Received: 26 December 2018 | Accepted: 1 April 2019

DOI: 10.1002/cbf.3397

RESEARCH ARTICLE

WILEY CELL BIOCHEMISTRY & FUNCTION

Ghrelin modulates intracellular signalling pathways that are critical for podocyte survival

Nabil El Zein[1] | Maya S. Abdallah[2,3] | Costantine F. Daher[4] | Mohammad Mroueh[5] | Joseph Stephan[2] | Sola Aoun Bahous[2] | Assaad Eid[6] | Wissam H. Faour[2]

[1] EDST, Lebanese University, Hadath, Lebanon

[2] Gilbert and Rose-Marie Chagoury, School of Medicine, Lebanese American University, Byblos, Lebanon

[3] Institut Européen des Membranes, Université de Montpellier, Montpellier, France

[4] School of Arts and Sciences, Natural Sciences Department, Lebanese American University, Byblos, Lebanon

[5] Department of Pharmaceutical Sciences, School of Pharmacy, Lebanese American University, Byblos, Lebanon

[6] Department of Anatomy, Cell Biology and Physiology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon

Correspondence
Wissam H. Faour, Gilbert and Rose-Marie Chagoury, School of Medicine, Lebanese American University, PO Box 36, Byblos, Lebanon.
Email:wissam.faour@lau.edu.lb

Funding information
Lebanese American University, Graduate Studies and Research, Grant/Award Number: R0215

Damage to podocytes is a key event in glomerulopathies. While energy dense food can contribute to kidney damage, the role of the orixegenic hormone "ghrelin" in podocyte biology is still unknown. In the present study, we investigated the effect of ghrelin on podocyte survival as well as the signalling pathways mediating ghrelin effect in immortalized cultured rat podocytes. RT-PCR analysis revealed that GHS-R1 is expressed in rat podocytes. Western blot analysis showed that ghrelin upregulated COX-2 protein expression in a time and dose-dependent manner. Additionally, ghrelin activated P38 MAPK, AKT, and ERK1/2 pathways and also induced P38 MAPK phosphorylation in high glucose conditions. Ghrelin induced ROS release and dose dependently reduced podocyte survival. Ghrelin mediated podocyte cell death was partially reversed by pharmacologically inhibiting P38 MAPK or phospholipase C (PLC). Furthermore, PLC inhibitor (U73122) inhibited ghrelin induced P38 MAPK activation. While PI3K inhibitor (LY294002) was without effect on cell survival or P38 MAPK activation, it inhibited ghrelin induced ERK1/2 phosphorylation. Finally, ghrelin induced TAU phosphorylation was reversed by pharmacologic inhibitors of either P38 MAPK or PKA. In conclusion, ghrelin activated harmful molecular pathways in podocytes that can be damaging to the glomerular filtration barrier

Significance of the study: Endocrine derangements secondary to obesity are major players in the aetiology of renal injuries. Furthermore, energy dense diet is thought to be the major element in developing obesity. Appetite and increase in energy intake are regulated by complex hormonal pathways which mainly include the orexigenic hormone "ghrelin" in addition to leptin. To date no study have highlighted a significant role for ghrelin in kidney biology, and therefore, it is thought that its endocrine effect is mostly limited to adipose tissue metabolism and appetite regulation. In this study, we first showed that ghrelin receptor is expressed on glomerular podocytes. Also, ghrelin showed negative impact on podocyte survival through modulating signalling pathways such as P38 MAPK and AKT known to play a key role in podocyte health. Moreover, the negative effects of ghrelin on podocytes were further exacerbated in hyperglycemic conditions. Of note, podocytes contribute to the formation and the maintenance of the glomerular filtration barrier and thus are important for

Nabil El Zein and Maya S. Abdallah contributed equally to this work.

normal renal function. Therefore, ghrelin secretion in the context of obesity could be involved in the aetiology of kidney injury, a well-known hallmark found in obese patients.

KEYWORDS

cell survival, ghrelin, P38MAPK, podocytes

1 | INTRODUCTION

Podocytes are essential component of a functional glomerular filtration barrier and when damaged leads to a defective filtration barrier and albuminuria.[1] Upregulation of cyclooxygenase (COX) enzyme and its metabolites, eg, prostaglandin E2 (PGE2), are reported in various kidney diseases.[2-4] Inhibition of COX reduced proteinuria in rodent models of kidney disease.[5] Finally, overexpression of COX predisposed to albuminuria and caused podocyte damage.[6] Prostaglandin E2 and mechanical stress can activate p38 MAPK, a harmful pathway in podocytes.[7,8] Also, stimulation of podocytes with PGE2 reduced AKT phosphorylation levels, a survival kinase that protects podocytes from cell death induced by advanced glycation end products.[9,10] Also, PGE2 upregulated COX-2 protein through a restricted EP4/cAMP/AMPK/P38 MAPK pathway.[8] Finally, ERK1/2 mediated high glucose-induced VEGF secretion plays a harmful role in DN.[11] Of note, podocyte lacking the EP4 receptor showed reduced proteinuria and renal damage when compared with their wild type littermates.[12] Recently, we showed that transforming growth factor-β1 (TGF-β1) and prostaglandin E2 significantly modulated TAU protein phosphorylation, and high glucose conditions potentiated TGF-β1 mediated COX-2 protein upregulation.[7] Ghrelin is an orexigenic gut-derived acylated peptide[13] that regulates energy homeostasis and stimulates growth hormone release.[14,15] It acts through various intracellular signalling cascades.[16-19] Ghrelin and its receptor, the growth hormone secretagogue receptor (GHS-R1a), are both expressed in the kidneys of humans and rodents, and preproghrelin mRNA is found in glomeruli, cultured mesangial cells, and podocytes.[20] Importantly, ghrelin inhibited glucose mediated secretion of insulin in animals and humans and antagonized insulin activity via stimulating the release of glucagon, GH, cortisol, and epinephrine.[13,21-23] Also, ghrelin deficiency or GHS-R knockout protected against high fat diet-induced hyperglycemia and hyperinsulinemia.[24] Furthermore, administration of ghrelin to humans induced hyperglycemia and hypoinsulinemia.[25] Finally, intrarenal infusion of ghrelin causes sodium reabsorption at the nephron level in rats.[26] Whilst ghrelin is known to modulate key endocrine events, its effect on kidney biology and particularly podocytes is fully unknown. Therefore, uncovering the signal transduction pathways activated by ghrelin in podocytes is essential toward understanding the molecular role played by ghrelin in glomerular biology. Our data revealed that ghrelin upregulated COX-2 protein expression and reduced podocyte survival through PLC/P38 MAPK pathway activation. In addition, ghrelin activated AKT and ERK pathways as well as increased phospho-TAU levels, all of which are known to modulate podocyte survival. In conclusion, this study suggests that ghrelin influences glomerular function possibly through modulating the activity of critical signalling pathways involved in podocytes health.

2 | MATERIALS AND METHODS

2.1 | Materials

RPMI-1460 medium, penicillin-streptomycin (PS) antibiotics, sodium dodecyl sulfate (SDS), ethanol, methanol, and isopropanol were purchased along with the Bradford solution and glycine from Sigma (Sigma-Aldrich, USA). The medium was supplemented with fetal bovine serum (FBS) obtained from Gibco, Life Technologies. Trypsin (1X) was obtained from LONZA (Basel, Switzerland). Thirty percent acrylamide/bisacrylamide, tetramethylethylenediamine (TEMED), Laemmli sample buffer, and ammonium persulfate (APS) were purchased from Bio-Rad (Hercules, USA). Nitrocellulose membranes were purchased from GE Healthcare LifeScience. Tris-base and complete Mini protease inhibitors cocktail tablets were purchased from Hoffman-La Roche (Basel, Switzerland). Prism ultra-protein ladder 10 to 245 KDa was purchased from abcam (Cambridge, UK). β-mercaptoethanol was purchased from Bio-Rad (Hercules, USA) as well as radio immunoprecipitation assay buffer (RIPA buffer). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), and prostaglandin E2 (PGE2) were purchased from Sigma.

