Fatty acids are novel nutrient factors to regulate mTORC1 lysosomal localization and apoptosis in podocytes

Mako Yasuda a, Yuki Tanaka a, Shinji Kume a,⁎, Yoshikata Morita a, Masami Chin-Kanashia, Hisazumi Araki a, Keiji Isshikia, Shin-ichi Arakia, Daisuke Koyab, Masakazu Haneda c, Atsunori Kashiwagi a, Hiroshi Maegawa a, Takashi Uzu a

a Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan
b Division of Diabetology and Endocrinology, Kanazawa Medical University, Kahoku-Gun, Ishikawa, Japan
c Department of Medicine, Asahikawa Medical University, Asahikawa, Hokkaido, Japan

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ABSTRACT

Podocyte apoptosis is a potent mechanism of proteinuria in diabetic nephropathy. More detailed mechanistic insight into podocyte apoptosis is needed to better understand the pathogenesis of diabetic nephropathy. An elevated level of serum free fatty acid (FFA), as well as hyperglycemia, is a clinical characteristic in diabetes, although its causal role in podocyte apoptosis remains unclear. This study examined the effect of three types of FFAs, saturated, monounsaturated and polyunsaturated FFAs, on podocyte apoptosis. Palmitate, a saturated FFA, induced endoplasmic reticulum (ER) stress-dependent apoptosis in podocytes. Oleate, a monounsaturated FFA, and eicosapentaenoic acid (EPA), an ω-3 polyunsaturated FFA did not induce apoptosis; rather, they antagonized palmitate-induced apoptosis. Palmitate activated mammalian target of rapamycin (mTOR) complex 1 (mTORC1), a nutrient-sensing kinase regulating a wide range of cell biology. Furthermore, inhibition of mTORC1 activity by rapamycin or siRNA for Raptor, a component of mTORC1, ameliorated palmitate-induced ER stress and apoptosis in podocytes. Activity of mTORC1 is regulated by upstream kinases and Rag/Ragulator-dependent recruitment of mTOR onto lysosomal membranes. Palmitate activated mTORC1 by enhancing recruitment of mTOR onto lysosomal membranes, which was inhibited by co-incubation with oleate or EPA. Inhibition of mTOR translocation onto lysosomes by transfection with dominant-negative forms of Rag ameliorated palmitate-induced apoptosis. This study suggests that saturated and unsaturated FFAs have opposite effects on podocyte apoptosis by regulating mTORC1 activity via its translocation onto lysosomal membranes, and the results provide a better understanding of the pathogenesis in diabetic nephropathy and a novel role of mTORC1 in cell apoptosis.

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1. Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease and is a serious health problem worldwide. Proteinuria is a major aspect of diabetic nephropathy, and is caused by disruption to the glomerular filtration barrier [1]. Glomerular epithelial cells (podocytes) are potent component cells that maintain glomerular filtration barrier function. Podocyte loss by apoptosis is a main cause of proteinuria in diabetic nephropathy [1,2], since podocytes are highly specialized, terminally differentiated and unable to proliferate. Protecting podocytes against apoptosis has therefore become a principal therapeutic target for diabetic nephropathy. However, detailed mechanisms of podocyte apoptosis in diabetes have not been fully elucidated.

Insufficiency of insulin action to skeletal muscle and the liver leads to hyperglycemia [3,4], which strongly associates with both the onset and progression of diabetic vascular complications [5–8]. As well as hyperglycemia, higher levels of serum free fatty acids (FFAs) resulting from insufficient insulin action to white adipose tissue have recently been suggested as a novel cause of organ dysfunction in diabetes [9–12]. Serum FFAs are composed of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids. The composition is modified by altered metabolic states, such as obesity and diabetes [13–15]. In addition to high levels of serum FFAs, modified FFA
composition has recently been the focus of study as they may be an additional causal factor of diabetes-related diseases [16–18]. However, the role of modified FFA composition in diabetes-related podocyte apoptosis remains unclear.

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved protein kinase and forms two functional complexes, termed mTOR complex 1 (mTORC1) and mTORC2 [19]. mTORC1 is a rapamycin-sensitive protein kinase complex of six proteins, including Raptor, that regulates a wide array of cellular processes including cell growth, proliferation and autophagy [19,20]. Some nutrient-related molecules, such as insulin, glucose and amino acids, activate mTORC1; although each regulatory mechanism is different. mTORC1 is finally activated by a small GTPase, the Ras homolog enriched in brain (Rheb), that is anchored to lysosomal membranes [21]. Insulin activates mTORC1 via Akt-dependent inactivation of tuberous sclerosis complex 1/2 (TSC1/2), a suppressor of Rheb [20,22,23]. A high glucose condition activates mTORC1 via inhibition of AMP-activated protein kinase (AMPK), an activator of TSC1/2 and a direct suppressor of Raptor [24]. Unlike these mechanisms, amino acids, glucose and glutaminolysis enhance translocation of mTORC1 onto lysosomal membranes in a Rag–Ragulator complex-dependent manner, leading to mTORC1 activation [25–29]. mTORC1 can act as a nutrient-sensing kinase regulating cellular processes; however, the relationship between FFA, a nutrient, and mTORC1 has not been fully elucidated.

