Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:753-764
DOI: 10.1159/000491904
Published online: July 19, 2018
Accepted: May 28, 2018

Original Paper

Activation of Cyclooxygenase-2 by ATF4 During Endoplasmic Reticulum Stress Regulates Kidney Podocyte Autophagy Induced by Lupus Nephritis

Juan Jin[a,b] Li Zhao[a,b] Wenli Zou[a,b] Wei Shen[a,b] Hongjuan Zhang[a,b] Qiang He[a,b]

[a]Department of Nephrology, Zhejiang Provincial People's Hospital, Zhejiang, [b]People's Hospital of Hangzhou Medical College, Zhejiang, China

Key Words
Lupus Nephritis • Autophagy • Endoplasmic reticulum stress • COX-2 • ATF4

Abstract
Background/Aims: Autophagy plays an essential role in lupus nephritis (LN)-induced kidney injury, although the mechanism of action remains obscure. We investigated the role of cyclooxygenase-2 (COX-2) and the ATF4 endoplasmic reticulum (ER) stress pathway in LN-induced podocyte autophagy. ***Methods:*** We evaluated podocyte autophagy in a mouse model of LN. Protein levels of COX-2 and ATF4, and markers of autophagy, were evaluated by immunofluorescence and western blotting. To evaluate apoptosis, levels of PGE2 were measured by enzyme-linked immunosorbent assay. ***Results:*** LN induced kidney damage and dysfunction, which was associated with podocyte autophagy. COX-2 and the ATF4 ER stress pathway were induced by LN in cultured podocytes. Inhibition of COX-2 inhibited LN-induced autophagy in podocytes. In addition, blocking ER stress with 4-phenylbutyrate or RNAi partially counteracted COX-2 overexpression and LN-induced autophagy, suggesting that ER stress is required for LN-induced kidney autophagy. Furthermore, LN activated ATF4 and induced its nuclear translocation. Knockdown of ATF4 inhibited LN-induced COX-2 overexpression. ***Conclusions:*** Our study suggests a novel molecular mechanism by which COX2 overexpression, induced by the ATF4 ER stress pathway, contributes to LN-induced kidney autophagy and injury. These data demonstrate that COX-2 may be a potential therapeutic target against LN-induced nephropathy.

Qiang He

Department of Nephrology, Zhejiang Provincial People's Hospital
Zhejiang 310014 (China)
Tel. 86-0571-85893689, Fax 86-0571-85893689, E-Mail qianghe1973@126.com

Introduction

Systemic lupus erythematosus (lupus; SLE) is a chronic autoimmune inflammatory disease characterized by loss of tolerance against self-antigens, polyclonal autoantibody production, and immune complex formation and deposition in different parts of the body, and can lead to damaging inflammation and multi-organ injury [1]. Lupus nephritis (LN) is one of the most common pathologies observed in patients with lupus, occurring in 50%–70% of patients with SLE in the first five years after diagnosis [2]. Although LN has been observed for centuries, the pathogenesis remains unclear.

Podocytes are highly specialized epithelial cells that line the urinary surface of the glomerular capillary tuft in the kidneys, and are involved in ultrafiltration of blood. Impairment of any of these functions following podocyte injury results in proteinuria and can lead to renal failure. Loss of glomerular podocytes is a key feature of the progression of renal diseases, and podocyte injury is thought to play a role in the pathogenesis of LN[3, 4]. Autophagy is a cellular process during which long-lived or aggregated proteins, defective organelles, and various soluble molecules are engulfed, digested, and recycled [5]. Autophagy is a precisely regulated process that plays a vital role in maintaining cell homeostasis, and is a protective, pro-survival response to cellular damage. Autophagy has many physiological roles and is involved in the pathogenesis of a variety of diseases, including LN[6, 7].

The endoplasmic reticulum (ER) is a cellular organelle that is involved in protein folding and in sustaining calcium equilibrium [8]. ER function can be altered by various intracellular or extracellular stimuli, such as Aldo [9] and some ER stress can trigger the unfolded protein response (UPR) pathway. ER stress responses involve three ER transmembrane proteins: inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6)[10]. Ordinarily, the ER lumenal domains of these proteins bind to glucose-regulated protein 78 (BiP or GRP78). When UPR is stimulated, GRP78 is activated, inhibits protein accumulation, and aids in correct protein folding. PERK directly phosphorylates eukaryotic initiation factor 2 α-subunit (eIF2α), resulting in a blockade of most mRNA transcription, and enhancing the translocation and activation of the ATF4 transcription factor. ER stress generates autophagy, a process by which unfolded proteins in cells are disposed [11-13]. The role of ER-stress in LN-induced autophagy remains unknown.

Cyclooxygenase-2 (COX-2) is upstream of the mPGES-1/ PGE2 cascade, and serves as a mediator prostaglandin induction under certain physiological and pathological conditions [14, 15]. In the kidneys, COX-2 is inducible in podocytes, interstitial cells, renal tubular epithelial cells, and mesangial cells [14]. There is mounting evidence indicating a detrimental role of COX-2 in mediating kidney injury [16-18]. Nevertheless, the role of COX-2 in autophagy induced by LN has not been explored.

In the present study, we discovered that LN-induced podocyte autophagy is accompanied by ER stress and COX-2 upregulation, both in mouse kidney tissues and in cultured podocyte cells. We hypothesized that ER stress and/or COX-2 regulates LN-induced podocyte autophagy. Our present study demonstrates, for the first time, that inhibiting COX-2 can decrease LN-induced autophagy. Furthermore, we discovered that LN-induced COX-2 overexpression and activation of autophagy is inhibited by RNAi or by antagonizing the ER stress response. Interestingly, we discovered that LN-induced podocyte autophagy is mediated by COX-2 through activation of ATF4. Our study sheds new light on potential molecular mechanisms that can be exploited as therapeutic interventions in LN.