2.2 | Cell culture

Culture of conditionally immortalized murine glomerular epithelial cells (podocytes), kindly provided by Dr K. Endlich, P. Mundel, and Dr B. Kasinath, was carried out as previously described.[27] Briefly, podocyte cells were propagated in complete RPMI medium supplemented with 10% FBS and 1% PS (100 U/mL penicillin, 100 μg/mL streptomycin; all from Sigma). These cells were plated onto 100-mm uncoated dishes with complete media supplemented with insulin 5 μg/mL. Confluent cells were trypsinized (Trypsin 1X), centrifuged at 1000 rpm for 5 minutes at 20°C, re-suspended in fresh complete media, and then passaged onto new dishes. Growth arrest and differentiation are induced through incubating semiconfluent podocytes without insulin, and cell morphology changed from cobblestone to arborized

appearance after few days of culture. The latter cells are then seeded onto uncoated six-well plates in a humidified incubator (5% CO_2) at 37°C. Cell counting was performed by mixing 20 µL of podocyte cells with an equal volume of trypan blue dye solution. A total volume of 10 µL of the mixture was placed under the cover slip of the haemocytometer chamber. The grid was visualized under the light microscope, and only white cells were counted as they represent viable cells, whereas stained cells (violet colour) indicating dead cells were disregarded. The obtained number was multiplied by the dilution factor (×2) and by ($\times 10^4$) to extrapolate the number of cells/mL. Then, the cells were seeded at a density of 0.5 million cells/well in duplicates on six-well plates in 1 mL of medium/well.

2.3 | Cell treatment protocol

Following overnight serum starvation in RPMI-1640 medium supplemented with 0.1% FBS, podocytes were subjected to stimulation with various concentrations of Ghrelin (Tocris) for 10 minutes to 16 hours as indicated. Control cells (designated as "nonstimulated") were cultured under identical conditions but were not exposed to bioactive chemicals. P38 MAPK inhibitors SB203580, (Sigma), phospholipase C (PLC) inhibitor U73122 (Sigma), and specific cell permeable phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Sigma) were employed each at 10 µM, respectively, when indicated, in RPMI-1640 + 0.1% FBS, with a 30-minute incubation prior to stimulation experiments. At indicated time points, both control and treated cells were washed in phosphate-buffered saline (PBS) and detached from tissue culture plates with either the TriPure reagent for RNA isolation or RIPA buffer for protein extraction.

2.4 | Protein isolation and protein concentration measurement

Cells cultured on the six-well plates were washed with ice-cold 1X PBS. The plate was placed on ice and 50 to 100-µL RIPA buffer (50 mM Tris-HCl pH = 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS), supplemented with protease inhibitors prior to the extraction, were added to each well. Cells were scraped gently, and the cell extracts were transferred into 1.5-mL microcentrifuge tubes and incubated on ice for 30 minutes. Subsequently, the cell lysate was cleared by centrifugation for 20 minutes at 4°C at 10 000 rpm. The supernatant was collected and transferred into a new 1.5-mL microcentrifuge tube and stored at −20°C. Determination of protein concentration was done before proceeding with western blotting. The concentration of the extracted proteins was measured using Nanodrop. Forty micrograms of protein was used for loading in the gel and western blot analysis.

2.5 | Western blot

The protein sample was mixed with lysis buffer, supplemented with 10% β-mercaptoethanol, to obtain a total volume containing 50-µg protein. This mixture was heated for 10 minutes at 95°C and then spinned down for 8 seconds, loaded and electrophoresed on 10% sodium-dodecyl sulfate-polyacrylamide (SDS-PAGE) separating gel. Electrophoresis was performed at 100 V for 90 minutes, in 1X running buffer (14.4-g glycine, 3.02-g Tris-base, and 1-g SDS dissolved in 1 L of H_2O). Proteins were transferred onto a nitrocellulose membrane in a cold transfer buffer (5.8-g glycine, 11.6-g Tris-base, and 0.74-g SDS; dissolved in 1.6 L of H_2O with 400 mL of methanol) at 100 V for 1 hour. The nitrocellulose membrane was subsequently blocked with blocking solution containing 5% BSA dissolved in TTBS wash buffer (TBS 1X containing: 10 mL of 1 M Tris-HCl pH = 8.8, 30 mL of 5 M NaCl, and 500-µL Tween-20 dissolved in 960 mL of H_2O) for 1 hour at room temperature and with continuous shaking. The membrane was then incubated with gentle agitation with primary rabbit polyclonal antibodies, anti-COX-2 (Abcam), anti-phospho-P42/44 (Thr202/Tyr204), anti-phospho-P38 (Thr180/Tyr182) or anti-phospho-AKT (Ser473) (Cell Signalling Technology, Inc.), anti-phospho-TAU (S396) (Abcam) at a dilution of 1:1000, and rabbit monoclonal anti-GAPDH or anti-β-actin (Santa Cruz, USA) diluted at 1:10 000 in TTBS (1X) wash buffer overnight at 4°C. The membrane is then washed three times (10 minutes each) with TTBS (1X) wash buffer and incubated for 1 hour at room temperature with secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (Cell Signalling Technology, Inc.) at a dilution of 1:10 000. Finally, the membrane is washed three times with TTBS (1X), and the bands were revelled using the enhanced chemiluminescence (ECL) reagent from Amersham GE Healthcare.

2.6 | Measurement of H_2O_2 production

Measurement of H_2O_2 is done as previously described.[28] Intracellular production of H_2O_2 was analysed on a Perkin Elmer LS50B luminescence spectrometer (Fluometer). Briefly, monocytes were incubated at 37°C for 20 minutes with 20 mM 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR). The acetate moieties of DCFH-DA are cleaved off intracellularly by esterases, liberating the membrane impermeable 2′,7′-dichlorofluorescin, which fluoresces when oxidized to 2′,7′-dichlorofluorescein (DCF) by H_2O_2. After labeling, cells were treated with inhibitors or stimulated with Ghrelin. Production of H_2O_2 was then monitored every 10 minutes on fluorimetry by measuring emission at 525 nm for DCF.

2.7 | Neutral red cell viability assay

The neutral red was prepared by measuring 10 mg of neutral red powder and dissolving them in 2.5-mL distilled water. The acetic acid solutions, on the other hand, were prepared as follows: solution A of 5-mL acetic acid and 95-mL distilled water and solution B consisting of 8 mL of solution A, 7-mL distilled water, and 25-mL ethanol (70%). After seeding the cells for 72 hours, the medium is discarded, and the cells are washed twice with 500-µL PBS (1X).

Then, 200 μL of neutral red was added to each well incubated for 3 hours, and then neutral red is removed by washing each well twice with 500-μL PBS (1X). We add solution B that consists of acetic acid in order to detach the cells, and 100 μL is transferred to a 96-well plate. Optical density is read using a spectrophotometer at a wavelength of 490 nm.