Hyperactivation of mTORC1 is likely to be associated with the pathogenesis of various diabetes- and age-related diseases [30–32]. Growing evidence suggests that mTORC1 is pathologically activated in podocytes of the diabetic kidney in both humans and rodent models, and inactivation of mTORC1 ameliorated podocyte injury in diabetic nephropathy [33,34]. mTORC1 inhibition has recently been suggested as a new therapeutic target to prevent diabetic nephropathy [35–37]. However, it is also likely that very strong inhibition of mTORC1 causes podocyte dysfunction leading to proteinuria, suggesting that a basal level of mTORC1 activity is required for maintaining the basic physiology of podocytes [38–40]. Therefore, more detailed mechanisms of diabetes-related hyperactivation of mTORC1 are required to establish an effective and safe therapy that targets mTORC1 inhibition.

We hypothesized that composite changes in serum FFAs contribute to diabetes-related mTORC1 hyperactivity, leading to podocyte apoptosis in diabetic nephropathy. To test this hypothesis, we examined the effect of each kind of FFA (saturated, monounsaturated and ω–3 polyunsaturated fatty acids) on mTORC1 activation and apoptosis in cultured podocytes. The results suggest that FFAs are potent nutrients regulating the pro-apoptotic function of mTORC1 in podocytes.

2. Materials and methods

2.1. Antibodies and reagents

Anti-PARP, anti-cleaved caspase 3 (Asp175), anti-phospho-P70S6K (Thr389), anti-phospho-P70S6K, anti-phospho-S6 (Ser235/Ser236), anti-S6, anti-CHOP, anti-phospho-PERK (Thr980), anti-PERK, anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473), anti-phospho-AMPK (Thr172), anti-AMPK, anti-phospho-Raptor (Ser792), anti-Raptor, anti-Rictor, anti-mTOR, anti-p18 and anti-RagC antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-LAMP2 antibody was purchased from Abcam (Cambridge, UK). Anti-Akt antibody was established by Peter Mundel (Mount Sinai School of Medicine) [41].

The plasmids for overexpression of Flag-RagA(TN), Flag-RagB(TN), Myc-RagC and Myc-4E-BP1–AA were as described previously [43,44]. ON-TARGETplus smart pool siRNA for Raptor, Rictor and CHOP were purchased from Dharmacon (Lafayette, CO, USA). One-hundred picomole of siRNA or control siRNA (non-targeting siRNA; Dharmacon) was transfected into 1.6 × 10⁶ podocytes using a Nucleofector Kit V according to the manufacturer’s guidelines (Amaxa Biosystems, Gaithersburg, MD, USA). Briefly, podocytes were suspended in 100 μl of Nucleofector solution provided with the Amaxa transfection reagent with 3 μg of plasmid DNA or 100 pmol of siRNA. Transfection was performed by electroporation using the T-20 program of the Amaxa electroporator. Transfected cells were incubated in RPMI medium containing 10% FCS for 48 h prior to serum starvation. ON-TARGETplus smart pool siRNA reagent was a mix of four different siRNAs for each gene. Sequences of siRNA were: for siRNA for CHOP: 5′-CACGACGGACAGGGAGACGA-3′, 5′-GCACAAAGACAGAGGACAGGC-3′, 5′-GGACCAAGAAGAGACAGGGAC-3′, 5′-GAGCAGACACGGAGACAGGGAC-3′, 5′-GAAGACAGACACGGAGACAGGGAC-3′, 5′-AGAAAGGACAGACACGGAGACAGGGAC-3′, 5′-CGACGACAGACACGGAGACAGGGAC-3′, and 5′-GUACACGACAGACACGGAGACAGGGAC-3′. For siRNA for Rictor: 5′-CUUACAGGGGAGACAGGAGACAGGGAC-3′, 5′-AUGUAGAAGUACUGUUGGAGAA-3′, 5′-AUGUAGAAGUACUGUUGGAGAA-3′, and 5′-AUGUAGAAGUACUGUUGGAGAA-3′.

2.4. Western blot analysis

Immunoblot analysis was performed as previously described [46]. Cell lysates in SDS sample buffers were electrophoresed and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA, USA).
MA, USA). After membranes were blocked, they were incubated with the indicated antibodies. Horseradish peroxidase-conjugated anti-rabbit, anti-mouse antibody (Amersham, Buckinghamshire, UK) and anti-goat antibodies (Santa Cruz, CA, USA) were used as secondary antibodies. Immunoreactive bands were detected with an enhanced chemiluminescence detection system (PerkinElmer Life Science, Boston, MA, USA). For cell fraction study, cytoplasmic and membrane fractions were isolated using a subcellular protein fractionation kit (Thermo Scientific, Cincinnati, OH, USA).

