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p47phox contributes to albuminuria and kidney fibrosis in mice

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Abstract

Reactive oxygen species (ROS) play an important pathogenic role in the development of many diseases, including kidney disease. Major ROS generators in the glomerulus of the kidney are the p47phox-containing NADPH oxidases NOX1 and NOX2. The cytosolic p47phox subunit is a key regulator of the assembly and function of NOX1 and NOX2 and its expression and phosphorylation are up-regulated in the course of renal injury, and have been shown to exacerbate diabetic nephropathy. However, its role in non-diabetic-mediated glomerular injury is unclear. To address this, we subjected p47phox-null mice to either adriamycin-mediated or partial renal ablation-mediated glomerular injury. Deletion of p47phox protected the mice from albuminuria and glomerulosclerosis in both injury models. Integrin $\alpha 1$ -null mice develop more severe glomerulosclerosis than wild type mice in response to glomerular injury mainly due to increased production of ROS. Interestingly, the protective effects of p47phox knockout were more profound in p47phox/integrin $\alpha 1$ double knockout mice. *In vitro* analysis of primary mesangial cells showed that deletion of p47phox led to reduced basal levels of superoxide and collagen IV production. Thus, p47phox-dependent NADPH oxidases are a major glomerular source of ROS, contribute to kidney injury, and are potential targets for antioxidant therapy in fibrotic disease.

Keywords

glomerulosclerosis; partial renal ablation; adriamycin; integrins; reactive oxygen species; NOX

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Introduction

Glomerulosclerosis, characterized by excessive extracellular matrix (ECM) 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 NADPH 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}, 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 p22^{phox}, however, is less stringent than 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 p22^{phox}, but not the cytosolic organizer subunit p47^{phox} or Rac for its stabilization and function.¹¹ Due to 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, since NOX4-null mice showed worse glomerular and tubular kidney injury than wild type 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, p47^{phox}-null mice have reduced kidney hypertrophy and mesangial matrix expansion in diabetic nephropathy.²²⁻²⁴ As p47^{phox} affects both NOX1 and NOX2, this finding suggests that simultaneous inactivation of both these NOXs is required to alleviate diabetic nephropathy. The role of p47^{phox}-containing NOXs in non-diabetic 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 $\alpha 1\beta 1$, 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 $\alpha 1$ -null mice develop more severe glomerulosclerosis than wild type 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 $\alpha 2\beta 1$, are protected from ROS-mediated injury.³¹ These findings suggest that integrin $\alpha 1\beta 1$ decreases, while integrin $\alpha 2\beta 1$ increases ROS production.³² The mechanism whereby integrin $\alpha 1$ -null glomerular cells produce increased basal levels of ROS is in part due to increased growth factor receptor-mediated Rac activation.²⁸ Thus, integrin $\alpha 1$ -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 p47^{phox}-dependent NOXs in non-diabetic glomerular injury, we subjected p47^{phox} null mice as well as p47^{phox} null mice crossed with the integrin $\alpha 1$ -null mice to adriamycin- and partial renal ablation-mediated glomerular injury. We provide evidence that deletion of p47^{phox} 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 $\alpha 1$ -null background. This protection is also accompanied by decreased production of free radicals, decreased activation of the EGF receptor/GTP-Rac axis and consequent decreased collagen production. Thus, our data indicate that p47^{phox}-containing NOXs are an important source of ROS in the kidney and point to p47^{phox} as a potential target for amelioration of albuminuria and renal fibrosis following injury.

Results

Loss of p47^{phox} ameliorates kidney injury

To identify the role of NADPH oxidase-mediated ROS production in glomerular injury, we used p47^{phox}-null (p47^{phox}KO) mice crossed onto the integrin $\alpha 1$ -null ($\alpha 1$ KO) mouse. This choice is dictated by the fact that integrin $\alpha 1$ KO mice develop exacerbated glomerulosclerosis following injury due in part to increased baseline and injury-mediated ROS production.²⁹ Glomerular injury was induced either by a single intravenous injection of adriamycin²⁹ or by partial renal ablation.³¹

Mice received a single intraperitoneal injection of adriamycin (10mg/kg) and their weight was assessed at 0, 1, 2, 3 and 4 weeks after injection. As previously reported, integrin $\alpha 1$ KO mice lost ~ 20% of body weight at 2 and 3 weeks after adriamycin injection.²⁹ In contrast, no body weight loss was observed in injured wild type control, p47^{phox}KO or p47^{phox}KO/integrin $\alpha 1$ KO mice (DKO) (Fig. 1A). At 4 weeks, ~ 50% mortality was observed in integrin $\alpha 1$ KO mice and ~ 20% in wild type mice, while no deaths were observed in either

p47^{phox}KO 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 adriamycin injection. One and four weeks were chosen because at 1 week albuminuria is observed in both wild type and integrin α 1KO 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 α 1KO group.²⁹ One week after adriamycin injection, albuminuria was evident in both wild type and integrin α 1KO mice, although it is more prominent in the latter group (Figs. 1B, C). In contrast, significantly lower albuminuria was evident in p47^{phox}KO and DKO compared to injured wild type and integrin α 1KO mice, respectively. Albuminuria decreased in all groups at 4 weeks after injury, although it was significantly lower in p47^{phox}KO and DKO mice (Figs. 1B, C). Similar results for the urine albumin-to-creatinine ratio were seen in mice post-partial renal ablation, with albuminuria in 12 weeks injured p47^{phox}KO or DKO mice significantly lower than that measured in injured WT and integrin α 1KO mice (Suppl. Fig. 1A). Thus, p47^{phox} deletion ameliorates both adriamycin- and partial renal ablation-induced albuminuria. To characterize and quantify the degree of adriamycin-mediated injury, the kidneys were subjected to pathological examination. Four weeks after adriamycin injection, integrin α 1KO mice showed more severe glomerular and tubular injury than injured WT mice. Injury was characterized by exacerbated mesangial matrix expansion, glomerulosclerosis, tubular accumulation of proteinaceous casts, and interstitial fibrosis (Figs. 2A-D). By contrast, the kidneys of injured p47^{phox}KO and DKO mice looked relatively normal with minimal matrix mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis (Figs. 2A-D). Similar differences in glomerular injury were seen among the different strains of mice 12 weeks after partial renal ablation (Suppl. Fig. 1B). These results show that loss of p47^{phox} in mice that either do or do not express integrin α 1 β 1 ameliorates adriamycin- and renal ablation-mediated glomerular injury.

Loss of p47^{phox} ameliorates kidney fibrosis

Increased collagen deposition is a key pathological feature of glomerular injury leading to glomerulosclerosis. Trichrome staining of kidney sections from 4 week adriamycin-injured mice revealed increased accumulation of fibrillar collagen in both glomeruli and tubules of integrin α 1KO mice compared to injured WT mice (Fig. 3A). In contrast, kidneys of injured p47^{phox}KO and DKO showed low levels of fibrillar collagen (Fig. 3A). Fibrillar collagen staining was not evident in uninjured mice independently of their genotype (Fig. 3A).

As collagens I and IV are the principal fibrillar and non-fibrillar collagens induced in glomerulosclerosis, we examined their expression levels in kidney either by immunohistochemistry or Western blot analysis. Increased levels of collagen IV were evident in injured integrin α 1KO mice compared to wild type mice and this was independent of the type of renal injury (Figs. 3B-D, Suppl. Figs. 1C, 1D). Moreover, decreased collagen IV deposition (especially in glomeruli) was detected in both p47^{phox}KO and DKO mice (Figs. 3B-D, Suppl. Figs. 1C, 1D). Similar to Trichrome staining, increased levels of collagen I were evident in injured integrin α 1KO mice compared to WT mice and these levels were significantly decreased in the kidneys of DKO mice (Figs. 3C, 3D). These

results suggest that p47^{phox}, at least in part, accounts for the increased injury and collagen deposition observed in the injured α 1KO mice.