2.8 | RNA isolation

Total RNA from cells was isolated using the TriPure reagent (Roche, USA). Briefly, 1 mL of TriPure per well was used to lyse cells cultured in a six-well culture plate. Then, the lysate was transferred into a clean RNase free 1.5-mL microcentrifuge tube. Then, 200 μL of chloroform was added per 1-mL TriPure, and the tubes were vigorously shacked and incubated at room temperature for 5 minutes. Samples were centrifuged at 10 000 ×g for 10 minutes at 4°C. After centrifugation, the mixture was separated into a lower red-organic phase, interphase, and an upper colourless-aqueous phase. The upper aqueous phase is transferred into a clean tube, and RNA was recovered by precipitation after adding 500 μL of isopropanol. Samples were incubated at room temperature for 15 minutes and centrifuged at 10 000 ×g for 25 minutes at 4°C thereafter. After removing the supernatant, the RNA pellet was washed twice with 75% ethanol and left to dry. At the end of the procedure, each pellet was resuspended in 12-μL RNase-free water. To determine the RNA concentration and purity, the ratio of the UV absorbance at 260 and 280 nm was measured by a spectrophotometer.

2.9 | RT-PCR

RNA was reverse-transcribed according to the manufacturers' instructions using the qScript cDNA SuperMix (Quanta BioSciences) that provides all the necessary components (except RNA template) for first-strand cDNA synthesis including buffer, dNTPs, $MgCl_2$, oligonucleotides (dTs) and random primers, RNase inhibitor protein, RNase H(+) derivative of MMLV reverse transcriptase, and stabilizers in a 20-μL reaction. Reverse transcription was performed as followed: 25°C for 5 minutes, 42°C for 30 seconds, 85°C for 5 minutes and held at 4°C. The resulting cDNA was diluted 1/5th for RT-PCR analysis and stored at −20°C. One microgram of RNA was used to synthesize cDNA using qScript cDNA Supermix (Quanta Biosciences) according to the manufacturer's protocol. PCR was then done using forward and reverse primers of EGFR and 5 μL of the prepared cDNA in each reaction. The enzyme used was Taq polymerase enzyme buffer, and $MgCl_2$ was added. The reaction started with a preheating step at 95°C for 3 minutes, followed by 32 cycles (95°C for 1-minute denaturation step, 60°C for 1-minute annealing step, and 72°C for 1-minute extension step), and finished with a final extension step at 72°C for 7 minutes. PCR products were analysed by 1% agarose gel electrophoresis (Life Technologies, Paisley, Scotland) and detected using Nancy-520 DNA Gel Stain (Sigma). To identify fragment sizes, we used 100 BP DNA Ladder Ready to Load (Solis Biodyne) and DNA molecular weight marker IV (Roche). Ghrelin primers: F: CCA TCT TCG TGC TAG TCG GG, R: GAG GTT GCA GTA CTG GCT GA. EGFR: Sense 5′-AAGG-CTGTCCAACGAATGGG-3′; antisense 5′-CCTC-TCCTGCAGCAGCCTC-3′.

2.10 | Measurement of intracellular calcium concentration

Free intracellular calcium was determined in podocytes cell line loaded with the calcium-sensitive dye Fura2/AM as previously described.[28] Cells were maintained with the appropriate media in an incubator (37°C, 5% CO2). Then, cells were grown to nearly 80% confluency, the cell medium was discarded, washed with PBS, and detached with trypsin. Cells were washed two times and suspended in buffer at a concentration of 1 × 106/mL; 1 mM Fura2 AM stock solution was added to cells to obtain a final concentration of 2-μM Fura2 AM Incubate for 30 minutes at 37°C and protected from light. The tube was centrifuged at 750 ×g for 5 minutes using a microcentrifuge, the supernatant was aspirated and discarded, and the pellet was resuspended in the appropriate volume of PBS buffer. The cells are now loaded with the dye; they remain viable (kept at 37°C and protected from light) for approximately 2 hours and may be used in fluorimetry techniques.

2.11 | Statistics

Data are expressed as means of duplicate determinations from individual experiments and are presented as means ± SEM where $n \geq 4$ or means ± SD where n = 3 experiments. Statistical significance was accepted at $P < .05$ as determined by ANOVA followed by a Newman-Keuls multiple comparisons test or alternatively determined by a paired *t*-test where appropriate.

3 | RESULTS

3.1 | Growth hormone secretagogue receptor-1a (GHS-R1a) expression, and ghrelin modulation of intracellular signalling kinase activation

Our results showed that rat podocytes GHR-1a (Figure 1A) and the epidermal growth factor receptor (EGFR) (Figure 1B) are expressed in podocytes. In order to evaluate the effect ghrelin on major signalling cascades known to modulate podocyte survival, podocyte cells were cultured in the presence or absence of ghrelin as indicated. Time course and dose response experiments showed that the levels of the phosphorylated activated forms of P38 MAPK, AKT, and ERK1/2 were significantly increased shortly after incubating podocytes with ghrelin, and these levels remained significantly elevated for up to 2 hours of stimulation. Furthermore, ghrelin was able to significantly increase phospho-p38 levels in high glucose conditions (Figure 2). Interestingly, pharmacologic inhibition of PLC but

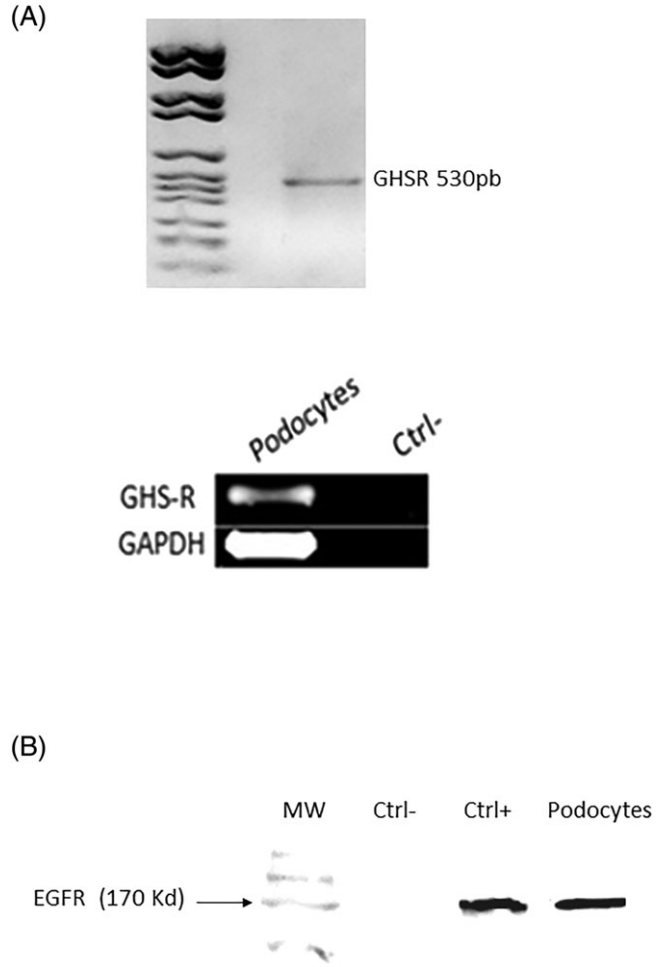

FIGURE 1 Cultured rat podocytes express GHS-R1a and EGFR. Total RNA from cells was extracted using the TriPure reagent (Roche, USA); 1 mL of TriPure was used for 1 million cells. Semiquantitative RT-PCR was performed to test EGFR gene expression in podocyte cells as described in materials and methods. In (A), expression profile of the GHS-R1a, note the presence of single band at 530 BP specific for GHS-R1a. GAPDH expression was used as a loading control. In (B), expression profile of EGFR. Positive control includes RNA isolated from cell expressing EGFR (lane 2), and RT-PCR on RNA isolated from podocytes (lane 3)

not ERK1/2 or PI3K significantly reversed ghrelin induced P38 MAPK activation, while pharmacologic inhibition of PI3K significantly reversed ghrelin induced ERK1/2 phosphorylation (Figure 3). Finally, ghrelin showed no significant effect on intracellular calcium release (data not shown).

