Free fatty acids cause podocytes dysfunction and inflammation

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Abstract

The mechanisms underlying obesity-related kidney disease are not well understood. Growing evidence suggests that free fatty acids, a cause of oxidative stress, play an important role in obesity and its related complications. So, we decided to investigate, in a human-conditioned immortalized podocyte cell line, the capacity of physiopathological concentrations of 27nM of nonconjugated palmitate to induce intracellular reactive oxygen species (ROS) production, podocytes endoplasmic reticulum stress, podocytes inflammation, and mitochondrial dysfunction. A conditionally immortalized human podocyte cell line was exposed to different percentages of palmitate conjugated to bovine serum albumin for 24h. We observed that palmitate, at the same concentrations seen in obese patients, caused overproduction of ROS in human podocytes and this oxidative stress induces dysfunctions in podocytes like inflammation and changes in profibrotic and lipotoxic markers. High-mobility group box 1 is likely known to be a major mediator of ROS damaging effects, as its pharmacological inhibition prevents all ROS effects on podocytes. Our study shows how, in podocytes, an unbounded fraction of 27nM of palmitate can induce dysfunctions similar to that observed in obesity-related glomerulopathy (ORG). These results could contribute to elucidating underlying mechanisms contributing to the ORG pathogenesis.

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Introduction

The prevalence of overweight [body mass index (BMI) ≥25 kg/m^2) and obesity (BMI≥30 kg/m^2) in the world has risen by over 27% in the last three decades, with approximately 2.1 billion affected people.[1] According to the World Health Organization, the number of obese people in the world has doubled since 1980. Furthermore, obesity is considered a serious primary health problem that impairs the quality of life due to its associated complications, including diabetes, cancer, sleep disorders, cardiovascular diseases, hepatic dysfunction, asthma, infertility, and renal dysfunction.[2] Obesity has a multifactorial mechanism and it is also considered an independent risk factor for chronic kidney disease (CKD) development and progression to end-stage renal disease (ESRD).[3] In particular, obesity-related glomerulopathy (ORG) is a renal complication of obesity that includes proteinuria and glomerulomegaly in obese patients who show any clinical and histopathological evidence of other renal diseases. It is known that about 30% of patients with ORG end up developing progressive renal failure or ESRD.[4,5] The pathophysiologic mechanism of CKD development in obese patients remains unclear, but many processes, like altered renal hemodynamics, insulin resistance, hyperlipidemia, inflammation, and oxidative stress seem to be linked to ESRD.[6] ORG primarily contributes to renal injury through multiple effectors such as adipokines, lipids, renin-angiotensin-aldosterone system (RAAS), sympathetic nervous system (SNS), inflammation, oxidative stress, and apoptosis.[7] In literature it is known how dyslipidemia, a critical component of metabolic syndrome, is often related to obesity and diabetes.[8] Several studies have demonstrated significant correlations between dyslipidemia and impaired renal function in diabetic subjects that may cause kidney damage and lipotoxicity.[9] It is also known that lipid accumulation increases mitochondrial reactive oxygen species (ROS), which causes further amassing of lipids in return.[10,11] Moreover, Szeto *et al.* demonstrated that a major cause

of renal pathology is mitochondrial dysfunction induced by high-fat diet (HFD).[12] This mitochondrial dysfunction generates ROS production and reduces mitochondrial β-oxidation inducing lipids accumulation resulting in a further mitochondrial ROS levels increase.[13] Podocytes, the cells of the Bowman's capsule of the kidney that envelop the capillaries of the glomerulus, play an important role in glomerular function. They form a filtration barrier together with endothelial cells of the glomerular capillary loop and the glomerular basement membrane. Injury of podocytes disrupts the stability of this structure leading to podocyte dysfunctions and apoptosis.[14] All these events are critical for the development of proteinuria and the pathogenesis of ORG. However, the damage mechanism of podocytes in ORG continues to be not very clear.[15] Excess of free fatty acids (FFAs), that is present in obesity, activates various inflammatory pathways involving endoplasmic reticulum stress, toll-like receptor, inflammasome, and nuclear factor-κB (NF-kB) signaling activation.[16] It has been demonstrated that *in vitro* exposure to high levels of FFAs is linked to lipotoxicity and causes cellular dysfunction and death.[17] Palmitate, one of the most common fatty acids in humans, is a potent inducer of ROS in several cell types, including pancreatic β cells, cardiomyocytes, vascular smooth muscle cells, endothelial cells, skeletal muscle cells, glomerular mouse podocytes, hepatocytes, and adipocytes.[18] Increased mitochondrial fatty acid oxidation has been proposed as the main process leading to ROS generation in lipotoxicity.[19] Anyway, the molecular mechanisms induced by palmitate in the cellular ROS generation remain to be clarified. It is known how high mobility group box 1 protein (HMGB1), a highly conserved chromatin-associated factor present in the nucleus of almost every cell type, is a potential biomarker of renal inflammation.[18] HMGB1 synthesis and its secretion are increased in obese patients in different types of tissue, especially in the adipose tissue. Moreover, it is known that HMGB1 has a role in the pathogenesis of different types of kidney disease as CKD and diabetic nephropathy.[20] It has been shown, in literature, that HMGB1 is secreted via a ROS-dependent mechanism.[21] On the other hand, palmitate, at concentrations of 27nM of free palmitate seen in the serum of obese patients, induces podocyte dysfunctions and podocyte insulin resistance, all events that are involved in the reduction of kidney function. All these effects are mediated by the palmitate-induced increase of ROS generation.