Loss of p47^{phox} ameliorates adriamycin-induced oxidative stress

p47^{phox} is a critical regulatory subunit of the NOX1 and NOX2 containing NADPH oxidases and deleting it reduces *in vivo* ROS production and therefore oxidative stress.^{33, 34} Thus, we examined markers of oxidative stress *in vivo* to determine whether reduced ROS production correlates with the decreased renal damage observed in the p47^{phox}KO background. Urinary levels of F2-isoprostane and nitrotyrosine in kidney tissues were analyzed as they are recognized markers of *in vivo* oxidative stress. The levels and/or expression of both markers peaked at 1 week after adriamycin injury in all groups analyzed (Figs. 4A-D). The nitrotyrosine staining in the kidneys of injured mice localized primarily to podocytes, mesangial cells, proximal tubule cells, and infiltrating cells (Fig. 4B) which have been all shown to express p47^{phox}. Both oxidative stress markers were significantly reduced in DKO mice compared to injured integrin α 1KO mice. Similarly, p47^{phox}KO mice showed significantly decreased oxidative stress markers in both urine and kidney tissues compared to injured WT mice (Figs. 4A-D). At 4 weeks, the overall levels of F2-isoprostane and nitrotyrosine were decreased in all four groups, although the levels of nitrotyrosine remained significantly higher in the kidneys of injured integrin α 1KO mice (Figs. 4A-D).

As the expression of p47^{phox} increases in the course of renal injury and correlates with the degree of injury, we analyzed the levels of p47^{phox} in the kidneys of uninjured (Control), 1 week and 4 weeks after adriamycin-injured mice. While no basal expression of p47^{phox} was detected in uninjured mice, increased expression of this NADPH regulatory subunit was evident primarily in 1 week injured integrin α 1KO mice and remained elevated at 4 weeks (Fig. 5A, 5B). The translocation of the p47^{phox} subunit to the plasma membrane is a key step for the formation of an active NOX2 containing complex.^{14,15} Thus, we analyzed the membrane localization of the p47^{phox} by performing Western blot analysis of membrane enriched fractions of kidneys from uninjured (control) and 4 week-adriamycin injured wild type and integrin α 1KO mice. Increased levels of membrane-associated p47^{phox} were detected in kidneys of injured integrin α 1KO mice (Fig. 5C).

Oxidative stress was still detected in the kidneys of adriamycin-treated DKO mice compared to p47^{phox}KO mice (Fig. 4B), suggesting that in addition to p47^{phox} other ROS generating enzymes might be responsible for the generation of nitrotyrosine in the kidneys of integrin α 1KO/p47^{phox} double KO mice. We therefore analyzed the levels of NOX4, a ROS-producing enzyme highly expressed in the kidneys and upregulated in kidney injury,^{35, 36} in the kidneys of control and adriamycin-treated mice. While no difference in the basal levels of NOX4 were observed in kidneys of uninjured mice, increased expression of this enzyme was observed in the kidneys of 1 and 4 week injured mice, although it was more significant in the kidneys of integrin α 1KO mice (Figs. 5A, 5B). Thus p47^{phox} and NOX4 contribute to the renal damage observed in the integrin α 1KO mice.

Loss of p47^{phox} attenuates podocyte injury and macrophage infiltration in integrin α 1KO mice

Podocytes are a major target of adriamycin-induced kidney injury.^{37, 38} Because these cells express p47^{phox} and NOX4 (reviewed in³⁹) they could contribute directly to podocyte and consequent glomerular injury by generating ROS in response to injury. To evaluate whether podocyte injury/loss directly correlates with the degree of renal damage observed in injured mice, we stained kidneys sections of control and injured mice with anti-WT1 antibodies. While no difference in the number of WT1 positive cells were observed in kidneys of uninjured mice, significantly less WT1 positive cells was observed primarily in the kidneys of 1 and 4 week injured wild type and integrin α 1KO mice, although it was more significant in the latter group (Figs. 6A, 6B).

Another feature associated to adriamycin-mediated injury is macrophage infiltration starting at 2 weeks after adriamycin treatment.³⁸ As interstitial fibrosis and collagen production are still evident in adriamycin-injured integrin α 1KO mice (despite decline in albuminuria and urinary ROS) at 4 weeks, we investigated whether infiltrating macrophages could account for these fibrotic features. No difference in macrophage number was observed among the 4 groups at baseline or 1 week after ADR treatment (Figs. 7A, 7B). This is consistent with the observation that infiltrating cells become evident at 2 weeks after adriamycin treatment and the primary cells affected by adriamycin are resident cells (e.g., podocytes and mesangial cells).^{29, 38} In contrast, at 4 weeks a significant increase in the number of F4/80 positive cells was detected only in the kidneys of integrin α 1KO mice (Figs. 7A, 7B), suggesting that, in addition to ROS and resident cells, macrophages might contribute to the late fibrotic phenotype observed in this group.

Loss of p47^{phox} leads to decreased glomerular activation of EGF receptor

We next determined the mechanism whereby loss of p47^{phox} protects against renal injury. The EGF receptor (EGFR) contributes to the development of kidney injury through multiple mechanisms.^{40, 41} We showed that increased basal activation of EGFR in integrin α 1KO mice contributes to kidney injury and matrix synthesis by promoting Rac-mediated activation of NADPH oxidase complex and in turn ROS production.^{28, 29} As ROS can potentiate the activation status of receptor tyrosine kinases⁴², we analyzed the levels of activated EGFR in glomeruli isolated from WT, α 1KO, p47^{phox}KO, or DKO mice 1 week after adriamycin injection. As expected, glomeruli from injured integrin α 1KO mice showed significantly higher levels of activated EGFR compared to injured WT mice, and this activation was significantly reduced in glomeruli of injured p47^{phox}KO and DKO mice (Figs. 8A, 8B). Thus, the p47^{phox}/ROS axis is required for activation of EGFR *in vivo*.

Crosstalk between the EGFR-Rac pathway and p47^{phox} regulates ROS and collagen IV production *in vitro*

In addition to podocytes, mesangial cells are a major glomerular cell type responsible for production of ROS and collagen IV. To further dissect the mechanisms whereby p47^{phox} regulates ROS and collagen IV production, the levels of activated EGFR, Rac, as well as the levels of ROS and collagen IV production were analyzed in mesangial cells isolated from WT, α 1KO, p47^{phox}KO, and DKO mice. Consistent with the *in vivo* data, integrin α 1KO

mesangial cells showed significantly higher levels of activated EGFR, activated Rac (GTP-Rac), ROS and collagen IV (Figs. 8C-E) than the other cell types analyzed. In contrast, deletion of p47^{phox} profoundly decreased activation of EGFR, GTP-Rac, ROS and collagen IV production (Figs. 8C-E).

We previously showed that integrin α 1KO mesangial cells have increased basal level of activated ERK⁴³. This MAPK kinase is not only downstream EGFR,⁴⁴⁻⁴⁶ but also promotes ROS production by regulating the expression and/or phosphorylation of p47^{phox}.^{47, 48} Therefore, we analyzed the levels of activated ERK in mesangial cells and found that, in contrast to the activated levels observed in integrin α 1KO mesangial cells, deletion of p47^{phox} resulted in decreased levels of activated ERK (Fig. 8D). These results suggest that integrin α 1 β 1-mediated downregulation of ROS production is mainly mediated by the p47^{phox}-dependent NADPH oxidase complex, and this complex also plays a role in regulating the activation of EGFR, Rac, ERK, and collagen IV production (Figs. 9A, 9B).

