Oxidatively stressed extracellular microenvironment drives fibroblast activation and kidney fibrosis

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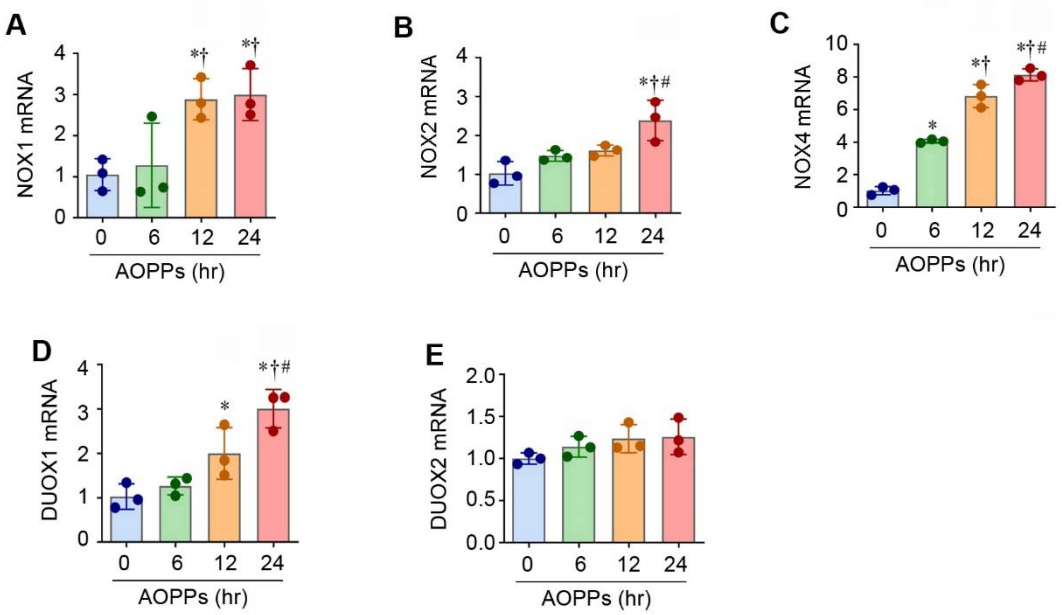

Fig. S1. AOPPs induces NADPH oxidase expression in renal interstitial fibroblasts in vitro. (A-E) qRT-PCR analyses show mRNA levels of NOX1, NOX2, NOX4, DUOX1 and DUOX2 in different groups as indicated. $^{*}P < 0.05$ versus controls, $^{\dagger}P < 0.05$ versus AOPP 6 h, $^{\#}P < 0.05$ versus AOPP 12 h (n = 3).