2.5. Immunofluorescence staining

Cells cultured on chamber slides coated with collagen I were fixed with 4% paraformaldehyde. Fixed cells were rinsed three times with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with 2% BSA-TBS for 30 min at room temperature. After rinsing three times with TBS containing 0.05% Tween (TBS-T), samples were incubated with primary antibodies in PBS for 3 h at room temperature, rinsed three times with TBS-T, incubated with secondary antibodies for 1 h at room temperature, washed twice with TBS-T, and rinsed twice with PBS [47]. Antibodies were used at the following dilutions: mTOR (1:400), LAMP2 (1:200), CHOP (1:1000), Alexa Fluor 488 anti-rabbit IgG (1:500), Alexa Fluor 594 anti-rat IgG (1:500) and Alexa Fluor 594 anti-mouse IgG (1:500). Before samples were mounted with mounting medium, slides were stained using DAPI and chamber borders were removed. Immunofluorescence staining was detected using a fluorescence microscope at original magnification with appropriate filters (BX61, Olympus, Tokyo, Japan). The ratios of merged area to LAMP2-positive area were digitally quantified using Image-Pro Plus 7.0 (Media Cybernetics, Bethesda, MD, USA).

2.6. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed with an in situ apoptosis detection kit (Takara-Bio Inc., Otsu, Japan). TUNEL-positive and -negative cells were counted in five randomly selected areas of a cultured slide. The results are expressed as a ratio of TUNEL-positive cells to total cell number in each field.

2.7. Fatty acid uptake assay

Cells plated on a 24-well plate were exposed to 50 μM of fluorescence-labeled palmitate (BODIPY C-16, Molecular Probes, Eugene, OR, USA) with 150 μM of non-fluorescence-labeled palmitate and co-incubated with/without oleate or EPA for 30 min. After washing with PBS, the uptake of fluorescence-labeled palmitate was measured using an Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

2.8. Statistical analysis

Results are expressed as mean ± SEM. Analysis of variance with subsequent Fisher’s test was used to determine significance of differences in multiple comparisons. Pearson’s correlation coefficients were calculated to investigate the association among indicated parameters. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Saturated and unsaturated fatty acids have opposite effects on podocyte apoptosis

To examine the effect of each kind of FFA on podocyte apoptosis, cultured podocytes were exposed to a saturated (palmitate), a monounsaturated (oleate) and an ω−3 polyunsaturated fatty acid (eicosapentaenoic acid; EPA) for the indicated hours. Of the FFAs, palmitate induced apoptosis, as determined by cleavage of caspase 3 and poly(ADP-ribose) polymerase (PARP), after 9 h of stimulation (Fig. 1A) in a concentration-dependent manner up to 150 μM (Fig. 1B and C). Oleate and EPA did not induce apoptosis (Fig. 1A), and more likely co-incubation with either oleate or EPA significantly ameliorated palmitate-induced cleavage of caspase 3 and PARP (Fig. 1D–F). The effect of each kind of FFA on apoptosis was validated by TUNEL assay. Palmitate significantly increased TUNEL-positive cell numbers, which was significantly ameliorated by co-incubation with oleate or EPA (Fig. 1G and H).

We next examined the additive effects of other nutrient factors such as the serum and/or glucose condition on palmitate-induced podocyte apoptosis. Under glucose-containing conditions, palmitate induced apoptosis regardless of glucose concentration and whether 10% FCS was present (Fig. 1I and J). However, under glucose-free conditions, the pro-apoptotic effect of palmitate weakened in 10% FCS-containing conditions, and more likely rescued podocytes from nutrient-depletion-induced apoptosis in FCS-free conditions (Fig. 1I and J). These results suggest that palmitate is an essential energy source in glucose-free conditions for cell survival, whereas palmitate becomes toxic and induces apoptosis when in co-existence with other nutrient factors including glucose.

3.2. Palmitate induced podocyte apoptosis via activation of mTORC1 signaling

To rule out the possibility that oleate and EPA interfered with palmitate uptake into cells, we analyzed the effects of oleate and EPA on the uptake of palmitate with fluorescence-labeled palmitate. Both oleate and EPA had no effect on palmitate uptake (Fig. 2A), suggesting that both inhibited palmitate-induced apoptosis via the alteration of some intracellular process.

We next tested the relationship between palmitate-induced apoptosis and mTORC1 signaling, as hyperactivation of mTORC1 has recently been suggested to be a causal factor of podocyte dysfunction in diabetic nephropathy [33,34]. Palmitate increased phosphorylation of P70S6K and S6K235/236, downstream substrates of the mTORC1 signal, within the first 5 min of exposure. This phosphorylation significantly increased after 30 min of palmitate stimulation and was sustained for 9 h (Fig. 2B and C). Thus, in a similar way to insulin and amino acids, palmitate was able to begin to activate mTORC1 within a few minutes. Furthermore, co-incubation with either oleate or EPA inhibited palmitate-induced phosphorylation of P70S6K and S6K235/236 (Fig. 2D and E), suggesting that both diminished palmitate-induced mTORC1 activation.

Rapamycin, a specific pharmacological inhibitor of mTORC1, decreased phosphorylation of P70S6K and S6K235/236, and significantly diminished palmitate-induced podocyte apoptosis in a dose-dependent manner (Fig. 2F and G). The anti-apoptotic effect of rapamycin was confirmed by TUNEL staining (Fig. 2H).