Discussion

Increased ROS production or oxidative stress has been documented in various human diseases, and studies using animal models have demonstrated that increased ROS levels are an important pathogenic mechanism in the development of fibrotic injuries. The overall goal of this study was to evaluate the role of p47^{phox}, the regulatory subunit of NOX1 and NOX2, in the development and progression of glomerular kidney disease. To do this, we used p47^{phox}-null mice crossed to integrin α 1-null mice, a model of increased ROS production and exacerbated glomerulosclerosis following injury.^{28, 29} Using both ROS-mediated and non-ROS mediated glomerular injury models; we provide evidence that deletion of p47^{phox} protects mice from albuminuria and kidney fibrosis which is consistent with reduced *in vivo* oxidative stress in the p47^{phox}-null background. In addition, we provide evidence that deletion of p47^{phox} in mesangial cells leads to significantly reduced ROS and collagen IV production. Thus, our study indicates that p47^{phox}-dependent NOXs are an important source of ROS in the kidney and suggests that p47^{phox} can be viewed as a potential target for amelioration of albuminuria and renal fibrosis following injury.

NOX1, NOX2, and NOX4 are major sources of ROS in various organs, including the kidneys. The finding that the expression of these oxidases is upregulated in the course of fibrosis has initiated a series of studies aimed to determine their contribution to fibrotic disease. NOX4, for example, has been implicated in the initiation and progression of diabetic nephropathy.¹⁸ The finding that glucose induces upregulation of NOX4 and oxidative stress in kidney tubular cells, and this effect is prevented by inhibition of the protease ADAM17, suggests that the ADAM17/NOX4 axis is a key mediator of glucose-promoted kidney tubular injury.⁴⁹ Moreover, the recent finding that inhibition of mTOR prevents NOX-4-mediated ROS generation and glomerular podocyte apoptosis in a model of type 1 diabetes, suggests that NOX4 mediates mTOR-induced podocyte instability in the course of glomerular injury.¹⁹ Consistent with a deleterious role of NOX4 protein in glomerular injury, we show that the kidneys of adriamycin-injured integrin α 1KO mice have increased levels of NOX4 and p47^{phox} levels, both of which could contribute to the severe glomerular injury observed in these mice. At present, it is unclear how loss of integrin α 1 β 1

might lead to increased NOX4 production, although MAPK kinases have been implicated in the regulation of NOXs under physiological and pathological conditions (reviewed in⁵⁰). Thus, further studies are needed to determine whether the increased ERK activation we detected in integrin α 1KO mesangial cells might contribute directly to the regulation of NOX4.

Although studies indicate that activation of NOX4 plays a deleterious role in fibrotic diseases, recent studies have cast a shadow on the pathological role of NOX4 in kidney injury. In this context, NOX4-null mice show worse pathology than wild type mice following glomerular and/or tubular injury.²⁰ Moreover, NOX4-null mice develop exaggerated cardiac contractile dysfunction, hypertrophy, and dilatation during exposure to chronic overload.⁵¹ Collectively, these results not only suggest that NOX4 might paradoxically play a protective role in stress-induced kidney injury, but also indicate that other sources of ROS-generating enzymes might play a role in disease, especially NOX1 and NOX2 complexes. p47^{phox}-null mice have been used to investigate the role of these complexes, as well as factors able to regulate their assembly, to disease. In this regard, p47^{phox}-null mice are protected from angiotensin-2 mediated liver fibrosis due to the inability of angiotensin-2 to promote phosphorylation and consequent activation of p47^{phox}.⁵² Consistent with this finding, loss of p47^{phox} ameliorates the susceptibility to heart disease in mice lacking the angiotensin converting enzyme 2, a negative regulator of the renin-angiotensin system, clearly indicating a positive correlation between angiotensin-2 and p47^{phox} in fibrosis and disease.⁵³ Despite these promising findings, it has been recently shown that loss of p47^{phox} leads to cytoskeleton destabilization and decreased activation of kinases found in focal adhesions, thus making the heart more susceptible to overload-induced biomechanical stress.⁵⁴ In addition, mice lacking p47^{phox} show increased neutrophil alveolitis and exacerbated lung injury following LPS treatment due to enhanced NF- κ B activity.³⁴ All together these results indicate that the beneficial and/or deleterious role of p47^{phox} is highly dependent on the type of injury and the cell type affected.

In renal disease, the contribution of p47^{phox} has been investigated primarily in diabetes-mediated injury. Its genetic deletion reduces kidney hypertrophy, oxidative stress and mesangial matrix expansion in a spontaneous model of type 1 diabetes.²⁴ However, no protection from albuminuria was observed in this study.²⁴ In contrast to this finding, we show that mice lacking p47^{phox} expression show decreased oxidative stress which is accompanied by decreased albuminuria and kidney fibrosis compared to wild type mice in two different models of glomerular injury. The beneficial effects on albuminuria are more evident when p47^{phox}-null mice are crossed onto the integrin α 1-null mouse, a model of increased NADPH-mediated oxidative stress and kidney injury.^{29, 30} Our results suggest that p47^{phox} and NOX4 contribute primarily to the early phase of adriamycin-mediated renal injury. In this context, at 1 week, proteinuria, matrix accumulation, podocyte loss and glomerular injury directly correlated to the levels of renal p47^{phox} and NOX4 expression and oxidative stress markers. This was particularly evident in 1 week injured integrin α 1-null mice which showed the highest levels of p47^{phox} and NOX4 proteins, F2 isoprostanes, and nitro-tyrosine. At 4 weeks, however, we observed an overall decrease in albuminuria and oxidative stress markers in all groups, although the p47^{phox} and NOX4 protein levels remained elevated in the kidney of injured α 1-null mice. Despite an overall decrease in

oxidative stress, significantly more glomerular injury (as determined by podocyte loss) as well as glomerular and tubular interstitial sclerosis were detected in integrin $\alpha 1$ -null mice at 4 weeks. This suggests that factors independent of ROS might contribute to the severity of injury in these mice at late phases of adriamycin-induced renal injury. A plausible explanation is that the high albuminuria detected in 1 week injured integrin $\alpha 1$ -null mice could have triggered tubule cell death with consequent generation of pro-fibrotic and pro-inflammatory mediators.⁵⁵ This possibility is supported by the significantly increase in collagen deposition and numbers of infiltrating macrophage particularly evident in the kidneys of 4 weeks injured integrin $\alpha 1$ -null mice. Another feature of 4 week injured integrin $\alpha 1$ -null mice was an overall increase in podocyte loss despite decreased albuminuria compared to 1 week injured integrin $\alpha 1$ -null mice. Although this seems to be a counter intuitive result, our finding agrees with studies in humans and rodents showing that proteinuria is not uniformly associated with podocyte injury. To this end, podocyte injury does not directly correlate to the levels of proteinuria in human glomerulopathies⁵⁶ and injection of antibodies to nephrin leads to rapid proteinuria in the presence of intact podocyte foot processes.^{57, 58}

p47^{phox} is required for both NOX1- and NOX2-mediated ROS production, suggesting that deletion of p47^{phox} might affect both NOX pathways. Surprisingly, the NOX2-null mice show no overall renal protection upon induction of type 1 diabetes.¹³ On the other hand, inhibition of the NOX1/NOX4 axis with the dual inhibitor GKT136901 protects mice from renal injury induced by type 2 diabetes.⁵⁹ Thus, it is conceivable that the p47^{phox}/NOX1 axis might be a major generator of ROS in kidney injury. Although more studies are needed in order to determine which of the two NOXs is activated by p47^{phox} in the course of renal injury, our study shows that p47^{phox}-dependent NADPH oxidase plays a key role in the progression of kidney injury and suggests that targeting p47^{phox} is sufficient to ameliorate both fibrotic responses and albuminuria.

