Intrarenal Renin-Angiotensin System Mediates Fatty Acid-Induced ER Stress in the Kidney

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Abstract

Obesity-related kidney disease is related to caloric excess promoting deleterious cellular responses. Accumulation of saturated free fatty acids in tubular cells produces lipotoxicity involving significant cellular dysfunction and injury. The objectives of this study were to elucidate the role of renin-angiotensin system (RAS) activation in saturated fatty acid-induced endoplasmic reticulum (ER) stress in cultured human proximal tubule epithelial cells (HK2) and in mice fed with high-fat diet. Treatment with saturated fatty acid palmitic acid (PA) (0.8mM) for 24h induced ER stress in HK2, leading to an unfolded protein response as reflected by increased expressions of the ER chaperone BiP and proapoptotic transcription factor CHOP protein as evaluated by immunoblotting. PA treatment also induced increased protein expression of IRE1α, phosphorylated eIF2α, and ATF4 as well as activation of caspase 3. PA treatment was associated with increased angiotensin II levels in cultured medium. The AT1R blocker valsartan or renin inhibitor aliskiren dramatically suppressed PA-induced upregulation of BiP, CHOP, IRE1α, p-eIF2alpha, and ATF4 in HK2 cells. In contrast, valsartan or aliskiren did not prevent ER stress induced by tunicamycin. C57BL/6 mice fed with high-fat diet for 14 weeks exhibited increased protein expressions of BiP and CHOP in comparison to control mice, which were significantly attenuated by the valsartan treatment. Increased angiotensin II levels in serum and urine were observed in mice fed with high-fat diet when compared with controls. It is suggested that the intrarenal RAS activation may play an important role in diabetic kidney injury via mediating ER stress induced by saturated fatty acid.

Key words: saturated fatty acid, angiotensin II, valsartan, kidney
INTRODUCTION

Diabetic nephropathy (DN) is the major cause of end-stage renal disease in many industrialized and developing countries. Type 2 diabetes (T2D) mellitus is characterized by hyperglycemia and dyslipidemia with increased plasma levels of long-chain free fatty acids (FFAs) (53). Recently, elevated levels of FFA have been detected in the urine of patients suffering from DN (43). In proteinuric kidney disease, FFAs binding to albumin are filtered and reabsorbed by the proximal tubule, which may contribute to tubulointerstitial inflammation and fibrosis (49, 50).

Accumulation of saturated FFA and their metabolites within cells produces lipotoxicity resulting in significant cellular dysfunction and injury (58). It is now widely accepted that lipid accumulation in the kidney critically contributes to the pathogenesis of DN.

The endoplasmic reticulum (ER) is the organelle where transmembrane, secretory, and ER resident proteins are folded and matured (2, 51). Accumulation of unfolded or misfolded protein results in ER stress and activates the unfolded protein response (UPR). In mammalian cells, there are three major UPR pathways, activated by transmembrane sensors located in the ER membrane. These three major pathways are the inositol requiring protein 1 (IRE1), activating transcription factor 6 (ATF6) and the protein kinase-like ER kinase (PERK)-mediated response (64). ER-resident chaperones, including the 78-kDa glucose regulated protein (GRP78, also known as binding immunoglobulin protein, BiP), assist in folding newly synthesized proteins, prevent the accumulation of misfolded proteins, and enhance ER-associated protein degradation (31, 59). However, prolonged or severe ER stress activates PERK-mediated phosphorylation of eukaryotic initiation factor (eIF2α), which leads to expression of activating transcription factor 4 (ATF4) and the transcription factor C/EBP homologous protein (CHOP) (also known as growth arrest and DNA damage (GADD)) — leading to cell death via apoptosis (42).
ER stress could be induced in podocytes in vitro and in a rodent model by protein accumulation (15) or by exposure of renal proximal tubular cells to high albumin concentrations resulting in apoptosis (37). It has been shown that during initial ER stress the tubular epithelial cells can undergo adaptive and protective response, but the persistent proteinuria and hyperglycemia can lead to an epithelial apoptosis (28), followed by an inflammatory response and fibrosis.

It is well known that angiotensin II play most crucial roles in DN through activating angiotensin type 1 receptors. The angiotensin converting enzyme inhibitors (ACEis) and angiotensin receptor blockers (ARBs) are widely used to delay the progression of DN. We (55) and others (23, 46) have demonstrated that RAS blockade with ACEi, ARB, or renin inhibitor effectively attenuated ER stress in kidneys of streptozotocin-induced type 1 diabetic animals, suggesting angiotensin II as a regulator of ER stress and apoptosis in diabetic kidneys.

Type 2 diabetes is usually associated with obesity and excessive tissue lipid accumulation. Palmitic acid (PA), a dietary saturated FFA and the most abundant circulating fatty acid in vivo (41), induces ER stress (10, 32, 44, 57) and is a proapoptotic factor in podocytes, proximal tubule cells and mesangial cells (20, 26, 34, 44). However, the intracellular pathways involved in the development of fatty acid-mediated ER stress within the kidney are not fully understood.

The objectives of the present study were to investigate whether the activation of local RAS directly mediated ER stress induced by palmitic acid in human HK2 cells, and whether the treatment with valsartan, angiotensin II type 1 receptor (AT1R) blocker, inhibited renal ER stress in mice fed with a high-fat diet.
MATERIALS AND METHODS

Materials

Palmitic acid, fatty acid-free BSA, tunicamycin, and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich; anti-BiP (3177), anti-CHOP (2895), anti-pelF2α (3597) /elF2α (9722), anti-IRE1α (3294) and anti-activated caspase 3 (9664) antibodies from Cell Signaling; anti-GRP78 (BiP) (SC-13968 for immunohistochemistry) from Santa-Cruz Biotechnology; anti-ATF4 from Abcam (ab50546) and anti-β-actin from Sigma. Renin was obtained from Cayman Chemical, Michigan USA, and angiotensin II was purchased from Sigma-Aldrich. The horseradish peroxidase-conjugated secondary antibodies were obtained from Dako. Valsartan and aliskiren were kindly provided by Novartis (Basel, Switzerland).