Materials and Methods

Reagents and antibodies

4-phenylbutyrate (4-PBA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were used: anti-COX-2, anti-ATF4, anti-Total-elF2a, anti-p-elF2a, anti-ß-actin (Abcam, England)； anti-LC3, anti-BiP, anti-AKT, anti-p-AKT(ser473), anti-p62 (Proteintech Group, USA).

Experimental animals

C57BL/6 (control) and MRL/lpr (LN) mice (aged 8 weeks; body weight 18–20g) were purchased from the Experimental Animal Center of Zhejiang University. Prior to the study, the animals were maintained under standard conditions with a 12-h/12-h day/night cycle and access to food and water *ad libitum*. All mice were sacrificed after 4 weeks. All experiments were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols using animals were approved by the Institutional Animal Care and Use Committee of Zhejiang Provincial People's Hospital.

Podocyte preparation

Glomeruli were resuspended in 2 ml digestion buffer and incubated for 40 min at 37°C on a thermomixer shaking at a rate of 1400/min. During this incubation period, glomeruli were sheared with a 27 G needle. After 40 min, the solution was vortexed and the digestions were evaluated by fluorescence microscopy. Samples were put on a magnetic particle concentrator to eliminate beads and glomerular structures lacking now devoid of podocytes. The supernatant was pooled and the magnetic particles discarded. The cell suspension (2 ml) was passed through a 40 μm pore filter on top of a 50 ml Falcon tube, and was rinsed with 10 ml of HBSS. Cells were collected by centrifugation at 1500 rpm for 5 min at 4°C, and were resuspended in 0.5 ml of HBSS supplemented with 0.1% bovine serum albumin plus 4′6-diamidino-2-phenylindole (1 μg/ml). To separate GFP-expressing (GFP+) and GFP-negative (GFP-) cells, glomerular cells were sorted with a Mo-Flo cell sorter (Beckman Coulter, Krefeld, Germany) with a laser excitation at 488 nm (Power 200 mW) and a sheath pressure of 60 PSI. Cells were kept at 4°C before entering the FACS machine and thereafter, whereas temperature during the sorting procedure (~3 min) was 22°C. Only viable (4'6-diamidino-2-phenylindole negative) cells were sorted (laser excitation 380 nm, power 80 mW). On average, 500, 000 podocytes were obtained per mouse. Efficiency was monitored by evaluating small podocyte aliquots under a fluorescence microscope.

Cell culture, RNA interference and cell transfection

Murine podocytes from C57BL/6 and MRL/lpr mice were differentiated and identified using immunofluorescence methods, and were cultured as previously described [19]. Podocytes were maintained in RPMI 1640 medium (Hyclone CA, USA,) supplemented with 10% fetal calf serum (Hyclone), 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Podocytes were propagated at 33°C in culture medium supplemented with 10 U/mL recombinant mouse interferon-γ (IFN-γ; PeproTech EC, London, UK) to enhance the expression of a thermosensitive T-antigen (under permissive conditions). Subsequently, the podocytes were incubated for 7 days at 37°C in the absence of IFN-γ (non-permissive conditions) to induce differentiation. In all experiments, podocytes were used after 7–14 days in culture. For COX-2 activation experiments, cells were cultured in the presence of 4-PBA (5 μM) for 48 h. To silence COX-2 and AFT4 expression by RNA interference, podocytes were transfected with siRNA using Lipofectamine 2000 in accordance with the manufacturer's instructions. A non-specific scramble siRNA sequence (siRNA-Scr) was used as a negative control.

Periodic Schiff-methenamine (PAS) Staining

Kidney samples were fixed with 10% buffered formalin overnight and embedded in paraffin. For histological assessment, sections (3 μm) were deparaffinized, hydrated, and stained with Periodic Schiff-methenamine (PAS).

Immunocytochemical staining

Cells were fixed in 4% paraformaldehyde on ice for 30 min, washed, and permeabilized using PBS containing 0.02% Triton X-100, followed by blocking with 5% BSA in PBS. Primary antibodies (1:200 dilution) were added to the cells and incubated for 2 h at room temperature. After extensive washing, cells were incubated with FITC-conjugated secondary antibodies (10 μg/mL) for 1 h at room temperature and then washed. Images were acquired by immunofluorescence microscopy (Olympus FV1000, Tokyo, Japan). The number of positive punctate signals that were observed in the cells was analysed using software designed for evaluation of immunofluorescence microscopy (FV1000, Olympus, Tokyo, Japan).

Western blot analysis

The protein levels of COX-2, P62, LC3, P-AKT, AKT, mTOR, p-mTOR, and ATF4 were detected by western blot analysis with the corresponding antibodies.

Cells were lysed in RIPA buffer (50 Mm Tris-Cl [pH 7.6], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 0.5% Triton X-100) containing 1 mg/mL protease inhibitors (leupeptin, aprotinin, and antipain), 1 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride. The protein concentration was measured using the Bradford assay. Proteins separation (total 50 mg) was performed by 6–15% SDS-PAGE; proteins were subsequently transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skimmed milk, the membranes were probed with primary antibodies overnight at 4˚C, and incubated with horseradish peroxidase-conjugated secondary antibodies. Band intensities were quantified using WCIF Image J software; the results were expressed relative to control [20, 21].

Cell viability assay

The viability of cells was measured using CCK-8 assays. Briefly, cells were seeded in 96-well plates (1×10^4/well) and incubated overnight. The cells were then treated with growth medium and conditioned media for specified time-periods. After treatment, culture medium was replaced with 10% CCK-8 reagent dissolved in PBS, and cells were incubated for 3 h at 37°C in the dark. Absorbance at 450 nm was measured with a Multiskan FC Microplate Reader (Thermo Scientific, USA) and the results were normalized to CCK-8 absorbance of untreated cells.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (Stanford University, Stanford, CA, USA). Results are expressed as mean ± standard deviation. Group means were compared using Student's *t*-test for independent data. All *P*-values are two-tailed and $P < 0.05$ was considered to indicate statistical significance.

