio toxicity by uremic toxins has been on the rise in recent years. Thus, it is imperative to find strategies to prevent uremic toxin accumulation in the early stages of CKD. Novel and more integrated approaches need to be developed to mount a combined attack on not only the known CV risk factors, such as hypertension, diabetes, and metabolic syndrome, but also to include uremic toxins as important targets for treatment of cardio renal syndrome.

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