In recent years several researchers have focused on the role of inflammation in ORG podocyte injury, therefore, we decided to investigate, in human-conditioned immortalized podocyte cell line, the capacity of physiopathological concentrations of 27 nM of unconjugated palmitate to induce intracellular ROS production, podocytes endoplasmic reticulum (ER) stress, podocytes inflammation, mitochondrial dysfunction and to evaluate the role played by HMGB1 expression in palmitate-induced podocytes dysfunctions.

Materials and Methods

Cell culture conditions

The conditionally immortalized human podocyte cell line AB 8/13 was kindly provided by the University of Bristol. The cell line was developed by transfection using a large T antigen (SV40) that allows cells to proliferate at the "permissive" temperature of 33°C. When transferred to the "non-permissive" temperature of 37°C the inactivation of large T antigen with minor changes in gene expression occurs. At this point, podocytes enter in growth arrest and express differentiated podocytes markers such as podocytes proteins, nephrin, podocin, CD2AP, synaptopodin, and the other known molecules of the slit diaphragm such as ZO-1, alpha-, beta-, and gamma-catenin and P-cadherin.[22] The cells were grown on dishes in RPMI-1640 medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO, USA), 100 µg/mL penicillin/streptomycin (Sigma Aldrich, St. Louis, MO, USA), 1 g/L D-Glucose Solution (Sigma Aldrich, St. Louis, MO, USA) and Insulin-Transferrin-Selenium 100× (Invitrogen, Massachusetts, USA) in 5% of CO_2 at 33°C. At 40-60% confluence, the cells were cultured at 37°C for 14 days changing medium 3 times per week until podocyte differentiation, and then the experiments were performed.

Preparation of free fatty acids solution

The total plasma FFA concentration does not reflect the physiological active FFA concentration because FFAs are physiologically bound to albumin. *In vitro* studies have used FFAs-albumin complexes to produce the soluble unbound FFAs (FFAus), which are believed to be at the concentration of 20 nM of FFAs in physiological conditions. However, as FFAus concentration increases with increasing FFAs/albumin ratio starting from the mole ratios of 4:1, the level of reliable FFAus can only be assumed.[23] Recently the new method of Oliveira *et al*., proposed to generate FFAs-albumin complex, showed the formation of physiopathological concentrations of 27 nM FFAus.[24] Following Oliveira *et al.* method, palmitate (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 50% (volume/volume) ethanol to a concentration of 50 mM. This stock solution was diluted in medium containing 0.75% or 1% (weight/volume) FFA-free BSA (Roche, Germany) to a final concentration of 0.5 mM and then incubated for 1 h at 37°C. However, it is necessary to evaluate the type of BSA used in this model, since differences in isolation methods, purity, and contaminants can interfere with the biological effects of FFAs. Also, the solvent used for palmitate must be well-evaluated. It usually includes the use of organic solvents and heating. We used ethanol, at an elevated temperature of 50-70°C, to dissolve palmitate. Conversely, due to its high solubility in aqueous solutions, BSA is normally dissolved directly in water or cell culture media.[25]

Adenoviral vector

Uncoupling protein 1 (UCP-1) was cloned into the shuttle vector pAd5CMVK-NpA and adenoviral vector. Gene Transfer Vector Core, at the University of Iowa, was used to prepare this empty control virus, as described previously.[26,27] Four hours before the addition of either medium, containing 0.75% or 1% (weight/volume) FFA-free BSA, podocytes were infected with UCP-1 or mannitol as osmotic control at a multiplicity of infection of 500.

Inhibition of nicotinamide adenine dinucleotide phosphate oxidase activity

Cells were cultured with or without nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor GKT137831 (GKT) (Cayman Chemical, Ann Harbor, MI). GKT was dissolved in 0.1% ethanol and then, 20 min before palmitate addition, it was added to the cells at 10 µM concentration.

Inhibition of high mobility group box 1

To clarify the role of HMGB1 in causing palmitate damage, we stimulated cells for 20 min with or without Glycyrrhizin (GLYZ) (Sigma Aldrich, St. Louis, MO, USA), a natural anti-inflammatory

and antifungal factor that inhibits HMGB1 chemoattractant and mitogenic activities through direct binding to HMGB.[28] GLYZ was dissolved in dimethyl sulfoxide (DMSO), at a final concentration of 130 µM before palmitate addition.

Measurement of reactive oxygen species in podocytes

Palmitate-treated cells were incubated, for 45 min at 37°C, with 10 mmol/L of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Molecular Probes-Life Technology, Brooklyn, NY) and ROS intracellular formation was measured.[29] Wallac 1420 Fluorescent Plate Reader instrument with an excitation/emission wavelengths of 485/535 nm was used.