Results

Lupus nephritis causes kidney injury

The renal hypertrophy index in the LN group was significantly higher than in the control group (Fig. 1A), and protein concentrations were significantly higher in urine from LN mice than control mice (Fig. 1B). Elevated urine protein concentrations prompted us to assess serum renal function parameters, and we discovered that serum blood urea nitrogen (BUN) and serum creatinine (Scr) were elevated in the LN group compared to the control group (Fig. 1C, 1D). Histological changes in renal tissue were examined by hematoxylin and eosin (HE) staining, revealing a thickening of capillary basement membrane and mesangial cells, and a noticeable increase in endothelial cell proliferation in the LN group compared with the control group (Fig. 1E).

While podocytes only account for ~20% of glomerular cell mass, they play an important role in maintaining the functionality of glomerular filtration. In our study, the number of podocytes were significantly decreased in LN mice compared with control mice, as indicated by optical microscopy (Fig. 1F). Immunofluorescent staining showed a significant decrease in the expression of nephrin, a podocyte marker protein, in the LN group (Fig. 1G). In addition, CCK-8 assays indicated that podocyte cell proliferation was inhibited in LN mice compared with control group (Fig. 1H). These data indicate that LN causes kidney damage.

Lupus nephritis induces kidney cell autophagy both in vivo and in vitro

Major characteristics of autophagy include the formation of autophagosomes and processing of LC3-I to LC3-II. We investigated the role of autophagic activity in kidneys from control C57BL/6 mice and MRL/lpr mice (a model of LN) by analysing the levels of p62, a selective substrate of autophagy, and by measuring LC3-II/LC3-I ratios (LC3 levels). Compared to the control group, LC3-II/LC3-I ratios were significantly increased and p62 levels were decreased in kidney tissue from the LN group (Fig. 2A).

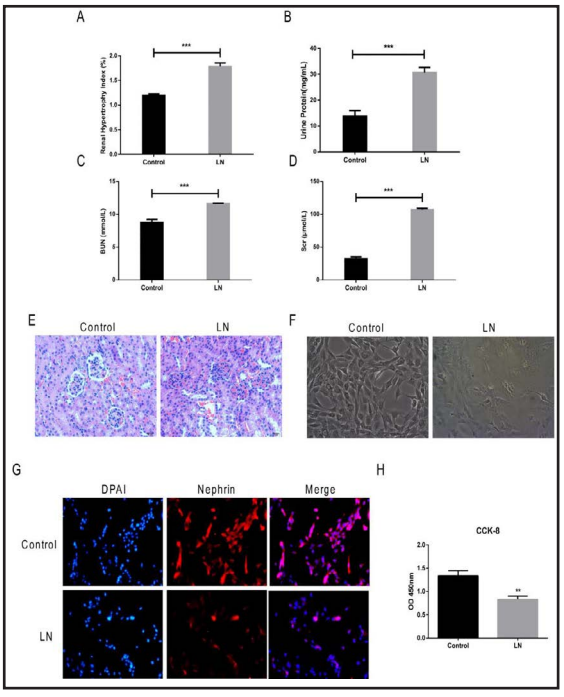

Fig. 1. Lupus nephritis (LN) causes kidney injury. (A) Renal hypertrophy index. (B) The level of urine protein. (C) BUN. (D) Scr. (E) Histopathological features (HE staining) of kidney tissue from control and LN mice (original magnification ×200). (F) Optical microscopy showing the features and numbers of podocytes from control and LN mice (original magnification ×400). (G) The expression of nephrin (a podocyte marker protein) in podocytes from control and LN mice (original magnification ×200). (H) Cell proliferation of podocytes from control and LN mice determined by CCK-8 assay. (***P<0.001).

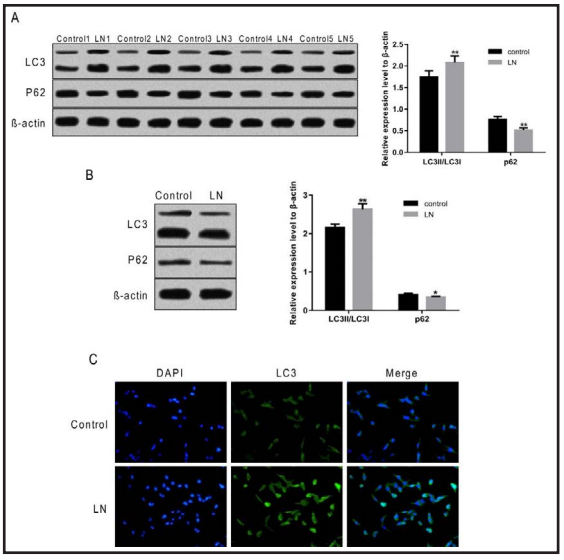

Fig. 2. LN induces kidney autophagy both in vivo and in vitro. (A) Autophagy-related proteins (LC3 and p62) expression levels in tissue. (B) Autophagy-related proteins (LC3 and p62) levels in differentiated mouse podocytes. (C) Immunofluorescent staining of LC3 (original magnification ×400) (**P<0.01 vs. control).

We characterized autophagic activity in differentiated mouse podocytes from the control and LN groups by analysing p62 levels and LC3-II/LC3-I ratios. Western blot analysis showed significantly increased LC3-II/LC3-I ratios and decreased p62 levels in primary podocytes from the LN group (Fig. 2B). In addition, we measured the expression of podocyte marker proteins, such as LC3, by immunofluorescence [22]. LC3 expression was greatly increased in the cultured podocytes from the LN group compared to the control group (Fig 2C). These data suggest that LN activates autophagy in podocytes.

Lupus nephritis elevates COX-2 expression in podocytes

To explore the role of COX-2 in LN, COX-2 expression was assessed in primary podocytes from control and LN mice. The levels of COX-2 protein, as well as PGE2 concentration, were increased in LN podocytes (Fig. 3B). Furthermore, immunofluorescence analysis revealed that COX-2 expression was higher in the cytoplasm and perinuclear space of LN podocytes

than in control podocytes (Fig. 3C). These results are consistent with other reports that COX-2 is expressed in the perinuclear space in granulocytes [22] and HaCaT cells [23].

