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$PM_{2.5}$ exposure induced renal injury via the activation of the autophagic pathway in the rat and HK-2 cell

Xiaoliu Huang[1,2], Zhitong Zhou[1], Xinwen Liu[1], Jue Li[1*] and Lijuan Zhang[1*]

Abstract

Background: Exposure to airborne fine particulate matter ($PM_{2.5}$) has been declared to be harmful to human kidney. However, whether activation of the autophagic pathway plays key roles in the nephrotoxicity caused by $PM_{2.5}$ exposure is still poorly understood. The aim of this study was to explore the mechanism of kidney damage after $PM_{2.5}$ exposure in vivo and in vitro.

Results: In the present study, statistically significant alterations in water intake, urine flow rate and mean blood pressure were observed between the concentrated $PM_{2.5}$ ($PM_{2.5}$) group and the filtered air (FA) group. Exposed animals showed severe edema of renal tubular epithelial cells, capillary congestion, reduction of the glomerular urinary space and early pro-fibrotic state. Moreover, significant increases in the levels of early kidney damage markers were observed in the exposed rats and these animals exhibited more apoptosis rate in kidney cells. In addition, $PM_{2.5}$ exposure activated the autophagic pathway, as evidenced by LC3-I to LC3-II conversion, activation of P62 and beclin-1. All of these effects are in concurrence with the presence of more autophagosomes both in vivo and in vitro after $PM_{2.5}$ exposure.

Conclusions: Taken together, our findings indicated that $PM_{2.5}$ induced renal function impairment via the activation of the autophagic pathway in renal tubular epithelial cells.

Keywords: $PM_{2.5}$, Renal injury, Autophagic pathway

Background

A report on the World Health Organization (WHO) in 2016 estimated that nearly 3 million people die every year due to air pollution-related diseases [43]. $PM_{2.5}$ refers to the complex mixture small particles and liquid droplets with aerodynamic diameter ≤ 2.5 μm in the atmosphere, and is an important indicator for assessing air pollution [43, 55]. $PM_{2.5}$ mainly deposits in lung tissues after inhaling the respiratory tract, and can even diffuse in the blood circulation system through the alveolar-capillary barrier, affecting distal organs such as liver and kidneys [25, 36]. Zhang et al. [51] found that severe histopathological changes in hepatocyte edema and glomerular atrophy occurred in rats induced by intratracheal instillation of $PM_{2.5}$. Recently, some epidemiological studies have reported a strong and consistent association between $PM_{2.5}$ exposure and renal function decline [5, 30]. Bowe et al. [2] estimated that the global burden of incident chronic kidney disease (CKD) attributable to $PM_{2.5}$ was about 6.95 million in 2016, and air pollution may be an important risk factor for the prevalence of kidney disease. Experimental studies have also shown that mid-/long-term exposure to high levels of $PM_{2.5}$ can induce kidney damage in rodent models [1, 16].

Although the respiratory system is the primary target organ for toxicity of $PM_{2.5}$, epidemiological evidence has indicated that $PM_{2.5}$ exposure is an important risk

*Correspondence: jueli1959@163.com; zhangxiaoyi@tongji.edu.cn
[1] Shanghai Pudong New Area Mental Health Center, Tongji University School of Medicine, Shanghai 200124, China
Full list of author information is available at the end of the article

factor for cardiovascular morbidity and mortality [3]. The strong correlation between chronic kidney disease and cardiovascular disease has been demonstrated by both observational studies and a meta-analysis [33]. Renal insufficiency and patients undergoing dialysis are prone to cardiovascular diseases have been reported in clinical practice, which indicates a potential interaction between the kidney and the cardiovascular system [1]. Tavera Busso et al. observed more severe alterations of fibrosis, mesangial expansion, tubular epithelial cells detachment, decrease glomerular and tubular lumen volumes in a spontaneously hypertensive rat (SHR) model after sub-chronic exposure to $PM_{2.5}$ than healthy animals [43]. Moreover, histological analysis performed by Yan et al. [49] showed $PM_{2.5}$ advanced glomerulosclerosis and a punctual tubular damage of the kidney in a diabetic rat model after subchronic exposure to $PM_{2.5}$. Endothelial dysfunction is viewed as one of the common pathophysiological mechanisms in cardiovascular disease and chronic kidney disease [40]. Aztatzi-Aguilar et al. [1] revealed that early kidney damage was induced by subchronic exposure to $PM_{2.5}$ in rats due to angiotensin/bradykinin systems' imbalance and a statistically significant increment in median blood pressure. At present, studies have confirmed that autophagy of cardiovascular endothelium was the potential mechanism of $PM_{2.5}$-induced cardiovascular dysfunction [12, 45, 56]. In contrast, the contribution of $PM_{2.5}$ exposure to endothelial damage in kidneys via the autophagic pathway has not been fully clarified at the cellular and molecular levels.

Autophagy is a physiological process whereby eukaryotic cells undergo self-digestion, which allows the degradation and recycling of unnecessary intracellular proteins and dysfunctional organelles via the autophagosome and lysosomes [9, 10, 41]. The formation of the autophagosome involves the action of multiple autophagy-related genes (Atgs), such as beclin-1 (Atg6), and microtubule-associated proteins light chain 3 (LC3) [9, 52]. Sequestosome1/P62 (SQSTM1) is a regulatory autophagy protein originally identified as a binding protein for nonreceptor-type tyrosine kinase $P56^{Lck}$ [19, 56]. Moreover, P62 has been found play key roles in the selective autophagy signaling pathway because of the interaction with both Keap1 and LC3. Autophagy is considered to be an adaptive response to stress, and plays an important role in maintaining cellular homeostasis during pathogenic conditions and diseases, as well as regulating caspase-independent apoptotic cell death [10, 56].

The kidney is an organ with rich and diverse endothelial cells [44]. Ding et al. [11] found that gold nanoparticles could induce autophagy of hypoxic human proximal tubule epithelial (HK-2) cells. The basal autophagy in the kidney is vital for the normal homeostasis of proximal tubules, but abnormal autophagy can impair renal function and increased p62 levels and oxidative stress [23, 28]. Therefore, we hypothesized that $PM_{2.5}$ exposure induced autophagy of renal endothelial cells leading to the deterioration of the renal function. This work was designed to study the effect of $PM_{2.5}$ exposure on kidney function of Sprague Dawley (SD) rats using a physiological inhalation exposure system. HK-2 cell line was also employed as an in vitro model to further investigate the potential mechanism triggered by $PM_{2.5}$ exposure to renal dysfunction. Moreover, kidney injury molecule type-1 (KIM-1), a specific biomarker of damage to tubular cells, was detected to assess renal injury after $PM_{2.5}$ exposure. Our findings would provide important insight into the involvement of $PM_{2.5}$ pollution in kidney damage.

Materials and methods

Animal maintenance

A total of 20 SD male rats of 4-week-old were purchased from Jiexijie experimental animal co., LTD. (Shanghai, China). All animals were raised in the specific pathogen-free (SPF) environmental conditions at a temperature of 22–24 °C with 50–60% relative humidity and a 12-h day/night cycle. Rats had been allowed to drink and eat freely until when they were kept in metabolic cages. After 1-week acclimation, rats were randomly divided in two groups (10 per group): one group was exposed to filtered air and the other group was exposed to concentrated $PM_{2.5}$. The study was subject to approval by the institutional animal care and use committees of Tongji University.