Reverse transcription reaction and real-time quantitative polymerase chain reaction

Total RNA from treated and control cells was extracted using the RNeasy Mini Kit (Invitrogen, Massachusetts, USA), following the manufacturer's instructions. SuperScript IV First-Strand Synthesis System (Life Technology, Brooklyn, NY, USA) was used for the reverse transcription of mRNA. Experiments were performed in optical 96-well reaction plates and the CFX96 TouchTM Real-Time polymerase chain reaction (PCR) Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used according to the manufacturer's instructions. Real-Time PCR Detection System was performed with Sso Advanced TM Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). To ensure that fluorescence signals exclusively reflected specific amplicons, Melting curves have been analyzed.[30] Expression levels of binding immunoglobulin protein/GRP78 (BiP/GRP78), X-box binding protein 1(sXBP1), NF-kB subunit p65, monocyte chemoattractant protein-1 (MCP-1), transforming growth factor beta (TGF-β), sterol regulatory element-binding protein 1 (SREBP1-c), cytochrome b (CytB), NADH: ubiquinone oxide- reductase core subunit 1, 4 and 4L (ND1, ND4, ND4L) and HMGB1 mRNA were normalized to Wilms tumor-1 protein (WT-1) levels in the same sample. The used primers are validated and purchased by Qiagen, Primm, and Invitrogen. WT-1 was chosen as the internal control as a marker of mature podocytes.

Statistical analysis

Data results passed Bartlett's test and then were analyzed using one-way ANOVA to compare the average of all groups. We used the Turkey-Kramer multiple-comparisons procedure to determine differences between pairs of means. All analyses were performed by using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). For the statistical significance, a p-value <0.05 was used.

Results

Mitochondrial and cytosolic mechanisms activated by palmitate induce reactive oxygen species production in human podocyte cell line

In palmitate-treated podocytes, the concentration of 0.5 mM of PAL/1% BSA produced an unbound palmitate concentration of 20 nM comparable to the concentration seen in healthy humans and for this reason, we used it as a control; this concentration, as expected, did not affect ROS production in human podocytes (Figure 1, bar 1). Instead, when the cells were exposed to PAL/0.75% BSA conjugate, corresponding to a physiopathological concentration of unbound FFAs of 27 nM, ROS production increased significantly to 1.5±0.01 standard error means (SEM) compared to the control PAL/1% BSA conjugate (Figure 1, bar 5). Mannitol, used as osmotic control did not affect intracellular ROS generation (data not shown). As previously described in another of our work,[27] both mitochondrial and cytosolic mechanisms can participate in intracellular ROS generation; therefore, we decided to overexpress a specific oxidative phosphorylation uncoupler protein, UCP-1, which breaks down the proton electrochemical gradient of superoxide production. Overexpression of UCP-1 prevented ROS production in cells exposed to 27 nM palmitate (Figure 1, bar 7). Next, since the major cytosolic sources of superoxide in podocytes are NADPH oxidases, we treated immortalized human podocytes with the most specific Nox1/4 inhibitor currently available, GKT.[31] As shown in Figure 1, bar 8, GKT also prevented ROS production in cells exposed to PAL/0.75% BSA conjugate.

Reactive oxygen species induced by free fatty acids cause endoplasmic reticulum stress, pro-inflammatory changes, lipotoxicity, and overexpression of renal fibrosis marker in human podocyte cell line

After treatment of podocytes with PAL/0.75% BSA, the mRNA gene expression levels of XBP-1 and BiP/GRP78 resulted increased by 2.7±0.04 (Figure 2 A, bar 2) and by 1.4±0.03 (Figure 2B, bar 2), respectively. Treatment with GKT inhibitor prevented

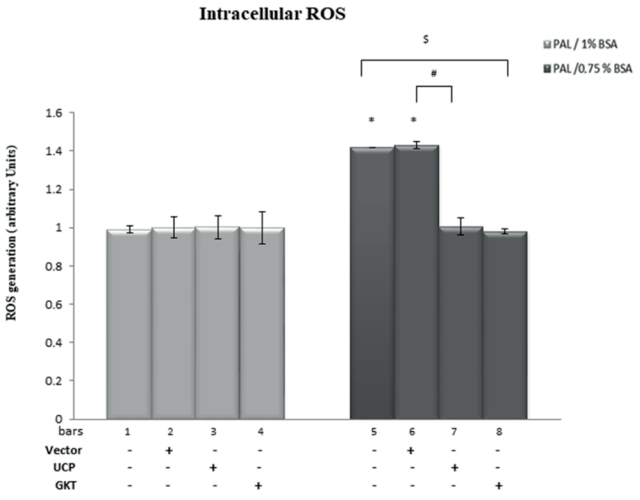

Figure 1. Palmitate induces reactive oxygen species in human podocytes. Reactive oxygen species production was measured, after 24h, in human podocytes incubated with different ratios of conjugated PAL/BSA or after infection with adenoviral vectors expressing uncoupling protein 1 or of nicotinamide adenine dinucleotide phosphate oxidase inhibitor GKT. Each bar represents the mean ± standard error means of 3 separate experiments. *P≤0.05 compared to control. # P≤0.05 cells transfected with the empty vector compared to cells overexpressing uncoupling protein 1, $ P≤0.05 cells not transfected or not treated with the inhibitor compared to cells treated with GKT.