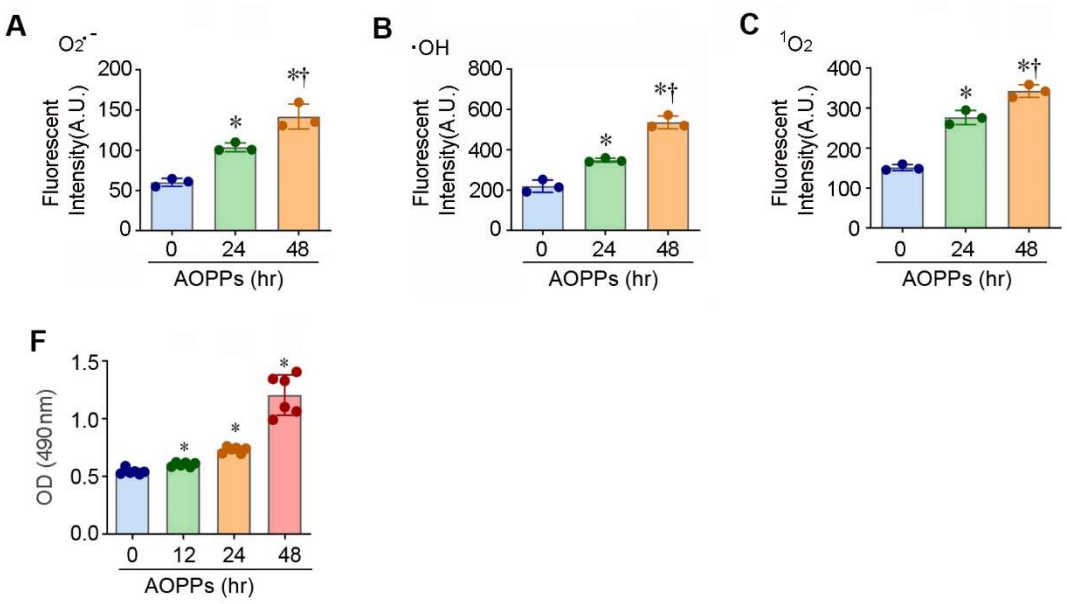

Fig. S2. AOPPs increased ROS production in renal fibroblasts and promotes cell proliferation. NRK-49F cells were treated with AOPPs (50 μg/ml) for various periods of time as indicated. The fluorescence intensity of superoxide radical anion ($O_2•^-$) (A), hydroxyl radical (•OH) (B) and singlet oxygen ($^1O_2$) (C) in cell supernatants were measured by a fluorescence spectrophotometer. $^*P< 0.05$ versus controls, $^†P < 0.05$ versus AOPP 24 h (n = 3). (D) Quantitative colorimetric MTT assay shows that AOPPs promoted NRK-49F cells proliferation in a time-dependent manner. OD, optical density. NRK-49F cells were treated with AOPPs (50 μg/ml) for various periods of time as indicated. $^*P < 0.05$ versus controls.

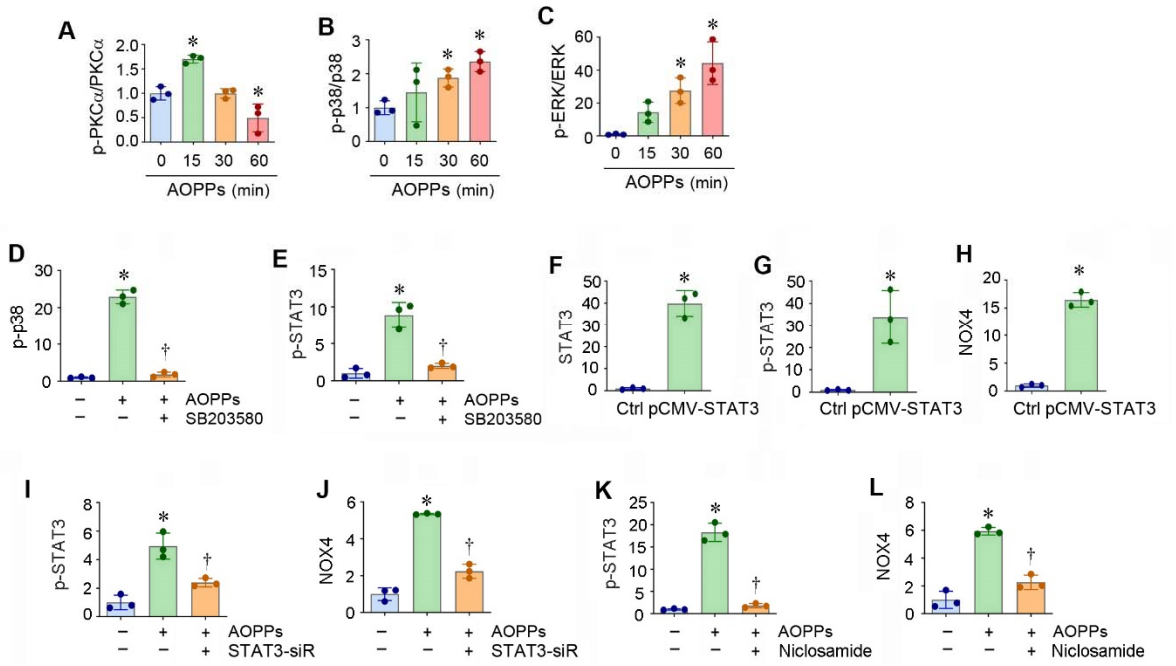

Fig. S3. Activation of NOX4 by AOPPs activates PKCα/MAPK/STAT3/NOX4 signal cascade in vitro. (A-C) Quantitative data of Western blot analyses show that AOPPs activated PKCα/MAPK signal cascade. NRK-49F cells were treated with AOPPs (50 μg/ml) for various periods of time as indicated. Cell lysates were subjected to Western blot analyses for p-PKCα, PKCα, p-p38, p38, p-ERK1/2, ERK1/2 and α-tubulin. $^{*}P < 0.05$ versus controls (n=3). (D, E) Quantitative data of Western blot analyses show that blockade of p-p38 suppressed p-STAT3 expression in NRK-49F cells. NRK-49F cells were pretreated with p38 inhibitor SB230580 (10 μM) for 1 h, then treated with AOPPs (50 μg/ml) for 30 min. $^{*}P < 0.05$ versus controls, $^{\dagger}P < 0.05$ versus AOPPs (50 μg/ml) (n=3). (F-H) Quantitative data of Western blot analyses show that overexpression of STAT3 induced the expression of NOX4 in NRK-49F cells. $^{*}P < 0.05$ versus controls. (I, J) Quantitative data of Western blot analyses show that knockdown of STAT3 inhibited AOPPs-induced NOX4 expression in NRK-49F cells. $^{*}P < 0.05$ versus controls, $^{\dagger}P < 0.05$ versus AOPPs (50 μg/ml) (n=3). (K, L) Quantitative data of Western blot analyses show that blockade of p-STAT3 suppressed AOPP-induced NOX4 expression in NRK-49F cells. NRK-49F cells were pretreated with STAT3 inhibitor Niclosamide (5 μM) overnight, then treated with AOPPs (50 μg/ml) for 24 h. $^{*}P < 0.05$ versus controls, $^{\dagger}P < 0.05$ versus AOPPs (50 μg/ml) (n=3).