Palmitic acid preparation

Palmitic acid was prepared as described previously (20, 27). Briefly, a stock solution of sodium palmitate (5M) was conjugated to fatty-acid-free bovine serum albumin (BSA) at 37 °C for 1 h prior to addition cultured cells. The palmitic acid concentration used in this study was 0.8mM complexed to BSA (0.2%), which is within the reported nutritional and metabolic disorders ranges of 720 to 3,730µmol/L (25). BSA with vehicle (see below) used for control experiments was prepared and handled exactly the same as BSA to which PA was conjugated to.

Cell culture and treatment

The immortalized human proximal tubular cell line, HK2, was obtained from ATCC and grown in 100-mm culture dishes (Corning, Corning, NY). The cells were cultured in DMEM containing glutamax (Gibco/Invitrogen), supplemented with 10% FCS, 100 units/ml penicillin, and 100 g/ml streptomycin in an atmosphere of 5% CO2 in air at 37℃ up to approximately 90% confluence. All experiments were performed after overnight cell starvation by adding culture medium without FCS. Cells were either fixed in 4% paraformaldehyde for immunofluorescence or lysed with RIPA
buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Thermo Scientific) plus a protease inhibitor cocktail for western blotting. HK2 cells were pretreated for 1 h with valsartan (10^{-6}M) or aliskiren (10^{-7}M) (stock solution was prepared in DMSO) followed by treatment with PA (0.8mM) or tunicamycin (2µg/mL) for 24 h. HK2 cells treated with vehicle (DMSO) and BSA were used for control. For angiotensin II study, HK2 cells were treated with Ang II (10^{-7}M) or renin (10^{-8}M) for 24h.

**Cell viability assay**

The MTT assay was performed to examine the effect of PA on cell viability. Briefly, HK2 cells were seeded into a 96-well culture plate at a density of 8,000 cells/well. After 24-hour incubation, the cells were treated with PA at concentrations ranging from 0, 0.1, 0.2, 0.4, 0.8, and 1.2mM for a further 24 hours. Cell viability was determined by the reduction of MTT. The absorbance was measured by using a Geniosplus microplate reader (Genius-basic, Tecan, Austria) at wavelengths of 560 nm (MTT formazan) and 670 nm (background). The experiment was conducted in triplicate. Results of the experiments are expressed as percentage of viable cells that was observed in PA treated cells.

**In situ cell death detection (TUNEL assay)**

Apoptotic cell death was detected by terminal deoxynucleotidyl transferase-mediated deoxyuridinriphosphate nick end labeling (TUNEL) assay, using an DeadEnd™ Colorimetric TUNEL System Kit (Promega, Madison, WI, cat# 7130) according to the manufacturer’ instructions. Brown labeled TUNEL positive cells were counted under a ×400 magnification in at least 10 random fields. The apoptotic index was calculated as the percentage of TUNEL-positive cells/total number of HK2 cells. All counting procedures were performed in a blinded fashion.

**Measurement of Ang II**

Blood and urine samples as well as culture medium were collected and angiotensin II concentrations were determined using commercially available Iodine
[\textsuperscript{125}I] Ang II radioimmunoassay kit (Beijing North Institute of Biological Technology, China, catalogue number: D02PJB) following the manufacturer’s protocol. The assay is based upon the competition of \textsuperscript{125}I-Ang II and Ang II (standard or samples) binding to the limited quantity of antibodies specific for Ang II in each reaction mixture. The standard range of the kit is 25-800pg/mL and the sensitivity is 10pg/mL. The concentration of AngII was extrapolated from the standard curve constructed in the same plate using curve-fitting software capable of four parameter logistics.

\textit{Animals and treatments}

Eight-week-old male C57BL/6 mice were obtained from the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. All mice were housed in an animal facility with a 12-h light–dark cycle and water ad lib. The mice were fed with a low fat diet (10\% of total calories from fat) or a high-fat diet (60\% of total calories from fat, Guangdong Medical Lab Animal Center, China) for fourteen weeks. The diets are otherwise identical in their protein, mineral and ion content. The mice were assigned to three groups, control with standard diet (CTL), high-fat diet group (HFD), and high-fat diet with valsartan treatment (HFDV, 8 mg/kg per day). Valsartan was administered in drinking water for ten weeks. Mice were placed in metabolic cages for 24-h urine collection at week 14, and the body weight and the blood glucose were measured at these time points. On week 14, all mice were anesthetized with pentobarbital, and the kidneys were rapidly excised from mice to perform biochemical and histological examinations, as described in following sections. All animal procedures were in accordance with the policies of the Animal Care and Use Committee, Sun Yat-Sen University, and conformed to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health in China.

\textit{Blood and urine chemistry}

The blood concentration of glucose was measured by blood glucose meter (Bayer Healthcare LLC, USA), creatinine level was measured by using QuantiChrom\textsuperscript{TM} Creatinine Assay Kit (Bioassay Systems). Urinary kidney injury molecule 1 (KIM-1)
levels were measured by using CLIA Kit (Cloud-Clone Corp, Houston). The urinary protein was measured by using the BCA Protein Assay Kit (Thermo Scientific). Urinary protein excretion rate (UPER) was calculated by the following formula: 24-hour total volume of urine (ml/100g body weight) × urinary protein levels (µg/ml) (39).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from the kidney cortex or cultured cells according to the manufacturer’s instructions for Trizol reagent (Invitrogen, CA, USA). Total RNA (1000 ng) was used for reverse transcription using PrimeScript® RT reagent Kit Perfect Real Time kit (Takara Bio Inc., Japan). The cDNA was used for quantitative real-time PCR analysis (qPCR) using SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara Bio Inc., Japan). All samples were analyzed in triplicate. Average CT values in each group are shown in Table 1. The calibrator sample was selected from PBS-treated tissue or cell samples, and GAPDH was used as an internal control. Relative amounts of mRNA were normalized by GAPDH and a control sample and calculated by using the comparative Ct (2−ΔΔCt) (cycle threshold) method. Signals from the control group were assigned a relative value of 1.0. Primers were designed based on previous publications or on the primerbank. Primer sequences used are provided in Table 2 and 3.