ER stress in cells exposed to conjugated PAL/BSA (Figure 2 A-B, bars 4). Next, we evaluated, in our model, the mRNA expression of two major inflammatory mediators: monocyte chemoattractant protein-1 (MCP-1) (Figure 2C) and NF-κB subunit p65 (Figure 2D), a NF-κB-specific inflammatory target gene. We observed that in cells exposed to pathological concentrations of 27 nM of free palmitate for 24h, mRNA gene expression levels of MCP-1 and subunit p65 were increased by 3±0.04 (Figure 2C, bar 2) and by 2.5±0.03 (Figure 2D, bar 2), respectively, indicating that the palmitate/BSA coupled complex can induce podocyte inflammation. All these palmitate effects were significantly reduced when the cells were treated with GKT inhibitor (Figure 2 C-D, bars 4). Therefore, after 24h of treatment with different percentages of conjugated FFA (PAL/1% BSA or PAL/0.75% BSA conjugate), we observed that TGF-β mRNA expression (Figure 2E, bar 2) and SREBP-1c (Figure 2F, bar 2) increased to 1.7±0.02 and 2.2±0.01, respectively. GKT inhibitor treatment reverted all these results in a significant way (Figure 2 E-F, bars 4).

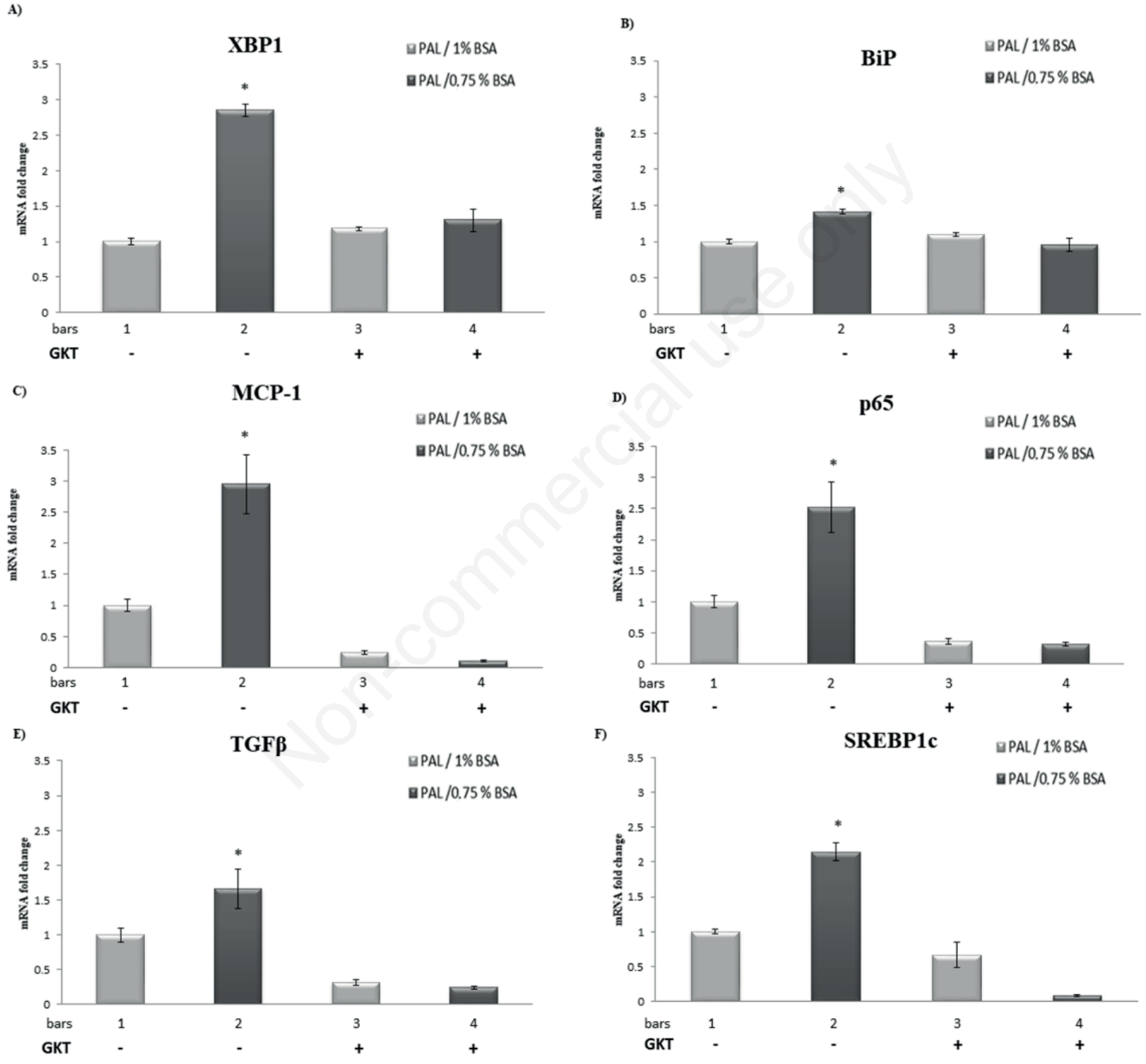

Figure 2. Effects of palmitate on endoplasmic reticulum stress, podocyte inflammation, renal fibrosis, and lipotoxicity in human podocytes. Evaluation of BiP mRNA expression (A), mRNA sXBP1 alternative splicing (B), Monocyte chemoattractant protein-1 (C), NFκB subunit p65 (D), Transforming growth factor-β (E) and Sterol regulatory element binding protein1c (F) in cells treated with physiopathological concentrations of palmitate for 24h, before or after treatment with nicotinamide adenine dinucleotide phosphate oxidase inhibitor GKT. Each bar represents the mean ± standard error means of 3 separate experiments. *P≤0.05 compared with control. All these effects were prevented by GKT.