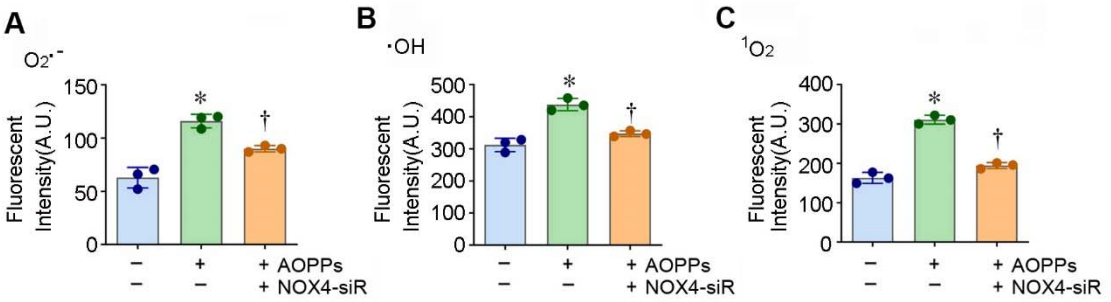

Fig. S4. Knockdown of NOX4 reduces ROS production in renal fibroblasts. NRK-49F cells were transfected with either control siRNA or NOX4-siRNA for 24 h, and then cells were treated with AOPPs (50 μg/ml) for additional 24 h. The fluorescence intensity of superoxide radical anion ($O_2^{\bullet -}$) (A), hydroxyl radical (•OH) (B) and singlet oxygen ($^1O_2$) (C) in cell supernatants were measured by a fluorescence spectrophotometer. $^{*}P < 0.05$ versus controls, $^{\dagger}P < 0.05$ versus AOPPs (50 μg/ml) (n = 3).