Western blotting

Cell protein was extracted with RIPA buffer supplemented with protein inhibitor cocktail. Protein lysate was prepared from the kidney cortex as described previously (55). The total protein concentrations of the samples were measured by the BCA method. Equal amounts of protein samples were subjected to SDS-PAGE, and they were then transferred to PVDF membranes. After being blocked with 5% fat-free milk poHFDer in 0.1% Tween 20 in Tris-buffered saline (20 mmol/l Tris•HCl and 150 mmol/l NaCl, pH 7.4), the blots were incubated with primary antibodies. Corresponding secondary antibodies were visualized using enhanced
chemiluminescence (Pierce, Bradford, IL). Signals were quantified by densitometry and normalized by corresponding value of β-actin and control samples.

**Immunohistochemistry and immunofluorescence**

Sections (4-μm thick) cut from 4% paraformalin-fixed, paraffin-embedded kidney samples were used for H&E staining and immunostaining of BiP (55). Briefly, after dewaxing and rehydration, a microwave pretreatment in citrate buffer (pH 6.0) was performed to unmask antigens present in the renal tissue. Tissue sections were then incubated overnight at 4°C with BiP antibodies. After rinsing in phosphate-buffered saline, slides were exposed for 1h to the secondary antibody. Kidney sections were finally incubated with ABC complex (Vector Laboratories, Burlingame, CA) for 30 min and bound peroxidase activity was detected with the 3, 30-diaminobenzidine kit. Samples were imaged with a light microscope (Leica DM 2000, Germany).

HK2 cells were seeded onto sterile glass cover-slips in 6-well dishes. The following days, cells were treated as indicated above. Cells were fixed with 1 mL 4% paraformaldehyde in PBS for 20 min prior to permeabilization in 0.25 % Triton X-100 in PBS for 15 min at room temperature with gentle agitation. Cells were blocked with 10 % normal goat serum for 30 min followed by incubation with primary (4°C, overnight) and secondary (1h, at room temperature) antibodies. Primary antibodies were detected with fluorescently labeled anti-mouse Alexa 488 and anti-rabbit Alexa 594 diluted 1:200 (Invitrogen, Burlington, ON). Nuclei were counterstained with DAPI (1 mg/mL in PBS), and cover-slips mounted onto slides and visualized with a confocal fluorescent microscope (Leica, DMI4000B, Germany). Quantifications were performed in a blinded manner. Image J software was used to quantify the BiP or CHOP immunofluorescence intensity by measuring the positive staining area.

**Statistical analysis**
Results are presented as the means ± SD. Data were analyzed by one-way ANOVA and Student-Newman-Keuls tests for multiple comparisons. Statistical significance was accepted at the P < 0.05 level.
RESULTS

Cell viability after palmitic acid treatment

As a vehicle BSA treatment alone did not induce increases in protein abundance of ER stress markers BiP and CHOP in HK2 cells, and valsartan or aliskiren had no effects on these markers (Fig. 1A and B). To investigate HK2 cell viability in response to PA treatment, MTT assay (uptake directly correlates with the number of viable cells) was employed. As shown in Figure 1C, there was a dose-dependent decrease in cell viability after PA treatment when compared with non-treatment (Fig. 1C). The concentration of 0.8mM was chosen for further experiments.

Valsartan or aliskiren blocks palmitic acid-induced ER stress in HK2 cells.

Palmitic acid produced a significant increase in the expression of markers of ER stress by Western blotting. Specifically, incubation of HK2 cells with 0.8 mM PA produced significantly increased levels of BiP, IRE1α, p-eIF2α, ATF4 proteins after 24 h (Fig. 1D and E), whereas eIF2α protein abundance was unchanged. The proapoptotic transcription factor CHOP, which is typically upregulated during severe ER stress, was detectable at low levels in controls and was increased ninefold after incubation of PA (Fig. 1D and E). Valsartan or aliskiren treatment significantly decreased expression of BiP, IRE1α, p-eIF2α, and ATF4 proteins induced by PA; and dramatically reduced CHOP expression (Fig. 1D and E). Immunofluorescence confirmed prevention of increased expression of BiP (Fig. 2A and 2C) and CHOP proteins (Fig. 2B and 2C) by RAS blockade with valsartan or aliskiren. mRNA levels of BiP in HK2 cells were significantly increased after PA treatment for 24h (6.05 ± 1.36 vs. 1.0 ± 0.16 in controls, p<0.05), which was unaffected by either valsartan (7.89 ± 1.62, p<0.05 compared with controls) or aliskiren (8.22 ± 1.42, p<0.05 compared with controls). X-box-binding protein 1 (XBP-1), another ER stress marker downstream of IRE1 pathway, was significantly upregulated at mRNA levels in PA-treated HK2 cells (3.54 ± 0.19 in PA group vs. 1.0 ± 0.28 in controls, p<0.05), which was significantly inhibited by valsartan (2.12 ± 0.17, p<0.05 when compared with PA
or controls) or aliskiren (2.58 ± 0.11, p<0.05 when compared with PA or controls). In contrast to increased protein expression, mRNA levels of CHOP in HK2 cells didn’t change after 24h PA incubation (0.84 ± 0.2 in PA group vs. 1.0 ± 0.1 in controls, p>0.05) with valsartan (0.76 ± 0.21, p>0.05 compared with controls) or with aliskiren (1.19 ± 0.49, p>0.05 compared with controls).