In addition to p47^{phox}, the activity and assembly of NOX1 and NOX2 containing NADPH oxidase are mediated by the small GTPase Rac.^{11, 32, 60-62} We previously showed that integrin $\alpha 1\beta 1$ acts as a negative regulator of ROS production by preventing EGFR phosphorylation and consequent Rac activation.²⁸ Consistent with this finding, transfection of integrin $\alpha 1$ KO cells with dominant negative Rac significantly decreases basal levels of ROS.²⁸ In addition, we here show that injured integrin $\alpha 1$ -null mice crossed onto the p47^{phox}-null background produces less *in vivo* markers of oxidative stress than integrin $\alpha 1$ -null mice. However, the levels of oxidative stress in the integrin $\alpha 1$ /p47^{phox}-null mice were still higher than those detected in injured p47^{phox}- mice mice. This difference suggests that integrin $\alpha 1\beta 1$ might also negatively regulate ROS productions in a Rac/p47^{phox} independent manner. In this context, mitochondria and 5-lipoxygenase have been found to be involved in integrin-mediated ROS production.⁶³ Moreover, the presence of integrin $\alpha v\beta 3$ at focal adhesion sites inhibits local ROS production,⁶⁴ suggesting that integrin-mediated adhesion is important in regulating the local redox environment.

In conclusion, our study suggests that p47^{phox} contributes to the generation of ROS in kidney glomeruli and contributes to kidney injuries. This, together with the finding that the

assembly of Rac-dependent NOXs is negatively regulated by integrin $\alpha 1\beta 1$, points to integrins and p47^{phox} as potential targets for fibrotic diseases.

Methods

In vivo Injury Models

All *in vivo* experiments were performed according to institutional animal care guidelines and conducted in Association for Assessment and Accreditation of Laboratory Animal Care–accredited facilities. p47^{phox}-null (p47^{phox}KO) mice on C57/B6 background were backcrossed onto the BALB/c background for 8 generations and then were crossed with integrin $\alpha 1$ -null ($\alpha 1$ KO) mice onto the BALB/c background²⁹ to generate integrin $\alpha 1$ het/p47^{phox}het. The integrin $\alpha 1$ het/p47^{phox}het mice were then crossed among themselves in order to generate wild type (WT), integrin $\alpha 1$ KO, p47^{phox}KO, and integrin $\alpha 1$ KO/p47^{phox}KO (DKO) mice, all on the BALB/c background. For adriamycin-mediated glomerulosclerosis, male mice (6 weeks old, 24 g body weight) received a single intravenous injection of adriamycin (10 mg/kg; Sigma Aldrich), and mice were sacrificed 0, 1 or 4 weeks after injection. One and 4 weeks were chosen because at these time points most of the integrin $\alpha 1$ KO mice develop severe albuminuria and glomerular injury.²⁹ Six to 18 mice/genotype were used for this set of experiments. For the remnant kidney model (5/6 nephrectomy), WT, integrin $\alpha 1$ KO, p47^{phox}KO, and DKO BALB/c male mice (6–7 weeks old, 20–23 g body weight) underwent 5/6 nephrectomy or sham operation as described.³¹ Mice were then sacrificed 12 weeks after injury. Twelve mice/genotype were used for this set of experiments.

Clinical Parameters and Morphological Analysis

Body weight was measured weekly and expressed in grams. For the analysis of albuminuria, spot urine was collected at 0, 1, and 4 weeks after adriamycin injection or at 0 and 12 weeks after partial renal ablation and the concentration of urine albumin and creatinine was measured using the ELISA Albuwell M kit (Exocel Inc., Philadelphia, PA). The albumin-to-creatinine ratio (ACR) was expressed as micrograms per milligram.

Kidneys, removed immediately at sacrifice, were fixed in 4% formaldehyde for paraffin section for both morphological and immunohistochemistry analysis. Paraffin tissue sections were stained Masson's Trichrome Blue for the evaluation of kidney fibrosis. Paraffin sections were stained with periodic acid Schiff (PAS) to assess glomerular mesangial expansion and sclerosis, acute tubular injury, and interstitial fibrosis. The total number of glomeruli per each kidney section was counted with a total of ~ 150 glomeruli/kidneys and 7-13 mice per each group analyzed. Global sclerosis and segmental sclerosis are presented as a percentage. A semi-quantitative index was used to evaluate the degree of glomerular mesangial expansion. Each glomerulus on a single section was graded from 0 to 4, where 0 represents no lesion, and 1, 2, 3 and 4 represent mesangial matrix expansion involving <25, 25-50, 51-75, and >75% of the glomerular tuft area, respectively. Tubular injury was scored according to the percentage of damaged tubules (loss of brush border, shedding of both necrotic and viable epithelial cells into the tubular lumen, tubular dilation, cast formation, and cell lysis): 1) <25% damaged; 2) 25–50% damaged; 3) 51–75% damaged; and 4) >75%

damaged, respectively. A semi-quantitative index was used to evaluate the degree of interstitial fibrosis: 0: no interstitial fibrosis; 1) <25%; 2) 25– 50%; 3) 51–75%; and 4) >75% interstitial fibrosis, respectively. Histologic analysis was performed in a blind fashion by a renal pathologist (PP).

F2-isoprostane Assay

The levels of urinary F2-isoprostane were determined using an EIA kit following the manufacturer's instruction (Cayman Chemical Company, USA). Briefly, spot urines were diluted 100 times with water. 50 μ l of standard or urine samples were placed in 96-well plate pre-coated with mouse anti-rabbit IgG. Then, 50 μ l F2-isoprostane tracer and F2-isoprostane antiserum were added into each well and incubated for 18 h at 4 °C. The plates were then washed to remove all unbound reagents and captured F2-isoprostane was detected with Ellman's reagent followed by absorbance at 420 nm using multiskanascnt analyzer (Thermo Labsystems). All samples were assayed in duplicate. The values of F2-isoprostane were calculated using a computer spreadsheet available from Cayman Chemical Company and expressed as a ratio to creatinine (pg/ μ g).

Cell Culture

Primary mesangial cells from WT, α 1KO, p47^{phox}KO, and DKO BALB/c mice were isolated, cultured, and immortalized by retroviral-mediated expression of T-antigen as previously described.⁶⁵

Immunohistochemistry

Immunohistochemistry staining for the evaluation of collagen IV and Nitro-tyrosine (N-Tyr) levels, as well as macrophage and podocyte numbers in uninjured and injured kidneys was performed on paraffin kidney sections as reported.^{29, 66-68} Briefly, kidney sections were incubated with rabbit anti-mouse collagen IV (1:500, AbD Serotec), rabbit anti-N-Tyr antibody (1:400, Santa Cruz), mouse anti-WT1 antibody (1:100, Santa Cruz), and rat anti-F4/80 (AbD Serotec) overnight at 4°C followed by 1 hour incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:100, Santa Cruz) at room temperature. Slides were developed using Sigma Fast DAB chromogenic tablets (Sigma) and then counterstained with toluidine blue in order to increase the contrast with DAB staining.

In vitro ROS Detection

ROS production was measured with dihydro-rhodamine as previously described²⁹. Briefly, 1.5×10^5 mesangial cells were plated in six-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal calf serum (FCS). After 2 days, the cells were washed with PBS and then incubated in serum-free medium with 2 μ M dihydro-rhodamine (Sigma) for additional 2 hours. The cells were then trypsinized and the generation of fluorescent rhodamine 123 was analyzed by a FACScan (BD Bioscience). Three independent experiments were performed in triplicate.