3.2 | Ghrelin induced COX-2 protein expression and ROS release in cultured rat podocytes

Time course and dose response experiments revealed that ghrelin upregulated COX-2 protein expression after 3 hours of stimulation, reaching maximum levels after 6 hours, and COX-2 protein levels remained significantly elevated up to 16 hours of stimulation. Finally, podocytes exposed to ghrelin released significant amount of ROS shortly after 5 minutes of incubation time and reached a maximum after 30 minutes and remained significantly elevated for up to 2 hours of stimulation (Figure 4).

3.3 | Ghrelin induced podocytes cell death is mediated though PLC/P38 MAPK pathways

The next step was to assess the role of ghrelin on podocyte survival via modulating the above studied signalling pathways. Interestingly, cell survival was significantly reduced when podocytes were exposed to a range of ghrelin concentrations for up to 72 hours. Moreover, inhibition of either PLC with U73122 or P38MAPK with SB203580 partially reversed ghrelin effect (Figure 5).

3.4 | Ghrelin modulated TAU protein phosphorylation in cultured rat podocytes

The effect of ghrelin on TAU protein phosphorylation is also evaluated. As shown in "Figure 6," ghrelin significantly increased the levels of phospho-TAU levels at serine-214 immediately after 5 minutes of stimulation, and the levels of phospho-TAU (Ser-214) remained elevated for up to 2 hours of stimulation. Furthermore, ghrelin induced TAU phosphorylation on serine-214 was abrogated when podocytes were incubated with either SB203580 a specific p38 MAPK inhibitor or H89 a specific PKA inhibitor (Figure 6).

4 | DISCUSSION

Glomerular injury secondary to obesity and diabetes is a major health concern worldwide.[29] Damage to podocytes is shown to play a cardinal role in the aetiology of many types of glomerular diseases.[30] While obesity and increased energy intake are significantly linked to kidney damage, the endocrine mechanisms by which obesity induces kidney dysfunction are still largely unknown. Ghrelin is an appetite inducing hormone that acts oppositely to leptin in regulating feeding and satiety.[13] Interestingly, previous and recent data reported conflicting results regarding the physiologic effect of ghrelin. The latter discrepancies are attributed to the fact that circulating ghrelin exists in blood as both the "acylated active" and the "nonacylated inactive" forms of the hormone. Recent evidence showed that both forms produced physiologic effects explaining the physiologic discrepancies in ghrelin effect. Cardiovascular physiologic effect of ghrelin is the most documented in the literature. Accordingly, ghrelin improved cardiovascular health by inhibiting osteogenetic differentiation of smooth muscle cells,[31] vascular calcification,[32] as well as improving pulmonary hypertension and vascular remodeling.[33] Oppositely, Li et al found that plasma acylated ghrelin positively correlated with pulmonary hypertension associated with congenital heart disease.[34] Furthermore, ghrelin showed important antiinflammatory properties in carotid artery cells.[35] Recently, Xu et al showed that ghrelin inhibited vascular

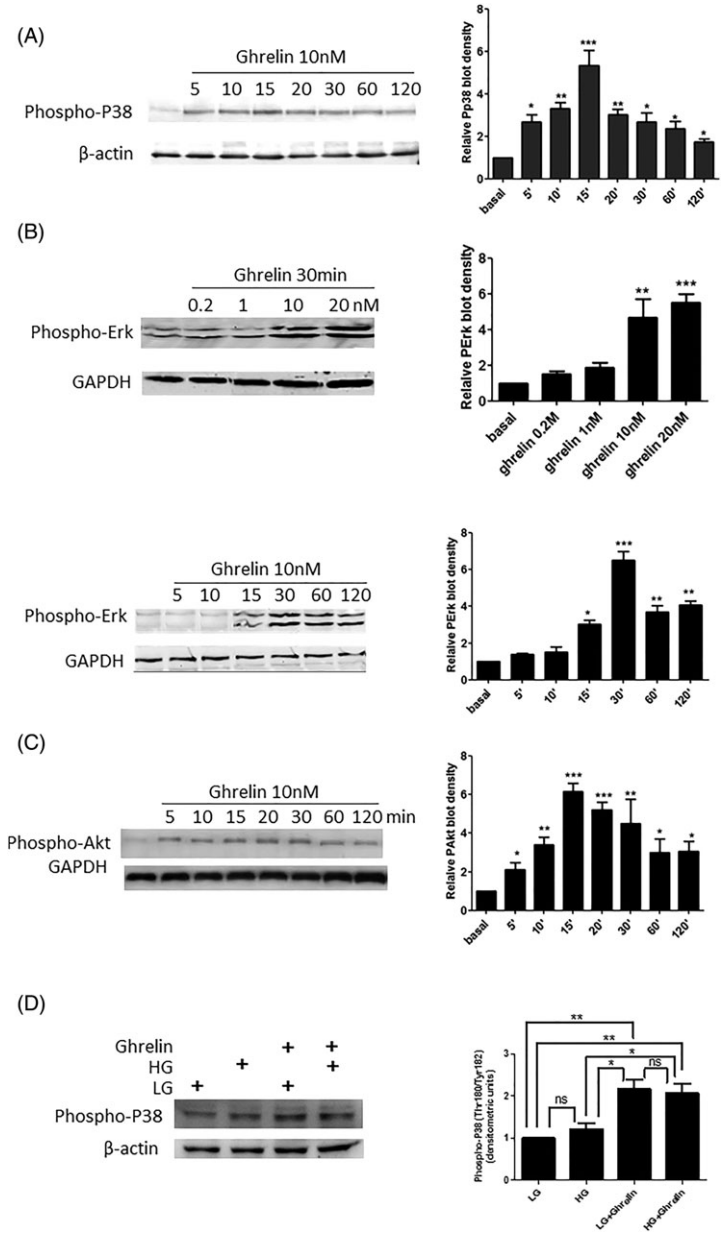

FIGURE 2 Ghrelin induced P38 MAPK, ERK1/2, and AKT activation. In (A), ghrelin induced p38 MAPK activation, western blot analysis showing the phosphorylated activated levels of the p38 MAPK pathway induced in rat podocytes incubated with 10 nM of ghrelin. Cells were treated with ghrelin for 5, 10, 15, 20, 30, 60, or 120 min as indicated. Cell extract (50 μg) was analysed by western blotting using anti-phospho-p38 antibody. Levels of phospho-p38 were normalized to β-actin, and signal intensity is quantified by densitometry. The first lane represents untreated cells which show basal phospho-P38 MAPK levels. In (B), ghrelin increased ERK activation in a concentration and time-dependent manner. Cells were treated with 10 nM ghrelin for 5, 10, 15, 30, 60, and 120 min or with various ghrelin concentrations (0.2, 1, 10, and 20 nM) for 30 minutes. Cell protein extract (50 μg) was analysed by western blot and levels of phospho-ERK1/2 were recovered using anti-phospho-ERK1/2 antibody and normalized to GAPDH protein content and signal intensity is quantified by densitometry. The first lane represents untreated cells which show basal phopho-ERK1/2 levels. In (C), ghrelin induced AKT activation. Western blot analysis showing the levels of the phosphorylated activated form of AKT kinase induced by ghrelin (10 nM) in podocyte cells. Cells were treated with ghrelin (10 nM) for various time points of 5, 10, 15, 20, 30, 60, or 120 min as indicated. Cell extract (50 μg) was analysed by western blotting using anti-phospho-AKT antibodies. Levels of phosphor-AKT levels were normalized to GAPDH protein content, and signal intensity quantified is by densitometry. The first lane represents untreated cells which show basal phospho-AKT levels. In (D), ghrelin induced P38 MAPK phosphorylation in high glucose condition. Western blot analysis showing the phosphorylated activated levels of the p38 MAPK pathway induced in rat podocytes incubated with either low glucose (5 mM D-glucose + 25 mM L-glucose) alone, or high glucose (5 mM L-glucose + 25 mM D-glucose) alone, or with 10 nM of ghrelin + low glucose (5 mM D-glucose + 25 mM L-glucose), or with 10 nM of ghrelin + high glucose (5 mM L-glucose + 25 mM D-glucose). Cell extract (50 μg) was analysed by western blotting using anti-phospho-p38 antibody. Levels of phospho-p38 were normalized to β-actin, and signal intensity is quantified by densitometry. Each blot is representative of at least three similar experiments and $^{*}P < .05$, $^{**}P < .005$, and $^{***}P < 0.001$ as significant differences with respect to the control ($n \geq 3$)