**Valsartan or aliskiren prevents HK2 cell apoptosis induced by palmitic acid**

To investigate whether PA induced HK2 cell apoptosis, we examined the activation of an effector caspase 3 by immunoblotting using an antibody specific for cleaved and activated caspase 3. Incubation with PA increased cleaved caspase-3 protein abundance, indicating that PA induced HK2 cell apoptosis. This was significantly prevented by either valsartan or aliskiren treatment (Fig. 3A and B). The percentage of HK2 cells containing TUNEL-positive nuclei significantly increased in response to PA treatment, which was attenuated by valsartan or aliskiren (Fig. 3C and D).

**Angiotensin II is involved in palmitic acid-induced ER stress in HK2 cells**

Since RAS blockade with valsartan or aliskiren attenuated ER stress, angiotensin II was therefore considered to play a role in PA-induced ER stress in HK2 cells. Accordingly, we measured angiotensin II content in medium of cultured HK2 cells. As shown in Fig. 3E, levels of angiotensin II was significantly higher in all PA-treated groups than controls. Angiotensin II level showed about 22% decrease in aliskiren treated cells (44.89 ± 1.78pg/mL) when compared to PA treated cells (58.65 ± 5.93pg/mL), although this decrease did not reach statistical significance (p=0.06).

To further investigate the role of RAS in ER stress, we examined whether exposure to renin (10^{-8}M) or angiotensin II (10^{-7}M) directly caused ER stress in HK2 cells. As seen in Fig. 3G and H, angiotensin II treatment was associated with strong upregulation of BiP in HK2 cells; CHOP expression was also significantly increased. In contrast, neither BiP nor CHOP protein expression was changed in response to renin treatment.
Effects of valsartan or aliskiren on mRNA levels of RAS components in HK2 cells treated with palmitic acid

The mRNA expression of angiotensinogen, renin, and AT1R was significantly increased in all PA-treated cells; single treatment with valsartan or aliskiren has no effect on such an increase. ACE mRNA levels were reduced in PA-treated HK2 cells, whereas valsartan or aliskiren treatment was associated with recovery of ACE mRNA expression (Fig. 3F).

Valsartan or aliskiren does not block tunicamycin-induced ER stress in HK2 cells

To test whether RAS was universally involved in ER stress, we examined the effect of RAS inhibition on ER stress induced by other inducers, such as tunicamycin (TM 2µg/ml). Consistent with previous studies, incubation of HK2 cells with TM for 24 h produced sequential increases in GRP78 and CHOP and then IRE1α, p-eIF2α, ATF4. However, neither valsartan nor aliskiren was able to reduce these ER stress marker expressions, indicating that RAS activation is unlikely involved in TM-induced ER stress in HK2 cells (Fig. 4).

Valsartan prevents tubular injury in the kidneys of mice fed with high-fat diet

Metabolic data of mice fed a normal, standard chow (CTL) or high-fat diet with (HFDV) or without (HFD) valsartan treatment are shown in Table 4. The body weight and kidney weight in mice fed with high-fat diet were significantly higher than that of CTL mice. The level of plasma glucose was significantly higher in high-fat diet fed mice with (7.96 ± 0.43 in HFDV vs. 6.74 ± 0.21mmol/L in controls, p<0.05) or without valsartan (7.49 ± 0.18mmol/L, p<0.05 when compared with CTL) than control mice. Urine protein excretion rate was higher in HFD mice (47 ± 4.3 in HF vs. 31 ± 3.8µg/100gBW in controls, p<0.05) than controls, and valsartan treatment slightly improved urinary protein excretion (35 ± 4.5µg/100gBW in HFDV). mRNA levels of two tubular injury markers were examined in the cortex of kidneys. Renal mRNA expression levels of neutrophil gelatinase-associated lipocalin (NGAL) and KIM-1 were significantly increased in mice with high-fat diet, valsartan treatment
was associated with reduced mRNA expression of KIM-1 compared with HFD mice. Urinary KIM-1 levels was increased in mice with high-fat diet, but decreased after valsartan treatment (Fig. 5A and B).

Serum and urine angiotensin II levels are increased in mice fed with high-fat diet

In order to examine whether high-fat diet was associated with renal RAS activation, angiotensin II levels were therefore examined. Serum angiotensin II concentration was significantly increased in mice fed with high fat diet when compared with controls, and valsartan treatment induced even higher level than that in non-treated mice (Figure 6A). Compared to controls, urine angiotensin II levels showed significant increases in mice fed with high fat diet, valsartan treatment was associated with a higher level than non-treated mice, but it did not reach statistical significance (Figure 6B).

Effects of valsartan on mRNA levels of RAS components in mice fed with high-fat diet

The renal mRNA expression of angiotensinogen was not different among three groups, however, renin mRNA expression was dramatically increased in mice fed with high-fat diet and this increase was greater after valsartan treatment. Renin receptor mRNA level was decreased and ACE mRNA expression was increased in HFD mice. Valsartan treatment was associated with increased mRNA level of ACE and AT1R when compared with controls (Fig. 6C).

Valsartan reduces BiP and CHOP expression in the kidneys of mice fed with high-fat diet

As shown in Fig. 7, Western blotting demonstrated significantly increased expression of BiP and CHOP proteins in the cortex of mice with high-fat diet as compared with controls and valsartan treatment prevented such an increase (Fig. 7A and B). mRNA levels of BiP did not change in mice with high-fat, whereas CHOP mRNA levels was significantly increased in HFD mice and almost fully returned to control levels in response to valsartan treatment.
An overall increase in vacuolated tubular cells was detected in the cortex of the HFD mice (Fig. 8B). The vacuoles were as large as the nucleus or some vacuoles were even larger and some proximal tubular cells appeared to be completely filled with these vacuoles. Chronic valsartan treatment largely suppressed the development of vacuolated tubular cells (Fig. 8C). BiP protein was mainly observed in the cytoplasm of renal proximal tubular cells, with occasional glomerular staining in both control and high-fat diet fed mice. Importantly, labeling of BiP in HFD mice (Fig. 8E and H) was more extensive than that in control mice, which was partially attenuated by valsartan treatment (Fig. 8F and I).
DISCUSSION

In the present study, we found that RAS activation is involved in saturated fatty acid palmitic acid-induced ER stress and apoptosis in HK2 cells. Angiotensin II type 2 receptor blocker valsartan significantly attenuated obesity-associated ER stress in proximal tubule cells in the kidney of mice fed with high-fat diet.