Free fatty acids-induced mitochondrial dysfunction is responsible for the mitochondrial reactive oxygen species production in podocytes after exposure to high free fatty acids concentrations

In the cells incubated with high palmitate for 24 hours, the expression of CytB was reduced by 0.7±0.02 fold (Figure 3 A bar 2) compared to the control. At the same time, palmitate reduced the expression of ND1, ND4, and ND4L by 0.5±0.01; 0.6±0.03, and 0.4±0.02 fold, respectively (Figure 3 B-C-D bars 2). GKT was able to reverse the effects of palmitate on the expression of proteins encoded within the mitochondrial genome (Figure 3 A-B-C-D bars 4).

Palmitate increases high mobility group box 1 expression in human podocytes

As shown in Figure 4, in podocytes treated with PAL/0.75% BSA for 24h there was an increase by 2±0.02 of HMGB1 mRNA expression level (bar 2), while the addition of GKT inhibitor prevented this increase in HMGB1 expression (bar 4).

Palmitate causes podocyte dysfunction by increasing high mobility group box 1 expression

As shown in Figure 5, the increase of podocyte inflammation (A), renal fibrosis marker (C), and lipotoxic effect (D), induced by physiopathological concentrations of palmitate, was prevented by GLYZ inhibitor.

Discussion

In this study, we investigated the effect of physiopathological concentration of palmitate conjugated to albumin, in human-conditioned immortalized podocytes cell line, following the method of Oliveira *et al.*[24] If podocytes are injured, mutated, or lost, the elaborate structure of podocytes is physically altered and this results in many proteinuric kidney diseases.[14] One of the major pathogenic mediators in ORG and its complications is dyslipidemia and this leads to elevated levels of saturated FFA concentrations. Palmitate is the most abundant saturated FFA present in the plasma of humans and rodents representing ~ 25% of total fatty acids. In literature, it is known that the presence of FFAs in the cytosol leads to the gen-