$PM_{2.5}$ exposure system

A physiologically inhaled $PM_{2.5}$ exposure system (Shanghai-MRTAS, patent #201510453600.8) was provided by the meteorological service of Shanghai. Concentrated particulate matter of the exposure system was generated using a versatile aerosol concentration enrichment system (VACES) as previously described [26, 47]. The $PM_{2.5}$ exposure system which basically keeps the chemical properties of $PM_{2.5}$ before concentration was located at the school of public health, Fudan University (130 Dong'an Road, Shanghai, China), where ambient $PM_{2.5}$ particles come mainly from traffic exhaust. The exposure experiments were performed for 8 h per day, 5 consecutive days per week from October 2018 to January 2019. The two $PM_{2.5}$ monitors (PDR-1500, Thermo Scientific) were connected to the air inlets of the exposure and control chambers, respectively, and the real-time concentrations of $PM_{2.5}$ in the atmosphere were determined by spectrophotometry [13].

Metabolic cage

After 5-day exposure, rats were placed in metabolic cages (Yuyan instrument, China) for 24 h each week. During the 24-h period, foods were not given to avoid contamination of the urine. The urine was harvested and water intake was estimated, then the urinary flow was calculated. These data were adjusted for body weight.

Measurement of blood pressure

MedLab biological signal acquisition and processing system (Nanjing Calvin Biotechnology, China) was used to record blood pressure. Animals were first fixed in the sleeve and warmed to a suitable temperature prior to each measurement to ensure adequate diastolic blood pressure. Then, more than three blood pressure measurements using a cutoff ring and a transducer placed on the proximal vein of the tail were performed. Basal measurement was evaluated 1 day before the initiation of the 12-week exposure and on the 7th day after every weekly exposure, with a metabolic cage period of 1 day for the animals to rest and hydrate. The mean blood pressure (MBP) was calculated as follows [1]:

$$\mathrm{MBP} = \text{diastolic pressure} + 0.33(\text{systolic pressure} - \text{diastolic pressure}).$$

Histology

Renal tissues were fixed with formaldehyde for more than 24 h, and then were dehydrated with alcohol, clarified by xylene, and embedded in paraffin. Slides were cut and stained with hematoxylin and eosin (H&E) stain, as well as Masson's Trichrome stain (Sigma Aldrich, USA). Images of three slides per animal were analyzed, were captured and determined by an optical microscope (Olympus, Japan); the median was obtained for each animal.

Transmission electron microscopy

Treated cells and kidney tissues were immediately fixed in 2.5% glutaraldehyde at 4 °C, then washed 3 times with 0.1 M phosphate-buffered saline (PBS) and underwent in osmic acid for 2 h after post-fixation at room temperature [18]. Subsequently, cells were washed 3 times with 0.1 M PBS, then dehydrated in a graded alcohol series and embedded in epoxy resin. Then, ultrathin serial sections (60–100 nm) of embedded samples were cut using ultra-microtomy (Leica, EM UC7, Germany), stained with uranyl acetate and lead citrate, and examined under an electron microscope (Tecnai G^2 20 TWIN, FEI Company, USA) at 200 kv.

Apoptosis assay

The TdT-mediated dUTP nick labeling (TUNEL) technique was used for the determination of cell apoptosis in kidney tissues. Slides of kidney were deparaffinized, and a TUNEL assay kit was used to detect apoptosis according to the manufacture's instructions (Roche, Shanghai, China). Images were observed and captured using a fluorescent microscope (Nikon, Japan).

Cells and culture

HK-2 cells provided by Professor Andong Qiu, School of Life Sciences and Technology, Tongji University, Shanghai, China, were cultured in DMEM/F12 (Biological Industries, Israel) supplemented with 10% (*v/v*) fetal bovine serum (Gibco, Grand Island, NY) and 1% (*v/v*) penicillin/streptomycin (Solarbio, China). Exponentially growing cells were maintained at 37 °C in a humidified incubator containing 5% CO_2, with daily replacement of the cell culture medium. Cells were washed with PBS, digested with 0.25% trypsin (Solarbio, China) and seeded in new culture flasks/dishes after they reached 80% confluence.

Real-time quantitative PCR analysis

Total RNA of the kidney cortex was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was used as a template to examine the mRNA expression levels of target genes using SYBR® Green mixture (Takara, Dalian, China) on an ABI QuantStudio 7 detection system (Applied Biosystems, USA). GAPDH was taken as an internal control and the gene expressions were assessed using the $2^{-\Delta\Delta Ct}$ method. The PCR cycle was as follows: initial denaturant at 95 °C for 6 min, followed by 40 cycles of denaturing at 95 °C for 10 s, and annealing at 60 °C for 34 s. The primer sequences for real-time PCR were shown as following: (5′ → 3′): Rat-GAPDH forward GCCTTCCGTGTTCCT ACC reverse CCTGCTTCACCACCTTCTT; Rat-KIM-1 forward GAGGTGGAGACTCTGGTTGA reverse TGG AGATTCCTGGATGGT; Rat-TGF-β forward CTAATG GTGGACCGCAACAAC reverse CACTGCTTCCCG AATGTCTGA; Rat-Smad2 forward ACCACTCTCTCC CCTGTCAATCA reverse AACCTAAGCAGAACCTCT CCGA.

Western blot analysis

Total protein of kidney tissues and cells was lysed in ice-cold NP40 buffer (Beyotime, China) containing protease and phosphatase inhibitors. Then, the liquid supernatants were collected by centrifugation at 12000*g* for 15 min at 4 °C, and the protein concentrations were calculated using a BCA protein quantification kit (Beyotime, China). The protein samples were subjected to 15%

sodium dodecyl sulfate polypropylene gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). The PVDF membranes were then blocked in 5% non-fat milk at room temperature for 1 h, incubated with specific primary antibodies KIM-1 (Cell Signaling Technology, USA), LC3, beclin-1, P62, GAPDH, and β-actin (Proteintech, USA) at 4 °C overnight, and subsequently incubated with HRP-conjugated secondary antibodies (Proteintech, USA) at room temperature for 1 h. After washing with TBST, the protein bands were visualized using an enhanced chemiluminescence system (Image Quant LAS, 4000 mini). Protein expression was quantified using ImageJ software (version 1.4.2b, USA) and standardized to the expression of a housekeeping gene and is given in the fold change compared to that in the control samples.

Data analysis

Data was expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS Statistical 19.0 software (IBM, USA). Independent-sample *t* test was used to compare the difference between $PM_{2.5}$ and FA groups. Statistical analysis between multi-groups were analyzed by one-way analysis of variance (ANOVA) followed Duncan's multiple-comparison tests. p value < 0.05 was considered statistically significant.