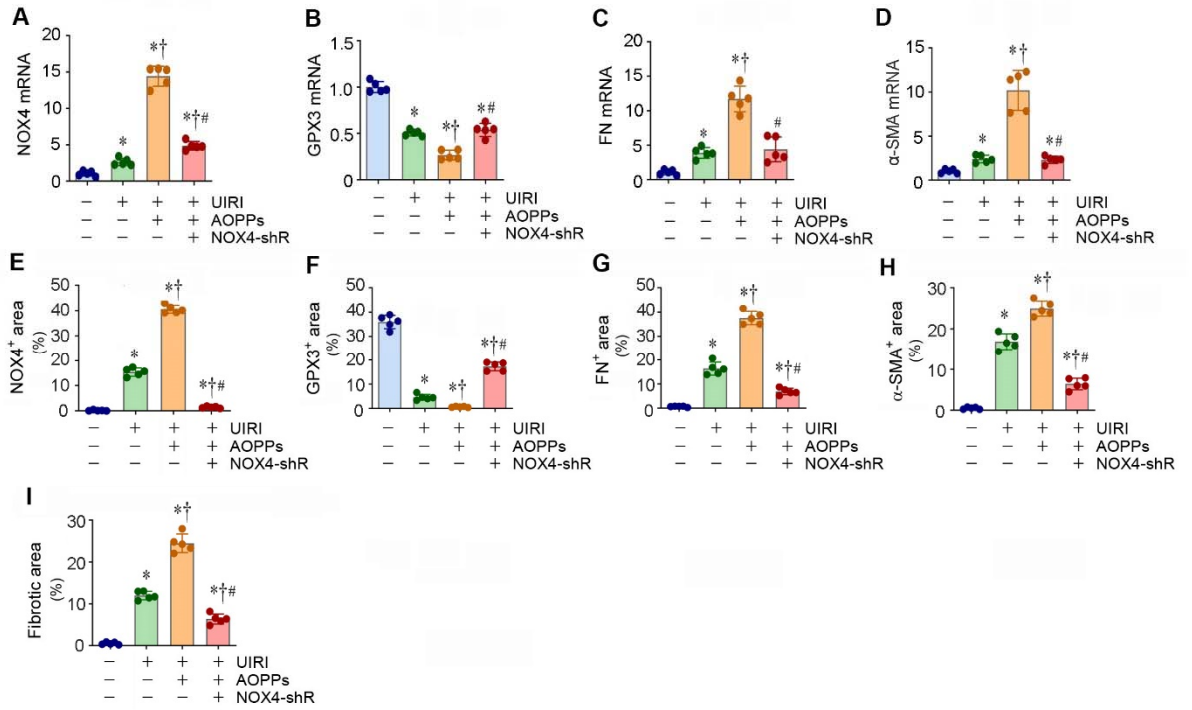

Fig.S5. Knockdown of NOX4 ameliorates fibrosis-related gene expression induced by AOPPs in vivo. (A-D) qRT-PCR analyses show mRNA levels of NOX4, GPX3, FN and α-SMA in different groups as indicated. $^{*}P < 0.05$ versus sham; $^{\dagger}P < 0.05$ versus UIRI; $^{\#}P < 0.05$ versus UIRI+AOPPs (n=5). (E-I) Graphic presentation shows the semi-quantitative determination of renal $NOX4^{+}$, $GPX3^{+}$, FN^{+} and α-SMA^{+} area and fibrotic lesions in different groups. At least 10 randomly selected fields were assessed, and results averaged for each kidney. $^{*}P < 0.05$ versus sham; $^{\dagger}P < 0.05$ versus UIRI; $^{\#}P < 0.05$ versus UIRI+AOPPs (n=5).

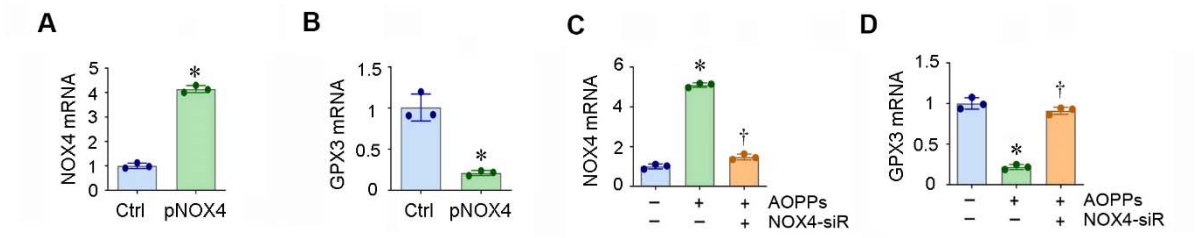

Fig. S6. Overexpression of NOX4 inhibits GPX3 mRNA expression and knockdown of NOX4 restores GPX3 mRNA expression in HK-2 cells. (A, B) qRT-PCR analyses show that overexpression of NOX4 inhibited GPX3 mRNA in HK-2 cells. HK-2 cells were transfected with pcDNA3 or pCMV-NOX4 plasmids for 24 h. $^{*}P < 0.05$ versus controls. (C, D) qRT-PCR analyses show that knockdown of NOX4 restored GPX3 gene expression after AOPPs treatment in HK-2 cells. HK-2 cells were transfected with either control siRNA or NOX4-siRNA for 24 h, and then cells were treated with AOPPs (50 μg/ml) for additional 24 h. $^{*}P < 0.05$ versus controls, $^{\dagger}P < 0.05$ versus AOPPs (50 μg/ml) (n=3).