To further confirm the role of mTORC1 in palmitate-induced apoptosis, we employed RNA interference to knockdown Raptor, an essential component of mTORC1. Transfection with siRNA for Raptor inhibited palmitate-induced phosphorylation of P70S6K and S6K235/236 and apoptosis, as determined by cleavage of PARP and caspase 3, and TUNEL staining (Fig. 2I–K). To rule out the involvement of mTORC2 on palmitate-induced podocyte apoptosis [48], we employed RNA interference to knockdown Rictor, an essential component of mTORC2. Knockdown of Rictor had no effect on palmitate-induced apoptosis (Fig. 2L), suggesting that palmitate-induced podocyte apoptosis and the anti-apoptotic effect of rapamycin were dependent on the mTORC1 signal.

To determine whether the pro-apoptotic function of mTORC1 was specific to palmitate, we examined the relationship between apoptosis and mTORC1 in cultured podocytes stimulated with insulin, a potent activator of mTORC1 signaling. Unlike palmitate, insulin did not induce apoptosis, although it did increase phosphorylation of P70S6K and S6K235/236 (Fig. 2M). Neither oleate nor EPA altered insulin-induced
phosphorylation of P70S6K\(_{\text{Thr}389}\) and S6\(_{\text{Ser}235/236}\) (Fig. 2N). These results suggest that FFA-mediated, but not insulin-mediated, alteration of mTORC1 activity is associated with cell apoptosis.

### 3.3. Palmitate induced endoplasmic reticulum stress-triggered apoptosis via mTORC1 in podocytes

We investigated the downstream mechanisms of mTORC1-dependent apoptosis in cultured podocytes stimulated with palmitate, with a focus on endoplasmic reticulum (ER) stress, since it is suggested to be related to mTORC1-dependent cell dysfunction and apoptosis [49].

Palmitate induced phosphorylation of PKR-like kinase (PERK) and overexpression of C/EBP homologous protein (CHOP), a potent pro-apoptotic transcription factor associated with ER stress [50], in a time-dependent manner (Fig. 3A). We also confirmed that both oleate and EPA inhibited palmitate-induced phosphorylation of PERK\(_{\text{Thr1461/1462}}\) and overexpression of CHOP (Fig. 3B–D). Nuclear overexpression of CHOP was confirmed by immunofluorescence study (Fig. 3E). To test whether ER-

![Image](https://example.com/figure1.png)

**Fig. 1.** Saturated and unsaturated fatty acids have opposite effects on podocyte apoptosis. (A) Cleavage of PARP and caspase 3 in cultured podocytes exposed to palmitate, oleate or EPA for the indicated hours; \(\beta\)-actin was used as the loading control. (B) Cleavage of PARP and caspase 3 in cultured podocytes exposed to palmitate at the indicated concentration for 9 h. (C) Quantitative analysis of the ratios of cleaved caspase 3 to \(\beta\)-actin (n = 3). (D) Cleavage of PARP and caspase 3 in palmitate-stimulated podocytes co-incubated with/without oleate or EPA. (E, F) Quantitative analysis of the ratios of cleaved caspase 3 to \(\beta\)-actin (n = 3). All data are expressed as mean ± SEM. PARP, poly(ADP-ribose) polymerase; PAL, palmitate; OLE, oleate; EPA, eicosapentaenoic acid; FCS, fetal calf serum; Glu, glucose; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; NS, not significant.

![Image](https://example.com/figure2.png)

