



Pulmonary, Gastrointestinal and Urogenital Pharmacology

 α -Linolenic acid protects renal cells against palmitic acid lipotoxicity via inhibition of endoplasmic reticulum stressElias Katsoulis^a, Jon G. Mabley^a, Mohamed Samai^b, Irene C. Green^a, Prabal K. Chatterjee^{a,*}^a Renal Research Group, Centre for Biomedical and Health Science Research, School of Pharmacy and Biomolecular Sciences, University of Brighton, UK^b College of Medicine and Allied Health Sciences, University of Sierra Leone, Freetown, Sierra Leone

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ABSTRACT

Unsaturated fatty acids may counteract the lipotoxicity associated with saturated fatty acids. Palmitic acid induced endoplasmic reticulum (ER) stress and caused apoptotic and necrotic cell death in the renal proximal tubular cell line, NRK-52E. We investigated whether α -linolenic acid, an unsaturated fatty acid, protected against ER stress and cell death induced by palmitic acid or by other non-nutrient ER stress generators. Incubation of NRK-52E cells for 24 h with palmitic acid produced a significant increase in apoptosis and necrosis. Palmitic acid also increased levels of three indicators of ER stress – the phosphorylated form of the eukaryotic initiation factor 2 α (eIF2 α), C/EBP homologous protein (CHOP), and glucose regulated protein 78 (GRP78). α -Linolenic acid dramatically reduced cell death and levels of all three indicators of ER stress brought about by palmitic acid. Tunicamycin, which induces ER stress by glycosylation of proteins, produced similar effects to those obtained using palmitic acid; its effects were partially reversed by α -linolenic acid. Salubrinal (a phosphatase inhibitor) causes increased levels of the phosphorylated form of eIF2 α – this effect was partially reversed by α -linolenic acid. Palmitoleate, a monosaturated fatty acid, had similar effects to those of α -linolenic acid. These results suggest that part of the mechanism of protection of the kidney by unsaturated fatty acids is through inhibition of ER stress, eIF2 α phosphorylation and consequential reduction of CHOP protein expression and apoptotic renal cell death.

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1. Introduction

Type 2 diabetes, which is on the increase world-wide, is characterized by increased blood glucose and fatty acid levels. Prolonged exposure of cells to nutrient overload results in pathological alterations which contribute to the development of microvascular complications such as diabetic nephropathy (Dronavalli et al., 2008; Kanwar et al., 2008). Recently, elevated levels of free fatty acids (e.g. linoleic and arachidonic acid) have been measured in the urine of patients suffering from diabetic nephropathy and this correlated with increased tubulointerstitial damage possibly caused by overload of free fatty acids in the proximal tubule (Sasaki et al., 2009).

Endoplasmic reticulum (ER) stress, which involves disruption of protein folding, lipid and sterol biosynthesis and intracellular calcium stores and ultimately cell death via apoptosis, is involved in the pathogenesis of many diseases and conditions including ischemia–reperfusion injury and diabetes (Lin et al., 2008; Schroder, 2008). Several intracellular pathways contribute to this terminal response (Cnop et al., 2007; Cunha et al., 2008; Ji et al., 2005; Kim et al., 2006)

and depending on the severity or longevity of ER stress, may result in intracellular signaling for either cytoprotection or cellular dysfunction within the kidney (Kitamura, 2008). For example, mild ER stress can mediate cell protection via the phosphorylation of eukaryotic initiation factor (eIF2 α) subsequent to activation of protein kinase-like ER kinase (PERK) pathway which produces a temporary shutdown of protein synthesis (Ron and Walter, 2007). Within the kidney, induction of ER stress by tunicamycin or thapsigargin in an anti-Thy1 rat model of nephritis has been shown to reduce mesangioproliferative glomerulonephritis (Inagi et al., 2008). However, prolonged or severe ER stress activates PERK-mediated eIF2 α phosphorylation which leads to expression of activating transcription factor 4 (ATF4) and the transcription factor C/EBP homologous protein (CHOP) – also known as growth arrest and DNA damage (GADD)153 – leading to cell death via apoptosis (Ron and Walter, 2007). Recent reports have described ER stress in the kidneys of diabetic rats (Liu et al., 2008) and in humans with nephropathy (Lindenmeyer et al., 2008). Glucose regulating protein (GRP)78, which acts as a chaperone and a common regulator/sensor of ER stress, can also be activated by PERK as well as by inositol-requiring ER-to-nucleus signal kinase (IRE)-1 upon its dissociation from its transmembrane site on the ER.

Accumulation of saturated free fatty acids and their metabolites within cells produces lipotoxicity involving significant cellular dysfunction and injury (Weinberg, 2006). The lipotoxicity of palmitic

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(16:0) acid, a dietary saturated free fatty acid, has been shown to involve both ER stress and/or apoptotic cell death in many cell types including pancreatic β cells (Beeharry et al., 2003; Diakogiannaki et al., 2008; Martinez et al., 2008), preadipocytes (Guo et al., 2007), ovary cells, cardiomyocytes (Borradaile et al., 2006), retinal pericytes (Cacicedo et al., 2008), liver (Wei et al., 2006) and neuronal cells (Almaguel et al., 2009). In contrast, the beneficial effects of unsaturated fatty acids, such as α -linolenic acid, in health (Fuentes et al., 2008) and disease has been studied in human (Zatonski et al., 2008) and animal models of cardiomyopathy (Fiaccavento et al., 2006) spinal cord ischemia (Lang-Lazdunski et al., 2003) and inflammation (Ren et al., 2007). α -Linolenic acid has been shown to lower apoptosis in human umbilical cord endothelial cells exposed to high glucose (Zhang et al., 2007) in neuronal cells incubated with etoposide, a classical inducer of apoptosis (Wu et al., 2007) and in murine enteroendocrine cells subjected to serum deprivation (Katsuma et al., 2005). Similar protection has been observed using mono-unsaturated fatty acids such as palmitoleate (C16:1) and oleate (C18:1) which were able to protect BRIN-BD11 pancreatic β cells against apoptosis induced by saturated fatty acids (palmitic acid), serum withdrawal or exposure to cytokines (Welters et al., 2004). Recently, a diet rich in polyunsaturated fatty acids, specifically, a canola (rapeseed)-supplemented diet high in levels of α -linolenic acid, has been shown to reduce the degree of diabetic renal disease in streptozotocin-induced diabetic rats (Garman et al., 2009).