GTP-Rac Assay

PAK1-p21Rac binding domain (PBD)-conjugated glutathione Sepharose beads were prepared and used as previously described.²⁸ 24 hour serum starved cells were scraped in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 200 mM NaCl, 1% NP-40, 5% glycerol, 0.05% Tween 20, 1 mM NaF, 1 mM Na₃VO₄, and proteinase inhibitor (Roche). Equal amount of cell lysates were incubated with 30 µl of PAK1-PBD-conjugated glutathione Sepharose beads at 4 °C for 1 hour. Beads were then centrifuged, washed with 25 mM Tris-HCl (pH 7.6)-30 mM MgCl₂-40 mM NaCl-1 mM dithiothreitol-1% NP-40, suspended in 20 µl of Laemmli's sample buffer and analyzed by Western blotting as indicated below.

Western Blot Analysis

For analysis of collagen IV, collagen I, p47^{phox}, NOX4, and N-Tyrosine levels, total kidney lysates were prepared in 62.5 mM Tris, pH 6.8, 2% SDS, and 10% glycerol by homogenization. For p47^{phox} membrane localization, kidneys were homogenized in 20 mM Hepes pH 7.2 using a Polytron homogenizer. After addition of NaCl (100 mM final concentration) the homogenates were centrifuged at 3,000 rpm for 5 minutes to remove debris. The supernatant was collected and centrifuged at 55,000 rpm for 50 minutes in order to separate membrane (pellet) and cytosol (supernatant) rich fractions. For analysis of phosphorylated EGF receptor, glomeruli were isolated 1 week after adriamycin injection as described⁶⁹ and lysed in the buffer described above. For analysis of phosphorylated and total ERK, as well as collagen IV levels in mesangial cells, 24 hour serum starved cells were lysed in RIPA buffer (50mM Tris, pH 7.4, 150mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1× Roche complete mini protease inhibitor cocktail, and 4 mM Na Orthovanadate).

Equal amounts of total proteins (20-40 µg/lane) were resolved in 8% SDS-PAGE, transferred onto nitrocellulose, and probed with anti-collagen IV (MD Bioproducts), anti-collagen I (MD Bioproducts), anti-p47^{phox} (Santa Cruz), anti-NOX4 (Abcam) anti-β-actin (Santa Cruz), anti-phosphorylated-EGF receptor and anti-EGF receptor (Santa Cruz), anti-Rac1 (Cell Signaling Technology), anti-ERK and anti-phosphorylated ERK (Cell Signaling Technology), and anti-N-Tyrosine (Saint Cruz) followed by the appropriate horseradish peroxidase-conjugated secondary antibodies.

In some experiments, collagen IV, collagen I, p47^{phox}, NOX4, N-Tyrosine, phosphorylated and total EGF receptor bands, and β-actin bands were quantified by densitometry analysis and the levels of collagen IV, collagen I, N-Tyrosine, p47^{phox}, NOX4, and phosphorylated EGF receptor expressed as collagen IV/β-actin, N-Tyrosine/β-actin, p47^{phox}/β-actin, NOX4/β-actin or phosphorylated EGF receptor/EGF receptor.

In some experiments 1 µl spot urine was analyzed for presence of albuminuria on 10% SDS-PAGE and stained with SimplyBlue (Invitrogen)

Statistical Analysis

We used ANOVA and Tukey's Post Hoc Test to analyze the statistical differences between multiple groups. $p < 0.05$ was considered statistically significant. Unpaired two tailed t-test was used for comparison of two groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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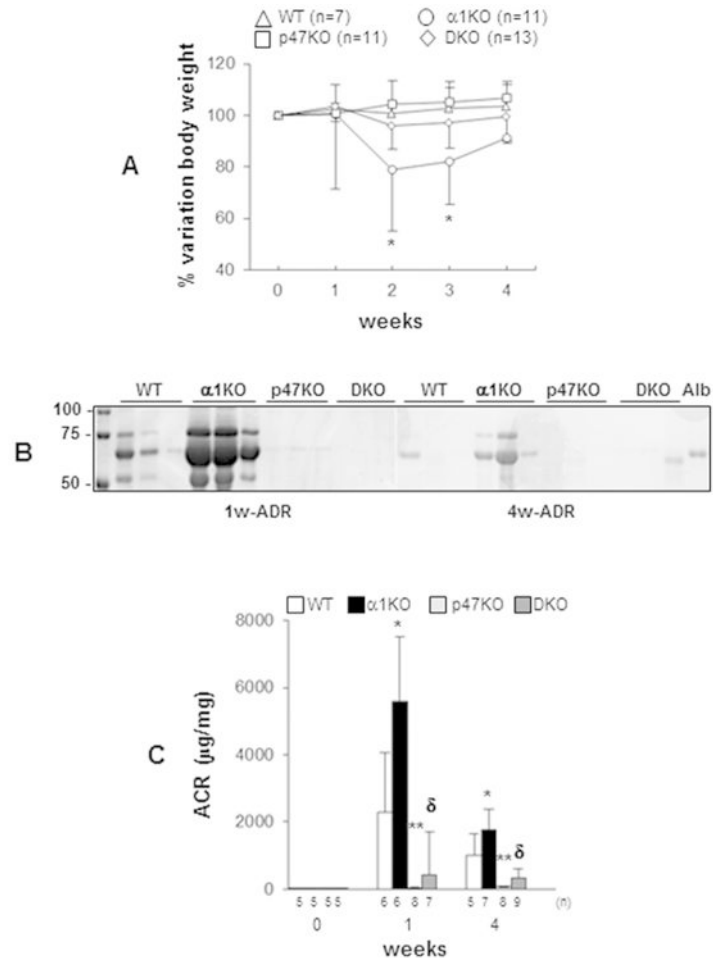


Figure 1. Loss of p47^{phox} improves adriamycin-mediated albuminuria

Kidney injury was induced by a single tail vein injection of adriamycin (10 mg/Kg b.w.) in wild type (WT), integrin α 1KO, p47^{phox}KO, integrin α 1KO/p47^{phox}KO (DKO) mice and changes in body weight (A) and urine albuminuria (B, C) were examined over time. (*) indicates significant differences ($p < 0.05$) between α 1KO and WT, or α 1KO and p47^{phox}KO, or α 1KO and DKO. Values are the mean \pm SD of the number of mice indicated. (B) SimplyBlue staining of 1 μ l urine showing elevated albuminuria in adriamycin-treated integrin α 1KO mice compared to WT, p47^{phox}KO or DKO mice. Alb = mouse serum albumin (1.25 μ g/lane). (C) Urine albumin excretion expressed as albumin-to-creatinine ratio (ACR) in untreated or adriamycin-treated WT, α 1KO, p47^{phox}KO, and DKO mice. Values represent the mean \pm SD of the mice indicated. Difference between injured WT and α 1KO (*) or injured WT and p47^{phox}KO (**) or injured α 1KO and DKO (δ) mice were significant ($p < 0.05$).