**Fig. 2.** Palmitate-induced podocyte apoptosis via activation of mTORC1 signaling. (A) Uptake of fluorescence-labeled palmitate with/without oleate or EPA. Cells were exposed to 50 \(\mu\)M of fluorescence-labeled palmitate with 150 \(\mu\)M of non-fluorescence-labeled palmitate co-incubated with/without oleate or EPA for 30 min. Results are expressed in arbitrary units (n = 3). (B) Phosphorylated P70S6K\(_{\text{Thr}389}\), P70S6K, phosphorylated S6\(_{\text{Ser}235/236}\) and S6 in cultured podocytes exposed to palmitate for the indicated hours. (C) Quantitative analysis of the ratios of phosphorylated P70S6K\(_{\text{Thr}389}\) to P70S6K and phosphorylated S6\(_{\text{Ser}235/236}\) to S6 at the indicated time (n = 3). (D) Phosphorylated P70S6K\(_{\text{Thr}389}\), P70S6K, phosphorylated S6\(_{\text{Ser}235/236}\) and S6 in cultured podocytes exposed to palmitate with/without co-incubation with oleate or EPA. (E) Ratios of phosphorylated S6\(_{\text{Ser}235/236}\) to S6 and phosphorylated P70S6K\(_{\text{Thr}389}\) to P70S6K at the indicated concentrations. (F) Ratios of cleaved PARP to full-length PARP and cleaved caspase 3 protein to \(\beta\)-actin in cultured podocytes exposed to palmitate at a concentration of 0.5 \(\mu\)g/ml (n = 6). (H) Representative immunoblotting showing TUNEL staining of cultured podocytes stimulated with palmitate with/without rapamycin. Original magnification: ×200. Percentage of TUNEL-positive cell numbers from these three groups (n = 5). (I) Raptor, phosphorylated P70S6K\(_{\text{Thr}389}\), P70S6K, phosphorylated S6\(_{\text{Ser}235/236}\), S6, and cleavage of PARP, caspase 3 and \(\beta\)-actin in palmitate-stimulated podocytes transfected with scrambled (Si-control) or siRNA for Raptor (Si-Raptor). (J) Cleavage of PARP, caspase 3 and \(\beta\)-actin in palmitate-stimulated podocytes transfected with scrambled (Si-control) or siRNA for Rictor (Si-Rictor). (K) Cleavage of PARP, caspase 3 and \(\beta\)-actin in palmitate-stimulated podocytes transfected with scrambled (Si-control) or siRNA for Rictor (Si-Rictor). (M) Cleavage of PARP, caspase 3, \(\beta\)-actin, phosphorylated P70S6K\(_{\text{Thr}389}\), P70S6K, phosphorylated S6\(_{\text{Ser}235/236}\) and S6 in cultured podocytes treated with insulin for the indicated hours. (N) Phosphorylated P70S6K\(_{\text{Thr}389}\), P70S6K, phosphorylated S6\(_{\text{Ser}235/236}\) and S6 in cultured podocytes exposed to insulin with/without co-incubation with oleate or EPA. All data are expressed as mean ± SEM. PARP, poly(ADP-ribose) polymerase; PAL, palmitate; OLE, oleate; EPA, eicosapentaenoic acid; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; Rapa, rapamycin; Si-Riap, Si-Raptor; NS, not significant.
related CHOP activation was involved in palmitate-induced podocyte apoptosis, we employed RNA interference to knockdown CHOP. Knockdown of CHOP significantly ameliorated palmitate-induced cleavage of PARP and caspase 3, and increased the number of TUNEL-positive cells (Fig. 3F–H). These results suggest that ER stress was involved in mTORC1-dependent apoptosis in cultured podocytes exposed to palmitate.

Downregulation of mTORC1 by both rapamycin and siRNA for Raptor significantly suppressed palmitate-induced phosphorylation of PERK Thr980 and nuclear overexpression of CHOP (Fig. 3I–M). Furthermore, transfection with a non-phosphorylatable mutant form of 4E-BP1 (4E-BP1-4A), which works as a constitutive active form of 4E-BP1 and can inhibit cap-dependent initiation of translation [45], diminished palmitate-induced ER stress and apoptosis (Fig. 3N). These results suggest that
4E-BP1-related regulation of translation is involved in mTORC1-dependent increases in ER stress and subsequent apoptosis in podocytes stimulated with palmitate.

3.4. Palmitate activates mTORC1 by enhancing translocation of mTORC1 onto lysosomal membranes

With regard to the main regulatory mechanisms of mTORC1 activity, it has been reported that mTORC1 activity is upregulated by insulin-induced activation of Akt and translocation of mTORC1 onto lysosomal membranes mediated by some nutrient factors such as amino acids, glucose, and glutaminolysis [25–29], and is suppressed by AMPK-dependent phosphorylation of Raptor Thr792 (Fig. 4A) [24]. We therefore investigated whether these upstream regulatory signals were involved in the mechanism underlying palmitate-induced activation of mTORC1. In Fig. 4B, the representative figure from the short exposure time shows that insulin stimulation as a positive control to activate Akt was able to induce maximal phosphorylation of Akt Thr308 and Akt Ser473 and that the podocyte cell line had a very low basal level of Akt activity under FCS-free conditions. The representative figure from the long exposure time visualizes the basal levels of Akt in the cultured podocytes, as phospho-Akt levels were already high at the time of exposure.

In Fig. 4B, the representative figure from the short exposure time shows that insulin stimulation as a positive control to activate Akt was able to induce maximal phosphorylation of Akt Thr308 and Akt Ser473 and that the podocyte cell line had a very low basal level of Akt activity under FCS-free conditions. The representative figure from the long exposure time visualizes the basal levels of Akt in the cultured podocytes, as phospho-Akt levels were already high at the time of exposure.
activation, we examined palmitate-induced mTORC1 activation in podocytes cultured in Krebs–Ringer buffer not containing any amino acids. Palmitate significantly increased phosphorylation of P70S6K and SG253/256 even in Krebs–Ringer buffer such as amino acids (Fig. 4C).

These observations led us to hypothesize that palmitate stimulation can recruit mTORC1 onto the lysosomal surface like other nutrients (Fig. 4A). To investigate this hypothesis, we performed a double immunofluorescence study using antibodies for mTOR and lysosome-associated membrane protein-2 (LAMP2) [47], a protein localized on the lysosomal membranes. In podocytes cultured in Krebs–Ringer buffer without any amino acid and palmitate, the green fluorescent signals of mTOR were diffusely distributed in the cytosol and little merged with the red fluorescent signals of LAMP2 (Fig. 4D and F). Both amino acids as a positive control and palmitate increased the co-localization of mTOR with LAMP2 around the nucleus (Fig. 4D). Since Roswell Park Memorial Institute (RPMI) medium contains amino acids, mTOR was partly coupled with the lysosomal membranes as defined by the LAMP2-positive component even under unstimulated conditions (Fig. 4E). In podocytes cultured in RPMI medium, palmitate, but not insulin, further accelerated the translocation of mTOR onto lysosomal membranes (Fig. 4E and F). This finding was confirmed by cell fraction study. Protein expression levels of Raptor and mTOR increased in the membrane fraction containing the lysosomal membrane, characterized by LAMP2 expression in podocytes cultured in Krebs–Ringer buffer stimulated with amino acids and palmitate (Fig. 4G). These results suggest that palmitate enhances translocation of mTOR onto the lysosomal membranes, leading to mTORC1 activation.

