p47^{phox} contributes to albuminuria and kidney fibrosis in mice

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Reactive oxygen species (ROS) have an important pathogenic role in the development of many diseases, including kidney disease. Major ROS generators in the glomerulus of the kidney are the p47^{phox}-containing NAPDH oxidases NOX1 and NOX2. The cytosolic p47^{phox} subunit is a key regulator of the assembly and function of NOX1 and NOX2 and its expression and phosphorylation are upregulated in the course of renal injury, and have been shown to exacerbate diabetic nephropathy. However, its role in nondiabetic-mediated glomerular injury is unclear. To address this, we subjected p47^{phox}-null mice to either adriamycin-mediated or partial renal ablation–mediated glomerular injury. Deletion of p47^{phox} protected the mice from albuminuria and glomerulosclerosis in both injury models. Integrin α1–null mice develop more severe glomerulosclerosis compared with wild-type mice in response to glomerular injury mainly due to increased production of ROS. Interestingly, the protective effects of p47^{phox} knockout were more profound in p47^{phox}/integrin α1 double knockout mice. *In vitro* analysis of primary mesangial cells showed that deletion of p47^{phox} led to reduced basal levels of superoxide and collagen IV production. Thus, p47^{phox}-dependent NADPH oxidases are a major glomerular source of ROS, contribute to kidney injury, and are potential targets for antioxidant therapy in fibrotic disease.

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Glomerulosclerosis, characterized by excessive extracellular matrix deposition in the glomeruli of the kidneys, is the common final pathway of many chronic kidney diseases irrespective of their etiologies.[1,2] The development of glomerulosclerosis is modulated by multiple factors including reactive oxygen species (ROS) and integrins.[3,4]

ROS are a group of active intermediate oxygen molecules produced under both physiological and pathological conditions. Although low levels of ROS are essential for physiological processes, excessive production leads to oxidative stress, which is an important mediator of pathological conditions, including kidney injury.[5–8] ROS are produced during many intracellular processes, and major cellular sources of ROS are the mitochondrial respiratory chain, the NOX family nicotinamide adenine dinucleotide phosphate oxidases, the xanthine oxidase, the uncoupled endothelial nitric oxide synthase, and the cytochrome P450 systems.[9,10]

The NOX family NADPH oxidases are superoxide-generating enzymes that catalyze electron transfer from NADPH onto molecular oxygen. The first NADPH oxidase described was the neutrophil phagocytic NADPH oxidase that contains the membrane-bound catalytic subunit gp91^{phox}/NOX2.[11] NOX2 is expressed in many different cell types and tissues including the kidney.[12,13] The phagocytic NADPH oxidase requires the assembly of five subunits to function properly: the two membrane-bound gp91^{phox}/NOX2 and p22^{phox} subunits and the cytosolic regulatory subunits p40^{phox}, p47^{phox}, and p67^{phox}. In addition, assembly of this NADPH oxidase is regulated by the balance between inactive (GDP-bound) and active (GTP-bound) small GTPase Rac. GTP-bound Rac leads to the translocation of cytosolic subunits to the membrane, association with the membrane subunits, and formation of an active NOX2-containing complex.[14,15]

In addition to the gp91^{phox}/NOX2, six other catalytic subunits have been identified, including NOX1, NOX3, NOX4, NOX5, Duox1, and Duox2.[11] NOX1, like NOX2, is expressed in many different tissues including the kidney[12,16] and requires p22^{phox} and the regulatory subunit p47^{phox}, as well as active Rac for its stabilization and function. Its

dependence on p22phox, however, is less stringent compared with that observed for NOX2.[11] NOX4, originally identified as a NADPH oxidase homolog, is also highly expressed in the kidney.[17,18] It requires interactions with p22phox but not the cytosolic organizer subunit p47phox or Rac for its stabilization and function.[11] Because of its selective expression in the kidney, NOX4 has been suggested to be the major NOX in kidney and the major source of ROS in animal models of diabetic nephropathy.[18,19] However, recent studies have cast doubt on the pathological role of NOX4 in kidney injury, as NOX4-null mice showed worse glomerular and tubular kidney injury compared with wild-type (WT) mice.[20,21] Interestingly, deleting NOX2 did not change the severity of tubulointerstitial injury in the kidney nor did it alter the phenotype of NOX4-null mice subjected to the same injury.[13,21] In contrast to the data showing no role for NOX2 in tubulointerstitial disease, p47phox-null mice have reduced kidney hypertrophy and mesangial matrix expansion in diabetic nephropathy.[22–24] As p47phox affects both NOX1 and NOX2, this finding suggests that simultaneous inactivation of both these NOXs is required to alleviate diabetic nephropathy. The role of p47phox-containing NOXs in nondiabetic glomerular injury is unknown.