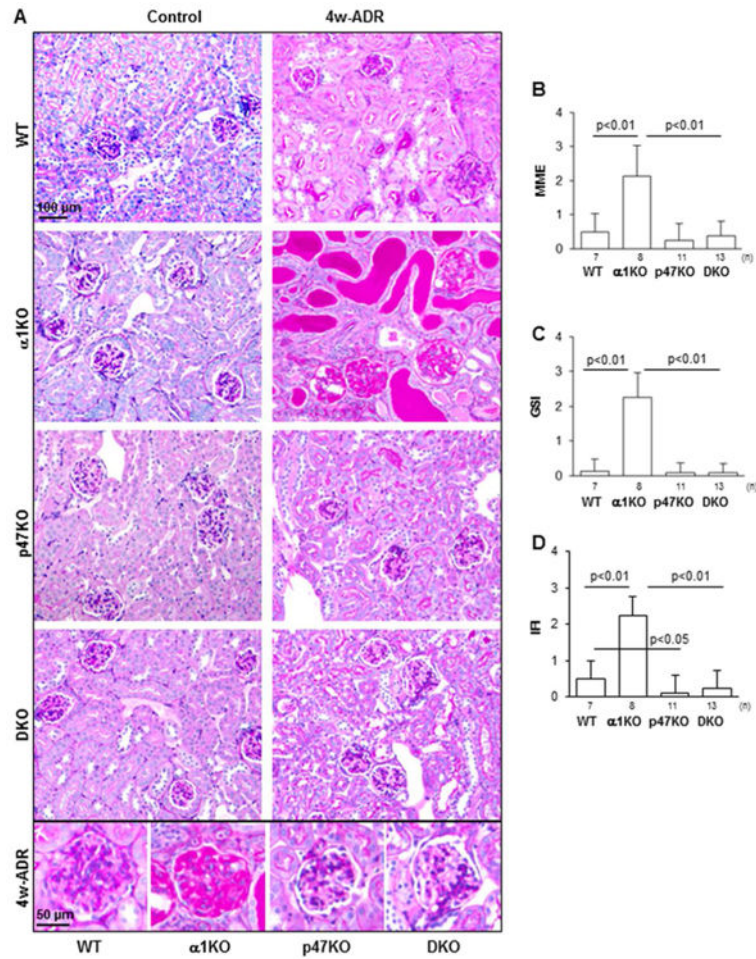


Figure 2. Loss of p47^{phox} improves adriamycin-induced kidney injury

(A) Representative light micrographs of periodic-acid-Schiff (PAS) stained kidneys from uninjured (Control) or adriamycin-treated (4w-ADR) WT, $\alpha 1$ KO, p47^{phox}KO, and DKO mice. Loss of p47^{phox} rescues the severe glomerular and tubular damage observed in injured integrin $\alpha 1$ KO mice. (B-D) Matrix mesangial expansion (MME) (B), global glomerulosclerosis index (GSI) (C), and interstitial fibrosis index (IFI) (D) were evaluated 4 weeks after adriamycin injection and scored as described in the Methods. Values represent the mean \pm SD of the number of mice indicated.

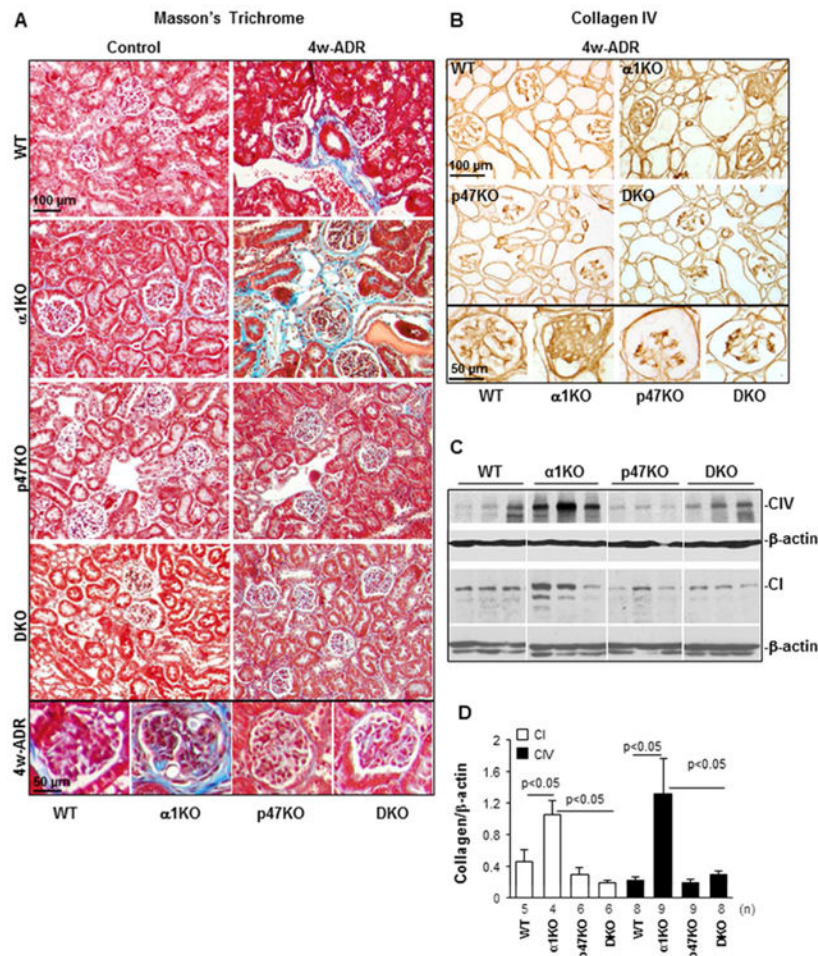


Figure 3. Loss of p47^{phox} improves adriamycin-induced matrix deposition

(A) Masson's Trichrome staining of kidneys from uninjured (Control) or adriamycin-treated (4w-ADR) WT, α 1KO, p47^{phox}KO, and DKO mice. Note the presence of fibrillar collagen (blue) in both glomeruli and tubules of integrin α 1KO mice which was reduced in kidneys of p47^{phox}KO and DKO mice. (B) Collagen IV staining of kidney sections from 4 weeks injured WT, α 1KO, p47^{phox}KO and DKO mice. Note the high levels of glomerular collagen IV in integrin α 1KO mice which were reduced in kidneys of p47^{phox}KO and DKO mice. (C) Equal amount of kidney lysates (20 μ g/lane) from WT, α 1KO, p47^{phox}KO, and DKO mice (n=3 shown) 4 weeks after adriamycin injection were analyzed by Western blot for levels of collagen IV and collagen I. (D) The collagen I, collagen IV and β -actin bands were analyzed by densitometry analysis and the levels of collagen IV are expressed as collagen I/ β -actin or collagen IV/ β -actin ratio. The values represent the mean \pm SD of the number of kidneys indicated.