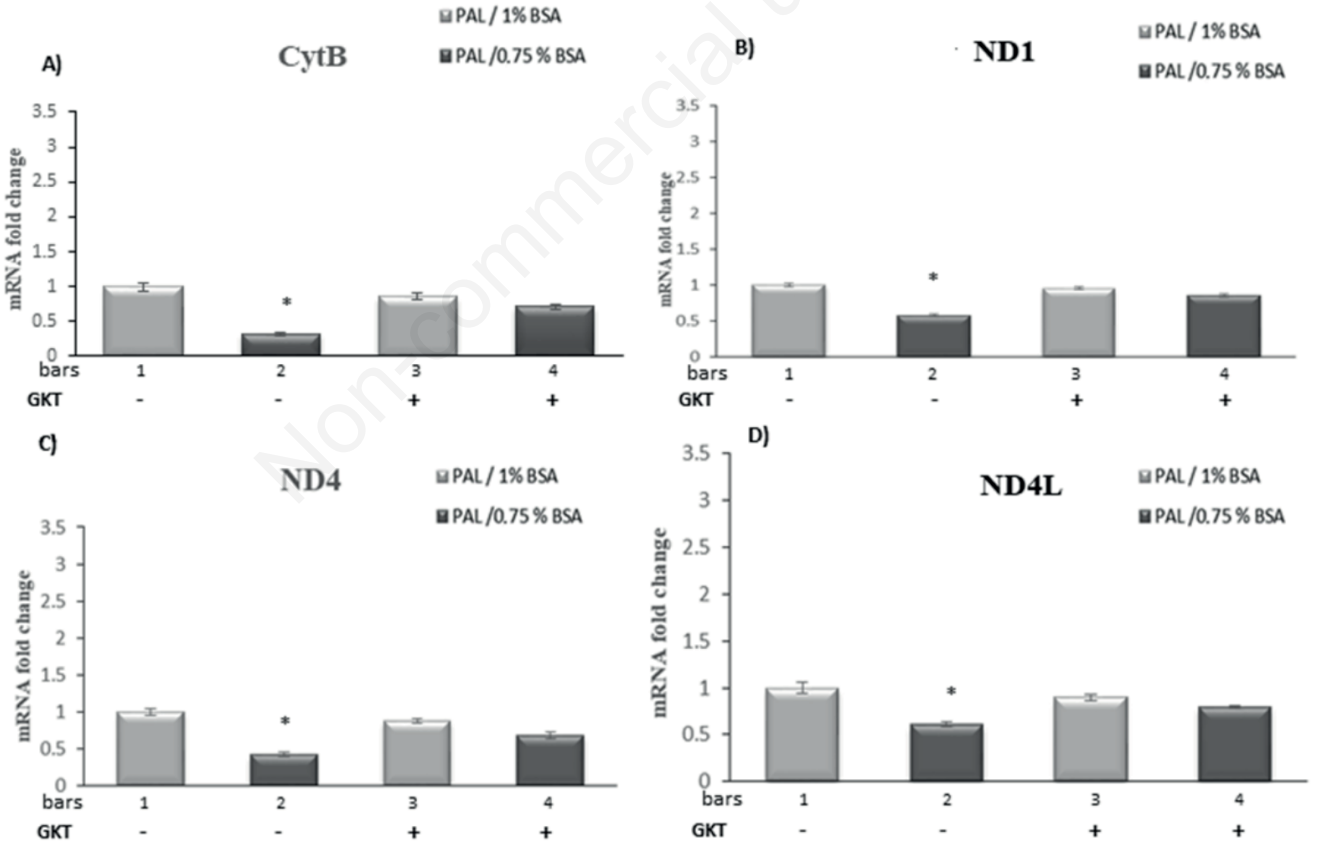

Figure 3. Exposure to high free fatty acids causes a reduction of mitochondrial electron transport chain gene expression. Podocytes were exposed to high free fatty acids for 24 h. In the indicated groups, podocytes were treated with GKT during the exposure to palmitate. The mRNA expression of (A) CytB, (B) ND1, (C) ND4, and (D) ND4L were measured by real-time polymerase chain reaction. The exposure to high free fatty acids caused a reduction of mitochondrial electron transport chain gene expression. Data are the mean ± standard error means from 3 independent experiments. *P<0.05 compared with cells not treated with high free fatty acids.

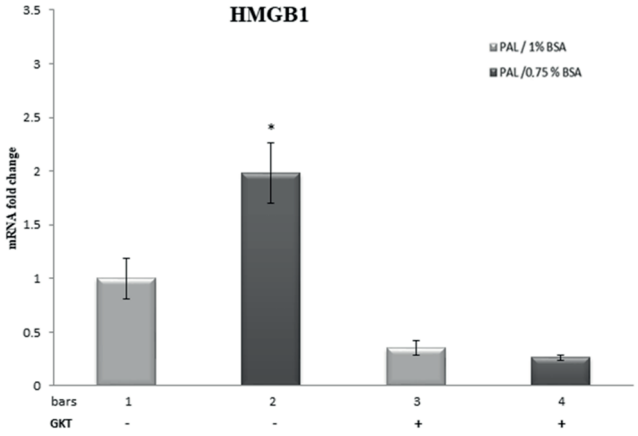

Figure 4. Palmitate induces an increase in high mobility group box 1 expression. The expression of high mobility group box 1 protein was evaluated in cells treated with palmitate by measuring mRNA gene expression. Palmitate induced an increase in high mobility group box 1 expression and the increase in the mRNA expression was prevented by GKT inhibitor. Each bar represents the mean ± standard error means of 3 separate experiments. *P≤0.05 compared to control.