During UPR, PERK phosphorylation activates eIF2α which reduces ER stress by reducing the load on the ER through inhibiting translation of new proteins from mRNA via PERK-eIF2α-ATF4 pathway, leading to a reduction in overall protein synthesis (11, 64). IRE1 mediates a frame shift of XBP1 mRNA in the coding sequence, leading to the manufacture of the potent transcriptional activator XBP1s, which activates genes encoding ER chaperones, promoting protein folding, maturation, and secretion in an attempt to decrease ER load and relieve ER stress (9, 13, 64).

ER stress has been proposed to propagate development of DN. A number of studies have documented activation of the ER stress in cultured cells, diabetic animal kidneys, and kidney biopsy samples of patients with diabetes (12, 23, 30, 35, 46, 48, 55). In humans, increased levels of XBP1 and ER chaperones are shown in patients with established DN when compared with those with mild diabetes (28). In streptozotocin (STZ)-treated rats, increased expression of BiP and CHOP in glomerular and tubular cells was observed (30). Additionally, high glucose or fatty acid has been reported to induce ER stress in human mesangial cells (40), podocytes, and tubular cells (27). Aberrant metabolic conditions such as hyperlipidemia in obese individuals promoting an environment of systemic ER stress could synergistically contribute to the pathophysiology of both insulin resistance and hyperglycemia in obesity and T2D.

Palmitic acid is the predominant circulating saturated FFA. PA induces insulin resistance in human podocytes (25) and causes proximal tubular and tubulointerstitial damage (43), indicating its deleterious role in the development of DN. PA has been demonstrated to induce ER stress in the proximal tubular cells (20,
The ability of palmitic acid to increase ER stress in HK2 cells was confirmed in this study via measurement of increased ER stress makers, such as BiP, IRE1α, p-eIF2α, ATF4 and CHOP. Our data showed that both PERK and IRE1 pathways inducing ER stress were activated by PA in proximal tubule cells. Importantly we for the first time demonstrated that ER stress induced by palmitic acid in renal proximal tubular cells can be significantly reduced by AT1R blocker valsartan or renin inhibitor aliskiren, suggesting an involvement of local RAS in maintaining homeostasis of kidney cells during metabolic alterations such as increased fatty acid overload. In HK2 cells, increased mRNA level of angiotensinogen and renin as well as Ang II secretion was observed after PA treatment, providing evidence for activation of local RAS by lipids overloading. We therefore treated HK2 cells with exogenous Ang II or renin to examine their direct effect on ER stress. Ang II clearly induced an increase of BiP and CHOP in HK2 cells, but renin did not. These data suggested that saturated fatty acid PA induces ER stress and apoptosis in proximal tubule cells, at least partly, by activating local RAS that releases Ang II via yet to be determined intracellular pathways. It is noted that aliskiren, at current concentration, induced a reduction of Ang II secretion from HK2 cells treated with PA, although such a reduction was not statistically significant (p=0.06). Nevertheless, aliskiren indeed prevented ER stress which is likely induced by increased Ang II in cultured HK2 cells in response to PA treatment.

Involvement of Ang II in obesity-associated ER stress in the kidneys was also supported by our in vivo studies. Upregulation of both BiP and CHOP was observed in the kidney cortex from the mice fed with high-fat diet, which was significantly attenuated by valsartan treatment. In particular, increased urine Ang II level, together with elevated mRNA levels of renin and ACE in the kidney of the mice fed with high-fat diet indicates a lipid-induced activation of intrarenal RAS. Consistent with previous studies (5, 6, 16, 22), we also found accumulation of lipid vacuoles in the proximal tubules and impairment of tubular structure, a prominent feature of high-fat diet-induced kidney disease. Several studies highlighted an increase of
neutral lipid in the kidney after a high-fat diet (17, 48-50, 56). A recent study (5), however, demonstrated that the majority of vacuoles are phospholipids and cholesteryl esters. The observed impairment of tubular morphology in high-fat diet mice was associated with increased kidney and urine KIM-1 levels, a transmembrane protein expressed in proximal renal tubular epithelial cells in damaged regions and a sensitive biomarker for the detection of early stage of nephropathy in T2D animals and patients (1, 14). Interestingly valsartan treatment showed a preventive effect in decreasing KIM-1. Therefore, the finding that valsartan treatment improved tubular morphology and attenuated ER stress in the proximal tubule supported a role of local RAS in lipid overload induced structural and functional impairment in the kidneys after high-fat diet.

Recently the presence of local organ specific RAS has been demonstrated in kidney, heart, aorta, and several other organs. Their activation lead to structural and functional changes, which are independent of those elicited by the classical renin–angiotensin endocrine system (4). Components of these local RAS have been found in cells and tissues and some of their local functions play an important role in cellular homeostasis (4, 65). Local intracrine/intracellular Ang II is profoundly involved in cell proliferation, oxidative stress, nitric oxide production and energy metabolism in the proximal tubules, as reviewed by (65). In addition, local RAS in adipocytes is activated during obesity in humans and obesity-prone rats show increased levels of Ang II and hypertension (61). Adipocyte-specific deficiency of angiotensinogen prevented the obesity-induced increase in plasma levels of Ang II (61). These studies likely indicate a potential role of fatty acid in activation of local RAS, as seen in the present study.