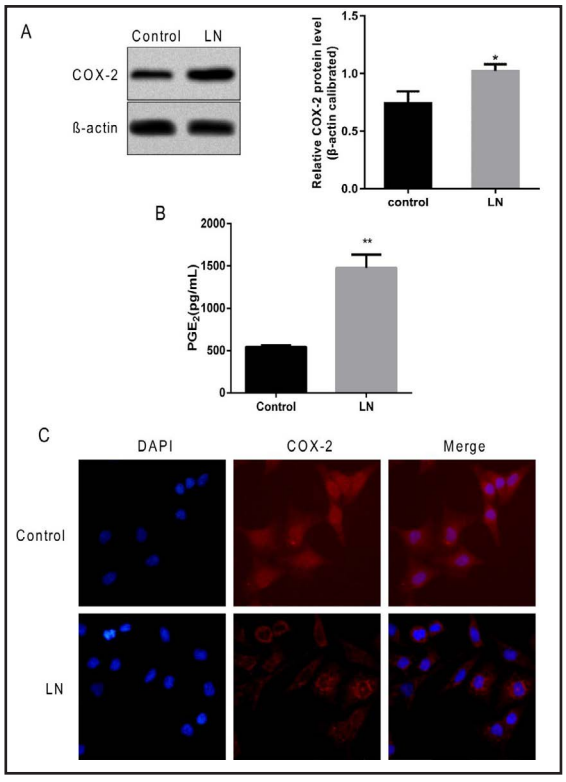

Fig. 3. Lupus nephritis elevates the COX-2 expression in podocytes. (A) COX-2 protein levels. (B) PGE2 production and (C) Immunofluorescence analysis indicates COX-2 expression is elevated in LN podocytes. (*P<0.05, **P<0.01 vs. control)

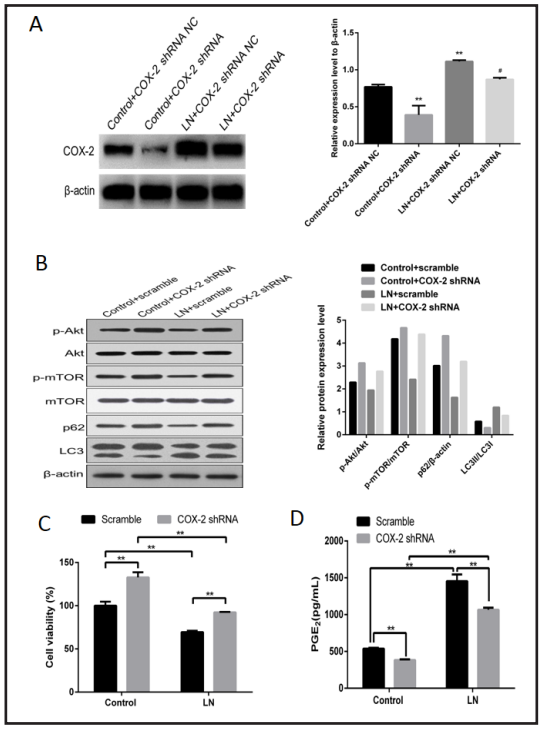

Fig. 4. LN-induced autophagy can be reversed by inhibition of COX-2. (A–D) Results of COX-2 silencing using COX-2 shRNA: (A) COX-2 levels (**P<0.01 vs. Control+COX-2 shRNA NC; #P<0.05 vs. LN+COX-2 shRNA NC). (B) Expression of autophagy-related proteins (p-AKT, AKT, p-mTOR, mTOR, LC3 and p62) analysed by western blot (**p<0.01 vs Control+COX-2 shRNA NC, ##p<0.01 vs LN+COX-2 shRNA NC, @@ p<0.01 vs Control+COX-2 shRNA). (C) alteration of cell growth (**P<0.01). (D) production of PGE2 (**P<0.01).

Inhibition of COX-2 reverses LN-induced autophagy

To elucidate whether COX-2 overexpression is required for LN-induced autophagy, COX-2 was inhibited using siRNA-mediated silencing of COX-2. Silencing COX-2 with siRNA resulted in a significant decrease in COX-2 protein levels (Fig. 4A). Western blot analysis revealed that silencing COX-2 in LN podocyctes resulted in an increase in p62, p-AKT, and p-mTOR, and a decrease in LC3-II/LC3-I ratios (Fig. 4B), indicating that silencing of COX-2 impairs autophagic activity in LN podocytes. In addition, CCK-8 assays indicated that autophagy blockade by COX-2 siRNA alleviated LN-induced podocyte damage (Fig. 4C). Additionally, PGE2 was also decreased in LN podocytes after transfection with COX-2-specific siRNA (Fig. 4D).

LN-induced COX-2 overexpression is dependent on ER stress

We evaluated the ER stress response in the LN and control groups. In the LN group, the levels of p-elF2, CHOP, and Bip were significantly higher than in the control group (Fig. 5A). Moreover, immunofluorescence analysis confirmed that Bip and CHOP were significantly increased in the nuclear space of LN podocytes compared to control podocytes (Fig. 5B, 5C).

To further characterize the role of ER stress in podocyte damage in LN, we treated podocytes with 4-PBA, an inhibitor of ER stress [24] that is widely used to decrease ER stress. 4-PBA treatment decreased expression of ATF4, CHOP, COX-2, BiP, and p-elF2, and reduced LC3-II/LC3-I ratios (Fig. 6A, 6B). In addition, CCK-8 assays indicated that ER stress blockade alleviated LN-induced podocyte damage (Fig. 6C).

