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Accumulation of advanced oxidation protein products induces podocyte apoptosis and deletion through NADPH-dependent mechanisms

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The accumulation of plasma advanced oxidation protein products (AOPPs) is prevalent in diverse disorders such as diabetes, metabolic syndromes, and chronic kidney disease. To study whether accumulated AOPPs have an important role in the progression of proteinuria and glomerulosclerosis, we chronically treated normal Sprague–Dawley rats with AOPP-modified rat serum albumin. Podocyte apoptosis was significantly increased coincident with the onset of albuminuria and preceded significant losses of glomerular podocytes. Increasing the amount of AOPPs in the media of conditionally immortalized podocytes rapidly triggered the production of intracellular superoxide by activation of NADPH oxidase and this, in turn, led to an upregulation of p53, Bax, caspase 3 activity, and apoptosis. Chronic inhibition of NADPH oxidase by apocynin prevented podocyte apoptosis, ameliorated podocyte depletion, and decreased albuminuria in AOPP-challenged rats. Our study demonstrates that accumulation of AOPPs promotes NADPH oxidase–dependent podocyte depletion by a p53-Bax apoptotic pathway both *in vivo* and *in vitro*.

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Progression to renal parenchymal damage and end-stage renal disease, which seems to be largely independent of the initial insults, is the common pathway for chronic kidney disease (CKD) in both animals and humans.[1,2] The key event of the pathway is the impairment of glomerular permeability to proteins, which permits excessive amounts of proteins to reach the lumen of the proximal tubule and contributes to renal interstitial injury by activating inflammatory and fibrogenic cascades.[3,4]

Glomerular visceral epithelial cell, or podocyte, is one of the major cell types in the glomerulus. The podocyte forms a critical part of the glomerular filtration barrier and functions to prevent urinary protein leakage and to maintain glomerular capillary loop integrity.[5] Podocyte depletion leads to areas of denuded glomerular basement membrane, culminating in proteinuria and development of glomerulosclerosis.[6] There is a growing body of literature showing that podocyte loss is related to increasing proteinuria and contributes to the progression of kidney disease in both diabetic and nondiabetic CKD.[7–13]

Numerous factors have been implicated in the pathogenesis of podocyte injury and depletion: hyperglycemia, angiotensin II, reactive oxygen species, and transforming growth factor-β have been extensively characterized.[14–18] Recently, a family of oxidized protein compounds, termed 'advanced oxidation protein products' (AOPPs), has emerged as a novel class of renal pathogenic mediators. AOPPs are a class of dityrosine-containing protein products formed during oxidative stress and carried mainly by albumin *in vivo*.[19,20] Accumulation of plasma AOPPs was first identified in patients who underwent dialysis [19] and was subsequently found in subjects with diabetes,[21,22] metabolic syndrome,[23] and nondiabetic CKD.[20] Our recent studies have shown that chronic accumulation of plasma AOPPs significantly increases urinary protein excretion and accelerates glomerulosclerosis in a remnant kidney model.[24] An increase in the concentration of plasma AOPPs to the level that has been found in diabetic patients increases urinary excretion of albumin in both normal and streptozotocin-induced diabetic rats.[24,25] Consistent with these observations, data from a clinical study have shown that plasma AOPP level

is a strong predictor for the prognosis of IgA nephropathy.[26] Although these observations suggest that AOPP accumulation has an important role in the progression of proteinuria and glomerulosclerosis, the underlying cellular and molecular mechanism(s) have not been clarified. It remains unknown whether AOPPs affect structure and function of the podocyte.

Two underlying mechanisms for podocyte loss are apoptosis and detachment. Apoptosis in glomerular cells has been demonstrated in animal models as well as in patients with chronic renal insufficiency, diabetes, and hypertension nephrosclerosis,[27–30] in which the accumulation of AOPPs is implicated. Therefore, this study was designed to determine the contribution of AOPPs to podocyte loss. Our data show that the accumulation of AOPPs results in podocyte loss through the induction of apoptosis. AOPP-induced podocyte apoptosis is mainly mediated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent superoxide generation, which activates the p53–Bcl-2-associated X (Bax)–caspase-3 proapoptotic pathway.

RESULTS

Chronic administration of AOPPs increased urinary albumin and 8-hydroxydeoxyguanosine excretion

To examine the renal pathogenic effects of AOPPs *in vivo*, normal Sprague–Dawley rats were randomly assigned to four groups and were treated with intravenous injection of AOPP-modified rat serum albumin (RSA) in the presence or absence of the NADPH oxidase inhibitor apocynin for 5–12 weeks. Plasma AOPP levels increased approximately onefold in AOPP-RSA-treated rats compared with that in vehicle- or RSA-treated controls (Table 1). Chronic administration of AOPP-RSA, but not that of native RSA, significantly enhanced the renal deposition of AOPPs, increased urinary albumin excretion, and elevated the level of urinary 8-OHdG (8-hydroxydeoxyguanosine), a well-known biomarker of oxidative stress *in vivo*.[1] Intervention of apocynin significantly attenuated albuminuria and decreased urinary 8-OHdG level in AOPP-challenged rats. There was no significant difference in serum creatinine levels among the groups (Table 1).