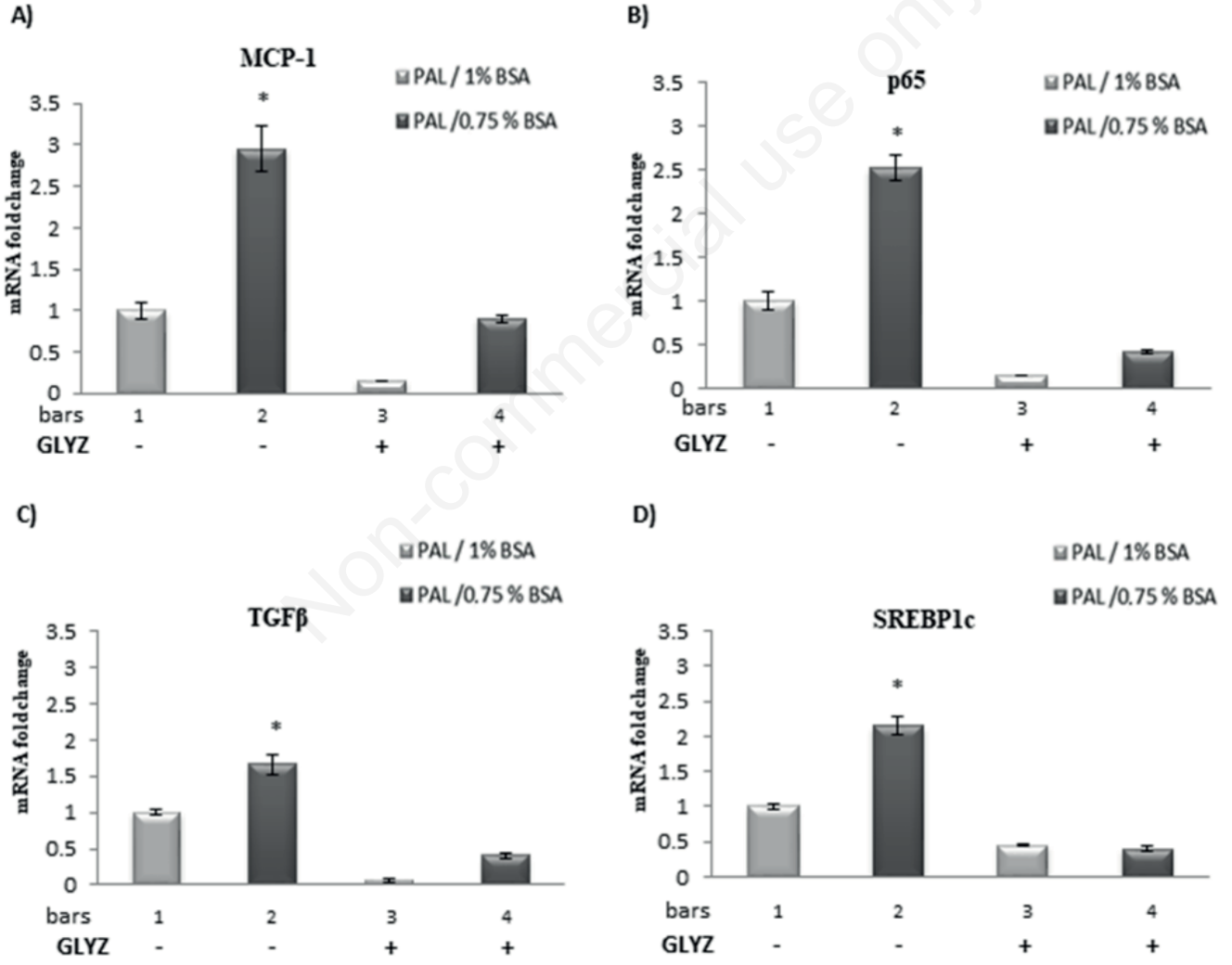

Figure 5. Palmitate causes podocyte dysfunction by increasing high mobility group box 1expression. Gene expression of (A) monocyte chemoattractant protein-1, (B) NFκB subunit p65 serum pro-inflammatory cytokines, (C) Transforming growth factor-β, and (D) Sterol regulatory element binding protein1c was evaluated in cells treated with palmitate for 24h by measuring mRNA expression. All the increased effects were prevented by Glycyrrhizin 130μM for 30 min. Each bar represents the mean ± standard error means of 3 separate experiments. *P≤0.05 compared to control.