We first investigated if palmitic acid, a dietary saturated free fatty acid and the most abundant circulating fatty acid *in vivo* (Richieri et al., 1993), could generate ER stress in a renal proximal tubular (NRK-52E) cell-line and compared its effects with those of a known inducer of ER stress (tunicamycin). Secondly, we investigated whether α -linolenic acid, an unsaturated fatty acid, could provide protection against the cell death induced by palmitic acid. Finally, once the beneficial actions of α -linolenic acid were confirmed, this study investigated whether these effects were mediated via modification of the ER stress process with specific attention given to the role of GRP78 expression, eIF2 α phosphorylation and induction of CHOP.

2. Materials and methods

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK). Palmitic acid was prepared as described previously (Beeharry et al., 2004). Briefly, a stock solution of sodium palmitate (10 mmol/l) was conjugated to fatty-acid-free bovine serum albumin (BSA) in a 3:1 molar ratio at 37 °C for 1 h prior to addition cultured cells at a dilution of 1:100. Salubrinal (Axxora, Nottingham, UK) and tunicamycin were initially dissolved in 100% dimethylsulfoxide (DMSO) and then diluted using Dulbecco's Modified Eagle's medium (DMEM).

2.1. Culture of NRK-52E cells

The immortalized rat renal cell-line NRK-52E was obtained from European Collection of Cell Cultures (ECACC) (www.ecacc.org.uk). Cells were maintained in 75 cm² flasks in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, PAA Laboratories, Yeovil, Somerset, UK), 1% non-essential amino-acid solution, 5.5 mmol/l glucose, 100 U/ml penicillin and 50 μ g/ml streptomycin (supplemented DMEM) and grown at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. For experiments involving Western blotting and cytotoxicity studies, NRK-52E cells were passaged using a combination of 0.1% (w/v) trypsin and 0.02 % (w/v) ethylenediaminetetraacetic acid (EDTA, versene) and cultured in 25 cm² flasks or on 24 well plates, respectively, in supplemented DMEM containing 5% (v/v) FBS. Final incubations were performed on sub-confluent cultures of NRK-52E cells in the passage range 16 to 52 in supplemented DMEM containing 1% (v/v) FBS.

2.2. Incubation of NRK-52E cells

Cultured NRK-52E cells at 80–90 % confluence, were incubated with palmitic acid (300 μ mol/l) for up to 24 h. NRK-52E cells were also incubated with tunicamycin (10 μ g/ml) or salubrinal (3 μ mol/l). Further incubations were also performed in which NRK-52E cells were incubated with palmitic acid (300 μ mol/l) in the absence or presence of α -linolenic acid (100 or 300 μ mol/l) or palmitoleate (300 μ mol/l) for up to 24 h.

2.3. Measurement of cell viability and death

Cell viability and death were assessed as described previously by measurement of the enzymatic conversion of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into purple formazan and the release of lactate dehydrogenase (LDH) from lysed cells, respectively (Samai et al., 2007; Samai et al., 2008). NRK-52E cells were stained with Hoechst 33342 and propidium iodide (HPI) to assess cell death by apoptosis and necrosis (Beeharry et al., 2004). Specifically, apoptotic cells were distinguished as those with characteristic nuclear fragmentation and intense staining of condensed chromatin. Propidium iodide does not enter cells with intact plasma membranes, however, after entering damaged apoptotic or non-apoptotic cells it stains nuclear DNA pink. One thousand, randomly distributed nuclei were counted per sample and were scored as morphologically normal, apoptotic and necrotic using an inverted fluorescence microscope (Axiovert 25, Zeiss) set at excitation and emission wavelengths of 365 and 397 nm, respectively.

2.4. Western immuno blotting for CHOP, p-eIF2 α and β actin

Cell protein was extracted using modified protein extraction (RIPA) buffer consisting of 150 mmol/l NaCl, 50 mmol/l Tris-HCl (pH 7.4), 1 mmol/l EDTA, 1 mmol/l phenylmethylsulphonyl fluoride (PMSF), 1% (v/v) Triton-X 100, 1% (w/v) sodium deoxycholate and 0.1% (w/v) sodium dodecyl sulfate (SDS) and containing protein inhibitor cocktail (Hasko et al., 2000). Following centrifugation, supernatants were assayed for determination of total protein content using the Bradford assay with BSA used as standard (Bradford, 1976). Subsequently, 30 μ g protein was loaded to each lane of a SDS gel and proteins were allowed to separate at 120 V for 90 min. Transfer of proteins to nitrocellulose membranes was performed at 4 °C at 100 V for 80 min. The membranes were then blocked using 5 % (w/v) fatty acid-free powdered milk for 1 h and incubated with the primary antibodies overnight at 4 °C. The primary antibodies used and final concentrations (which were based on manufacturer's recommendations) were: rabbit monoclonal anti-phospho-eIF2 α (New England Biolabs, Hitchin, Hertfordshire, UK), 1:1000 dilution; mouse monoclonal anti-CHOP/GADD153 (Santa Cruz/Insight Biotechnology, Wembley, Middlesex, UK), 1:500 dilution; rabbit polyclonal anti-GRP78 (BiP) (Stressgen via Cambridge Bioscience, Cambridge, UK), 1:2000 dilution and mouse monoclonal anti- β 2actin, 1:10,000 dilution. For p-eIF2 α immunodetection, the blocking solution consisted of 5% (w/v) BSA rather than milk, respectively, according to the manufacturer's protocol. Immunodetection was performed at room temperature for 1 h using an appropriate secondary antibody (e.g. anti-mouse IgG) diluted 1:5000 in Tween-Tris buffered saline containing 1 or 5% (w/v) milk or BSA. Visualization of protein bands was performed using an ECL Plus chemiluminescent detection system (GE Healthcare, Amersham, Buckinghamshire, UK). Densitometric analysis was performed using a scanning densitometer (Gel Doc-2000, Bio-Rad, Hertfordshire, UK) and the results were normalized using β 2actin readings (Starkel et al., 2007; Xie et al., 2002).