LN-induces COX-2 overexpression via ATF4 transcriptional regulation

To investigate the molecular mechanisms of LN-induced overexpression of COX-2 accompanying ER stress, we evaluated the role of the transcription factor ATF4. ATF4 protein levels were significantly increased in the LN group compared to the control group (Fig. 7A). Transfection of LN and control cells with ATF4 siRNA resulted in a significantly decreased in ATF4 levels, and attenuated LN-induced COX-2 and CHOP expression (Fig. 7B,7C). These results indicate that the transcriptional activation of ATF4 is essential for COX-2 overexpression in LN-induced podocyte damage. Moreover, immunofluorescence analysis confirmed that ATF4 was significantly increased in the nuclear space of LN podocytes compared to control podocytes (Fig. 7D).

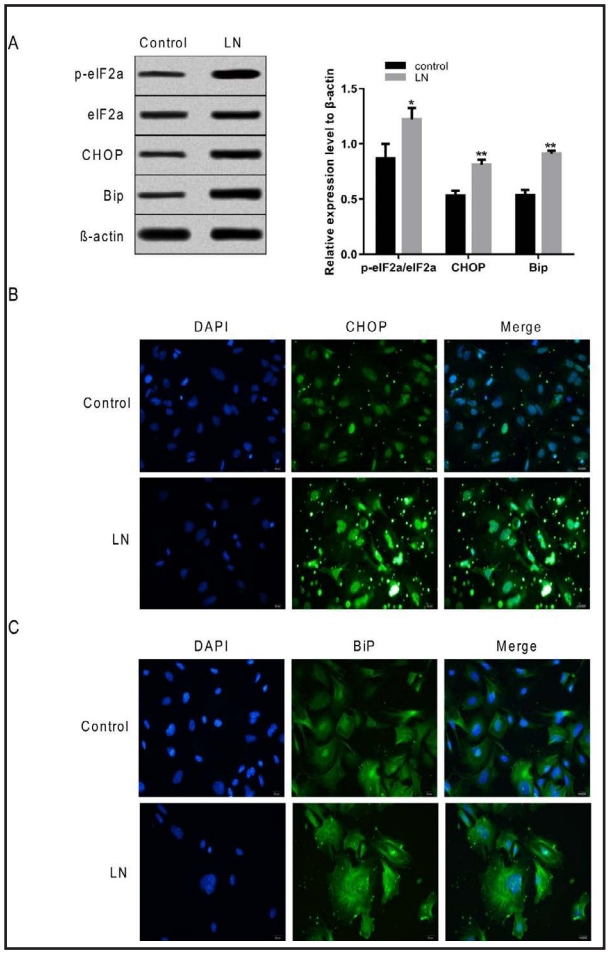

Fig. 5. LN induces ER stress. (A) Levels of proteins related to ER stress (p-eIF2α, eIF2α, CHOP and Bip) induced by LN. (B-C) Immunofluorescence analysis reveals that CHOP and Bip are elevated in LN podocytes. (*P<0.05, **P<0.01).

Discussion

In this study, we demonstrate that LN leads to kidney damage and

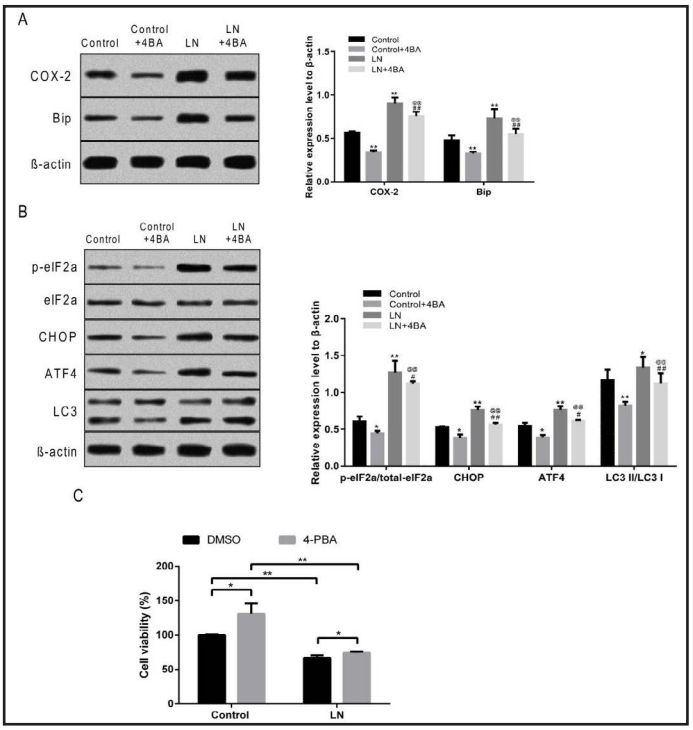

Fig. 6. COX-2 induction in podocytes by LN requires ER stress. (A-C) 4-PBA treatment partially counteracts LN-induced ER stress, autophagy and cell growth inhibition. (A) Levels of proteins COX-2 and Bip (*P<0.05, **P<0.01 vs. control. #P<0.05 vs. LN). (B) Levels of proteins related to ER stress and autophagy (p-eIF2α, Total-eIF2α, CHOP, ATF4 and LC3) induced by LN(**p<0.01 vs Control+COX-2 shRNA NC, ##p<0.01 vs LN+COX-2 shRNA NC, @@p<0.01 vs Control+COX-2 shRNA). (C) Alteration of cell growth (*P<0.05,**P<0.01).

