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Inhibition of eNOS phosphorylation mediates endothelial dysfunction in renal failure: new effect of asymmetric dimethylarginine

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Patients with chronic kidney disease have elevated circulating asymmetric dimethylarginine (ADMA). Recent studies have suggested that ADMA impairs endothelial nitric oxide synthase (eNOS) by effects other than competition with the substrate L-arginine. Here, we sought to identify the molecular mechanism by which increased ADMA causes endothelial dysfunction in a chronic kidney disease model. In wild-type mice with remnant kidney disease, blood urea nitrogen, serum creatinine, and ADMA were increased by 2.5-, 2-, and 1.2-fold, respectively, without any change in blood pressure. Nephrectomy reduced endothelium-dependent relaxation and eNOS phosphorylation at Ser1177 in isolated aortic rings. In transgenic mice overexpressing dimethylarginine dimethylaminohydrolase-1, the enzyme that metabolizes ADMA, circulating ADMA was not increased by nephrectomy and was decreased to half that of wild-type mice. These mice did not exhibit the nephrectomy-induced inhibition of both endothelium-dependent relaxation and eNOS phosphorylation. In cultured human endothelial cells, agonist-induced eNOS phosphorylation and nitric oxide production were decreased by ADMA at concentrations less than that of L-arginine in the media. Thus, elevated circulating ADMA may be a cause, not an epiphenomenon, of endothelial dysfunction in chronic kidney disease. This effect may be attributable to inhibition of eNOS phosphorylation.

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The prevalence of chronic kidney disease (CKD) is increasing worldwide.[1,2] Approximately 50% of the end-stage renal disease patients die from cardiovascular causes[3–5] and their cardiovascular mortality is 500-fold greater compared with that of age-matched controls with normal renal function.[5] The Framingham Heart Study revealed that mild renal failure was associated with increased prevalence of death and cardiovascular events even in the general population.[6] Endothelial dysfunction was documented from the early stage of renal failure,[7] suggesting that endothelial dysfunction is one of the initial mechanisms that lead to cardiovascular complications in CKD patients.

Asymmetric dimethylarginine (ADMA) is generated during the process of protein turnover and is actively degraded by the intracellular enzyme, dimethylarginine dimethylaminohydrolase (DDAH).[8–13] ADMA is an endogenous inhibitor of all types of nitric oxide synthases (NOSs).[14,15] It has long been thought that the NOS inhibition by ADMA is attributable to its competitive inhibition as an L-arginine analog.[10,16] However, there is increasing evidence that ADMA may have additional effects that are independent of the competitive inhibition of NOS although the precise mechanisms are unknown.[17–19]

We have shown that circulating ADMA levels are correlated with the thickness of the carotid artery in a general healthy population.[20] Thereafter, ADMA has been increasingly recognized as a putative biomarker in cardiovascular diseases.[21] It was reported that circulating ADMA levels were elevated in CKD patients.[16,22,23] Reduced bioavailability of nitric oxide (NO) has been documented in CKD patients, concurrently with endothelial dysfunction.[24–26] However, it remains undetermined whether the elevation of circulating ADMA level is a cause or an epiphenomenon of endothelial damages in patients or animal models of CKD.

The aims of this study were to examine whether increased circulating ADMA causes endothelial dysfunction in a mouse model of CKD and, if so, to investigate the molecular mechanism. To address the contribution of circulating ADMA to endothelial dysfunction in CKD, we created 5/6

nephrectomized (Nx) models in DDAH-1 overexpressed mice having reduced circulating ADMA levels. The effects of ADMA on endothelial NOS (eNOS) activity were investigated in cultured human umbilical vein endothelial cells (HUVECs).

RESULTS

5/6 Nx causes renal failure without hypertension

Nx increased blood urea nitrogen (BUN) and serum creatinine levels in wild-type (WT) mice. The elevations of BUN and serum creatinine in human DDAH-1–transgenic (TG) mice receiving Nx (TG + CKD) were similar to those in WT mice receiving Nx (WT + CKD) 4 weeks after the operation (Figure 1a and b). There were no differences in systolic blood pressure and heart rate among the four groups (Figure 1c and d). We did not observe apparent morphological and histological changes in the heart and aorta in WT + CKD, TG + sham, and TG + CKD mice (data not shown).

Serum ADMA levels

In WT + CKD mice, serum ADMA levels were increased by 20% compared with those in WT + sham mice (Figure 1e). TG + sham mice showed a 50% reduction in serum ADMA levels compared with WT + sham mice. In TG mice, Nx did not increase the ADMA levels.