2.5. Statistical analysis

Results are expressed as mean \pm standard error of the mean (S.E.M.) for n independent observations as indicated. Statistical differences between mean values of groups have been determined using one way analysis of variance (ANOVA) followed by a Dunnett's post-significance test for comparison of multiple means using a commercially available software (Graphpad Prism, version 4.0, Graphpad Software, San Diego, CA, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Palmitic acid causes significant cellular dysfunction and death of renal cells – protection by α -linolenic acid

Incubation of subconfluent cultures of the NRK-52E cells with 300 $\mu\text{mol/l}$ palmitic acid for 24 h produced a significant loss of cell viability as demonstrated by decreased reduction of MTT and increased LDH release (Fig. 1A). The mode of cell death observed at this concentration of palmitic acid was a combination of apoptosis and necrosis as determined using a combination of HPI staining (Fig. 1B). However, co-incubation of NRK-52E cells with 300 $\mu\text{mol/l}$ palmitic acid and 100 or 300 $\mu\text{mol/l}$ α -linolenic acid restored cell viability to levels observed in untreated cells (Fig. 1A). As expected, this beneficial effect of α -linolenic acid was reflected in a significant reduction of both the apoptotic and necrotic cell death caused by palmitic acid (Fig. 1B).

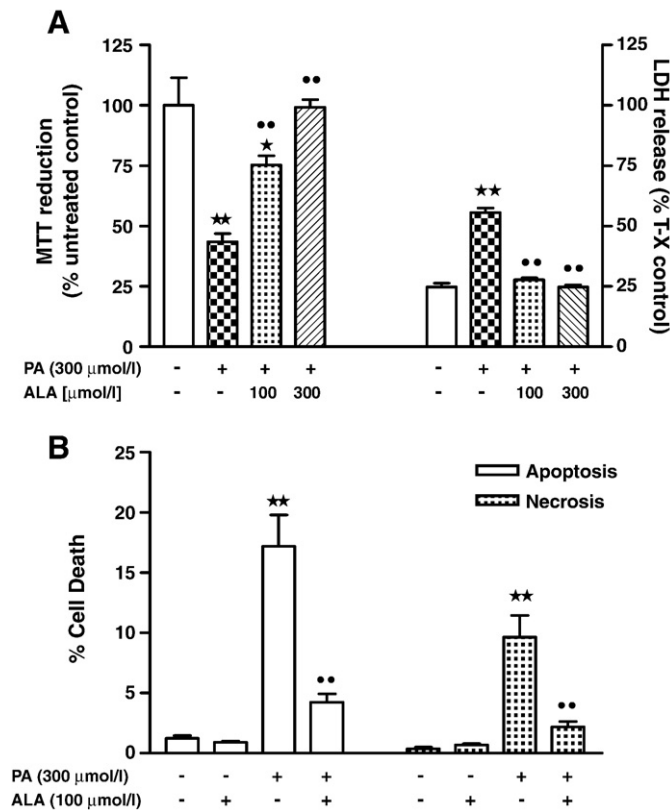


Fig. 1. α -Linolenic acid protects NRK-52E cells against toxicity mediated by palmitic acid. (A) MTT reduction and LDH release from cells treated with 300 $\mu\text{mol/l}$ palmitic acid (PA) for 24 h in presence of 100 or 300 $\mu\text{mol/l}$ α -linolenic acid (ALA). (B) HPI analysis for cells treated with 300 $\mu\text{mol/l}$ palmitic acid (PA) for 24 h in presence of 100 $\mu\text{mol/l}$ α -linolenic acid (ALA). Data represent mean \pm S.E.M., $n = 3$, * $P < 0.05$, ** $P < 0.01$ vs. untreated controls (0 $\mu\text{mol/l}$ palmitic acid), •• $P < 0.01$ vs. palmitic acid-only cells.

3.2. α -Linolenic acid reduces ER stress mediated by palmitic acid

Palmitic acid produced a significant increase in the expression of markers of ER stress. Specifically, incubation of NRK-52E cells with 300 $\mu\text{mol/l}$ palmitic acid produced an early increase in levels of phosphorylated eIF2 α (p-eIF2 α) after 6 h (Fig. 2A, B) which was detected using Western blotting. After 24 h incubation with palmitic acid, increased levels of CHOP were also detected (Fig. 2C, D). Tunicamycin, which inhibits early protein glycosylation and promotes ER stress in many cell types (Diakogiannaki et al., 2008; Peyrou and Cribb, 2007), was used as a positive control in this study. Incubation of NRK-52E cells with 10 $\mu\text{g/ml}$ of tunicamycin for 24 h produced an increase in p-eIF2 α which was comparable to that obtained using 300 $\mu\text{mol/l}$ palmitic acid after 6 h (Fig. 2A, B). In the presence of α -linolenic acid, ER stress mediated by palmitic acid was significantly reduced. Co-incubation of NRK-52E cells with 300 $\mu\text{mol/l}$ palmitic acid and 300 $\mu\text{mol/l}$ α -linolenic acid produced a significant reduction in levels of p-eIF2 α after 6 h (Fig. 2A, B) and CHOP after 24 h (Fig. 2C, D).