Angiotensin II has been shown to contribute to the induction of ER stress and ER stress-associated apoptosis in the hearts of diabetic mice (60). Treatment of adult rat cardiac myocytes with angiotensin II induced cardiac myocyte apoptosis along with the inductions of ER chaperones and CHOP, either of which was inhibited by
AT1R blockers (38). ARB (23) or renin inhibitor treatment (55) significantly attenuated the ER stress markers and ER stress-induced apoptosis in STZ-induced diabetic kidneys, although detailed sites where ER stress occurred were not specified. It is therefore reasonable to assume that Ang II may mediate PA-induced ER stress and apoptosis in kidney cells. Another interesting outcome of the current study was the finding that RAS blockade with valsartan or aliskiren was not able to prevent the ER stress induced by tunicamycin, a typical chemical inducer, indicating that RAS activation is unique and specific in PA-induced ER stress in the proximal tubule cells. Tunicamycin causes ER stress likely by specifically inhibiting N-linked glycosylation of peptides in ER (36, 45). The mechanism by which RAS blockade failed to prevent tunicamycin-induced ER stress is yet unknown.

Emerging evidence suggest an association between ER stress and DN, two characteristics of which are fibrosis and inflammation. A recent data demonstrate that lipid accumulation occurs in the kidney after a high-fat caloric exposure, leading to impairment of tubular cell structure and associated inflammation and fibrosis (5), as increased gene expression of profibrotic and proinflammatory molecules were observed in the kidneys by high-fat diet (17, 56). ER stress facilitates fibrotic remodeling through activating proapoptotic pathways and inducing epithelial-mesenchymal transition (47). Prolonged ER stress may lead to fibrosis through the activation of CHOP-mediated apoptosis followed by the release of profibrotic cytokines (24), such as release of transforming growth factor TGF-α and expression of collagen I (19). ER stress is also able to induce inflammatory responses through the activation of NFκB and MAPK/JNK pathways (62) and NLRP3 inflammasome (33), which regulates the maturation and secretion of proinflammatory cytokines IL-1β and IL-18 (8).

In summary, our present data demonstrate that local RAS activation mediates FA-induced ER stress and FA-associated impairment of the proximal tubular cells. These findings contribute to the understanding of the cellular mechanisms
underlying the development of chronic kidney disease caused by saturated fatty acids with interactions between ER and RAS.
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DISCLOSURE

The authors declare no conflicts of interest.

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DISCLOSURE

The authors declare no conflicts of interest.
REFERENCES


FIGURE LEGENDS

Figure 1: Valsartan or aliskiren prevented palmitic acid (0.8mM)-induced ER stress in cultured HK2 cells after 24h treatment. A and B. Protein abundance of BiP and CHOP was unchanged in HK2 cells treated with BSA (2mg/mL and 10mg/mL) and with or without valsartan (10^-6M) or aliskiren (10^-7M). C. MTT assays of HK2 cells. HK2 cells were incubated with palmitic acid at different concentrations for 24 h. After the cells were incubated with tetrazolium salt solution for 2 h, the quantity of formazan product was determined from the absorbance at 560 nm. * p<0.05 compared with PA concentration 0; # p<0.05 compared with PA concentration 0.8mM. D. PA induced upregulation of the ER stress markers: BiP, p-eIF2α/eIF2α, ATF4, and CHOP expression in HK2 cells, whereas pretreatment with valsartan (10^-6M) or aliskiren (10^-7M) attenuated ER stress induced by PA. E. Quantitative analysis of ER stress marker levels normalized to β-actin. For the control condition (CTL), the BSA concentration was equivalent to cells exposed to 0.8mM palmitic acid complexed to BSA. Representative results of three independent experiments are shown. * p<0.05 compared with controls; # p<0.05 compared with PA. BiP, ER chaperone immunoglobulin heavy chain binding protein; IRE1α, inositol requiring protein 1α; eIF2α, eukaryotic initiation factor 2α; ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein. CTL, controls; PA, palmitic acid treatment group; PA+Val, palmitic acid plus valsartan treatment; PA+Ali, palmitic acid plus aliskiren treatment.

Figure 2: Valsartan or aliskiren treatment decreased BiP (A) and CHOP (B) expression in HK2 cells treated with PA. A. immunofluorescence staining of BiP in cultured HK2 cells. In controls (a, e, i), BiP scarcely localizes intracellular cytoplasm of HK2 cells, whereas PA (0.8mM) induced significantly increased labeling of BiP (b, f, j), which was clearly suppressed by either valsartan (10^-6M) (c, g, k) or aliskiren (10^-7M) (d, h, l) treatment. B. immunofluorescence staining of CHOP in cultured HK2 cells. The control group exhibited weak staining of CHOP in nuclei and cytoplasm of HK2 cells (a, e, i), in contrast, PA treatment was associated with abundant nuclear expression.
of CHOP (b, f, j), which was inhibited by either valsartan (c, g, k) or aliskiren treatment (d, h, l). C. Quantitative analysis of BiP and CHOP positive staining. * p<0.05 compared with controls; # p<0.05 compared with PA. CTL, controls; PA, palmitic acid treatment group; PA+Val, palmitic acid plus valsartan treatment; PA+Ali, palmitic acid plus aliskiren treatment.