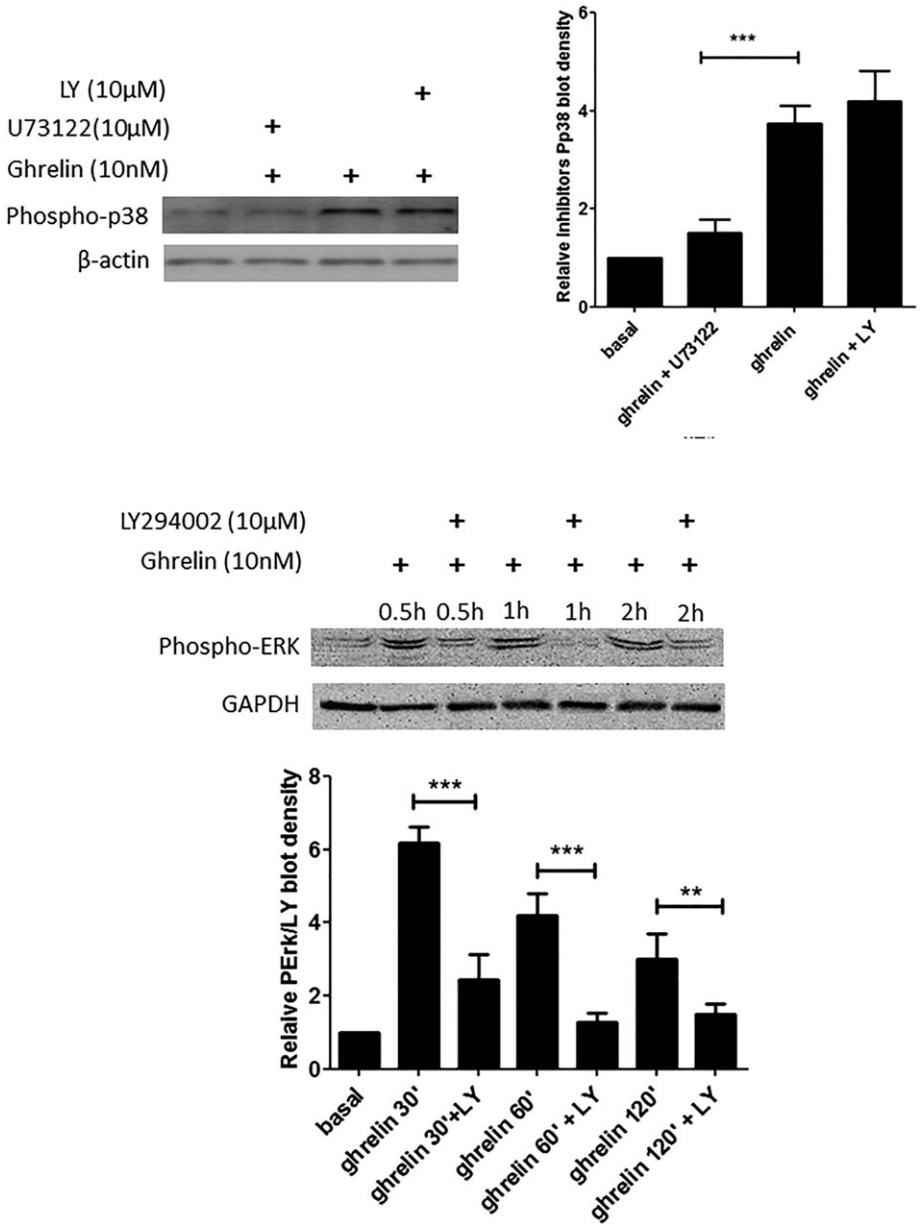

FIGURE 3 Ghrelin induced P38 MAPK and ERK activation is mediated through PLC and PI3K, respectively. In (A), conditionally immortalized differentiated rat podocytes were incubated for 30 min with inhibitors prior to stimulation with ghrelin as follow: Vehicle (ethanol) as control (C) or with ghrelin (10 nM), or with ghrelin (10 nM) + U73122 (10 μM), or with ghrelin (10 nM) + LY294002 (10 μM) as indicated and lysed with lysis buffer. Protein extracts were resolved by SDS-PAGE and immunoblotted with anti-phospho-P38 MAPK antibody. Expression levels were normalized to beta-actin protein content as assessed by densitometric analysis. Each blot is representative of at least three similar experiments and *P < .05, **P < .005, and ***P < .001 as significant differences with respect to the control ($n \geq 3$). In (B), conditionally immortalized differentiated rat podocytes were incubated with vehicle (ethanol) as control (C) or with ghrelin (10 nM) for 30 min, or with ghrelin (10 nM) + LY294002 (10 μM) for 30 min, or with ghrelin (10 nM) for 60 min, or with ghrelin (10 nM) + LY294002 (10 μM) for 60 min, with ghrelin (10 nM) for 120 min, or with ghrelin (10 nM) + LY294002 (10 μM) for 120 min as indicated and lysed with lysis buffer. Protein extracts were resolved by SDS-PAGE and immunoblotted with anti-phospho-ERK1/2 antibody

calcification in amputated patients.[36] All these data suggested a protective role of ghrelin over the cardiovascular system mainly though inhibiting vascular smooth cells induced vascular calcification. Interestingly, ghrelin agonists were shown to produce off target hypotension effects through novel receptors but not GHSR-1a.[37] While the effect of ghrelin on the cardiovascular system is well established, the effect of ghrelin on the kidney is still unknown. We first showed that ghrelin receptor is expressed in rat podocytes. Interestingly, ghrelin receptor expression in podocytes is described in only one report. Importantly, ghrelin produced harmful effect on podocyte and reduced cell survival. The role of various signalling pathways involved in podocyte damage is well described in the literature. For instance, TGF-beta induced cell