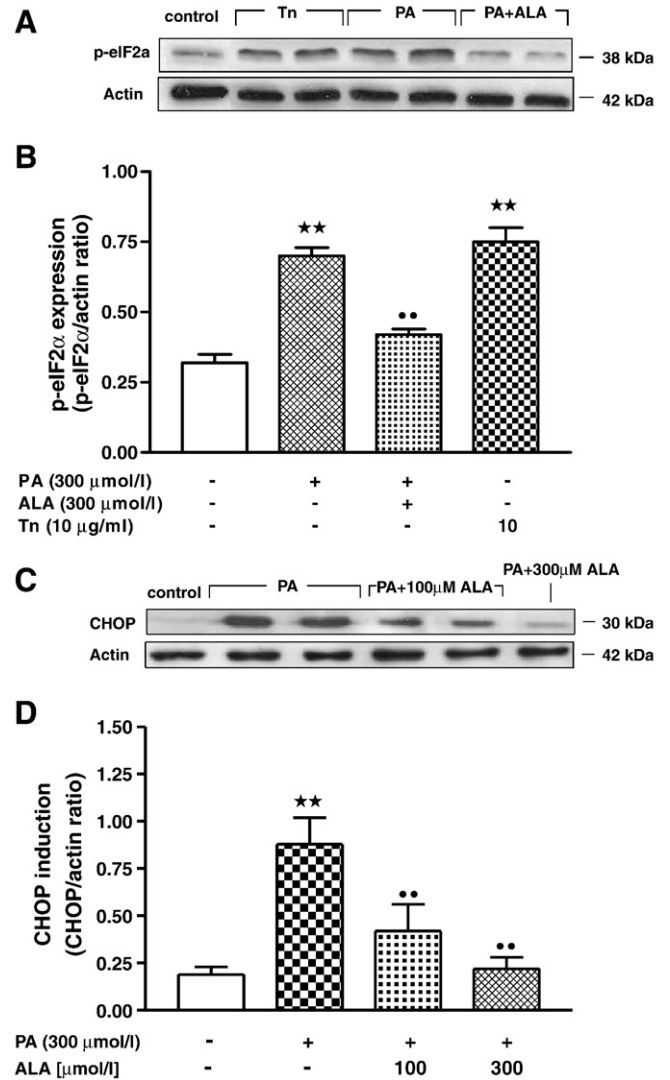


Fig. 2. α -Linolenic acid protects NRK-52E cells against ER stress induced by palmitic acid. (A) Western blot and (B) densitometric analysis demonstrating the reduction of palmitic acid (PA)-induced p-eIF2 α expression by 300 $\mu\text{mol/l}$ α -linolenic acid (ALA) after 24 h. (C) Western blot and (D) densitometric analysis of CHOP expression after 24 h incubation of cells with 300 $\mu\text{mol/l}$ palmitic acid (PA) in presence of 100 or 300 $\mu\text{mol/l}$ α -linolenic acid (ALA). Tunicamycin was used as a positive control in both (A) and (C) (Tn; 10 $\mu\text{g/ml}$). Data represent mean \pm S.E.M., $n = 3$, * $P < 0.05$, ** $P < 0.01$ vs. untreated controls (0 $\mu\text{mol/l}$ palmitic acid), •• $P < 0.01$ vs. palmitic acid-only cells.

3.3. Effects of α -linolenic acid on renal cell death mediated by tunicamycin

Incubation of NRK-52E cells with 10 μ g/ml tunicamycin for 24 h reduced cell viability (Fig. 3A) which was confirmed by significant increases in both apoptosis and necrosis (Fig. 3B). α -Linolenic acid at concentrations of 100 and 300 μ mol/l, was able to increase cell viability significantly but not completely (Fig. 3A) – an effect which was reflected by a significant reduction in the numbers of apoptotic cells generated by palmitic acid (Fig. 3B). In contrast, α -linolenic acid at a concentration of 300 μ mol/l increased significantly the necrotic cell death mediated by tunicamycin (Fig. 3B).

3.4. Effects of α -linolenic acid on ER stress induced by tunicamycin

Incubation of NRK-52E cells with 10 μ g/ml tunicamycin for 24 h produced a significant increase in levels of CHOP (Fig. 4A, B) as well as increased expression of GRP78 (Fig. 4A, C) subsequent to an increase in p-eIF2 α levels observed after 6 h (Fig. 2A, B). α -Linolenic acid, at concentrations of 100 and 300 μ mol/l, was able to reduce the increase in CHOP levels produced by 300 μ mol/l palmitic acid (Fig. 4A, B). However, neither concentration was able to reduce GRP78 expression mediated by palmitic acid (Fig. 4A, C).

3.5. Effects of α -linolenic acid on p-eIF2 α and CHOP levels after salubrinal

In order to clarify further the effects of α -linolenic acid on ER stress in NRK-52E cells, the effects of salubrinal, a phosphatase inhibitor which maintains p-eIF2 α in its phosphorylated form, on levels of p-eIF2 α and CHOP were also examined. As expected, 3 μ mol/l salubrinal produced a significant increase in p-eIF2 α after 6 and 24 h (Fig. 5A, B) and an increase in CHOP levels after 24 h (Fig. 5A, B). The increase in p-eIF2 α

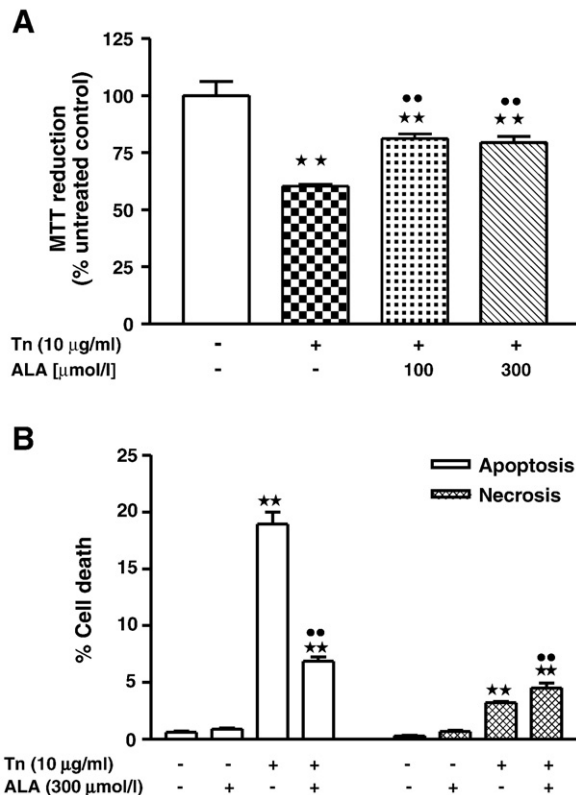


Fig. 3. α -Linolenic acid protects against dysfunction and apoptosis of NRK-52E cells induced by tunicamycin. Data represent mean \pm S.E.M., $n = 3$, ** $P < 0.01$ vs. untreated controls (0 μ g/ml tunicamycin), ●● $P < 0.01$ vs. tunicamycin-only cells.