Results

Exposure description and hydration state

During exposure periods, the mean concentration of $PM_{2.5}$ outdoor (Xujiahui District, Shanghai) was 41.48 μg/m^3 (19.2–83.8 μg/m^3), and the average concentration in the exposed chamber was 255.71 μg/m^3 (72.15–596.84 μg/m^3) and 8.24 μg/m^3 (4.52–13.54 μg/m^3) in the control chamber, respectively. The average concentration of particulate enrichment was 6.16 times (2.91–13.69 times). These results showed that the concentration of $PM_{2.5}$ in the exposed chamber was affected by $PM_{2.5}$ concentration in outdoor air, which was consistent with the dynamic change of outdoor concentration (Fig. 1a). During the 12 weeks of exposure, the animals' body weight was recorded every weekend. The results showed that there was no statistical difference between the FA and $PM_{2.5}$ groups (Fig. 1b). In addition, water intake and urinary flow rates were measured during the 24-h period. During the exposure period, the water consumption of animals in the $PM_{2.5}$ group was higher than that in the FA group, and there was a significant difference in the 8th, 10th and 11th weeks ($p < 0.05$) (Fig. 1c). Meanwhile, the results showed (Fig. 1d) that the urinary flow rate in the $PM_{2.5}$ group was higher than that in the FA group, along with significant difference in all weeks except the 1st, 2nd and 12th weeks ($p < 0.05$).

Changes of mean blood pressure

In this study, MBP was used as a physiological parameter of vascular tone, which can be an indicator of perfusion pressure of organs. We assessed the basic blood pressure of the animals in each group and measured the tail blood pressure of the animals after weekly exposure. The results indicated that in the 5th, 7th, 8th, 10th and 12th week after exposure, the MBP of SD rats in the $PM_{2.5}$ group was significantly higher than that in the FA group (all $p < 0.05$), while no significant differences were observed in other weeks (all $p > 0.05$) (Table 1). Several studies have linked $PM_{2.5}$ with increases in the blood pressure, and elevated blood pressure serves as an indicator of cardiovascular stress and disruption of normative vascular homeostasis [6, 8, 27]. These results indicated that $PM_{2.5}$ exposure affected vascular tone of experimental animals and probably the perfusion of organs.

Histology and pro-fibrotic state

After exposure to $PM_{2.5}$ for 12 weeks, pathological changes of H&E-stained renal tissue samples showed the severe edema of renal tubular epithelial cells, capillary congestion and reduction of the glomerular urinary space, whereas the normal structures of glomerulus and tubular can be observed in the FA group (Fig. 2a). In addition, to further evaluate renal injury, we used Western blotting and RT-PCR to examine the protein and gene mRNA expression of KIM-1, a marker of early renal injury. Shown as Fig. 2c, d, mRNA expression of KIM-1 in renal cortex of $PM_{2.5}$ group was significantly higher than those of FA group ($p < 0.01$), and levels of KIM-1 protein in serum of $PM_{2.5}$ group were significantly higher than those of FA group ($p < 0.01$).

Masson's Trichromic stain was further used to estimate the changes of collagen deposition in renal tissue induced by $PM_{2.5}$ exposure. As showing in Fig. 3a, compared with the control group, significant collagen deposition was observed in the kidney tissues of the $PM_{2.5}$ group. mRNA expression levels of TGF-β and Smad2 were analyzed as inducers of early pro-fibrosis (Fig. 3b). We observed that mRNA expression levels of TGF-β and Smad2 in renal cortex of $PM_{2.5}$ group were significantly higher than those of FA group ($p < 0.05$).

$PM_{2.5}$ induced renal cell apoptosis of rats

The TUNEL method was used to further determine whether $PM_{2.5}$ exposure could induce cell apoptosis in renal tissues. These results showed that green fluorescence points in renal tissues in the $PM_{2.5}$ group were significantly more than those in the FA group (Fig. 4a). Compared with FA group, the proportion of apoptosis cells in $PM_{2.5}$ group showed significantly statistical difference ($p < 0.01$) (Fig. 4b).

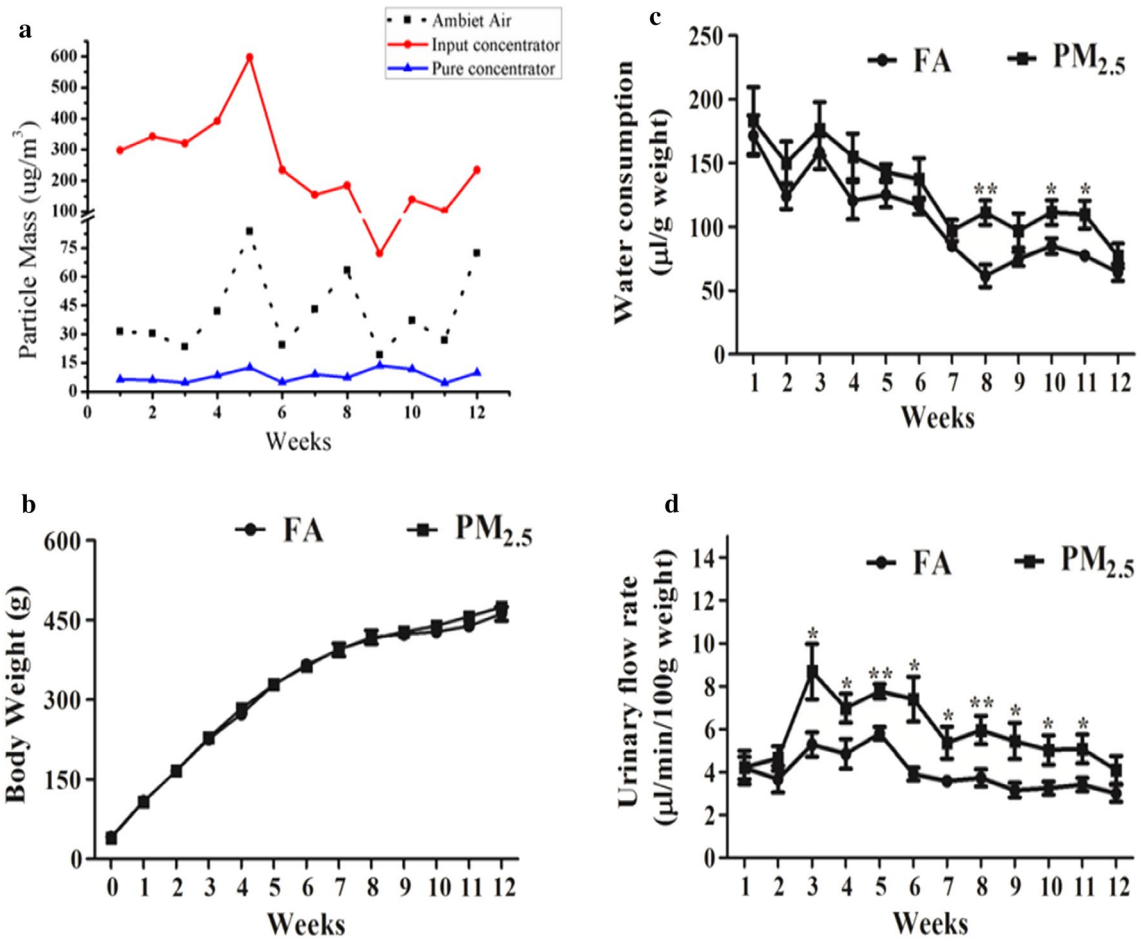

Fig. 1 $PM_{2.5}$ concentration monitoring and the effects of $PM_{2.5}$ on the physiological metabolism in rats. **a** During the exposure period, the mean $PM_{2.5}$ concentrations of ambient air, exposure chamber and control chamber were monitored simultaneously, 5 days per week and 8 h per day. **b** Changes in rats' body weight during exposure period. **c** The water consumption during 24-h period in the metabolic cages. **d** Urinary flow rates during 24 h in the metabolic cages. Values are significantly compared to FA group: $^{*}p < 0.05$, $^{**}p < 0.01$, independent-sample *t* test