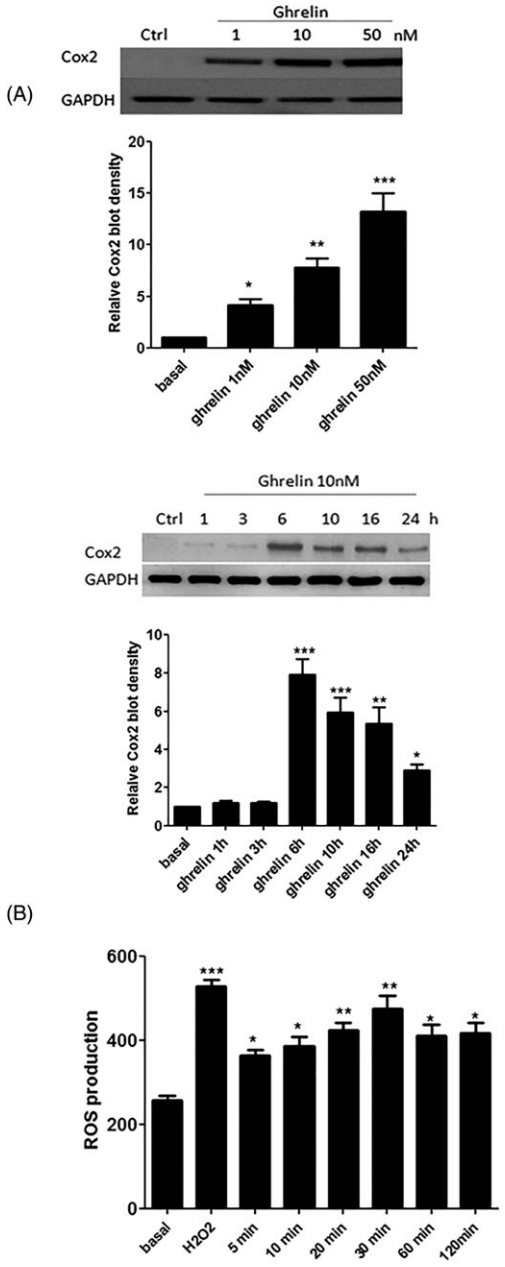

FIGURE 4 Ghrelin upregulates COX-2 protein and ROS release in rat podocytes. In (A), conditionally immortalized differentiated rat podocytes were incubated with vehicle (ethanol) as control (Ctr) or 10 nM of ghrelin for 1, 3, 6, 10, 16, and 24 h, as indicated or incubated for 6 h with either vehicle (ethanol) as control (Ctr), or various increasing concentrations of ghrelin (1, 10, or 50 nM) as indicated and lysed with lysis buffer. Protein extracts were resolved by SDS-PAGE and immunoblotted with a COX-2 antibody. Expression levels were normalized to GAPDH protein content as assessed by densitometric analysis. Each blot is representative of at least three similar experiments and $^{*}P < .05$, $^{**}P < .005$, and $^{***}P < .001$ as significant differences with respect to the control ($n \geq 3$). In (B), podocyte cells were incubated vehicle, or H2O2, or with 10 nM of ghrelin for up to 2 h as indicated, then levels of ROS released in the media were measured as described in the materials and methods. The experiment is representative of at least three similar experiments and $^{*}P < .05$, $^{**}P < .005$, and $^{***}P < .001$ as significant differences with respect to the control ($n \geq 3$)

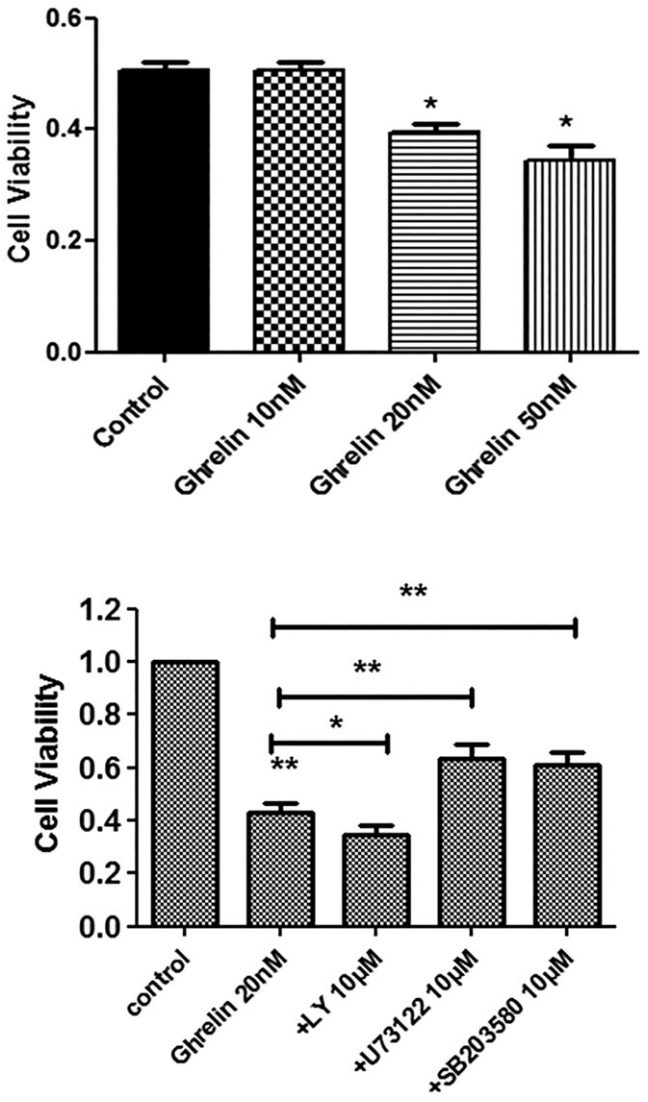

FIGURE 5 Ghrelin reduced podocyte cell survival through activation of PLC or P38 MAPK. In (A), podocytes cells were incubated with either vehicle, or ghrelin (10 nM), or ghrelin (20 nM), or ghrelin (50 nM) for up 72 h. Then, podocyte survival was measured as indicated in the materials and methods section. In (B), podocyte cells were incubated with either, vehicle, or ghrelin (20 nM), or ghrelin (20 nM) + LY (10 µM), or ghrelin (20 nM) + U73122 (10 µM), or ghrelin (20 nM) + SB203580 (10 µM). Each experiment is representative of at least three similar experiments and $^{*}P < .05$, $^{**}P < .005$, and $^{***}P < .001$ as significant differences with respect to the control ($n \geq 3$)

death is mediated via P38 MAPK activation.[38] Furthermore, ERK1/2 activation is also associated with podocyte injury induced by glycation end products.[9] In this study, we found that ghrelin signals in podocytes through activating the abovementioned pathways, and P38 MAPK inhibition reversed ghrelin induced podocyte cell death. Furthermore, ghrelin induced P38 MAPK in both low glucose and high glucose conditions raising the possibility that ghrelin could significantly contribute to podocyte damage in hyperglycemic conditions.

While COX-2 protein induction is well documented in multiple types of kidney diseases, we reported that ghrelin significantly