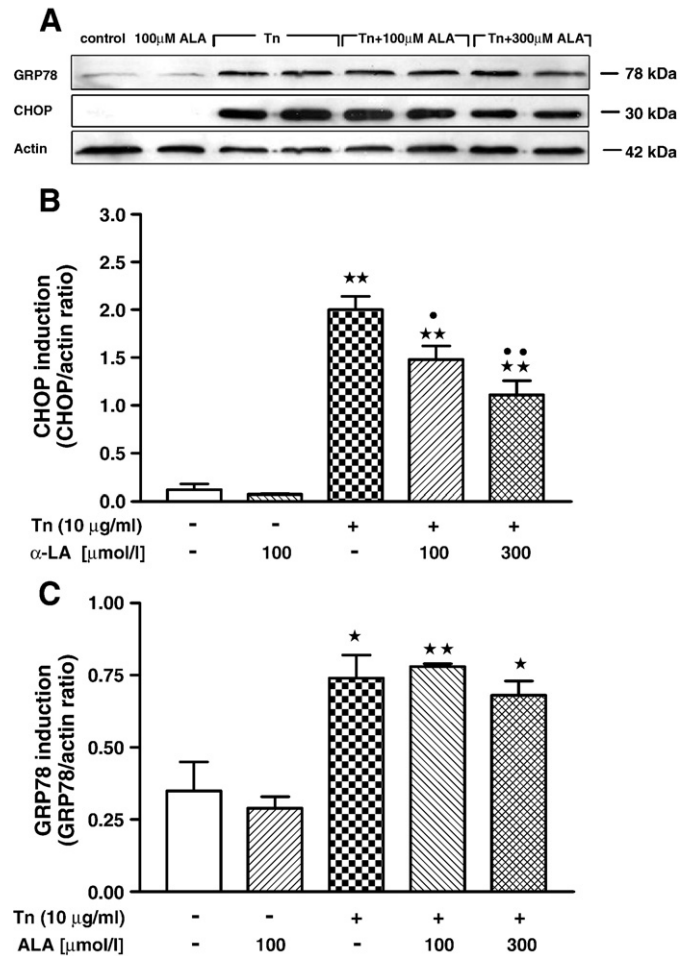


Fig. 4. α -Linolenic acid protects NRK-52E cells against ER stress induced by tunicamycin. (A) Western blot image and densitometric analysis of (B) CHOP and (C) GRP78 expression in cells treated with tunicamycin (Tn; 10 μ g/ml) in presence of increasing concentrations of α -linolenic acid (ALA) for 24 h. Data represent mean \pm S.E.M., $n = 3$, * $P < 0.05$, ** $P < 0.01$ vs. untreated controls (0 μ g/ml tunicamycin), ● $P < 0.05$, ●● $P < 0.01$ vs. tunicamycin-only cells.

levels after 6 h could be significantly reduced by 300 μ mol/l α -linolenic acid, however, α -linolenic acid did not have any effect on p-eIF2 α after 24 h (Fig. 5A, B). α -Linolenic acid, at a concentration of 300 μ mol/l, was also able to reduce CHOP subsequent to salubrinal administration (Fig. 5A, B). NRK-52E cells were incubated with 3 μ mol/l salubrinal in the presence of palmitoleate, a mono-unsaturated fatty acid, for comparison with α -linolenic acid. Although 300 μ mol/l palmitoleate was able to reduce salubrinal-mediated increases in p-eIF2 α observed after 6 h incubation (Fig. 5A, B), it had no effect on the increase in CHOP levels produced by salubrinal after 24 h (Fig. 5A, B).

4. Discussion

In this study, we report that (i) palmitic acid causes significant cell death in a renal proximal tubular cell-line (NRK-52E cells) and that the mode of cell death is both apoptosis and necrosis, (ii) palmitic acid causes a significant degree of ER stress in renal cells, (iii) α -linolenic acid protects NRK-52E cells against palmitic acid lipotoxicity by reducing ER stress and apoptosis and (iv) α -linolenic acid reduces the early phosphorylation of eIF2 α and the increase in levels of CHOP caused by palmitic acid. α -Linolenic acid was also able to reduce levels of p-eIF2 α and CHOP within cells incubated with the phosphatase inhibitor salubrinal. Palmitoleate, a mono-unsaturated fatty acid, was able to reduce salubrinal-mediated increases in p-eIF2 α levels but had no effect on CHOP. The effects of palmitic acid were similar to those observed using

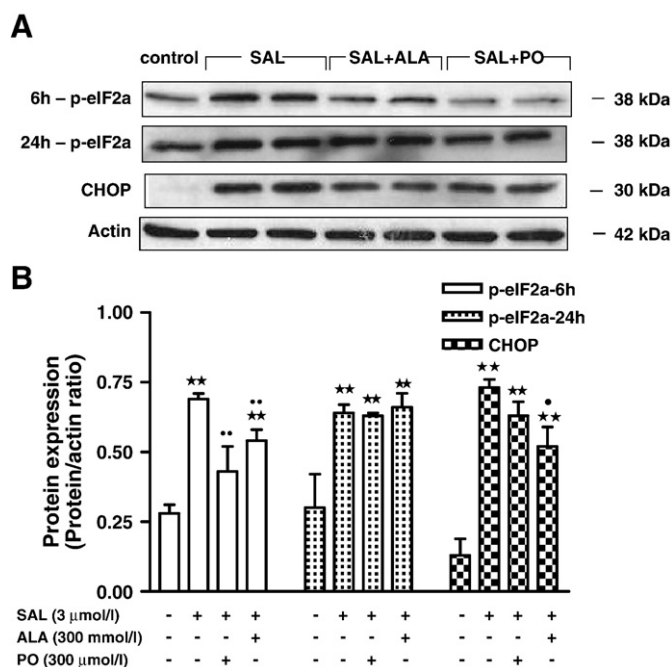


Fig. 5. Inhibition of salubrinal-induced increases in p-eIF2 α and CHOP levels by α -linolenic acid in NRK-52E cells. (A) Western blot and (B) densitometric analysis of p-eIF2 α expression 6 and 24 h and CHOP expression 24 h after administration of salubrinal (SAL; 3 μ mol/l) in presence of palmitoleate (PO; 300 μ mol/l) or α -linolenic acid (ALA; 300 μ mol/l). Data represent mean \pm S.E.M., $n = 3-4$, * $P < 0.05$, ** $P < 0.01$ vs. untreated controls (0 μ mol/l salubrinal), $\bullet P < 0.05$, $\bullet\bullet P < 0.01$ vs. salubrinal-only cells.

tunicamycin, an inhibitor of protein glycosylation which promotes ER stress. Taken together, these results suggest that unsaturated fatty acids such as α -linolenic may protect the kidney against lipotoxicity generated by saturated dietary free fatty acids such as palmitic acid.

α -Linolenic was also able to reduce cellular dysfunction and apoptosis caused by tunicamycin – a recognised inducer of ER stress. Interestingly, a concentration of 300 μ mol/l α -linolenic acid produced a small, but significant, increase in necrotic cell death caused by tunicamycin as indicated by HPI staining (Fig. 3B). We believe this reflects the toxic nature of tunicamycin which, as well as promoting ER stress and apoptosis, also causes necrotic cell death in other cells such as neuronal cells (Nakayama et al., 2009). The mechanism underlying the necrotic cell death produced by tunicamycin is not clear but it does not seem to involve the generation of oxidative stress (Zhang et al., 2006), rather, it is likely to be a consequence of major disruption of intracellular calcium homeostasis. It is possible that by protecting cells against tunicamycin-induced apoptosis, α -linolenic acid diverted damaged NRK-52E cells into a necrotic cell death pathway.