Since mTORC1 is recruited onto lysosomal membranes in a Rag–Ragulator complex-dependent manner (Fig. 4A) [26–27], we analyzed palmitate-induced apoptosis in cells transfected with the dominant-negative form of RagA or RagB together with co-transfection of RagC to examine the relationship between mTORC1 translocation and palmitate-induced apoptosis in cultured podocytes. Co-transfection with either the dominant-negative form of RagA or RagB with RagC diminished palmitate-induced overexpression of CHOP and cleavage of caspase 3 (Fig. 4H and I), suggesting that palmitate-induced recruitment of mTORC1 onto the lysosomal surface was essential for palmitate-induced apoptosis in cultured podocytes.

Ragulator is the trimeric p14, p18, and MP1 protein complex (Fig. 4A). Since palmitoylation of p18 is essential for anchoring the Rag–Ragulator complex to the lysosomal surface [51], we hypothesized that palmitate increased palmitoylation of p18, leading to an accumulation of the Rag–Ragulator complex on lysosomal membranes. However, palmitate did not alter localization of either p18 or RagC proteins (Fig. 4J).

3.5. Oleate and EPA inhibit palmitate-induced translocation of mTOR onto lysosomal membranes

We investigated whether unsaturated fatty acids affected palmitate-induced translocation of mTOR onto lysosomal membranes. Both oleate and EPA inhibited palmitate-induced translocation of mTOR onto lysosomal membranes (Fig. 5A and B). In contrast, oleate and EPA did not affect amino acid-induced mTOR translocation onto lysosomal membranes in podocytes cultured in Krebs–Ringer buffer (Fig. 5C and D). Nor did oleate or EPA alter the expression patterns of either p18 or RagC (Fig. 5E–H). In addition to the finding that both oleate and EPA did not inhibit insulin-induced mTORC1 activation (Fig. 2N), these results suggest that unsaturated FFAs can antagonize palmitate-induced mTORC1 translocation, but they do not have a unique mechanism to inhibit mTORC1 activity.

3.6. FFAs regulate the pro-apoptotic function of mTORC1 in HEK293 cells

To investigate whether the FFA-related pro-apoptotic function of mTORC1 was specific to podocytes, we conducted similar experiments to those above using cultured human embryonic kidney cell line 293 (HEK293) cells. Like the podocyte study, palmitate increased cleavage of PARP and caspase 3, and increased phosphorylation of P70S6K and SG253/256 and expression of CHOP (Fig. 6A). We also confirmed apoptosis by TUNEL assay, and ER stress by immunofluorescence study using an antibody for CHOP (Fig. 6B and C). These actions were all reversed by co-incubation with either oleate or EPA (Fig. 6A–C), and treatment with rapamycin (Fig. 6D–F). Furthermore, both oleate and EPA inhibited palmitate-induced enhancement of mTOR translocation onto lysosomal membranes in HEK293 cells (Fig. 6G).

4. Discussion

The results of this study suggest that palmitate, a saturated FFA, is a potent nutrient that can induce ER stress-related apoptosis by activating the pro-apoptotic function of mTORC1 in cultured podocytes and HEK293 cells. Saturated FFAs activate mTORC1 signaling by enhancing mTORC1 translocation onto lysosomal membranes. In contrast, unsaturated FFAs can antagonize palmitate-induced hyperactivation of mTORC1 and ER stress-related apoptosis by inhibiting mTORC1 translocation (Fig. 7). These results provide novel information on the role of FFAs in mTORC1-dependent cell biology and podocyte apoptosis in diabetic nephropathy.

We suggest that saturated FFA is a novel nutritional activator of mTORC1. There are at least five major activators of mTORC1, including insulin, stress, glucose, oxygen and amino acids, controlling many intracellular processes, such as protein and lipid synthesis, cell growth and proliferation, glycolysis, and autophagy [19–21]. Hyperglycemia has been suggested to be a major player in the activation of the mTORC1 pathway in various cells under diabetic conditions, leading to diabetic vascular complications [52,53]. Insufficiency of insulin action in diabetes causes the elevation of serum FFA levels as well as hyperglycemia. Based on our findings, an increased FFA level is also likely to be associated with diabetes-related mTORC1 activation leading to diabetic complications.