**Figure 3:** Valsartan or aliskiren attenuated palmitic acid-induced HK2 cell apoptosis by inhibiting RAS. A. Western blot analysis of palmitic acid-induced active, cleaved caspase-3 protein steady-state levels in HK2 cells, which was inhibited by valsartan or aliskiren treatment. B. Corresponding densitometric analyses of levels of cleaved caspase-3 protein normalized to β-actin. C and D. Representative TUNEL staining of palmitic acid treated HK2 cells and corresponding quantitative analysis. Apoptotic nuclei (arrowheads) were observed in PA-treated HK2 cells. The percentage of apoptotic cells (TUNEL-positive cells) was obtained in at least 10 fields from the PA treatment group (b), PA+Val group (c), and PA+Ali group (d). CTL, controls; PA, palmitic acid treatment group; PA+Val, palmitic acid plus valsartan treatment; PA+Ali, palmitic acid plus aliskiren treatment. * p<0.05 compared with controls; # p<0.05 compared with PA. E. Palmitic acid treatment was associated with increased release of angiotensin II (Ang II) in medium of cultured HK2 cells. * p<0.05 compared with controls (CTL). F. mRNA levels in PA-stimulated HK2 cells with pretreatment of valsartan or aliskiren. CTL, controls; PA, palmitic acid treatment group; PA+Val, palmitic acid plus valsartan treatment; PA+Ali, palmitic acid plus aliskiren treatment. * p<0.05 compared with controls; # p<0.05 compared with PA. G and H. Western blots and quantitative analysis of upregulated BiP and CHOP induced by angiotensin II (Ang II). Renin treatment didn’t induce ER stress in HK2 cells. * p<0.05 compared with controls (CTL).
Figure 4: Valsartan or aliskiren treatment didn’t prevent tunicamycin (2µg/ml) induced ER stress in HK2 cells. **A.** Tunicamycin induced upregulation of the ER markers: BiP, IRE1α, p-eIF2α/eIF2α, ATF4 and CHOP expression in HK2 cells, neither pretreatment with valsartan (10⁻⁶M) nor aliskiren (10⁻⁷M) attenuated ER stress induced by TM. **B.** Quantitative analysis of ER stress marker levels normalized to β-actin. Representative results of three independent experiments are shown. * p<0.05 compared with controls. CTL, controls; TM, tunicamycin treatment group; TM+Val, tunicamycin plus valsartan treatment; TM+Ali, tunicamycin plus aliskiren treatment.

Figure 5: Valsartan prevented increased KIM-1 in mice fed with high-fat diet (HFD). **A.** mRNA levels of NGAL and KIM-1 in the kidney cortex of HFD-fed mice with or without valsartan treatment. **B.** Urinary levels of KIM-1 measured by ELISA. * p<0.05 compared with controls; # p<0.05 compared with mice fed with HFD diet. n=6 in each group. NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1; CTL, control mice; HFD, mice fed with high-fat diet; HFDV, mice fed with high-fat diet and valsartan.

Figure 6: HFD diet was associated with RAS activation in mouse kidneys. **A** and **B.** Serum and urine Ang II concentration was significantly increased in mice fed with HFD diet. * p<0.05 compared with controls (CTL), # p<0.05 compared with mice without valsartan treatment. n=10-12 in each group. **C.** mRNA levels in the kidneys of HFD-fed mice with or without valsartan treatment. * p<0.05 compared with controls; # p<0.05 compared with mice fed with HFD diet. n=6 in each group. RAS: renin-angiotensin system, ATG: angiotensinogen; ReninR: renin receptor; ACE: angiotensin-converting enzyme; AT1R: angiotensin II type 1 receptor. CTL, control mice; HFD, mice fed with high-fat diet; HFDV, mice fed with high-fat diet and valsartan.
Figure 7: Valsartan inhibited increased BiP and CHOP protein expression in the kidney cortex of mice fed with high-fat diet (HFD). A. Western blots analysis of BiP and CHOP expression. High-fat diet treatment was associated with increased levels of BiP and CHOP protein expression in the kidney cortex, this was prevented by valsartan. B. Corresponding densitometric analyses of BiP and CHOP levels normalized to β-actin. C. Analysis of mRNA expression by quantitative real-time PCR for BiP and CHOP in HFD-fed mice with or without valsartan. * p<0.05 compared with controls; # p<0.05 compared with HFD. CTL, control mice; HFD, mice fed with high-fat diet; HFDV, mice fed with high-fat diet and valsartan, n=6 in each group.

Figure 8: Valsartan treatment improved tubular histology and BiP expression from mice on high-fat diet. Representative photomicrographs illustrated vacuolated proximal convoluted tubular cells (arrowheads) and impaired brush border in HFD mice (B, E and H), which were attenuated by valsartan treatment (C, F and I). Immunostaining of BiP (arrows) was observed in cytoplasm of proximal tubular cells, with typical perinuclear labeling in control mice (D and G). In HFD mice (E and H), immunolabeling of BiP was seen more extensive than controls and surrounding vacuoles in proximal tubule cells. There was clearly reduced BiP immunostaining in HFD mice treated with valsartan (F and I). CTL, control mice; HFD, mice fed with high-fat diet; HFDV, mice fed with high-fat diet and valsartan. Magnification: ×400 for A-F; ×1000 for G, H, and I.
Table 1. Average CT values for RAS component genes measured by qPCR in HK2 cells