Table 1 | Metabolic and biochemical parameters of rats at week 5 and week 12

Parameters	Week 5[a] (n = 10)	Week 12[a] (n = 10)
Body weight (g)		
Vehicle	329.24 ± 24.29	396.07 ± 17.50
RSA	325.33 ± 26.06	396.21 ± 13.58
AOPPs	306.60 ± 26.64	388.11 ± 33.42
AOPPs+apocynin	315.89 ± 24.44	390.27 ± 28.99
Systolic blood pressure (mm Hg)		
Vehicle	118.28 ± 3.47	123.56 ± 3.18
RSA	120.33 ± 4.16	126.45 ± 3.59
AOPPs	121.25 ± 3.79	125.97 ± 4.03
AOPPs+apocynin	120.40 ± 3.75	122.49 ± 4.15
Urinary albumin excretion (μg per 24 h)[b]		
Vehicle	152.95 ± 84.11	163.63 ± 116.63
RSA	205.28 ± 139.35	243.32 ± 130.24
AOPPs	759.69 ± 125.38[c]	1548.90 ± 324.16[c]
AOPPs+apocynin	203.52 ± 49.93[d]	273.86 ± 90.26[d]
Urinary 8-OHdG excretion (ng per 24 h)[e]		
Vehicle	475.10 ± 178.97	454.86 ± 185.47
RSA	445.04 ± 223.72	488.15 ± 313.82
AOPPs	1346.31 ± 263.56[c]	2279.34 ± 430.46[c]
AOPPs+apocynin	827.29 ± 235.82[c,d]	1076.07 ± 520.22[c,d]
Serum creatinine (μmol/l)[f]		
Vehicle	35.1 ± 2.1	35.3 ± 3.1
RSA	34.7 ± 4.0	34.3 ± 3.3
AOPPs	35.3 ± 2.6	35.2 ± 4.2
AOPPs+apocynin	35.5 ± 3.3	35.7 ± 2.7
Plasma AOPPs (μmol/l)[g]		
Vehicle	27.72 ± 3.51	27.90 ± 4.31
RSA	26.94 ± 6.83	28.93 ± 7.75
AOPPs	60.92 ± 14.67[c]	76.87 ± 18.73[c]
AOPPs+apocynin	43.19 ± 10.66[c,d]	58.86 ± 10.36[c,d]
AOPP level in renal tissue (μmol/g protein)[g]		
Vehicle	8.03 ± 1.43	8.16 ± 2.94
RSA	8.11 ± 2.31	9.94 ± 3.62
AOPPs	19.35 ± 3.93[c]	25.33 ± 6.23[c]
AOPPs+apocynin	15.65 ± 5.22[c,d]	18.95 ± 7.68[c,d]

ANOVA, analysis of variance; AOPP, advanced oxidation protein product; 8-OHdG, 8-hydroxydeoxyguanosine; ELISA, enzyme-linked immunosorbent assay; RSA, rat serum albumin.
[a]Data are mean ± s.d.
[b]Urinary albumin excretion was measured using an ELISA kit, ANOVA, $P<0.001$.
[c]$P<0.05$ vs vehicle.
[d]$P<0.05$ vs AOPPs.
[e]Urinary 8-OHdG level was analyzed using a commercial kit, ANOVA, $P<0.001$.
[f]Serum creatinine was measured by enzymatic assay (CRE-EN, Kynos, Tokyo, Japan).
[g]AOPP levels in the plasma and in renal tissue were measured as described in Li *et al.*[24], ANOVA, $P<0.001$.

Podocyte apoptosis increased in AOPP-challenged rats and was ameliorated by treatment with apocynin

To determine whether AOPP accumulation induces podocyte apoptosis and to delineate the relationship between apoptosis and changes in podocyte number, we first quantified rates of podocyte apoptosis using double-immunofluorescence labeling, including Wilms's tumor-1 and terminal deoxyuridine nick-end labeling (TUNEL) assay. TUNEL-positive podocytes per glomerular cross-section were significantly increased in AOPP-treated rats from week 5 compared with age-matched control animals (vehicle- or RSA-treated rats) (Figure 1a and b). Interestingly, apoptosis was also detectable in tubular epithelial cells, particularly in AOPP-treated rats (data not shown). Treatment of apocynin significantly prevented podocytes from AOPP-induced apoptosis (Figure 1a and b), indicating that the apoptotic processes are dependent on the activation of NADPH oxidase.

To further confirm the *in vivo* proapoptotic effect of AOPPs on podocytes, we next identified and quantified podocytes in urine sediments. Podocytes in cytospin urine sediments were identified as either nucleated or multinucleated cells expressing podocalyxin, using an immunofluorescence microscope. As shown in Figure 1c, no urinary podocytes were detected in rats treated with vehicle or native RSA. The mean value of podocytes per milliliter urine among