$PM_{2.5}$ induced increase of autophagy and changes of autophagic protein expression in renal tissues

As can be observed in Fig. 5, there were multiple layers (myeloid) of exposed animal renal tissue cells. To further clarify whether $PM_{2.5}$ activated autophagy signaling-related molecules in renal cells, Western blotting was used to analyze the expression levels of LC3, P62, and beclin-1 proteins. Our results showed that compared with the FA group, protein signals of LC3 and P62 in $PM_{2.5}$ group were significantly down-regulated, while protein signals of beclin-1 were significantly up-regulated (Fig. 6). The ratio of LC3II/LC3I and beclin-1 protein abundance in the $PM_{2.5}$ group were significantly higher than that in the FA group, and the protein abundance of P62 was significantly lower than that in the FA group. Collectively, $PM_{2.5}$ exposure triggered the intracellular autophagy signaling pathway in renal tissue.

Morphological changes following $PM_{2.5}$ treatment in HK-2 cells

We further examined the morphology of HK-2 cells following treatment with $PM_{2.5}$ using transmission electron microscopy (TEM). Untreated control HK-2 cells presented typical cellular morphology, including normal-sized nucleus, even distribution of microvilli on the cell surface. In contrast, HK-2 cells that were treated with $PM_{2.5}$ (400 μg/mL) displayed an absence of microvilli on the surface of the cell membrane, along with an obvious swelling of the nucleus, and destruction of the cell membrane lysis. Moreover, high-magnification images showed the presence of numerous autophagic vacuoles, early autophagic vacuoles, and degradation autophagic vacuoles (Fig. 7).

Table 1 Mean blood pressure measurements after exposure to $PM_{2.5}$ (independent-sample *t* test)

Weeks	The mean blood pressure (MBP)		
	$PM_{2.5}$	FA	*p* value
Basal	78.97 ± 0.96	77.51 ± 0.55	0.202
WK-1	77.72 ± 1.04	79.98 ± 0.94	0.131
WK-2	84.16 ± 0.89	84.10 ± 1.14	0.969
WK-3	84.92 ± 0.83	86.24 ± 0.83	0.281
WK-4	101.99 ± 3.37	102.86 ± 2.12	0.601
WK-5	109.53 ± 1.20	101.09 ± 1.76	0.002
WK-6	105.24 ± 1.79	104.97 ± 1.31	0.904
WK-7	112.41 ± 1.62	106.16 ± 1.05	0.006
WK-8	112.61 ± 1.38	105.91 ± 1.23	0.003
WK-9	105.34 ± 2.38	106.83 ± 3.36	0.727
WK-10	110.81 ± 1.33	104.93 ± 1.25	0.005
WK-11	108.50 ± 1.51	107.42 ± 1.41	0.641
WK-12	114.46 ± 1.34	108.09 ± 0.91	0.001

$PM_{2.5}$ induced the activation of autophagic pathways in HK-2 cells

To determine whether $PM_{2.5}$ treatment also induced the activation of autophagic pathways in HK-2 cells, we examined the expression of LC3, which contains two species including activated LC3-I and processed IL3-II, as well as P62 and beclin1 using Western blot analyses. $PM_{2.5}$ treatment resulted in a significant increase in the ratio of LC3-II to LC3-I content in a dose- and time-dependent manner, indicating that $PM_{2.5}$ exposure induced the conversion of LC3-I to processed LC3-II (Fig. 8). Moreover, $PM_{2.5}$ treatment significantly up-regulated protein expression levels of P62 and beclin1 compared to untreated control cells. Collectively, these findings demonstrated that $PM_{2.5}$ induced the activation of the cascade of LC3, P62 and beclin1 proteins involved in the autophagic pathway in HK-2 cells.

Discussion

In the present study, the artificial climatic environment exposure system (Shanghai-METAS) was used to study the effects of $PM_{2.5}$ exposure on kidney damage. This equipment which could maximally simulate "real world" $PM_{2.5}$ exposure is the first comprehensive animal exposure system established in China, and has been effectively used to assess the effects of $PM_{2.5}$ exposure on health and diseases development in rodent in several studies [13, 47,

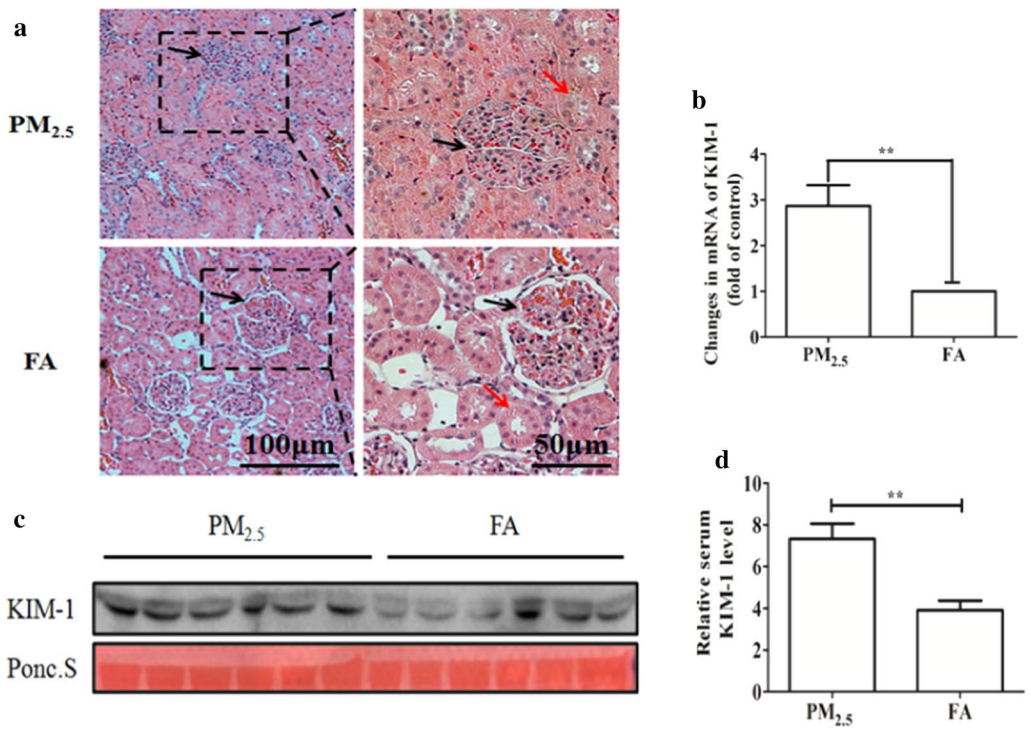

Fig. 2 Renal histopathologic changes caused by $PM_{2.5}$ exposure. **a** H&E stain of renal tissue (glomerulus marked by black arrow and renal tubules marked by the red arrow). **b** RT-PCR analysis of KIM-1 in kidney cortex, on histogram graphic** indicates statistical difference ($p < 0.01$), independent-sample *t* test. **c**, **d** WB analysis of KIM-1 in serum, on histogram graphic** indicates statistical difference ($p < 0.01$), independent-sample *t* test