Saturated fatty acids have been shown to produce deleterious effects on many cell types ranging from pancreatic β cells and preadipocytes (Cunha et al., 2008; Guo et al., 2007) to tumor cells (Zeng et al., 2008). The mechanisms involved include generation of oxidative stress (Beeharry et al., 2003; Cacicedo et al., 2005) altered mitochondrial permeability (Koshkin et al., 2008), changes in the morphology of the ER (Borradaile et al., 2006) and an increase in ER stress (Cunha et al., 2008; Lai et al., 2008; Wei et al., 2006). The ability of palmitic acid to generate oxidative stress is well known (Beeharry et al., 2003) as is its ability to increase levels of markers of ER stress such as p-eIF2 α and CHOP (Cunha et al., 2008; Diakogiannaki et al., 2008; Guo et al., 2007; Lai et al., 2008; Wei et al., 2006). The ability of palmitic acid to increase ER stress in NRK-52E cells was confirmed in this study via measurement of increased p-eIF2 α and CHOP. Using these divergent pathways, palmitic acid is able to induce apoptosis via activation of protein kinase C (Eitel et al., 2003), nuclear factor- κ B (Staiger et al., 2006) or JNK (Malhi et al., 2006; Pagliassotti et al., 2007).

ER stress has been associated with several pathological conditions which can affect the kidney including ischemia-reperfusion injury, glomerulonephritis and diabetic kidney disease (Bando et al., 2004; Inagi et al., 2005; Inagi et al., 2008; Liu et al., 2008; Ohse et al., 2006).

In this study, we have demonstrated for the first time that ER stress produced by palmitic acid in renal proximal tubular cells can be significantly reduced by α -linolenic acid, an unsaturated fatty acid. We also report here, for the first time, that the protection afforded by an unsaturated fatty acid involves a reduction of ER stress. The mechanism involves a reduction in the raised levels of p-eIF2 α and CHOP associated with palmitic acid, however the results of our investigation appears to rule out a protective mechanism mediated by GRP78 as α -linolenic acid did not significantly affect levels of this chaperone molecule whereas levels of CHOP were significantly reduced.

Specific unsaturated fatty acids such as linolenic (Beeharry et al., 2003; Beeharry et al., 2004) palmitoleic (Diakogiannaki et al., 2008) and oleate (Koshkin et al., 2008) have previously been shown to protect against the damaging actions of saturated fats in different cell types via reduction of mitochondrial permeability (Koshkin et al., 2008) and activation of phosphoinositide 3 kinase (Beeharry et al., 2004) in pancreatic β cells, reduction of phospholipase A₂ in liver cells (Wei et al., 2006) and reduction of JNK activation in cardiac myocytes (Miller et al., 2005). In 2005, Hufnagel and co-workers demonstrated that unsaturated fatty acids could activate protein phosphatase type 2C in endothelial cells leading to apoptosis (Hufnagel et al., 2005). However, we believe that our study is the first to report the protective effects of α -linolenic acid in renal proximal tubular cells.

To investigate the mechanisms involved in the protection afforded by α -linolenic acid, the effects of this unsaturated fatty acid on the pro-apoptotic PERK pathway were investigated. PERK activation results in phosphorylation of eIF2 α and as expected, salubrinal, which inhibits the phosphatase responsible for eIF2 α dephosphorylation increased levels of p-eIF2 α and produced apoptosis of NRK-52E cells. α -Linolenic acid was able to reduce the early phosphorylation of p-eIF2 α and reduce levels of CHOP. In our study, a similar effect on p-eIF2 α phosphorylation was obtained using palmitoleate – a mono-unsaturated fatty acid. A similar effect has recently been shown by Diakogiannaki and co-workers using pancreatic β cells (Diakogiannaki et al., 2008). We therefore speculate that the unsaturated fatty acids could enhance phosphatase activity in the kidney.

To summarize, the results presented here suggest that unsaturated fatty acids such as α -linolenic acid may be able to provide protection of renal cells against the detrimental effects of saturated fatty acids such as dietary palmitic acid and nutrient overload associated with type 2 diabetes and obesity.

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References

- Almaguef, F.G., Liu, J.W., Pacheco, F.J., Casiano, C.A., De Leon, M., 2009. Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid. *J. Neurosci. Res.* 87, 1207–1218.
- Bando, Y., Tsukamoto, Y., Katayama, T., Ozawa, K., Kitao, Y., Hori, O., Stern, D.M., Yamauchi, A., Ogawa, S., 2004. ORP150/HSP12A protects renal tubular epithelium from ischemia-induced cell death. *FASEB J.* 18, 1401–1403.
- Beeharry, N., Lowe, J.E., Hernandez, A.R., Chambers, J.A., Fucassi, F., Cragg, P.J., Green, M.H.L., Green, I.C., 2003. Linoleic acid and antioxidants protect against DNA damage and apoptosis induced by palmitic acid. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* 530, 27–33.
- Beeharry, N., Chambers, J.A., Green, I.C., 2004. Fatty acid protection from palmitic acid-induced apoptosis is lost following PI3-kinase inhibition. *Apoptosis* 9, 599–607.
- Borradaile, N.M., Han, X.L., Harp, J.D., Gale, S.E., Ory, D.S., Schaffer, J.E., 2006. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J. Lipid Res.* 47, 2726–2737.

- Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Cacicedo, J.M., Benjachareowong, S., Chou, E., Ruderman, N.B., Ido, Y., 2005. Palmitate-induced apoptosis in cultured bovine retinal pericytes – roles of NAD(P)H oxidase, oxidant stress and ceramide. *Diabetes* 54, 1838–1845.
- Cacicedo, J.M., Yagihashi, N., Adachi, T., Ruderman, N.B., Ido, Y., 2008. Activation of AMPK by AICAR inhibits palmitate-induced endoplasmic reticulum (ER) stress, increases in ceramide mass, and cytotoxicity in cultured bovine retinal pericytes (BRPC). *Diabetes* 57, A233.
- Cnop, M., Ladriere, L., Hekerman, P., Ortis, F., Cardozo, A.K., Dogusan, Z., Flamez, D., Boyce, M., Yuan, J.Y., Eizirik, D.L., 2007. Selective inhibition of eukaryotic translation initiation factor 2 alpha dephosphorylation potentiates fatty acid-induced endoplasmic reticulum stress and causes pancreatic beta-cell dysfunction and apoptosis. *J. Biol. Chem.* 282, 3989–3997.
- Cunha, D.A., Hekerman, P., Ladriere, L., Bazarra-Castro, A., Ortis, F., Wakeham, M.C., Moore, F., Rasschaert, J., Cardozo, A.K., Bellomo, E., Overbergh, L., Mathieu, C., Lupi, R., Hai, T., Herchuelz, A., Marchetti, P., Rutter, G.A., Eizirik, D.L., Cnop, M., 2008. Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. *J. Cell Sci.* 121, 2308–2318.
- Diakogiannaki, E., Welters, H.J., Morgan, N.G., 2008. Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids. *J. Endocrinol.* 197, 553–563.
- Dronavalli, S., Duka, I., Bakris, G.L., 2008. The pathogenesis of diabetic nephropathy. *Nat. Clin. Pract. Endocrinol. Metab.* 4, 444–452.
- Eitel, K., Staiger, H., Rieger, J., Mischak, H., Brandhorst, H., Brendel, M.A., Bretzel, R.G., Haring, H.U., Kellner, M., 2003. Protein kinase C delta activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. *Diabetes* 52, 991–997.
- Fiaccavento, R., Carotenuto, F., Minieri, M., Forte, G., Cossa, P., Di Nardo, P., 2006. alpha-linolenic acid-enriched diet restores myocardial contractile function and expands longevity in cardiomyopathic hamster. *J. Mol. Cell. Cardiol.* 40, 1001–1002.
- Fuentes, F., Lopez-Miranda, J., Perez-Martinez, P., Jimenez, Y., Marin, C., Gomez, P., Fernandez, J.M., Caballero, J., Delgado-Lista, J., Perez-Jimenez, F., 2008. Chronic effects of a high-fat diet enriched with virgin olive oil and a low-fat diet enriched with alpha-linolenic acid on postprandial endothelial function in healthy men. *Br. J. Nutr.* 100, 159–165.
- Garman, J.H., Mulrone, S., Manigrasso, M., Flynn, E., Maric, C., 2009. Omega-3 fatty acid rich diet prevents diabetic renal disease. *Am. J. Physiol., Renal Physiol.* 296, F306–F316.
- Guo, W., Wong, S., Xie, W.S., Lei, T.L., Luo, Z.J., 2007. Palmitate modulates intracellular signaling, induces endoplasmic reticulum stress, and causes apoptosis in mouse 3T3-L1 and rat primary preadipocytes. *Am. J. Physiol.: Endocrinol. Metab.* 293, E576–E586.
- Hasko, G., Kuhel, D.G., Chen, J.F., Schwarzschild, M.A., Deitch, E.A., Mabley, J.G., Marton, A., Szabo, C., 2000. Adenosine inhibits IL-12 and TNF-alpha production via adenosine A(2a) receptor-dependent and independent mechanisms. *FASEB J.* 14, 2065–2074.
- Hufnagel, B., Dworak, M., Soufi, M., Mester, Z., Zhu, Y., Schaefer, J.R., Klumpp, S., Krieglstein, J., 2005. Unsaturated fatty acids isolated from human lipoproteins activate protein phosphatase type 2C beta and induce apoptosis in endothelial cells. *Atherosclerosis* 180, 245–254.
- Inagi, R., Nangaku, M., Onogi, H., Ueyama, H., Kitao, Y., Nakazato, K., Ogawa, S., Kurokawa, K., Couser, W.G., Miyata, T., 2005. Involvement of endoplasmic reticulum (ER) stress in podocyte injury induced by excessive protein accumulation. *Kidney Int.* 68, 2639–2650.
- Inagi, R., Kumagai, T., Nishi, H., Kawakami, T., Miyata, T., Fujita, T., Nangaku, M., 2008. Preconditioning with endoplasmic reticulum stress ameliorates mesangioproliferative glomerulonephritis. *J. Am. Soc. Nephrol.* 19, 915–922.
- Ji, J., Zhang, L., Wang, P., Mu, Y.M., Zhu, X.Y., Wu, Y.Y., Yu, H., Zhang, B., Chen, S.M., Sun, X.Z., 2005. Saturated free fatty acid, palmitic acid, induces apoptosis in fetal hepatocytes in culture. *Exp. Toxicol. Pathol.* 56, 369–376.
- Kanwar, Y.S., Wada, J., Sun, L., Xie, P., Wallner, E.L., Chen, S., Chugh, S., Danesh, F.R., 2008a. Diabetic nephropathy: Mechanisms of renal disease progression. *Exp. Biol. Med.* 233, 4–11.
- Katsuma, S., Hatae, N., Yano, T., Ruike, Y., Kimura, M., Hirasawa, A., Tsujimoto, G., 2005. Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. *J. Biol. Chem.* 280, 19507–19515.
- Kim, J.E., Kim, E.B., Kim, Y.W., Kim, J.Y., Won, K.C., Park, S.Y., 2006. The mechanism of palmitic acid induced apoptosis in bovine arterial endothelial cell. *Diabetes* 55, A521.
- Kitamura, M., 2008. Endoplasmic reticulum stress in the kidney. *Clin. Exp. Nephrol.* 12, 317–325.
- Koshkin, V., Dai, F.F., Robson-Doucette, C.A., Chan, C.B., Wheeler, M.B., 2008. Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic beta-cells. *J. Biol. Chem.* 283, 7936–7948.
- Lai, E., Bikopoulos, G., Wheeler, M.B., Rozakis-Adcock, M., Volchuk, A., 2008. Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells. *Am. J. Physiol. Endocrinol. Metab.* 294, E540–E550.
- Lang-Lazdunski, L., Blondeau, N., Jarretou, G., Lazdunski, M., Heurteaux, C., 2003. Linolenic acid prevents neuronal cell death and paraplegia after transient spinal cord ischemia in rats. *J. Vasc. Surg.* 38, 564–575.