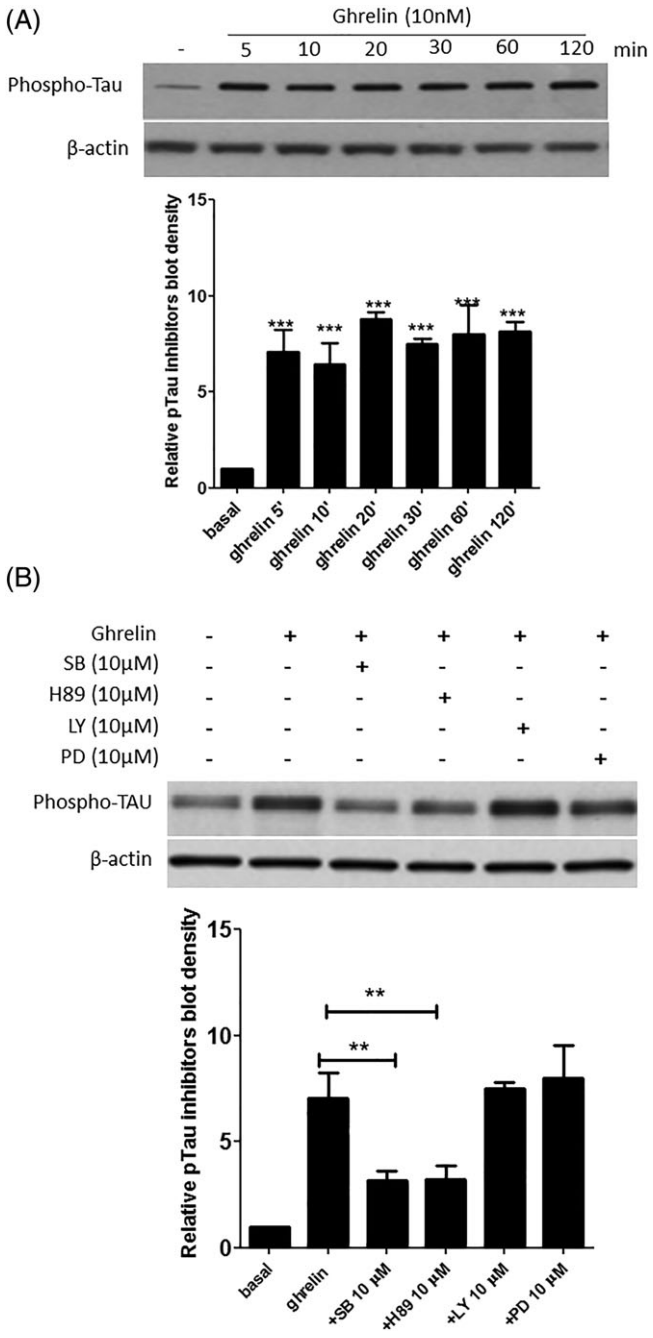

FIGURE 6 Ghrelin increased phospho-TAU levels through P38 MAPK and PKA. In (A), podocyte cells were stimulated with either vehicle, or ghrelin (10 nM) for 5, 10, 20, 30, 60, or 120 minutes. Cell extract (50 μg) was analysed by western blotting using anti-phospho-TAU antibody. Levels of phospho-TAU were normalized to β-actin, and signal intensity is quantified by densitometry (n = 3). **P* < .01 vs vehicle control. In (B), podocyte cells were stimulated with either vehicle, or ghrelin (10 nM), or ghrelin (10 nM) + SB (10 μM), or ghrelin (10 nM) + H89 (10 μM), or ghrelin (10 nM) + LY (10 μM), or ghrelin (10 nM) + PD (10 μM). Cell extract (50 μg) was analysed by western blotting using anti-phospho-TAU antibody. Levels of phospho-TAU were normalized to β-actin, and signal intensity is quantified by densitometry. Each blot is representative of at least three similar experiments and **P* < .05, ***P* < .005, and ****P* < .001 as significant differences with respect to the control (n ≥ 3)

induced COX-2 protein expression. Similarly, a previous study reported the gatroprotective effect of ghrelin through modulation of PGE2 production.[39,40] Furthermore, COX-2 activity was required for the gastroprotective effect of ghrelin.[41] We previously demonstrated that the arachidonic cascades played a crucial role in podocyte health. Accordingly, mice with podocyte specific PGE2 EP4 receptor subtype targeted deletion showed reduced proteinuria in 5/6 nephrectomized mice when compared with their wild type littermates.[12] Additionally, PGE2 further established a positive feedback loop and induced COX-2 protein expression through restricted activation of EP4/cAMP/AMPK/P38 MAPK pathway and inhibited AKT kinase activation.[8] Also, AKT inhibition is involved in podocyte detachment and loss in urine.[42] As such, ghrelin mediated induction of COX-2 protein expression will have profound effect on podocyte survival and adherence.

Podocyte foot processes are key player in the formation and maintenance of a functional glomerular filtration barrier. Podocyte injury is mostly associated with foot processes effacement leading to podocyte detachment or apoptosis.[43] Previous work in our lab showed that podocytes undergo significant cytoskeletal reorganization when exposed to mechanical stress through P38 MAPK activation.[44] Recently, we found that PGE2 can significantly modulate TAU protein phosphorylation.[7] In this study, we also found that ghrelin induced TAU phosphorylation. The fact that ghrelin modulates TAU protein phosphorylation is of considerable importance knowing that TAU is a crucial player in cytoskeleton rearrangement, a key process for podocyte foot processes maintenance. Finally, based on the abovementioned data, we conclude that ghrelin is a new and important player in podocyte health that might act as an endocrine link by which obesity and diabetes can cause kidney damage. Epidermal growth factor receptor (EGFR) activation found to promote glomerular injury in podocyte can promote kidney injury and deletion of EGFR in podocyte protected against podocyte damage and prevented DN.[45] Interestingly, ghrelin intestinal induction of epithelial cell proliferation is mediated though EGF receptor transactivation. We previously showed that mechanical strain activated ERK1/2 and SAPK/JNK but not P38 MAPK through EGF receptor transactivation.[10] Therefore, we believe that EGF receptor can be involved in ghrelin harmful effect on podocytes. However, uncovering the molecular interactions between ghrelin and EGFR requires further investigation. In conclusion, based on the above results, we believe that ghrelin is a new important player in podocyte biology that can act as a key link between endocrine derangements and kidney injury secondary to obesity or diabetes.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

FUNDING

This work has been supported by a grant from the Lebanese American University, Graduate Studies and Research, grant nb: R0215. Byblos, Lebanon, P.O. Box 36.

AUTHORS' CONTRIBUTIONS

N.E.Z., M.S.A., C.F.D., M.R., J.S., S.A.B., A.E., and W.H.F. were involved in the planning and design of the study. W.H.F. oversighted and coordinated the entire work as well as writing the manuscript. All authors were involved in interpreting the data, reviewing, and approving the final draft of the manuscript.

DATA AVAILABILITY STATEMENT

No data set is available.

ORCID

Mohammad Mroueh https://orcid.org/0000-0003-1572-7133
Wissam H. Faour https://orcid.org/0000-0002-0746-3687