eration of ROS. In particular, palmitate is a potent inducer of ROS in various cell types, including cardiomyocytes, endothelial cells, mouse glomerular podocytes, and adipocytes.[32] One of the major limitations in *in vitro* and *in vivo* studies is the low solubility of long-chain FFAs in aqueous solutions. A solution to this problem is to conjugate FFAs directly to BSA *in vitro* preparing a solution in the physiological concentration range. In the *in vitro* experiments, when using FFAs, particular attention should be paid to the concentrations and preparation of FFAs and BSA since the percentage of unbound FFAs, available for cellular uptake, depends on the conjugation protocol and the concentration of unbound FFAs is influenced by the relative affinity for BSA.[33] Physiologically, serum FFAs are bound to BSA and only a small portion of them is free. In pathological conditions, the amount of FFAs increases leading to cellular dysfunctions. Until now, in experimental conditions, different doses of palmitate were incubated with a fixed dose of albumin producing an unknown amount of free palmitate. So, following the protocol suggested by Oliveira *et al.*,[24] in our experiments, we manually prepared the PAL/BSA conjugate maintaining unvaried palmitate concentration and by varying the BSA percentage. In this protocol, the number of unbounded FFAs obtained reproduced the limit of physiopathological conditions of obese patients (in normal physiological conditions, an average of two FFAs molecules bind to each circulating albumin molecule). This corresponds to an unbound FFA concentration of less than 20 nM.[24] From the literature it is known that palmitate, at a concentration similar to that seen in patients with diabetic nephropathy (DN), can induce ROS production in cultured mouse podocyte cell line.[34] Since mouse podocytes do not preserve the human cell type's main metabolism, we used an *in vitro* model, instead, consisting of human conditionally immortalized podocytes incubated with palmitate. Our data show that physiopathological concentrations of palmitate stimulate ROS production, in human podocytes and induce ER stress. To identify the origin of this ROS production we treated podocytes with a specific oxidative phosphorylation uncoupler protein, UCP-1, and with the most specific Nox1/4 inhibitor currently available, GKT. In both cases, a reduction in ROS production was observed in human podocytes treated with palmitate underlying that physiopathological palmitate concentrations stimulate ROS generation in human podocytes through both mitochondrial and cytosolic mechanisms. It has been shown that chronic adipose inflammation is a major factor for ORG, resulting in an imbalance between pro-inflammatory and anti-inflammatory factors.[35] Therefore, in our model we investigated whether ROS production induced by physiopathological concentration of palmitic acid could lead to podocyte inflammation. So, we analyzed the mRNA gene expression of MCP-1 which is one of the key chemokines that regulate the migration and infiltration of monocytes/macrophages, the gene expression of the subunit p65, which is one of the five components that form the NF-κB transcription factor family, the mRNA expression of TGF-β that is considered a major culprit for renal cell injury in progressive CKD and contributes to both renal cell apoptosis and renal fibrosis, and the mRNA gene expression of SREBP-1c that plays an important role in regulating fatty acid synthesis and may also be involved in fatty liver disease.[36] Our data suggest that in cells exposed to physiopathological concentrations of palmitate for 24h, mRNA gene expression levels of MCP-1 and subunit p65 were increased such as the mRNA gene expression of TGF-β and SREBP-1c and all these palmitate effects were significantly reduced when the cells were treated with GKT inhibitor, proving that palmitate induces podocytes inflammation by increasing ROS production. Several studies have demonstrated that oxidative mtDNA damage can further increase the generation of superoxide by impairing the integrity and function of the electron transport chain.[18] In particular, in our cells high palmitate significantly reduces the transcription of the ND1, ND4, and ND4L subunits of Complex I. Also, we observed a reduction of the CytB subunit of Complex III-derived ROS. Complex I has been recently recognized to be the mitochondrial source of deleterious ROS and it can react readily with mitochondrial DNA and other matrix components that are susceptible to oxidative damage. In contrast, the CytB subunit of Complex III-derived ROS functions as a second messenger in cell signaling.[37] On the other hand, it is known that HMGB1, a nuclear DNA binding protein, is released under pathological conditions. It functions locally as a messenger of one of the potent damage-associated molecular patterns (DAMPs) that cause tissue damage and chronic inflammation. HMGB1 synthesis and its secretion is increased in obese patients in different types of tissues and it is considered to be implicated in the pathogenesis of a large range of kidney diseases.[20] Several studies have reported increased levels of serum HMGB1 in ORG patients or models and showed a positive correlation with the severity of the disease.[37] Decreasing HMGB1 levels by HMGB1-specific inhibitors can significantly decrease the release of related cytokines and reduce the inflammatory reaction in kidney disease.[28] The exact function of HMGB1 and its mechanism still needs to be elucidated. We demonstrated that HMGB1 mRNA gene expression, in the human podocytes treated with palmitate conjugated with different percentages of BSA for 24h, was increased, and adding a GKT inhibitor prevented this increase in HMGB1expression, proving that it is mediated by ROS production. Moreover, GLYZ, a natural anti-inflammatory and antifungal factor, demonstrated anti-inflammatory properties and is considered to inhibit the cytokine activity of HMGB1 with amelioration of inflammation during acute/chronic phases of hepatitis, myocarditis, and liver injury. For example, *Glycyrrhiza glabra* (more commonly known as Liquorice) is considered one of these inhibitors. This plant has been used as a natural medicine and as a flavour and sweetener food. The roots are used to prevent and treat several complications, especially microbial/viral infection, cancer, and dermatitis.[38] Various phytochemicals, including GLYZ, have been identified and associated with antioxidant, antiviral, antimicrobial, anticancer or anti-inflammatory activities.[38] Furthermore, to obtain HMGB1 inhibitors with higher activity and good pharmacokinetic properties, two different types of GLYZ analogues, containing C-N glycoside bonds, were synthesized and their anti-inflammatory, antioxidant, and anti-septic kidney injury were evaluated in a recent interesting study.[39] The authors demonstrated that the two compounds have activity against septic acute kidney injury so they can be potential therapeutic drugs.[39] To understand the role of HMGB1 in causing palmitate-induced glomerulopathy, we measured mRNA expression of inflammatory, fibrosis, and steatosis genes in treated podocytes stimulated with GLYZ for 20 min. Our results show that the increase of MCP-1, NF-kB subunit p65, TGF-β, and SREBP-1c, induced by physiopathological concentrations of palmitate, was prevented by GLYZ inhibitor. All these results show the key role played by HMGB1 in ORG and suggest a regulatory role of HMGB1 in inflammation, steatosis, and fibrosis. However, the mechanisms by which HMGB1 is released and the signaling pathways are activated are required to be elucidated. Moreover, it is known that the inhibition of HMGB1-mediated signaling may constitute a new strategy for the different types of kidney disease treatment.[20] Further *in vitro* and *in vivo* studies are required to evaluate the involvement of the extracellular and intracellular HMGB1 in the pathogenesis of different kinds of kidney diseases. However, the limit of our study remains to not perform the silencing of HMGB1 to prove its key role in the pathogenesis of ORG.

Conclusions

Our study shows that the concentration of 27 nM of unbound palmitate found in ORG patients causes ROS overproduction in human podocytes. In our study, HMGB1 seems to be the main mediator of ROS effects on podocytes. Indeed, the pharmacological inhibition of HMGB1 prevents all ROS effects on podocytes. Taken together, our results can give further insight into identifying the mechanisms that contribute to the development of ORG.

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