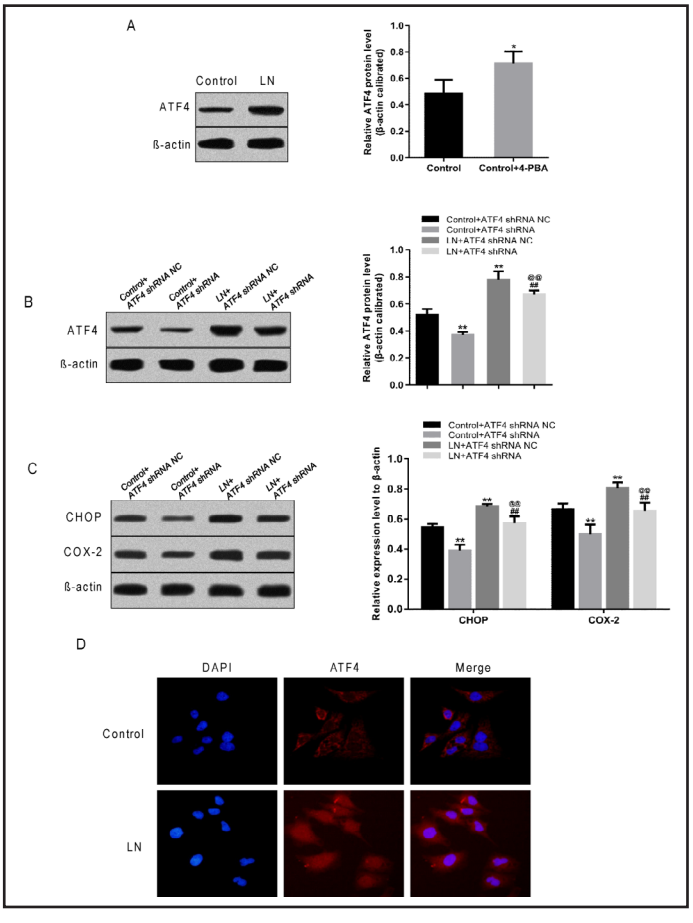

Fig. 7. COX-2 induction by LN is mediated by ATF4 transactivation. (A) LN increases the ATF4 protein levels in LN podocytes (**P<0.01 vs. control). (B) ATF4silencing decreases LN-induced ATF4 translocation; NC is the siRNA negative control (*P<0.05 vs. control+ATF4 shRNA NC; #P<0.05 vs. LN+ATF4 shRNA NC). (C) ATF4 silencing restores LN-induced CHOP and COX-2 protein overexpression (**p<0.01 vs Control+COX-2 shRNA NC, ##p<0.01 vs LN+COX-2 shRNA NC, @@p<0.01 vs Control+COX-2 shRNA). (D) In podocytes, LN causes ATF4 translocation from the cytoplasm to the nucleus, as detected by immunofluorescence microscopy.

dysfunction, and induces podocyte autophagy in mice. LN also caused renal hypertrophy and pathological changes. Furthermore, the number of podocytes was significantly decreased in LN mice compared with control mice. We also discovered that LN-induced podocyte autophagy is related to COX-2 overexpression. We showed that treatment with COX-2 inhibitors, such as 4-PBA, can reduce LN-induced podocyte injury and autophagy, which is further exemplified by protection against LN injury in podocyte cells caused by siRNA knockdown of COX-2. Moreover, we demonstrate that ER stress is required for LN-induced COX-2 overexpression in cultured podocytes. Finally, our research confirmed that the ATF4 pathway stimulates COX-2 transcription to mediate LN-induced podocyte autophagy. Our study reports, for the first time, a novel molecular mechanism of LN-induced podocyte autophagy and injury, which is dependent on COX-2 overexpression regulated by ER stress.

Kidneys are commonly involved in SLE. Auto-antibodies deposited in the kidneys activates inflammation, leading to LN, which can advance to end-stage renal failure [25]. The pathogenic mechanisms of LN are incompletely understood. Previous studies reported that long-lived memory plasma cells play a role in the production of autoantibodies and can be detected in the peripheral blood of SLE patients during a disease flare [26]. However, the exact contribution of these memory plasma cells to the development of LN is unclear. Glomerular immune-complex deposition leads to the influx of multiple immune cells, including macrophages. In SLE, sialoadhesin-expressing monocytes have been shown to correlate with disease severity [27].

Autophagy is an evolutionarily conserved catabolic process that degrades cytoplasmic contents through lysosomes; at least three

major types of autophagy have been identified [28]. Autophagy is active at a basal level in all cell types, can be further induced in response to different stresses, and can also affect diverse aspects of the immune system [29]. Genome-wide association studies (GWAS) have linked polymorphisms in autophagy-related genes to some autoinflammatory and autoimmune diseases, specifically in SLE and LN[7, 30]. In our study, LN increased serum CRE and BUN, and also caused histological alterations and dysfunction in kidney tissue.

The mTOR kinase is a key negative controller of autophagy [31], and is the main effector in the PI3K-AKT-mTOR pathway [32]. Inhibition of the PI3K-AKT-mTOR pathway can activate autophagy [33]. However, inhibition of mTOR by pharmacological agents, such as rapamycin, can prevent cellular apopotosis induced by HG-treatment [34] and promote AKT phosphorylation by blocking the phosphorylation of insulin receptor substrate 1 and p70 ribosomal S6 kinase [32, 35], which are part of a negative feedback mechanism of PI3K-AKT signal transduction [36]. Our results demonstrate that LN inhibits mTOR, but increases the phosphorylation of AKT, suggesting that LN inhibited mTOR independent of AKT activation. Another possible explanation for LN-induced increased AKT phosphorylation could be due to the loss of mTOR-mediated feedback inhibition on AKT activation, similar to rapamycin treatment. Consistently, we discovered that COX-2 knockdown increased p-mTOR, leading to a decrease of autophagy and p-AKT. Furthermore, we discovered that autophagy and ER stress are activated in LN podocytes. We confirmed that LN induces ER stress in podocyte cells using two ER stress reporter systems. We also demonstrated that an ER stress inhibitor counteracted LN-induced podocyte autophagy and LN-induced inhibition of proliferation in podocyte cells; this was verified by inhibition of ER stress with 4-PBA. Activation of ER stress has been shown to induce autophagy [37, 38], suggesting that ER stress may be required for LN-induced autophagy in the kidney. We demonstrate that LN-induced COX-2 overexpression was attenuated by ATF4 silencing, suggesting that COX-2-mediated autophagy induced by LN in podocytes requires ER stress.