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<thead>
<tr>
<th>Gene</th>
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<th>PA</th>
<th>PA+Val</th>
<th>PA+Ali</th>
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<tr>
<td>AGT</td>
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<td>27.92</td>
<td>28.67</td>
<td>28.41</td>
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<td>Renin</td>
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<td>28.94</td>
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<tr>
<td>AT1R</td>
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<td>28.00</td>
<td>28.67</td>
<td>28.23</td>
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<td>GAPDH</td>
<td>15.85</td>
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<td>16.64</td>
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AGT, angiotensinogen; reninR, renin receptor; ACE, angiotensin converting enzyme; AT1R, angiotensin II type 1 receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CTL, controls; PA, palmitic acid treatment group; PA+Val, palmitic acid plus valsartan treatment; PA+Ali, palmitic acid plus aliskiren treatment. Initial RNA amount in the reaction was 1000ng/ul.
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<thead>
<tr>
<th>target gene</th>
<th>primer sequence</th>
<th>reference No.</th>
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<tbody>
<tr>
<td>ACE F</td>
<td>TCCTGTTGGATATGGAAACCACCTA</td>
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</tr>
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<td>ACE R</td>
<td>GTGGCCCATCACATTCTGCAGA (29)</td>
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<tr>
<td>AGT F</td>
<td>TGCTGCAATGGAGTACAGTAGA</td>
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<td>AGT R</td>
<td>CACAAACAAGCTGGTGATGTCAGA (52)</td>
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</tr>
<tr>
<td>AT1R F</td>
<td>GCCCTTTGGCAATTACCTATGT</td>
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</tr>
<tr>
<td>AT1R R</td>
<td>CGTGAGTAGAAACACTAGCGT PBID 122939143c2</td>
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</tr>
<tr>
<td>BiP F</td>
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<tr>
<td>BiP R</td>
<td>GAAAAGCAGTAACAGGCCGC (3)</td>
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</tr>
<tr>
<td>CHOP F</td>
<td>AGTCTCTGCCCTTCGCTTTT</td>
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</tr>
<tr>
<td>CHOP R</td>
<td>GGTGCTTGATGACCTCTGCT (7)</td>
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</tr>
<tr>
<td>GAPDH F</td>
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</tr>
<tr>
<td>GAPDH R</td>
<td>GCTGTAAGCCAAT TCCTGCTC</td>
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</tr>
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<td>XBP-1 R</td>
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ACE, angiotensin converting enzyme; AGT, angiotensinogen; AT1R, angiotensin II type 1 receptor; BiP, ER chaperone immunoglobulin heavy chain binding protein; CHOP, C/EBP homologous protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; reninR, renin receptor; XBP-1, X-box-binding protein 1; PBID, Primer Bank ID
### Table 3. Primer sequences for RT-PCR (mouse)

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<td>ACE F</td>
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<tr>
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<tr>
<td>AGT F</td>
<td>ATGCACAGATCGGAGATGACT</td>
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<td>AGT R</td>
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<tr>
<td>AT1R F</td>
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</tr>
<tr>
<td>AT1R R</td>
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<td>(18)</td>
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</tr>
<tr>
<td>BiP R</td>
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<tr>
<td>CHOP F</td>
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<tr>
<td>reninR R</td>
<td>TAGCACTTTGCAGTTCGGAGA</td>
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</table>

ACE, angiotensin converting enzyme; ATG, angiotensinogen; AT1R, angiotensin II type 1 receptor; BiP, ER chaperone immunoglobulin heavy chain binding protein; CHOP, C/EBP homologous protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; reninR, renin receptor; KIM-1: kidney injury molecule-1; NGAL: XBP-1, X-box-binding protein 1; PBID, Primer Bank ID
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<th>Parameter</th>
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<th>HFDV</th>
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<tr>
<td>BW (g)</td>
<td>25.6 ± 0.5</td>
<td>36.3 ± 1.7*</td>
<td>38.9 ± 1.9*</td>
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<tr>
<td>KW (g)</td>
<td>0.15 ± 0.01</td>
<td>0.24 ± 0.02*</td>
<td>0.25 ± 0.01*</td>
</tr>
<tr>
<td>S-Cr (mg/dl)</td>
<td>0.36 ± 0.01</td>
<td>0.36 ± 0.01</td>
<td>0.32 ± 0.01</td>
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<td>BG (mmol/L)</td>
<td>6.74 ± 0.21</td>
<td>7.49 ± 0.18*</td>
<td>7.96 ± 0.43*</td>
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<td>UO (ml/day)</td>
<td>0.91 ± 0.11</td>
<td>0.72 ± 0.08</td>
<td>1.08 ± 0.19#</td>
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<td>UPER (µg/100g)</td>
<td>31 ± 3.8</td>
<td>45 ± 4.3*</td>
<td>37 ± 4.5#</td>
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</table>

Values are means ± SE; CTL, control mice; HFD, mice fed with high-fat diet; HFDV, mice fed with high-fat diet and valsartan. BW, body weight; KW, kidney weight; S-Cr, serum creatinine; BG, blood glucose; UO, urine output; UPER, urinary protein excretion rate; * p<0.05 when compared with control; # p<0.05 compared with HFD; n=6 in each group.
Figure 1

A. BiP, CHOP, β-actin

B. Protein abundance (% of control)

C. Cell viability (%)

D. BiP, IRE1α, p-eIF2α, eIF2α, ATF4, CHOP, β-actin

E. Protein abundance (% of control)
Figure 2

A. BiP

CTL | PA | PA+Val | PA+Ali
---|----|--------|--------
(a) | (b) | (c) | (d)

DAPI

(a) | (b) | (c) | (d)

Merge

(i) | (j) | (k) | (l)

B. CHOP

CTL | PA | PA+Val | PA+Ali
---|----|--------|--------
(a) | (b) | (c) | (d)

DAPI

(a) | (b) | (c) | (d)

Merge

(i) | (j) | (k) | (l)

C. Fluorescence intensity (% of control)

- CTL
- PA
- PA+Val
- PA+Ali

Bar graph showing the fluorescence intensity of BiP and CHOP for different treatments.
Figure 3
Figure 4

A. BiP, IRE1α, p-eIF2α, eIF2α, ATF4, CHOP, β-actin.

B. Protein abundance (% of control).

Figure 4
NGAL                                    KIM-1
mRNA relative levels (% of control)

A.  

B.  

Urinary KIM-1 levels (ng/mL)

Figure 5
Figure 6

A. Serum Ang II (% of control)

B. Urine Ang II (% of control)

C. mRNA levels of RAS components (% of control)

Figure 6
Figure 7

A. 

B. 

C. 

Protein abundance (% of control)

Protein abundance (% of control)

mRNA relative levels (% of control)

BiP

CHOP

BiP

CHOP

BiP

CHOP

* 

# 

* 

Figure 7
Figure 8