Saturated FFA induced apoptosis via hyperactivation of mTORC1 but not of mTORC2. Interestingly, there seems to be distinct biological roles of mTORC1 in cells treated with saturated FFAs and other stimuli. The results of the present study show that palmitate-induced mTORC1 activation leads to apoptosis, whereas insulin does not, and that unsaturated FFAs antagonized palmitate-induced, but not insulin-induced, mTORC1 activation. These results suggest that FFAs have unique roles in mTORC1-dependent cell biology, particularly in association with cell apoptosis. Furthermore, the findings were reproduced in cultured HEK293 cells, indicating that palmitate-induced mTORC1 activation and subsequent apoptosis are not specific to podocytes. This finding provides additional information about the physiological biology of mTORC1 in cell death.

mTORC1 activity is regulated by two distinct mechanisms: upstream signal transduction; and translocation onto lysosomal membranes (Fig. 4A) [20]. This study investigated which mechanism was involved in palmitate-induced mTORC1 activation. Our findings suggest that palmitate did not alter Akt- and AMPK-dependent regulation of mTORC1 activity, suggesting that upstream signals are not involved in palmitate-induced mTORC1 activation.

Of the original mTORC1 activators mentioned above, amino acids, glutaminolysis and glucose have been reported to be able to activate translocation of mTORC1 onto lysosomal membranes where Rheb is localized [25–29]. Palmitate-induced mTORC1 activation was observed to occur within minutes, suggesting that palmitate directly activates mTORC1, like amino acid-mediated activation; although we have not completely ruled out the possibility that mTORC1 was activated indirectly via some intracellular intermediate process such as increasing de novo synthesis of glucose or amino acid. This is the first study to report that palmitate is an additional nutrient that can translocate mTORC1 onto lysosomal membranes and this translocation is essential for palmitate-induced CHOP induction and apoptosis.
Fig. 5. Oleate and EPA inhibit palmitate-induced translocation of mTOR onto lysosomal membranes. (A) Representative photomicrographs showing co-immunostaining for mTOR (green) and LAMP2 (red) in cultured podocytes stimulated with palmitate with/without oleate or EPA in RPMI medium. Original magnification: ×1000. (B) The ratios of merged area (yellow) to LAMP2-positive area (yellow and red) in each group (n = 10). (C) Co-immunostaining for mTOR (green) and LAMP2 (red) in cultured podocytes stimulated with amino acids with/without oleate or EPA in Krebs–Ringer buffer. Original magnification: ×1000. (D) The ratios of merged area (yellow) to LAMP2-positive area (yellow and red) in each group (n = 10). (E) Co-immunostaining for p18 (green) and LAMP2 (red) in cultured podocytes stimulated with palmitate and co-incubated with oleate or EPA in RPMI medium. Original magnification: ×1000. (F) The ratios of merged area (yellow) to LAMP2-positive area (yellow and red) in each group (n = 10). (G) Co-immunostaining for RagC (green) and LAMP2 (red) in cultured podocytes stimulated with palmitate and co-incubated with oleate or EPA in RPMI medium. Original magnification: ×1000. (H) The ratios of merged area (yellow) to LAMP2-positive area (yellow and red) in each group (n = 10). All data are expressed as mean ± SEM. LAMP2, lysosome-associated membrane protein 2; PAL, palmitate; OLE, oleate; EPA, eicosapentaenoic acid; NS, not significant.

Fig. 4. Palmitate activates mTORC1 by enhancing translocation of mTOR onto lysosomal membranes. (A) Schematic representation of mTORC1 regulation. (B) Representative immunoblots of phosphorylated AktThr308 from short and long exposure times, phosphorylated AktSer473 from short and long exposure times, Akt, phosphorylated AMPKThr172, AMPK, phosphorylated RaptorSer792 and Raptor in cultured podocytes exposed to palmitate for the indicated hours in RPMI medium. Quantitative analysis of the ratios of phosphorylated P70S6KThr389 to P70S6K and phosphorylated S6S235/236 to S6 (n = 6). (C) Phosphorylated P70S6KThr389, P70S6K, phosphorylated S6S235/236 and S6 in cultured podocytes exposed to palmitate in Krebs–Ringer buffer for the indicated hours. Quantitative analysis of the ratios of phosphorylated P70S6KThr389 to P70S6K and phosphorylated S6S235/236 to S6 (n = 6). (D) Representative photomicrographs showing co-immunostaining for mTOR (green) and LAMP2 (red) in cultured podocytes stimulated with amino acids or palmitate for 6 h with 2 h of pre-incubation in Krebs–Ringer buffer. Original magnification: ×1000. (E) Co-immunostaining for mTOR (green) and LAMP2 (red) in cultured podocytes stimulated with insulin or palmitate in FCS-free RPMI medium. Original magnification: ×1000. (F) The ratios of merged area (yellow) to LAMP2-positive area (yellow and red) in each group (n = 10). (G) Raptor, mTOR and LAMP2 in cytoplasmic and membrane fractions of cultured podocytes with amino acids or palmitate for 6 h with 2 h of pre-incubation in Krebs–Ringer buffer. (H) Phosphorylated S6S235/236, S6, CHOP, cleavage of caspase 3 and β-actin in palmitate-stimulated podocytes transfected with dominant-negative forms of RagA or RagB with co-transfection with RagC. (I) Representative photomicrographs showing TUNEL staining of cultured podocytes transfected with dominant-negative forms of RagA or RagB with co-transfection with RagC exposed to palmitate for 9 h. Original magnification: ×1000. (J) Co-immunostaining for p18 (green), RagC (green) and LAMP2 (red) in cultured podocytes stimulated with palmitate in Krebs–Ringer buffer. Original magnification: ×1000. All data are expressed as mean ± SEM. Akt, serine/threonine kinase; AMPK, AMP-activated protein kinase; PARP, poly(ADP-ribose) polymerase; Krebs–Ringer buffer (KRB), amino acid free medium; RPMI medium, standard medium; PAL, palmitate; AA, amino acids; LAMP2, lysosome-associated membrane protein 2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; NS, not significant.
We also tried to identify a specific mechanism of palmitate-induced mTORC1 translocation. Unfortunately, our hypothesis that palmitate increases the recruitment of the Rag–Ragulator complex to the lysosomal surface by increasing p18 palmitoylation was not supported by the results. Similar to amino acid- or glucose-mediated translocation, it still remains to be discovered how palmitate activates the Rag–Ragulator complex leading to mTORC1 translocation. Nonetheless, it is of interest to examine whether there are any differences between the molecular mechanisms of amino acid-/glucose- and FFA-mediated translocation of mTORC1.