We found that LN increased COX-2 and PGE2, along with podocyte autophagy and ER stress, in cultured podocytes. Inhibition of COX-2 by 4-PBA or COX-2 RNAi reversed LN-induced podocyte autophagy. In support of these results, 4-PBA has been shown to inhibit LPS-activated ER stress. Moreover, it was reported that autophagy is decreased by treatment with 4-PBA in acute lung injury [39]. Furthermore, 4-PBA also attenuates transit ischemic brain injury [40]and right ventricular dysfunction during monocrotaline-induced rat

pulmonary arterial hypertension [41].

The molecular mechanisms by which ER stress regulates autophagy have been well-characterized. The ER stress response involves three transmembrane signal transducers including PERK, protein kinase IRE1, and the transcription factor ATF6[38]. Our results showed that LN affects Bip, ATF4, and CHOP gene expression, but not expression of IRE1 or ATF6 in podocytes. Furthermore, LN-induced podocyte autophagy was counteracted by ATF4 RNAi. Therefore, it is likely that the ATF4 pathway regulates LN-induced podocyte autophagy and injury.

The present research demonstrated that LN promotes ATF4 translocation from the cytoplasm to the nucleus. Silencing of ATF4 inhibited both its translocation and COX-2 overexpression induced by LN. When ATF4 is translocated into the nucleus, it binds to the promoter region of the PTGS2 gene and elevates *PTGS2* transcription [42]. Our study exposed a novel pathway by which LN causes autophagy via COX-2 overexpression, and demonstrates that COX-2 is transcriptionally regulated by ATF4.

In conclusion, the present research demonstrates that LN results in renal podocyte injury and autophagy. We report that LN-induced autophagy is mediated by the ATF4 pathway and its downstream effector, COX-2, suggesting that COX-2 is a potential molecular marker of LN-induced kidney injury. The present study also suggests that safer COX-2 inhibitors or other therapeutic strategies should be developed for treatment of LN-induced podocyte injury.

Acknowledgements

We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript.

This work was supported by grants from the National Natural Science Foundation of China (Grant Number：81641026), the Major Projects of Science and Technology Department of Zhejiang Province (Grant Number 2014C03047-2), the Natural Science Foundation of Zhejiang Province (Grant Number：LY16H050005, LZ17H050001), and the Project of Scientific Research Foundation of Chinese Medicine (Grant Number：2017ZA010) , the General Project of the Medical and Health of Zhejiang Province(Grant Number：2016KYB007).

Disclosure Statement

The authors have no competing interests to declare. All authors have approved the final version of the manuscript and agreed to submit it for publication.

References

1 Lech M, Anders HJ: The pathogenesis of lupus nephritis. J Am Soc Nephrol 2013;24:1357-1366.
2 Maroz N, Segal MS: Lupus nephritis and end-stage kidney disease. Am J Med Sci 2013;346:319-323.
3 Han TS, Schwartz MM, Lewis EJ: Association of glomerular podocytopathy and nephrotic proteinuria in mesangial lupus nephritis. Lupus 2006;15:71-75.
4 Kraft SW, Schwartz MM, Korbet SM, Lewis EJ: Glomerular podocytopathy in patients with systemic lupus erythematosus. J Am Soc Nephrol 2005;16:175-179.
5 Mizushima N: Autophagy: process and function. Genes Dev 2007;21:2861-2873.
6 Levine B, Kroemer G: Autophagy in the pathogenesis of disease. Cell 2008;132:27-42.
7 Wang L, Law HK: The Role of Autophagy in Lupus Nephritis. Int J Mol Sci 2015;16:25154-25167.
8 Krebs J, Agellon LB, Michalak M: Ca(2+) homeostasis and endoplasmic reticulum (ER) stress: An integrated view of calcium signaling. Biochem Biophys Res Commun 2015;460:114-121.