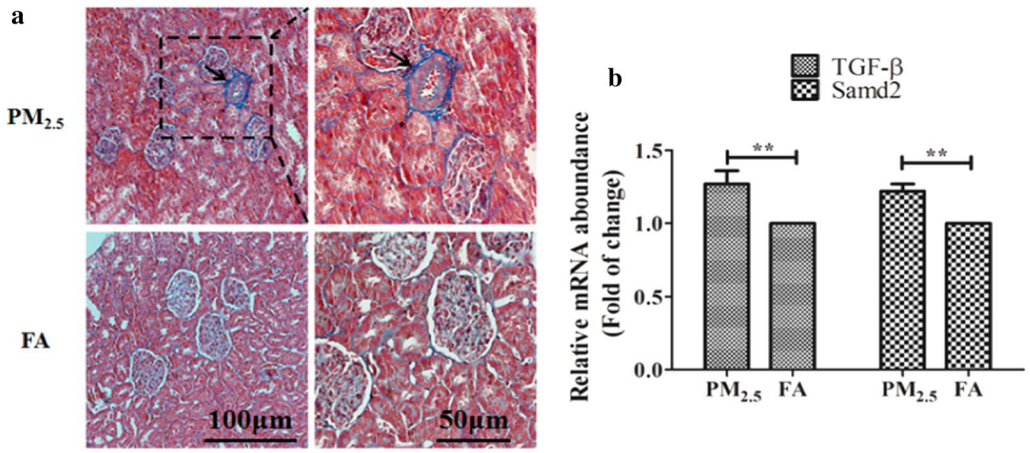

Fig. 3 Collagen deposition induced by $PM_{2.5}$ exposure. **a** Deposit of collagen by Masson's Trichromic stain. **b** mRNA expressions of TGF-β and Smad2, on histogram graphic** indicate statistical difference ($p < 0.01$), independent-sample *t* test

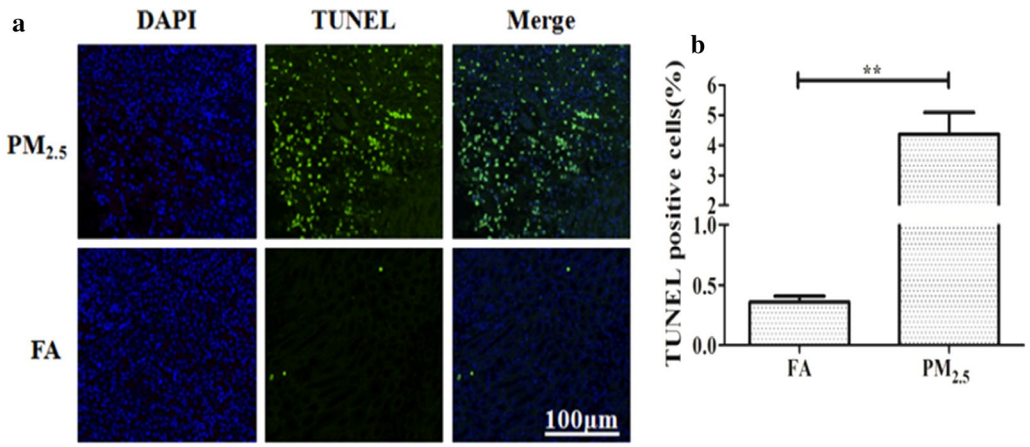

Fig. 4 TUNEL analysis of rats' kidney tissues. **a** Representative images are showing cell apoptosis in renal tissues induced by $PM_{2.5}$. **b** Statistical analysis of the proportion of apoptosis cells. Cells with green light presented TUNEL-positive cells, on histogram graphic** indicates statistical difference ($p < 0.01$), independent-sample *t* test

48]. Interestingly, the results of this study also confirmed that subchronic exposure to $PM_{2.5}$ led to kidney damage in SD rats.

The kidneys are complex organs, and they are vital in maintaining normal body functions such as urinary production, excretion/reabsorption, acid–alkaline homeostasis and endocrine function [22]. To investigate whether kidney adequate filtration was affected by $PM_{2.5}$ exposure, the water consumption and urine volume of the animals within 24 h once a week were recorded during the exposure period. As shown in Fig. 1c, d, the alterations of water intake and urinary flow rate were observed between $PM_{2.5}$ and FA groups after exposure to $PM_{2.5}$, and the results were consistent with the previous report [1]. The stimulating effects of $PM_{2.5}$ hygroscopic properties and nervous system, as well as adequate water needed in lung tissues to clean up the harm of $PM_{2.5}$ particle deposition for self-protection, might be the main reasons for the increases of water consumption in rodent after $PM_{2.5}$ exposure [1]. Moreover, it has been shown that administration of Cisplatin (CP) or CP + Cerium oxide nanoparticles (CeO_2 NPs) in rats increased the water intake and urine volume compared with saline, indicated that the damaged renal tubules could cause the deterioration of capacity of tubular cells to reabsorb water, and subsequent polyuria leading to dehydration [34]. Similarly, the increase of urine volume was one of the nephrotoxicity characteristics in an acute renal