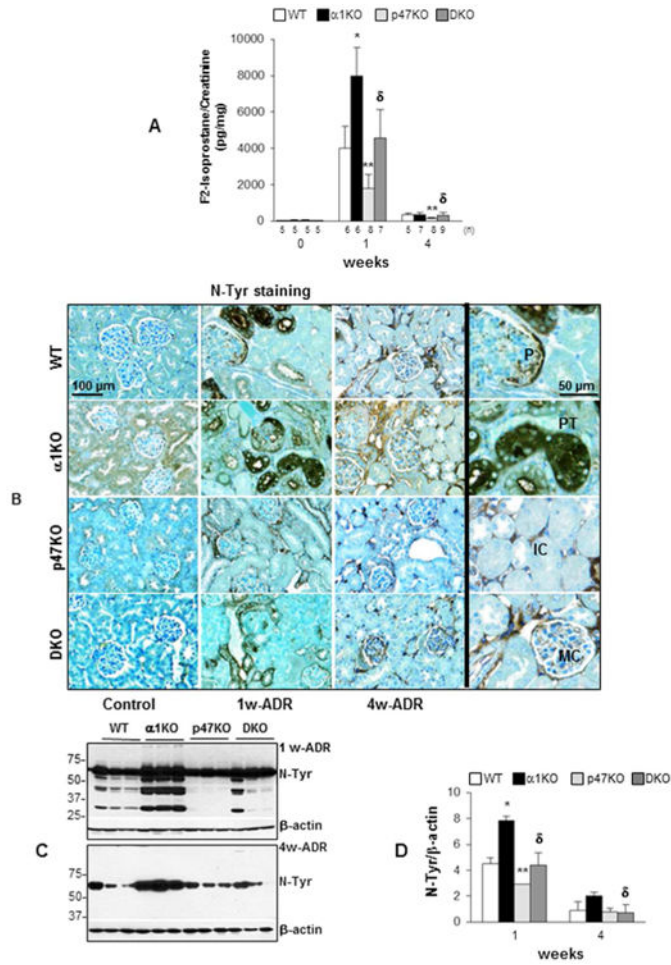


Figure 4. Loss of p47^{phox} improves adriamycin-induced oxidative stress
(A) The levels of urinary F2-isoprostane in the mice indicated were analyzed by ELISA before or after adriamycin injection and were expressed F2-isoprostane/urine creatinine ratio. Values are the mean \pm SD of the number of mice indicated. (*), (**), and (δ) are as in Fig. 1. **(B)** Paraffin sections of kidneys from uninjured (Control) or adriamycin-treated (1w-ADR and 4w-ADR) WT, α 1KO, p47^{phox}KO, or DKO mice were stained with anti-nitrotyrosine (N-Tyr) antibodies to evaluate the degree of oxidative stress-induced tyrosine nitration (brown staining). Slides were counterstained with toluidine blue (blue staining). The high-magnification images on the right show nitrotyrosine staining in infiltrating cells (IC), mesangial cells (MC), podocytes (P), and proximal tubules (PT). **(C)** Equal amount of kidney lysates (20 μ g/lane) from WT, α 1KO, p47^{phox}KO, and DKO mice (n=3) 1 and 4 weeks after adriamycin injection were analyzed by Western blot for levels of nitro-tyrosine. **(D)** The nitro-tyrosine and β -actin bands were analyzed by densitometry analysis and the levels of tyrosine nitration are expressed as N-Tyr/ β -actin ratio. The values represent the mean \pm SD of 3 kidneys/genotype. (*), (**), and (δ) are as in Fig. 1.

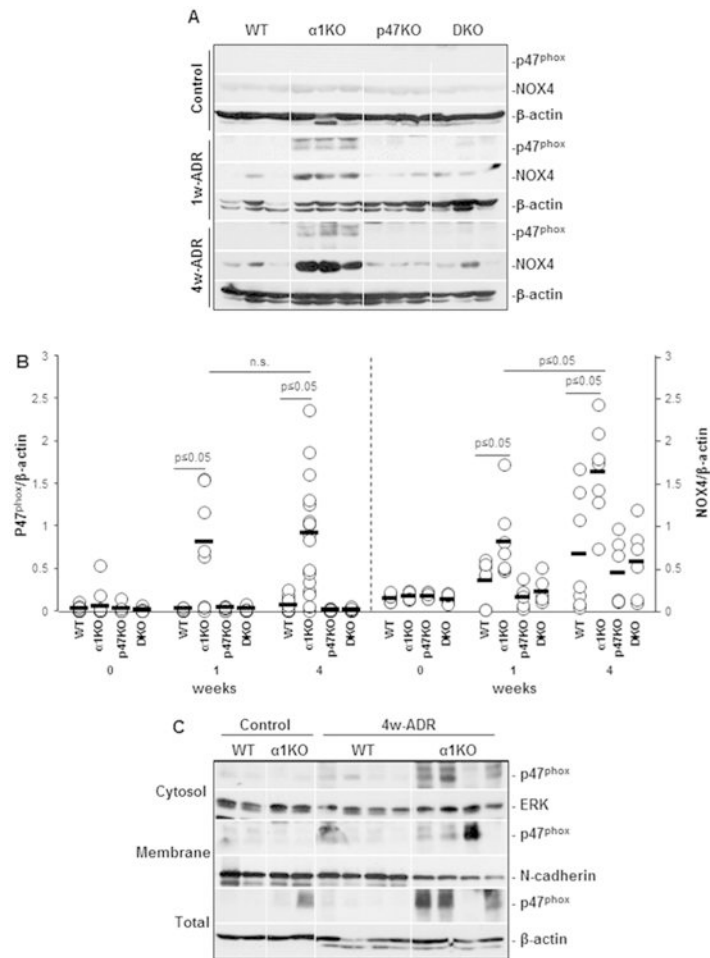


Figure 5. Analysis of p47^{phox} levels and localization in kidneys from uninjured and adriamycin-injured mice

(A) Equal amount of kidney lysates (20 μ g/lane) from uninjured (Control) or adriamycin-treated (1w-ADR and 4w-ADR) WT, α 1KO, p47^{phox}KO, and DKO mice (n=3 shown) were analyzed by Western blot for levels of p47^{phox} and NOX4. (B) The p47^{phox}, NOX4 and β -actin bands were analyzed by densitometry and the levels of p47^{phox} and NOX4 are expressed as p47^{phox}/ β -actin or NOX4/ β -actin ratio. Open circles represent values of individual kidneys, while the bar represents the mean value. (C) Equal amount of kidney lysates (Total) membrane enriched (Membrane) and membrane deprived (Cytosol) fractions (40 μ g/lane) from uninjured (Control) or adriamycin-treated (4w-ADR) WT and integrin α 1KO mice (n=3 shown) were analyzed by Western blot for p47^{phox} localization. Membranes were re-blotted with anti-ERK, anti-N-cadherin, and anti β -actin antibodies to verify the purity of various fractions.

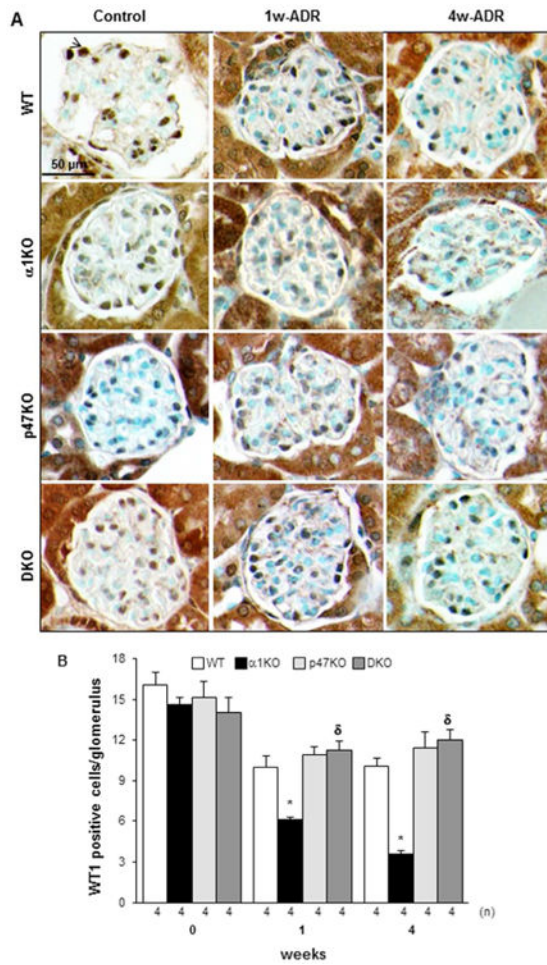


Figure 6. Loss of p47^{phox} improves adriamycin-induced podocyte loss
(A) WT-1 staining in kidneys from uninjured (Control) or adriamycin-treated (1w-ADR and 4w-ADR) WT, α1KO, p47^{phox}KO, and DKO mice. Slides were counterstained with toluidine blue (blue staining). **(B)** The number of WT1 positive cells (arrowhead) was evaluated in 20 randomly chosen glomeruli per kidney. The values represent the mean ± SD of the kidneys indicated. (*) and (δ) are as in Fig. 1. Note the decreased number of podocytes in injured integrin α1KO mice which was reduced in kidneys of p47^{phox}KO and DKO mice.