This study suggests that palmitate-induced apoptosis is mediated by mTORC1–ER stress pathway. Evidence of crosstalk between mTORC1 and ER stress has recently emerged, as growing research suggests that mTORC1-related ER stress is involved in the pathogenesis of various diabetes-related organ dysfunctions [34,49,54]. This study has provided an additional pathological role of the crosstalk in diabetes-related
Fig. 7. Schematic representation of differential roles of saturated and unsaturated FFAs in mTORC1 activation and subsequent apoptosis. Saturated fatty acids activate mTORC1 by enhancing translocation of mTORC1 onto lysosomal membranes, leading to ER stress-related apoptosis. In contrast, unsaturated FFAs can antagonize saturated fatty acid-induced apoptosis by inhibiting translocation of mTORC1 onto the lysosomal surface.

diseases. Several previous reports have revealed the mechanistic roles of the crosstalk in cell apoptosis, although the details have not been fully elucidated [49,55,56]. In this study, we observed that 4E-BP1-dependent inhibition of translation ameliorated palmitate-induced apoptosis. This finding suggests that mTORC1-dependent enhancement of translation may be involved in palmitate-induced ER stress and subsequent apoptosis in podocytes.

Inhibition of mTORC1 hyperactivation in podocytes is thought to be a potent therapeutic target for diabetic nephropathy [35–37]. However, strong inhibition of mTORC1 often causes proteinuria [38,39]. It is therefore desirable that the details and specific molecular mechanisms of diabetes-related hyperactivation of mTORC1 be elucidated. Insulin-, amino acid- and glucose-induced mTORC1 activation may play a fundamental role in podocyte biology. In contrast, based on our results, of the several nutritional activators of mTORC1, saturated FFAs could be associated with diabetes-related hyperactivation of mTORC1 in podocytes. If only saturated FFAs, but not other mTORC1 activators, play a pathological role in podocyte apoptosis in diabetic nephropathy, identification of detailed molecular mechanisms underlying saturated FFA-induced mTORC1 activation or saturated FFA-specific downstream apoptotic pathways related to mTORC1 may lead to novel and safer therapies to prevent podocyte apoptosis in diabetic nephropathy.

In this study, palmitate induced apoptosis in glucose-containing conditions and prevented nutrient-depletion-induced apoptosis in podocytes cultured in FCS-free medium without glucose. The reciprocal effect of palmitate on apoptosis is very interesting with regard to the pathogenesis of diabetic complications. A high plasma FFA concentration with the high glucose condition is a unique pathological condition only seen in the diabetic condition. Our results suggest that palmitate would be safe under nutrient-depleted conditions such as starvation since it is physiologically required for maintaining energy production, whereas it can become a toxic nutrient factor inducing apoptosis under nutrient- and glucose-rich conditions such as diabetes and an insulin-resistant state.

Our in vitro study suggests the possibility that alteration of serum FFA composition may be a pathogenesis of podocyte apoptosis in patients with diabetes, and supports the findings from more recent reports [57]. Furthermore, recent human studies have revealed that the concentrations of saturated fatty acids in overweight people or patients with type 2 diabetes are significantly higher than those in people of normal weight or non-diabetic people [13–15]. Our findings that saturated and unsaturated FFAs show opposite effects on mTORC1-dependent apoptosis in podocytes suggest that an altered serum FFA profile is associated with hyperactivation of mTORC1 and subsequent podocyte apoptosis in patients with type 2 diabetes. These results propose that some pharmacological or dietary intervention aimed at correcting the serum FFA profile may be useful for preventing podocyte apoptosis. However, further clinical studies are required.

5. Conclusion

Our data suggest that saturated and unsaturated FFAs show opposite effects on podocyte apoptosis, and that mTORC1 is a novel inducer of saturated FFA-related cell apoptosis. Ameliorating saturated FFA-mediated apoptosis with mTORC1 hyperactivation may be a new therapeutic target to protect podocytes in diabetic nephropathy.

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