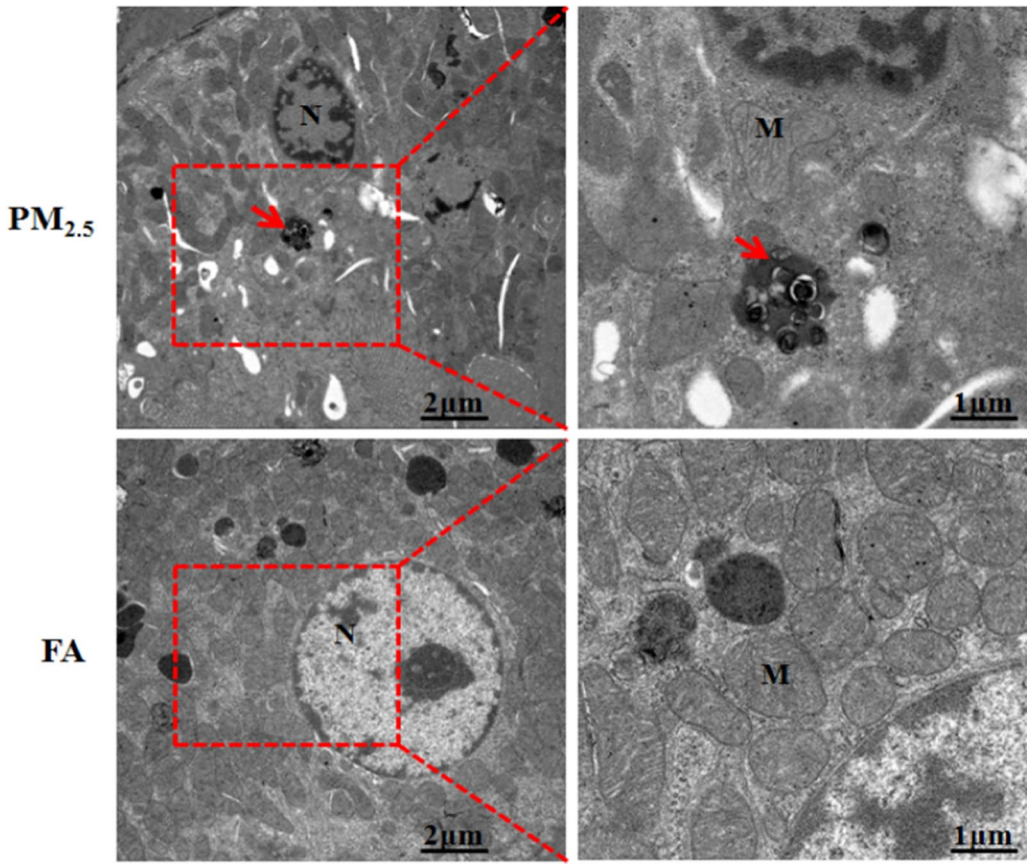

Fig. 5 TEM images of kidney tissues after $PM_{2.5}$ exposure. Representative images showing changes in the microscopic structure of renal tissue cells. N means the nucleus, M the mitochondria, and arrows the multilayered (myeloid) matter

failure (ARF) rat model induced by gentamicin in a study about plant extracts for the prevention and attenuation of ARF [14]. These results suggested that the physiological functions of the kidney were damaged, resulting in an imbalance of hydration state after exposure to $PM_{2.5}$. However, the body weight of rats in both groups has not been affected during the exposure to $PM_{2.5}$ period in this study (Fig. 1b).

$PM_{2.5}$ exposure increased the risk of cardiovascular disease [17]. Vascular endothelial cells are the primary vascular barrier to local damage factors induced by exposure to $PM_{2.5}$, such as inflammatory factors and free radicals, as well as toxic and harmful substances of $PM_{2.5}$ [15]. Vascular endothelium plays key roles in regulating blood pressure, atherosclerosis and thrombosis, and $PM_{2.5}$ exposure can lead to structural and functional impairment of vascular endothelial cells [37]. For this reason, we monitored MBP during animal exposure as an indicator of vascular response to $PM_{2.5}$ to assess the effects of $PM_{2.5}$ on peripheral blood pressure and organ blood perfusion. Our results showed that there was no difference in the basal blood pressure between the two groups. But in the 5th, 7th, 8th, 10th and 12th weeks, the MBP of the $PM_{2.5}$ group was significantly higher than that of the FA group after subchronic exposure to $PM_{2.5}$. Because of the close relationship between the physiological function of the kidney and systemic blood pressure, an increase in MBP could cause the renal peritubular capillaries damage.

Currently, a few studies have described that exposure to $PM_{2.5}$ could cause pathological alterations of kidney tissues [1, 16, 43]. Here, both in vivo and in vitro experiments clarified that the morphological structures of renal tissues and HK-2 cells were damaged after $PM_{2.5}$ exposure. H&E staining results of tissue sections indicated tubular and glomerular damage evidenced by the severe edema of renal tubular epithelial cells, capillary congestion and reduction of the glomerular urinary space (Fig. 2a). Moreover, $PM_{2.5}$ treatment resulted in significant changes in cellular morphology in HK-2 cells, including destruction of the cell membrane lysis and swelling of the nucleus (Fig. 7). Similar histopathologic changes have been reported in diabetic nephropathy and acute kidney injury [4, 49]. KIM-1 is a transmembrane

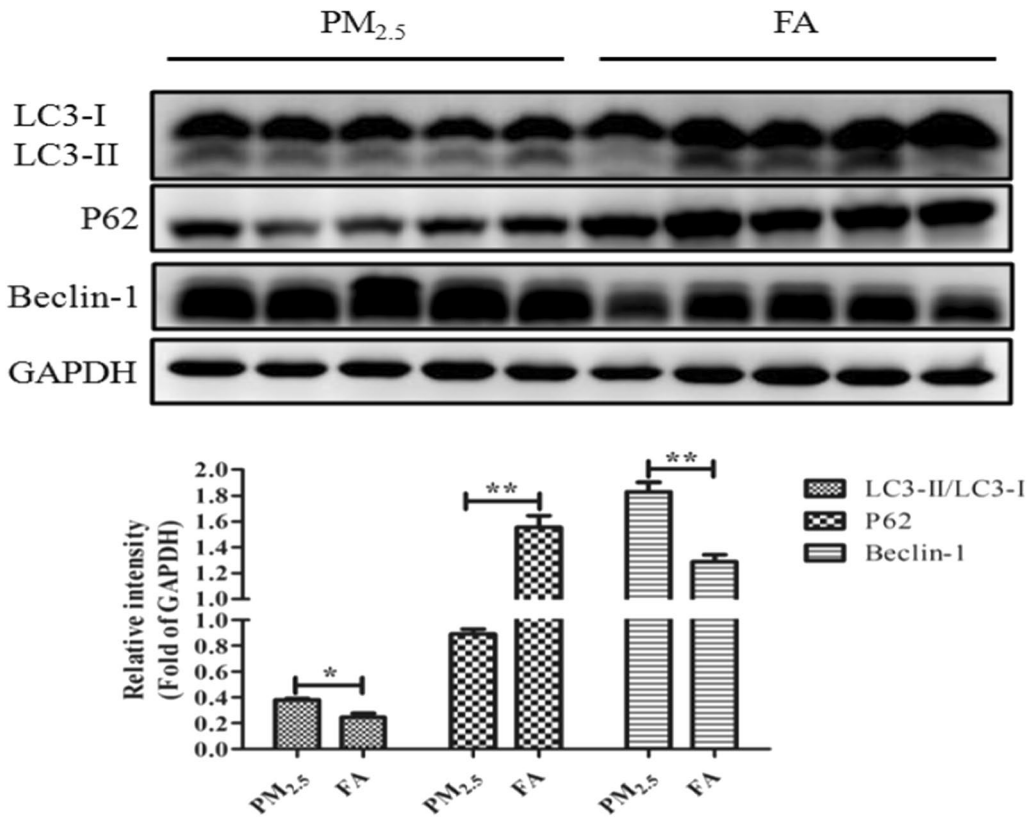

Fig. 6 Activation of autophagic pathway after $PM_{2.5}$ exposure in rats. Western blots and quantitative expressions of LC3, beclin-1 and P62. GAPDH was used as the loading control, on histogram graphic* and on histogram graphic** indicate statistical difference ($p < 0.05$) and ($p < 0.01$), respectively, independent-sample *t* test

glycoprotein whose extracellular segments can be shed, and the levels in urine are often detected in clinical or experimental studies to diagnose acute kidney injury or early kidney damage [24, 42, 51]. In this study, the significant increases of KIM-1 protein expression in serum and KIM-1 mRNA expression in kidney tissues were observed after $PM_{2.5}$ exposure in vivo experiment (Fig. 2b–d). Thus, these results indicated that exposure to $PM_{2.5}$ induced damage to the proximal tubule epithelium.