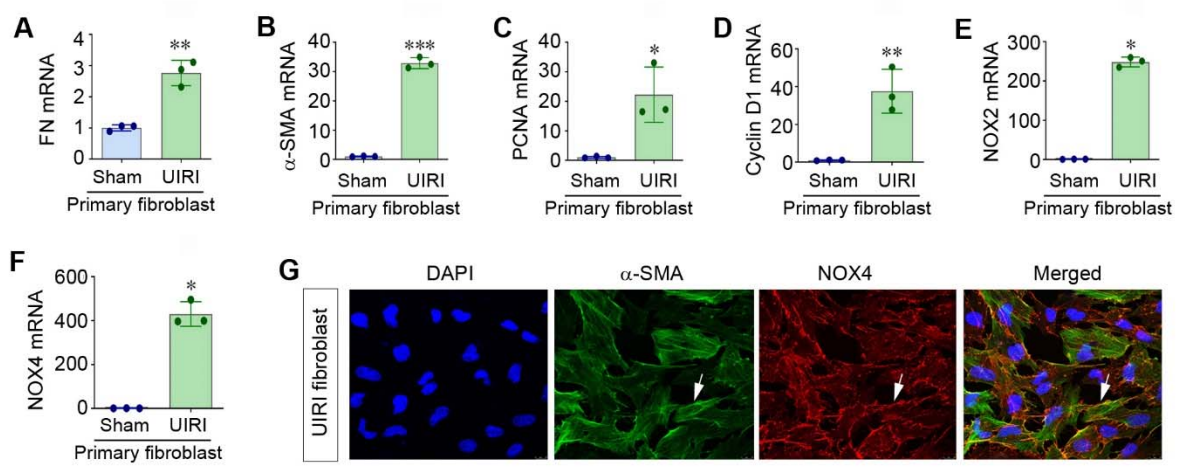

Fig. S7. Primary fibroblasts from the fibrotic kidney express high-level mRNA of fibrosis-related genes. (A-F) qRT-PCR analyses show mRNA levels of FN, α-SMA, PCNA, cyclin D1, NOX2 and NOX4 in different groups as indicated. Primary fibroblasts were extracted from sham and UIRI mice and then were cultured for 24 h. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus sham (n=3). (G) Representative micrographs show the expression and localization of α-SMA and NOX4 in UIRI primary fibroblasts by co- immunofluorescence staining. Arrows indicate positive staining.

Supplementary Table S1. The sources of antibodies used in this study

Antibodies	Catalogue number	Company	Location
Primary antibodies			
anti-Gpx3	Ab256470	Abcam	Cambridge, MA
anti-NOX4	BA2813	Boster Biological Technology	Wuhan, China
anti-PCNA	sc-56	Santa Cruz Biotechnology	Santa Cruz, CA
anti-Cyclin D1	sc-8396	Santa Cruz Biotechnology	Santa Cruz, CA
anti-fibronectin	F3648	Sigma-Aldrich	St. Louis, MO
anti-α-SMA	A2547	Sigma-Aldrich	St. Louis, MO
anti-c-fos	BA0207-2	Boster Biological Technology	Wuhan, China
anti-p-p38	9211S	Cell Signaling Technology	Danvers, MA
anti-p38	9212S	Cell Signaling Technology	Danvers, MA
anti-p-PKCα	sc-377565	Santa Cruz Biotechnology	Santa Cruz, CA
anti-PKCα	59754	Cell Signaling Technology	Danvers, MA
anti-p-ERK1/2	9101S	Cell Signaling Technology	Danvers, MA
anti-ERK1/2	4695S	Cell Signaling Technology	Danvers, MA
anti-p-STAT3	9145S	Cell Signaling Technology	Danvers, MA
anti-STAT3	9139S	Cell Signaling Technology	Danvers, MA
anti-Vimentin	5741s	Cell Signaling Technology	Danvers, MA
anti-PDGFR-β	sc-432	Santa Cruz Biotechnology	Santa Cruz, CA
anti-E-cadherin	3195s	Cell Signaling Technology	Danvers, MA
anti-α-Tubulin	RM2007	Ray Antibody Biotech	Peachtree Corners, GA
Secondary antibodies			
Goat anti-mouse	BA1050	Boster Biological Technology	Wuhan, China
Goat anti-rabbit	BA1054	Boster Biological Technology	Wuhan, China

Supplementary Table S2. Nucleotide sequences of the primers used for qPCR

Mouse gene	Primer Sequence 5' to 3'	
	Forward	**Reverse**
Fibronectin	GATGAGCTTCCCCAACTGGT	CTGGGTTGTTGGTGGGATGT
α-SMA	CATCGTGTTGGATTCTGGGG	GTCACGAAGGAATAGCCACG
Gpx3	CATCCTGCCTTCTGTCCCTG	CGATGGTGAGGGCTCCATAC
NOX4	TGTCTGCATGGTGGTGGTAT	CTTCAACAAGCCACCCGAAA
NOX1	CGAAGTGGCTGTACTGGTTG	AAAGGCACCCGTCTCTCTAC
NOX2	TGCACATCTGTTCAACGTGG	AACCGAGTCACAGCCACATA
Duox1	TGTGTACCAGCCCTTGAGAG	TGTTTCCACACTCACCAGGT
Duox2	CTGCGGTTTGGGTCATATGG	TACAATCAGCCAAGCCCAGA
PCNA	AAGTTTTCTGCGAGTGGGGA	ACAGTGGAGTGGCTTTTGTGA
CyclinD1	TCAAGTGTGACCCGGACTG	GACCAGCTTCTTCCTCCACTT
β-actin	AAGATCAAGATCATTGCTCCTCCTG	CGCAGCTCAGTAACAGTCCG

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