REFERENCES

1. Gassler N, Elger M, Kranzlin B, et al. Podocyte injury underlies the progression of focal segmental glomerulosclerosis in the fa/fa Zucker rat. *Kidney Int*. 2001;60(1):106-116.
2. Takano T, Cybulsky AV. Complement C5b-9-mediated arachidonic acid metabolism in glomerular epithelial cells: role of cyclooxygenase-1 and -2. *Am J Pathol*. 2000;156(6):2091-2101.
3. Tomasoni S, Noris M, Zappella S, et al. Upregulation of renal and systemic cyclooxygenase-2 in patients with active lupus nephritis. *J Am Soc Nephrol*. 1998;9(7):1202-1212.
4. Weichert W, Paliege A, Provoost AP, Bachmann S. Upregulation of juxtaglomerular NOS1 and COX-2 precedes glomerulosclerosis in fawn-hooded hypertensive rats. *Am J Physiol Renal Physiol*. 2001;280(4):F706-F714.
5. Fujihara CK, Antunes GR, Mattar AL, et al. Cyclooxygenase-2 (COX-2) inhibition limits abnormal COX-2 expression and progressive injury in the remnant kidney. *Kidney Int*. 2003;64(6):2172-2181.
6. Cheng H, Fan X, Guan Y, Moeckel GW, Zent R, Harris RC. Distinct roles for basal and induced COX-2 in podocyte injury. *J Am Soc Nephrol*. 2009;20(9):1953-1962.
7. Abdallah MS, Kennedy CRJ, Stephan JS, et al. Transforming growth factor-beta1 and phosphatases modulate COX-2 protein expression and TAU phosphorylation in cultured immortalized podocytes. *Inflamm Res*. 2018;67(2):191-201.
8. Faour WH, Gomi K, Kennedy CR. PGE(2) induces COX-2 expression in podocytes via the EP(4) receptor through a PKA-independent mechanism. *Cell Signal*. 2008;20(11):2156-2164.
9. Chuang PY, Yu Q, Fang W, Uribarri J, He JC. Advanced glycation endproducts induce podocyte apoptosis by activation of the FOXO4 transcription factor. *Kidney Int*. 2007;72(8):965-976.
10. Faour WH, Thibodeau JF, Kennedy CR. Mechanical stretch and prostaglandin E2 modulate critical signaling pathways in mouse podocytes. *Cell Signal*. 2010;22(8):1222-1230.
11. Hoshi S, Nomoto K-i, Kuromitsu J, Tomari S, Nagata M. High glucose induced VEGF expression via PKC and ERK in glomerular podocytes. *Biochem Biophys Res Commun*. 2002;290(1):177-184.
12. Stitt-Cavanagh EM, Faour WH, Takami K, et al. A maladaptive role for EP4 receptors in podocytes. *J Am Soc Nephrol*. 2010;21(10):1678-1690.
13. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*. 1999;402(6762):656-660.
14. Hataya Y, Akamizu T, Takaya K, et al. A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. *J Clin Endocrinol Metab*. 2001;86(9):4552.
15. Takaya K, Ariyasu H, Kanamoto N, et al. Ghrelin strongly stimulates growth hormone release in humans. *J Clin Endocrinol Metab*. 2000;85(12):4908-4911.
16. Favaro E, Granata R, Miceli I, et al. The ghrelin gene products and exendin-4 promote survival of human pancreatic islet endothelial cells in hyperglycaemic conditions, through phosphoinositide 3-kinase/Akt, extracellular signal-related kinase (ERK)1/2 and cAMP/protein kinase a (PKA) signalling pathways. *Diabetologia*. 2012;55(4):1058-1070.
17. Kurashina T, Dezaki K, Yoshida M, et al. The beta-cell GHSR and downstream cAMP/TRPM2 signaling account for insulinostatic and glycemic effects of ghrelin. *Sci Rep*. 2015;5(1):14041.
18. Murata M, Okimura Y, Iida K, et al. Ghrelin modulates the downstream molecules of insulin signaling in hepatoma cells. *J Biol Chem*. 2002;277(7):5667-5674.
19. Wang X, Wang XL, Chen HL, et al. Ghrelin inhibits doxorubicin cardiotoxicity by inhibiting excessive autophagy through AMPK and p38-MAPK. *Biochem Pharmacol*. 2014;88(3):334-350.
20. Mori K, Yoshimoto A, Takaya K, et al. Kidney produces a novel acylated peptide, ghrelin. *FEBS Lett*. 2000;486(3):213-216.
21. Nagaya N, Kojima M, Uematsu M, et al. Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. *Am J Physiol Regul Integr Comp Physiol*. 2001;280(5):R1483-R1487.
22. Reimer MK, Pacini G, Ahrén B. Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology*. 2003;144(3):916-921.
23. Salehi A, Dornonville de la Cour C, Håkanson R, Lundquist I. Effects of ghrelin on insulin and glucagon secretion: a study of isolated pancreatic islets and intact mice. *Regul Pept*. 2004;118(3):143-150.
24. Wortley KE, del Rincon J-P, Murray JD, et al. Absence of ghrelin protects against early-onset obesity. *J Clin Invest*. 2005;115(12):3573-3578.
25. Chapman IM, Bach MA, Van Cauter E, et al. Stimulation of the growth hormone (GH)-insulin-like growth factor I axis by daily oral administration of a GH secretogogue (MK-677) in healthy elderly subjects. *J Clin Endocrinol Metab*. 1996;81(12):4249-4257.
26. Kemp BA, Howell NL, Gray JT, Keller SR, Nass RM, Padia SH. Intrarenal ghrelin infusion stimulates distal nephron-dependent sodium reabsorption in normal rats. *Hypertension*. 2011;57(3):633-639.
27. Sun J, Li ZP, Zhang RQ, Zhang HM. Repression of miR-217 protects against high glucose-induced podocyte injury and insulin resistance by restoring PTEN-mediated autophagy pathway. *Biochem Biophys Res Commun*. 2017;483(1):318-324.
28. El Zein N, Corazza F, Sariban E. The neuropeptide pituitary adenylate cyclase activating protein is a physiological activator of human monocytes. *Cell Signal*. 2006;18(2):162-173.
29. Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999-2004. *JAMA*. 2006;295(13):1549-1555.
30. Asanuma K, Mundel P. The role of podocytes in glomerular pathobiology. *Clin Exp Nephrol*. 2003;7(4):255-259.
31. Liang Q-H, Jiang Y, Zhu X, et al. Ghrelin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the ERK pathway. *PLoS ONE*. 2012;7(4):e33126.
32. Li GZ, Jiang W, Zhao J, et al. Ghrelin blunted vascular calcification in vivo and in vitro in rats. *Regul Pept*. 2005;129(1-3):167-176.
33. Xu YP, Zhu JJ, Cheng F, et al. Ghrelin ameliorates hypoxia-induced pulmonary hypertension via phospho-GSK3 beta/beta-catenin signaling in neonatal rats. *J Mol Endocrinol*. 2011;47(1):33-43.

34. Li G, Xia J, Jia P, et al. Plasma levels of acylated ghrelin in children with pulmonary hypertension associated with congenital heart disease. *Pediatr Cardiol*. 2015;36(7):1423-1428.
35. Chow KB, Cheng CH, Wise H. Anti-inflammatory activity of ghrelin in human carotid artery cells. *Inflammation*. 2009;32(6):402-409.
36. Xu S, Ye F, Li L, et al. Ghrelin attenuates vascular calcification in diabetic patients with amputation. *Biomed Pharmacother*. 2017;91:1053-1064.
37. Callaghan B, Kosari S, Pustovit RV, et al. Hypotensive effects of ghrelin receptor agonists mediated through a novel receptor. *Br J Pharmacol*. 2014;171(5):1275-1286.
38. Schiffer M, Bitzer M, Roberts ISD, et al. Apoptosis in podocytes induced by TGF-β and Smad7. *J Clin Invest*. 2001;108(6):807-816.
39. Chen YT, Tsai SH, Sheu SY, Tsai LH. Ghrelin improves LPS-induced gastrointestinal motility disturbances: roles of NO and prostaglandin E2. *Shock*. 2010;33(2):205-212.
40. Sibilia V, Pagani F, Rindi G, et al. Central ghrelin gastroprotection involves nitric oxide/prostaglandin cross-talk. *Br J Pharmacol*. 2008; 154(3):688-697.
41. Warzecha Z, Ceranowicz P, Dembinski M, et al. Involvement of cyclooxygenase-1 and cyclooxygenase-2 activity in the therapeutic effect of ghrelin in the course of ethanol-induced gastric ulcers in rats. *J Physiol Pharmacol*. 2014;65(1):95-106.
42. Canaud G, Bienaime F, Viau A, et al. AKT2 is essential to maintain podocyte viability and function during chronic kidney disease. *Nat Med*. 2013;19(10):1288-1296.
43. Wharram BL, Goyal M, Wiggins JE, et al. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *J Am Soc Nephrol*. 2005;16(10):2941-2952.
44. Martineau LC, McVeigh LI, Jasmin BJ, Kennedy CRJ. p38 MAP kinase mediates mechanically induced COX-2 and PG EP4 receptor expression in podocytes: implications for the actin cytoskeleton. *Am J Physiol Renal Physiol*. 2004;286(4):F693-F701.
45. Bollee G, Flamant M, Schordan S, et al. Epidermal growth factor receptor promotes glomerular injury and renal failure in rapidly progressive crescentic glomerulonephritis. *Nat Med*. 2011;17(10):1242-1250.

How to cite this article: El Zein N, Abdallah MS, Daher CF, et al. Ghrelin modulates intracellular signalling pathways that are critical for podocyte survival.*Cell Biochem Funct*. 2019;37:245–255. https://doi.org/10.1002/cbf.3397