Integrins can also regulate the levels of ROS in both physiological and pathological conditions. Integrins are transmembrane receptors for matrix components formed by two non-covalently associated α and β subunits that combine to form 24 different heterodimers with different ligand specificity to matrix molecules.[25] Upon binding to matrix, integrins initiate multiple cell signaling pathways, which regulate critical cellular functions such as survival, proliferation, and matrix homeostasis.[26,27] Integrin α1β1, a major collagen-binding receptor, is highly expressed on all major cell types in the glomeruli of the kidney, and it has been identified as an important negative regulator of collagen synthesis.[4,28] In this context, integrin α1–null mice develop more severe glomerulosclerosis compared with WT mice in response to glomerular injury, and this is mainly due to increased production of pro-fibrotic ROS.[29,30] In contrast, mice lacking another collagen receptor, integrin α2β1, are protected from ROS-mediated injury.[31] These findings suggest that integrin α1β1 decreases, whereas integrin α2β1 increases ROS production.[32] The mechanism whereby integrin α1–null glomerular cells produce increased basal levels of ROS is in part due to increased growth factor receptor–mediated Rac activation.[28] Thus, integrin α1–null mice and cells are predisposed to produce excessive collagen because of increased activity of Rac-dependent NOXs (NOX1 and NOX2), and they are an excellent model to define the mechanisms whereby ROS production contributes to glomerulosclerosis.

To determine the role of p47phox-dependent NOXs in nondiabetic glomerular injury, we subjected p47phox-null mice as well as p47phox-null mice crossed with the integrin α1–null mice to adriamycin (ADR)- and partial renal ablation–mediated glomerular injury. We provide evidence that deletion of p47phox protects mice from renal injury–mediated albuminuria and glomerulosclerosis found in both injury models, and these effects are more profound in mice on the integrin α1–null background. This protection is also accompanied by decreased production of free radicals, decreased activation of the epithelial growth factor (EGF) receptor/GTP-Rac axis, and consequent decreased collagen production. Thus, our data indicate that p47phox-containing NOXs are an important source of ROS in the kidney and point to p47phox as a potential target for amelioration of albuminuria and renal fibrosis following injury.

RESULTS

Loss of p47phox ameliorates kidney injury

To identify the role of NADPH oxidase–mediated ROS production in glomerular injury, we used p47phox-null (p47phox knockout (KO)) mice crossed onto the integrin α1–null (α1KO) mouse. This choice is dictated by the fact that integrin α1KO mice develop exacerbated glomerulosclerosis following injury in part due to increased baseline and injury-mediated ROS production.[29] Glomerular injury was induced either by a single intravenous injection of ADR[29] or by a partial renal ablation.[31]

Mice received a single intraperitoneal injection of ADR (10 mg/kg), and their weight was assessed at 0, 1, 2, 3, and 4 weeks after injection. As previously reported, integrin α1KO mice lost ~20% of body weight at 2 and 3 weeks after ADR injection.[29] In contrast, no body weight loss was observed in injured WT control, p47phoxKO, or p47phoxKO/integrin α1KO mice (DKO; Figure 1a). At 4 weeks, ~50% mortality was observed in integrin α1KO mice and ~20% in WT mice, whereas no deaths were observed in either p47phoxKO or DKO mice. To determine whether there were differences in renal function among the various groups, we analyzed albuminuria by measuring urine albumin-to-creatinine ratio at 1 and 4 weeks after ADR injection. One and 4 weeks were chosen because at 1 week albuminuria is observed in both WT and integrin α1KO mice, but glomerular matrix deposition and injury are evident primarily in the latter group, and at 4 weeks albuminuria decreases in both groups, but it stays more elevated (together with overall kidney damage) in the integrin α1KO group.[29] One week after ADR injection, albuminuria was evident in both WT and integrin α1KO mice, although it was more prominent in the latter group (Figure 1b, c). In contrast, significantly lower albuminuria was evident in p47phoxKO and DKO compared with injured WT and integrin α1KO mice, respectively. Albuminuria decreased in all groups at 4 weeks after injury, although it was significantly lower in p47phoxKO and DKO mice (Figure 1b, c). Similar results for the urine albumin-to-creatinine ratio were seen in mice post partial renal ablation, with albuminuria in 12-week-injured p47phoxKO or DKO mice significantly lower compared with that measured in injured WT and integrin α1KO mice (Supplementary Fig. 1A). Thus, p47phox deletion ameliorates both ADR- and partial renal ablation–induced albuminuria.