9 Wang B, Xu X, He X, Wang Z, Yang M: Berberine Improved Aldo-Induced Podocyte Injury via Inhibiting Oxidative Stress and Endoplasmic Reticulum Stress Pathways both *In vivo* and *In vitro*. Cell Physiol Biochem 2016;39:217-228.
10 Rutkowski DT, Kaufman RJ: That which does not kill me makes me stronger: adapting to chronic ER stress. Trends Biochem Sci 2007;32:469-476.
11 Liu S, Sarkar C, Dinizo M, Faden AI, Koh EY, Lipinski MM, Wu J: Disrupted autophagy after spinal cord injury is associated with ER stress and neuronal cell death. Cell Death Dis 2015;6:e1582.
12 Taniguchi M, Yoshida H: Endoplasmic reticulum stress in kidney function and disease. Curr Opin Nephrol Hypertens 2015;24:345-350.
13 Yorimitsu T, Nair U, Yang Z, Klionsky DJ: Endoplasmic reticulum stress triggers autophagy. J Biol Chem 2006;281:30299-30304.
14 Hao CM, Breyer MD: Physiologic and pathophysiologic roles of lipid mediators in the kidney. Kidney Int 2007;71:1105-1115.
15 Paul AG, Chandran B, Sharma-Walia N: Cyclooxygenase-2-prostaglandin E2-eicosanoid receptor inflammatory axis: a key player in Kaposi's sarcoma-associated herpes virus associated malignancies. Transl Res 2013;162:77-92.
16 Agrawal S, Guess AJ, Chanley MA, Smoyer WE: Albumin-induced podocyte injury and protection are associated with regulation of COX-2. Kidney Int 2014;86:1150-1160.
17 Jia Z, Sun Y, Liu S, Liu Y, Yang T: COX-2 but not mPGES-1 contributes to renal PGE2 induction and diabetic proteinuria in mice with type-1 diabetes. PLoS One 2014;9:e93182.
18 Ranganathan PV, Jayakumar C, Mohamed R, Dong Z, Ramesh G: Netrin-1 regulates the inflammatory response of neutrophils and macrophages, and suppresses ischemic acute kidney injury by inhibiting COX-2-mediated PGE2 production. Kidney Int 2013;83:1087-1098.
19 Wu L, Feng Z, Cui S, Hou K, Tang L, Zhou J, Cai G, Xie Y, Hong Q, Fu B, Chen X: Rapamycin upregulates autophagy by inhibiting the mTOR-ULK1 pathway, resulting in reduced podocyte injury. PLoS One 2013;8:e63799.
20 Li LM, Hou DX, Guo YL, Yang JW, Liu Y, Zhang CY, Zen K: Role of microRNA-214-targeting phosphatase and tensin homolog in advanced glycation end product-induced apoptosis delay in monocytes. J Immunol 2011;186:2552-2560.
21 Zhu H, Wu H, Liu X, Li B, Chen Y, Ren X, Liu CG, Yang JM: Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells. Autophagy 2009;5:816-823.
22 Watanabe T, Nagai A, Sheikh AM, Mitaki S, Wakabayashi K, Kim SU, Kobayashi S, Yamaguchi S: A human neural stem cell line provides neuroprotection and improves neurological performance by early intervention of neuroinflammatory system. Brain Res 2016;1631:194-203.
23 Soliman E, Henderson KL, Danell AS, Van Dross R: Arachidonoyl-ethanolamide activates endoplasmic reticulum stress-apoptosis in tumorigenic keratinocytes: Role of cyclooxygenase-2 and novel J-series prostamides. Mol Carcinog 2016;55:117-130.
24 Luo B, Lin Y, Jiang S, Huang L, Yao H, Zhuang Q, Zhao R, Liu H, He C, Lin Z: Endoplasmic reticulum stress eIF2alpha-ATF4 pathway-mediated cyclooxygenase-2 induction regulates cadmium-induced autophagy in kidney. Cell Death Dis 2016;7:e2251.
25 de Zubiria Salgado A, Herrera-Diaz C: Lupus nephritis: an overview of recent findings. Autoimmune Dis 2012;2012:849684.
26 Hiepe F, Dorner T, Hauser AE, Hoyer BF, Mei H, Radbruch A: Long-lived autoreactive plasma cells drive persistent autoimmune inflammation. Nat Rev Rheumatol 2011;7:170-178.
27 Biesen R, Demir C, Barkhudarova F, Grun JR, Steinbrich-Zollner M, Backhaus M, Haupl T, Rudwaleit M, Riemekasten G, Radbruch A, Hiepe F, Burmester GR, Grutzkau A: Sialic acid-binding Ig-like lectin 1 expression in inflammatory and resident monocytes is a potential biomarker for monitoring disease activity and success of therapy in systemic lupus erythematosus. Arthritis Rheum 2008;58:1136-1145.
28 Levine B, Klionsky DJ: Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell 2004;6:463-477.
29 He C, Klionsky DJ: Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 2009;43:67-93.
30 Levine B, Deretic V: Unveiling the roles of autophagy in innate and adaptive immunity. Nat Rev Immunol 2007;7:767-777.

31 Heras-Sandoval D, Perez-Rojas JM, Hernandez-Damian J, Pedraza-Chaverri J: The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cell Signal 2014;26:2694-2701.
32 O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J, Rosen N: mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 2006;66:1500-1508.
33 Zhang C, Jia X, Wang K, Bao J, Li P, Chen M, Wan JB, Su H, Mei Z, He C: Polyphyllin VII Induces an Autophagic Cell Death by Activation of the JNK Pathway and Inhibition of PI3K/AKT/mTOR Pathway in HepG2 Cells. PLoS One 2016;11:e0147405.
34 Lei J, Zhao L, Zhang Y, Wu Y, Liu Y: High Glucose-Induced Podocyte Injury Involves Activation of Mammalian Target of Rapamycin (mTOR)-Induced Endoplasmic Reticulum (ER) Stress. Cell Physiol Biochem 2018;45:2431-2443.
35 Miyazaki M, McCarthy JJ, Esser KA: Insulin like growth factor-1-induced phosphorylation and altered distribution of tuberous sclerosis complex (TSC)1/TSC2 in C2C12 myotubes. FEBS J 2010;277:2180-2191.
36 Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, Barnett J, Leslie NR, Cheng S, Shepherd PR, Gout I, Downes CP, Lamb RF: The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol 2004;166:213-223.
37 Fouillet A, Levet C, Virgone A, Robin M, Dourlen P, Rieusset J, Belaidi E, Ovize M, Touret M, Nataf S, Mollereau B: ER stress inhibits neuronal death by promoting autophagy. Autophagy 2012;8:915-926.
38 Marciniak SJ, Ron D: Endoplasmic reticulum stress signaling in disease. Physiol Rev 2006;86:1133-1149.
39 Zeng M, Sang W, Chen S, Chen R, Zhang H, Xue F, Li Z, Liu Y, Gong Y, Zhang H, Kong X: 4-PBA inhibits LPS-induced inflammation through regulating ER stress and autophagy in acute lung injury models. Toxicol Lett 2017;271:26-37.
40 Zhang X, Yuan Y, Jiang L, Zhang J, Gao J, Shen Z, Zheng Y, Deng T, Yan H, Li W, Hou WW, Lu J, Shen Y, Dai H, Hu WW, Zhang Z, Chen Z: Endoplasmic reticulum stress induced by tunicamycin and thapsigargin protects against transient ischemic brain injury: Involvement of PARK2-dependent mitophagy. Autophagy 2014;10:1801-1813.
41 Wang JJ, Zuo XR, Xu J, Zhou JY, Kong H, Zeng XN, Xie WP, Cao Q: Evaluation and Treatment of Endoplasmic Reticulum (ER) Stress in Right Ventricular Dysfunction during Monocrotaline-Induced Rat Pulmonary Arterial Hypertension. Cardiovasc Drugs Ther 2016;30:587-598.
42 Cho HK, Cheong KJ, Kim HY, Cheong J: Endoplasmic reticulum stress induced by hepatitis B virus X protein enhances cyclo-oxygenase 2 expression via activating transcription factor 4. Biochem J 2011;435:431-439.