Endothelium-dependent relaxation of aortic rings

In WT + sham mice, acetylcholine (Ach) induced a dose-dependent relaxation of the aortic rings precontracted with phenylephrine (Figure 2 and Supplementary Table S1 online). The endothelium-dependent relaxation was significantly impaired in isolated aortic rings obtained from WT + CKD mice. In TG + sham mice, the endothelium-dependent relaxation was similar to that in WT + sham mice. The Nx-induced endothelial dysfunction was prevented in TG mice. These results were confirmed by the area under the curve analysis (Table 1). The half-maximal inhibitory concentration (IC_{50}) of Ach was significantly greater in WT + CKD mice compared with WT + sham mice, suggesting that ADMA reduced the sensitivity for Ach after Nx in WT mice. However, the IC_{50} levels in TG + sham and TG + CKD mice did not differ from that in WT + sham mice (Table 1).

Vascular eNOS expression and phosphorylation in CKD mice

The Ser1177 residue of eNOS is a key phosphorylation site that positively regulates eNOS enzyme activity independently of intracellular calcium concentrations.[27] We investigated the eNOS expression and phosphorylation levels in the aorta of WT and TG mice with or without Nx (Figure 3a and b). Nx did not affect eNOS protein expression levels in WT mice. However, eNOS phosphorylation at Ser1177 was attenuated by Nx, suggesting the inhibition of eNOS activity in WT + CKD mice. In TG + sham mice, the eNOS expression and phosphorylation levels were similar to those in WT + sham mice. The reduction in eNOS phosphorylation observed in WT + CKD mice was abolished in TG + CKD mice. In addition, we examined urinary nitrate/nitrite (NOx) excretion in 24 h as an indicator of NO production *in vivo* (Figure 3c). Urinary NOx excretion was reduced by 66% in WT + CKD mice compared with WT + sham mice. In TG + sham mice, urinary NOx excretion was much higher than that in WT + sham mice. The NOx excretion was significantly reduced in TG + CKD mice compared with TG + sham mice. But, the NOx levels in TG + CKD mice were similar to the levels in WT + sham mice and significantly higher than those in WT + CKD mice.

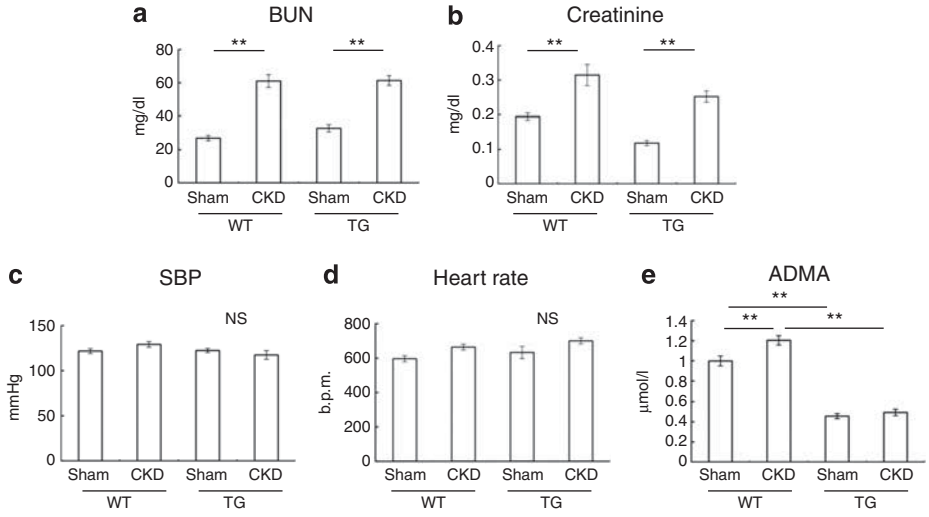

Figure 1 | Nephrectomy (Nx) causes moderate renal failure and increases circulating asymmetric dimethylarginine (ADMA). The effects of Nx on blood urea nitrogen (BUN) (**a**), serum creatinine (**b**), systolic blood pressure (SBP) (**c**), heart rate (**d**), and serum ADMA (**e**) at 4 weeks after Nx or sham operation in wild-type (WT) mice and dimethylarginine dimethylaminohydrolase-1 transgenic (TG) mice. WT mice receiving sham operation (WT + sham), $n = 9$; WT mice receiving Nx (WT + CKD), $n = 9$; TG mice receiving sham operation (TG + sham), $n = 6$; TG mice receiving Nx (TG + CKD), $n = 6$. Values are mean ± s.e.m. $^{**}P < 0.01$. b.p.m., beats per minute; CKD, chronic kidney disease; NS, not significant.