- Lin, J.H., Walter, P., Yen, T.S.B., 2008. Endoplasmic reticulum stress in disease pathogenesis. *Ann. Rev. Pathol. – Mech. Dis.* 3, 399–425.
- Lindenmeyer, M.T., Rastaldi, M.P., Ikehata, M., Neusser, M.A., Kretzler, M., Cohen, C.D., Schlondorff, D., 2008. Proteinuria and hyperglycemia induce endoplasmic reticulum stress. *J. Am. Soc. Nephrol.* 19, 2225–2236.
- Liu, G.G., Sun, Y.Y., Li, Z.H., Song, T., Wang, H.B., Zhang, Y., Ge, Z.M., 2008. Apoptosis induced by endoplasmic reticulum stress involved in diabetic kidney disease. *Biochem. Biophys. Res. Com.* 370, 651–656.
- Malhi, H., Bronk, W., Werneburg, N.W., Gores, G.J., 2006. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *J. Biol. Chem.* 281, 12093–12101.
- Martinez, S.C., Tanabe, K., Cras-Meneur, C., Abumrad, N.A., Bernal-Mizrachi, E., Permutt, M.A., 2008. Inhibition of FoxO1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. *Diabetes* 57, 846–859.
- Miller, T.A., LeBrasseur, N.K., Cote, G.M., Trucillo, M.P., Pimentel, D.R., Ido, Y., Ruderman, N.B., Sawyer, D.B., 2005. Oleate prevents palmitate-induced cytotoxic stress in cardiac myocytes. *Biochem. Biophys. Res. Com.* 336, 309–315.
- Nakayama, H., Zhao, J., Ei-Fakhrany, A., Isosaki, M., Satoh, H., Kyotani, Y., Yoshizumi, M., 2009. Neuroprotective effects of pramipexole against tunicamycin-induced cell death in PC12 cells. *Clin. Exp. Pharmacol. Physiol.* Jun 8 Epub ahead of print.
- Ohse, T., Inagi, R., Tanaka, T., Ota, T., Miyata, T., Kojima, I., Ingelfinger, J.R., Ogawa, S., Fujita, T., Nangaku, M., 2006. Albumin induces endoplasmic reticulum stress and apoptosis in renal proximal tubular cells. *Kidney Int.* 70, 1447–1455.
- Pagliassotti, M.J., Wei, Y., Wang, D., 2007. Insulin protects liver cells from saturated fatty acid-induced apoptosis via inhibition of c-Jun NH2 terminal kinase activity. *Endocrinology* 148, 3338–3345.
- Peyrou, M., Cribb, A.E., 2007. Effect of endoplasmic reticulum stress preconditioning on cytotoxicity of clinically relevant nephrotoxins in renal cell lines. *Toxicol. In Vitro* 21, 878–886.
- Ren, J., Han, E.J., Chung, S.H., 2007. In vivo and in vitro anti-inflammatory activities of alpha-linolenic acid isolated from *Actinidia polygama* fruits. *Arch. Pharm. Res.* 30, 708–714.
- Richieri, G.V., Anel, A., Kleinfeld, A.M., 1993. Interactions of long-chain fatty-acids and albumin – determination of free fatty-acid levels using the fluorescent-probe Adifab. *Biochemistry* 32, 7574–7580.
- Ron, D., Walter, P., 2007. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* 8, 519–529.
- Samai, M., Sharpe, M.A., Gard, P.R., Chatterjee, P.K., 2007. Comparison of the effects of the superoxide dismutase mimetics EUK-134 and tempol on paraquat-induced nephrotoxicity. *Free Radic. Biol. Med.* 43, 528–534.
- Samai, M., Hague, T., Naughton, D.P., Gard, P.R., Chatterjee, P.K., 2008. Reduction of paraquat-induced renal cytotoxicity by manganese and copper complexes of EGTA and EHPG. *Free Radic. Biol. Med.* 44, 711–721.
- Sasaki, H., Kamijio-Ikemori, A., Sugaya, T., Yamashita, K., Yokoyama, T., Koike, J., Sato, T., Yasuda, T., Kimura, K., 2009. Urinary fatty acids and liver-type fatty acid binding protein in diabetic nephropathy. *Nephron Clin. Pract.* 112, c148–c156.
- Schroder, M., 2008. Endoplasmic reticulum stress responses. *Cell. Mol. Life Sci.* 65, 862–894.
- Staiger, K., Staiger, H., Weigert, C., Haas, C., Haring, H.U., Kellner, M., 2006. Saturated, but not unsaturated, fatty acids induce apoptosis of human coronary artery endothelial cells via nuclear factor-kappa B activation. *Diabetes* 55, 3121–3126.
- Starkel, P., De Saeger, C., Leclercq, I., Horsmans, Y., 2007. Role of signal transducer and activator of transcription 3 in liver fibrosis progression in chronic hepatitis C-infected patients. *Lab. Invest.* 87, 173–181.
- Wei, Y.R., Wang, D., Topczewski, F., Pagliassotti, M.J., 2006. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am. J. Physiol. Endocrinol. Metab.* 291, E275–E281.
- Weinberg, J.M., 2006. Lipotoxicity. *Kidney Int.* 70, 1560–1566.
- Welters, H.J., Tadayyon, M., Scarpello, J.H.B., Smith, S.A., Morgan, N.G., 2004. Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett.* 560, 103–108.
- Wu, Y.M., Tada, M., Takahata, K., Tomizawa, K., Matsui, H., 2007. Inhibitory effect of polyunsaturated fatty acids on apoptosis induced by etoposide, okadaic acid and AraC in Neuro2a cells. *Acta Med. Okayama* 61, 147–152.
- Xie, S.H., Lin, H., Sun, T., Arlinghaus, R.B., 2002. Jak2 is involved in c-Myc induction by Bcr-Abl. *Oncogene* 21, 7137–7146.
- Zatonski, W., Campos, H., Willett, W., 2008. Rapid declines in coronary heart disease mortality in Eastern Europe are associated with increased consumption of oils rich in alpha-linolenic acid. *Eur. J. Epidemiol.* 23, 3–10.
- Zeng, L., Wu, G.-Z., Goh, K.J., Lee, Y.M., Ng, C.C., You, A.B., Wang, J., Jia, D., Hao, A., Yu, Q., Li, B., 2008. Saturated fatty acids modulate cell response to DNA damage: implication for their role in tumorigenesis. *PLoS ONE* 3, e2329.
- Zhang, Y., Soboloff, J., Zhu, Z., Berger, S.A., 2006. Inhibition of Ca²⁺ influx is required for – mitochondrial reactive oxygen species-induced endoplasmic reticulum Ca²⁺ depletion and cell death in leukemia cells. *Mol. Pharmacol.* 70, 1424–1434.
- Zhang, W., Wang, R., Han, S.F., Bu, L., Wang, S.W., Ma, H., Jia, G.L., 2007. alpha-Linolenic acid attenuates high glucose-induced apoptosis in cultured human, umbilical vein endothelial cells via PI3K/Akt/eNOS pathway. *Nutrition* 23, 762–770.