Our results also showed that during the 12-week period of exposure to $PM_{2.5}$, kidney tissue not only experienced sustained damage, but the body also activated a response to repair the damage. At the end of exposure, mRNA expression of TGF-β in renal tissues of the $PM_{2.5}$ group was increased. TGF-β plays an important role in participating in post-injury repair of tissues by promoting the deposition of extracellular matrix components such as collagen [20]. But prolonged, uncontrolled TGF-β activation can lead to an overdose of extracellular matrix, leading to tissue fibrosis [38]. An increase of collagen deposition in renal tissue was observed by Masson's Trichromic stain in this study. We speculated that long-term exposure to $PM_{2.5}$ could lead to renal damage, but the body could induce collagen deposition through activation of TGF-β components to promote damage repair. However, the increased mRNA expression of Smad2 gene in renal tissues suggested that long-term exposure to $PM_{2.5}$ may promote pro-fibrosis and renal dysfunction. Similarly, TGF-β1 and Smad2 played an important role in the process of renal fibrosis which has been confirmed in previous studies [31, 32].

To investigate the renal damage of SD rats exposed to $PM_{2.5}$ at the cellular level, we first used transmission electron microscopy to observe the changes in renal cell microstructure. The results showed that there were lysosome vacuoles and multilayer (myeloid) substances in the cytoplasm of the kidney tissues of the exposed group. Moreover, TUNEL analysis was used to further analyze the degree of cell damage in kidney tissues and the results indicated that apoptosis responses were observed significantly in $PM_{2.5}$ exposed rats. Previous studies suggested that apoptosis in renal tubular cells was considered as a causal factor in the development of kidney diseases/injury [39, 53]. Many in vivo and in vitro experiments

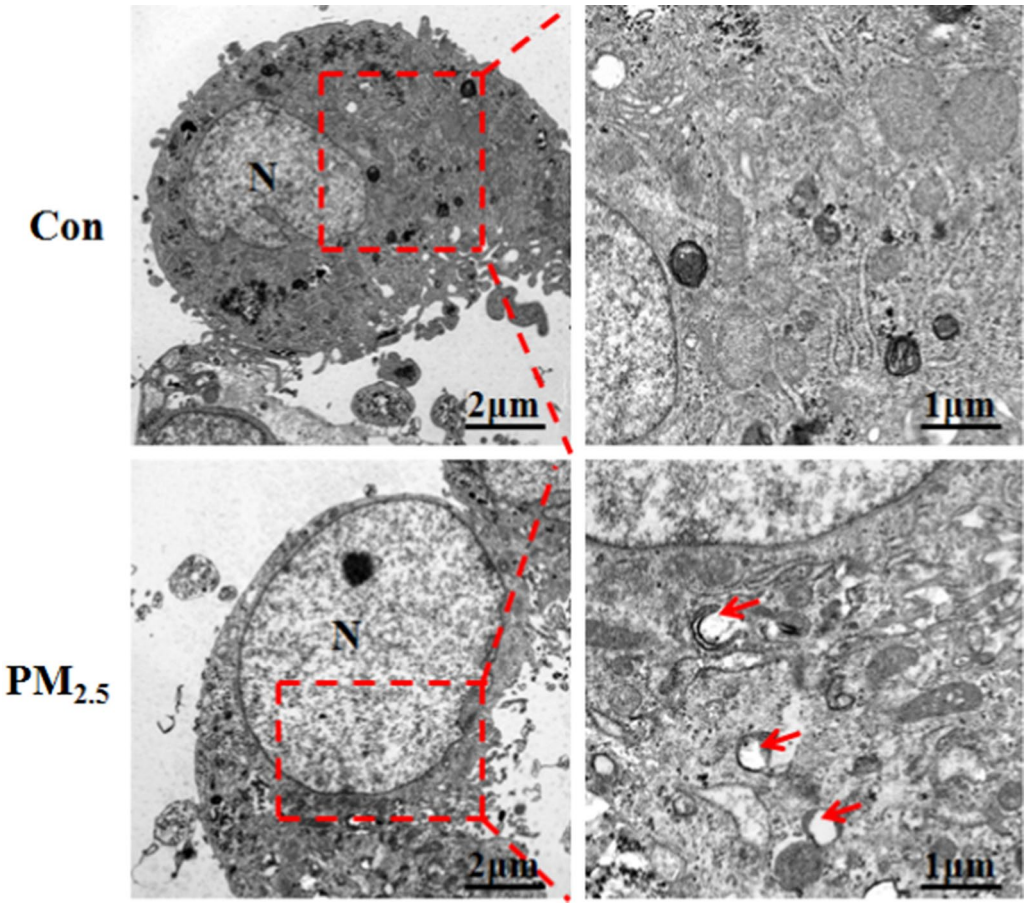

Fig. 7 Effects of $PM_{2.5}$ exposure on cell morphology and autophagy in HK-2 cells. TEM images showing morphological changes and autophagosomes in $PM_{2.5}$ (400 μg/mL)-treated HK-2 cells. Red arrows indicate autophagosomes. N, nucleus

have confirmed that $PM_{2.5}$-induced inappropriate apoptosis (too much) is one of the potential mechanisms of $PM_{2.5}$ health hazards [29, 46, 50, 54]. Therefore, we speculated that $PM_{2.5}$ may enter the kidney through blood circulation, and then accumulate in cells, resulting in kidney damage.

As previously known, obvious autophagosomes and lysosome vacuoles were found in the cytoplasm of HK-2 cells after exposure by transmission electron microscopy. Autophagy refers to the catabolic process of self-digestion of abnormal substances in the cytoplasm of cells by lysosomes [7]. Beclin-1 plays a key role in early autophagosome formation, followed by LC3 which is a significant feature of autophagy level. P62 is an intracellular multifunctional protein acting on selective autophagy, which is mainly induced by stress and involved in a variety of signal transduction, including in the formation process of autophagosomes, as a bridge between LC3 and polyubiquitin protein; it can regulate the transport of damaged mitochondria into autophagosomes and degradation, and has an important regulatory role in the Nrf2/Keap1 signaling pathway [19, 21]. Studies have found that autophagy induced by $PM_{2.5}$ in human umbilical vein endothelial cells (HUVECs) and human lung epithelial cells (A549) is characterized by significant increase in LC3-II and beclin-1 expression levels and LC3-II/LC3-I ratio [10, 12]. Our results showed that after exposure to $PM_{2.5}$, LC3-I to LC3-II conversion, P62, and beclin-1 were activated. The accumulation of P62 indicates that intracellular autophagic flux is compromised, Keap1 is sequestered by P62 and can no longer bind Nrf2, leading to activated Nrf2 signaling which functions to protect cells against oxidative stress, environmental toxicants, and harmful chemicals through the induction of cytoprotective genes [21]. These data in vitro suggested that autophagic pathway played key roles for the activation of the cell self-protection mechanism in the HK-2 cells' damage induced by $PM_{2.5}$ exposure. In vivo assay, the LC3-II/LC3-I ratio and beclin-1