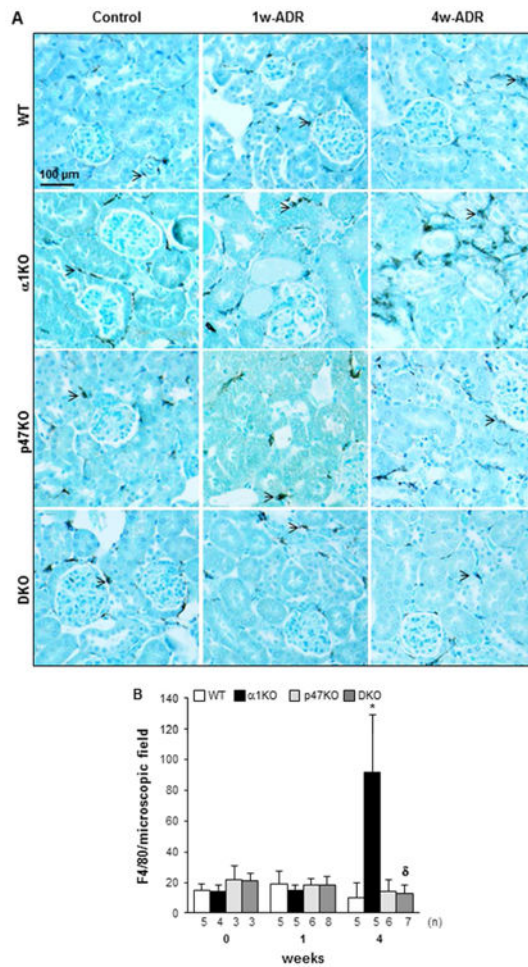


Figure 7. Loss of p47^{phox} improves adriamycin-induced macrophage infiltration

(A) F4/80 staining in kidneys from uninjured (Control) or adriamycin-treated (1w-ADR and 4w-ADR) WT, α 1KO, p47^{phox}KO, and DKO mice. Slides were counterstained with toluidine blue (blue staining). (B) The number of macrophages (arrowhead) was evaluated in 20 randomly chosen field per kidney. The values represent the mean \pm SD of the kidneys indicated. (*) and (δ) are as in Fig. 1. Note the increased number of macrophages in 4 week injured integrin α 1KO mice which was reduced in kidneys of p47^{phox}KO and DKO mice.

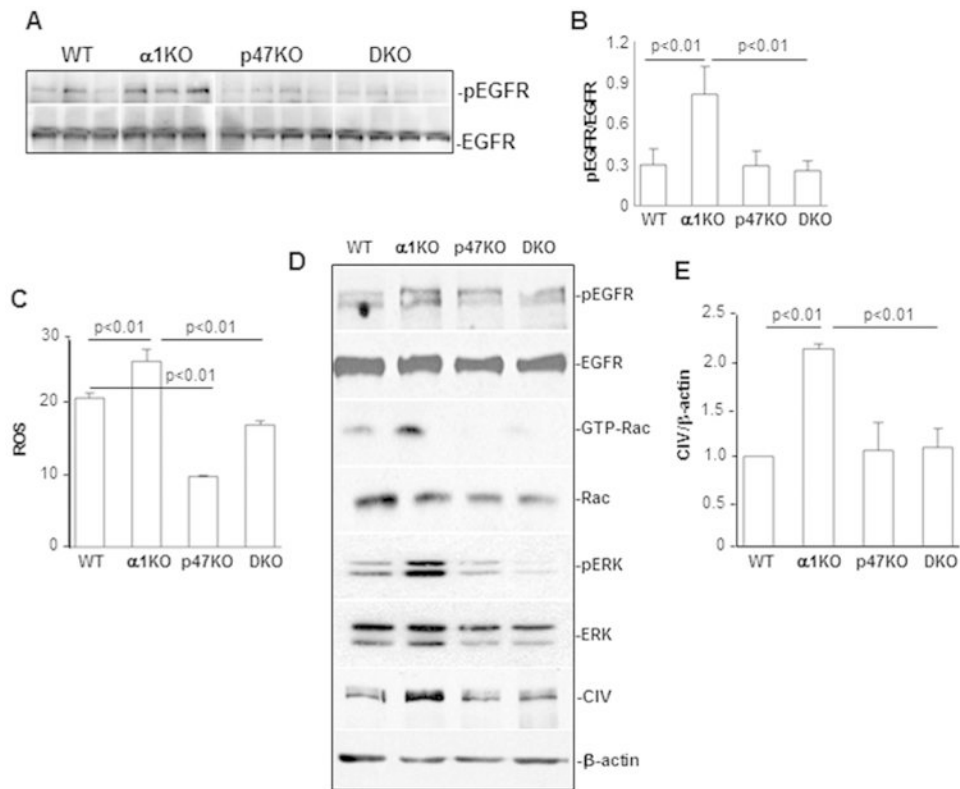


Figure 8. Loss of p47^{phox} improves adriamycin-mediated activation of pro-fibrotic pathways (A) Equal amount of glomerular lysates (20 μg/lane) from 1 week adriamycin-treated WT, α1KO, p47^{phox}KO, and DKO mice were analyzed by Western blot for levels of phosphorylated and total EGF receptor (EGFR). (B) pEGFR and EGFR bands were quantified by densitometry analysis and the levels of activated EGFR are expressed as pEGFR/EGFR ratio. Values are the mean ± SD of 3-4 mice/genotype. (C) Primary mesangial cells isolated from the mice indicated were cultured in serum free medium. Twenty-four hours later, cells were incubated with 2 μM dihydro-rhodamine and 2 hours later ROS generation was determined by FACS as described in the Methods. The level of ROS was expressed as a ratio of fluorescence intensity of cells with dihydro-rhodamine vs. that of cells without dihydro-rhodamine. Data represent the mean ± SD of 3 samples/genotypes. The same experiment was repeated twice with similar result. (D) Equal amount of cell lysates (20 μg/lane) from the serum starved cells indicated were analyzed by Western blot for the phosphorylated and total EGFR, activated and total Rac, phosphorylated and total ERK, as well as collagen IV (CIV) levels. (E) The collagen IV and β-actin bands were analyzed and expressed as described in Fig. 3. The values represent the mean ± SD of 3 independent experiments.

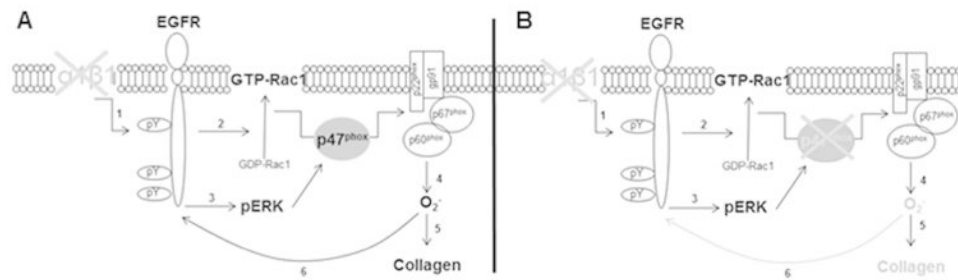


Figure 9.

Schematic reorientation of how loss of p47^{phox} ameliorates the fibrotic response in integrin $\alpha 1\beta 1$ KO cells or mice. **(A)** Loss of integrin $\alpha 1\beta 1$ leads to increased activation of EGFR **(1)** which in turn promotes p47^{phox} membrane translocation and the assembly of the NADPH oxidase system by activating Rac1 **(2)** and/or ERK **(3)**. NADPH-generated superoxide **(4)** can then exert a pro-fibrotic action by promoting collagen synthesis **(5)** and prolonging EGFR activation **(6)**. **(B)** Loss of p47^{phox} reduces this fibrotic response by dampening ROS production **(4)**, collagen synthesis **(5)**, and EGFR activation **(6)**.