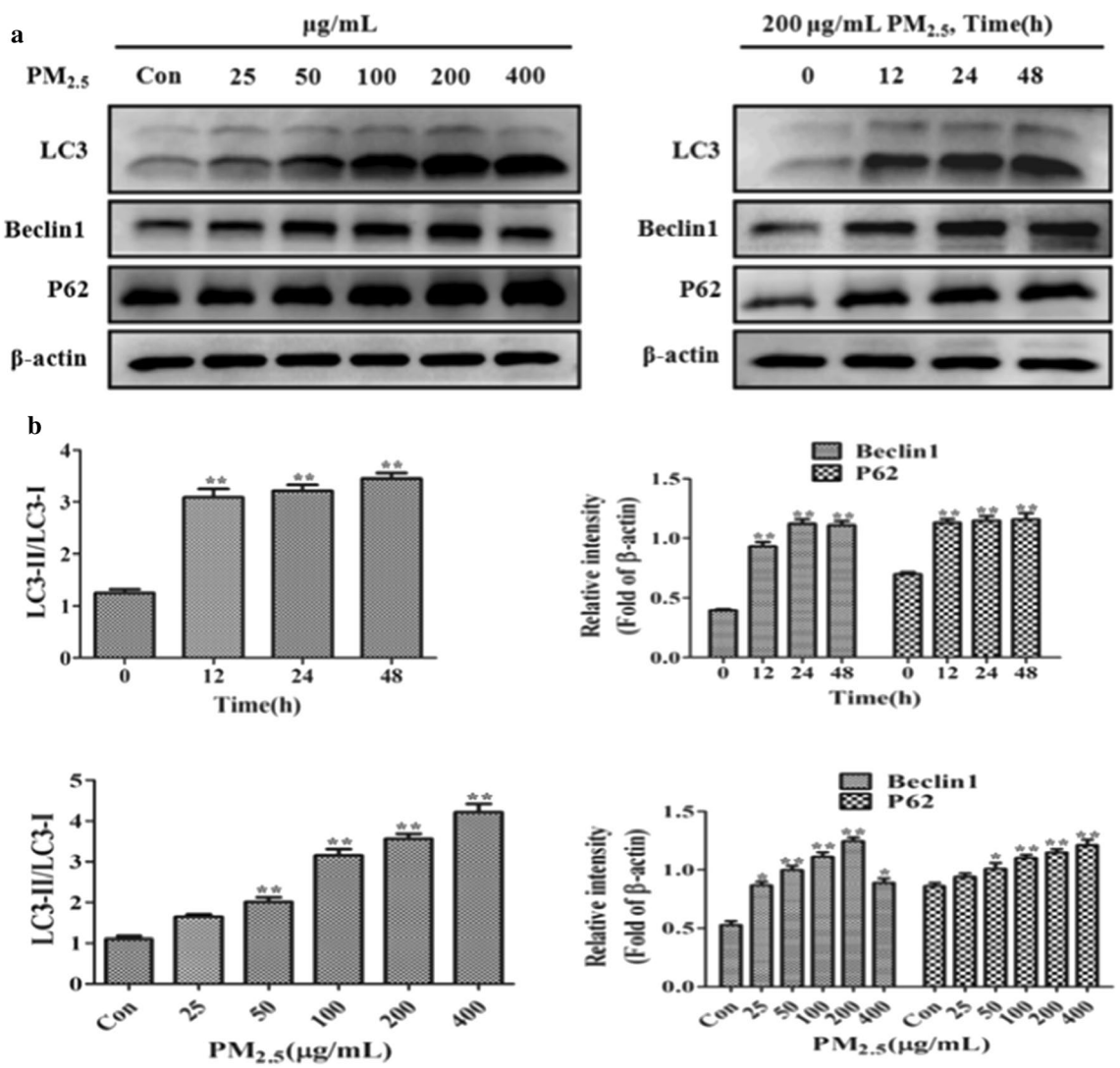

Fig. 8 Activation of autophagic pathway caused by $PM_{2.5}$ in HK-2 cells. **a**–**d** Western blotting and quantitative expressions of LC3, beclin-1 and P62. β-actin was used as the loading control, $^{**}p < 0.01$ compared with the control group or 0 h, one-way analysis of variance

protein expression level in the $PM_{2.5}$ group were significantly higher than those in the FA group, while the P62 protein expression level in the $PM_{2.5}$ group was significantly lower than that in the FA group. These results revealed that $PM_{2.5}$ exposure led to the occurrence of autophagy and autophagic flux in renal tissues, and P62 was a selective regulatory protein, and autophagy played an important role in $PM_{2.5}$-induced HK-2 cytotoxicity and renal injury. The previous study confirmed that oxidized proteins-triggered autophagosome formation could prevent further oxidative stress to protect other cells [35]. The increased levels of ROS in HK-2 cells induced by $PM_{2.5}$ exposure have been observed in our previous study [18]. Moreover, the expressions of Nrf2 and NQO1 proteins in the kidneys of the exposed animals were significantly lower than those of the FA group after $PM_{2.5}$ exposure (data not displayed). Therefore, we considered that autophagy may be an adaptation process to prompt self-repair of kidney damage, and avoid ending up in a kidney failure process in the current study.

Conclusion

In summary, the present study demonstrated that $PM_{2.5}$ exposure could promote autophagy in kidneys, especially renal tubular epithelial cells both in vitro and vivo. We further provided evidence that autophagy pathway was crucial for the progression of kidney damage induced by

$PM_{2.5}$ exposure, which was most likely via the activation of LC3, beclin-1 and P62 expressions. Our findings provided insight about the effects and mechanism of $PM_{2.5}$ on renal injury.

Abbreviations
SD: Sprague–Dawley; FA: Filtered air; PBS: Phosphate-buffered saline; $PM_{2.5}$: Fine particulate matter; HK-2: Human proximal tubule epithelial cells; KIM-1: Kidney injury molecule-1; TUNEL: TdT-mediated dUTP nick labeling; MBP: Mean blood pressure; Atgs: Autophagy-related genes; LC3: Microtubule-associated proteins light chain 3; P62: Sequestosome1; qPCR: Quantitative Real-time PCR.

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Authors' contributions
LZ and JL designed the research; XH, ZZ and XL conducted the in vivo and in vitro studies and data analyses; XH, JL and LZ wrote the draft of initial manuscript; XH, LZ and JL contributed to the interpretation of the data and preparation of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets obtained and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study was subject to approval by the institutional animal care and use committees of Tongji University.

Consent for publication
Not applicable.

Competing interests
The authors declare that all authors have no conflicts of interest related to this manuscript.

Author details
[1] Shanghai Pudong New Area Mental Health Center, Tongji University School of Medicine, Shanghai 200124, China. [2] School of Medicine, Jinggangshan University, Ji'an 343009, Jiangxi, China.

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