Inhibition of Autophagy Rescues Palmitic Acid Induced Necroptosis of Endothelial Cells*

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*Running Title: Lipotoxic signaling of palmitic acid in endothelial cells

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Background: Accumulation of palmitic acid in endothelial cells induces cellular dysfunction and death.

Results: Palmitic acid triggers Ca2+-dependent autophagy, which results in programmed necrotic death (necroptosis) of endothelial cells.

Conclusion: Autophagy promotes lipotoxic signaling of palmitic acid in endothelial cells leading to necroptosis.

Significance: Showing a new molecular mechanism of palmitic acid induced cytotoxicity may reveal novel strategies in the treatment of diseases related to lipid overload.

SUMMARY

Accumulation of palmitic acid (PA) in cells from non adipose tissues is known to induce lipotoxicity resulting in cellular dysfunction and death. The exact molecular pathways of PA-induced cell death are still mysterious. Here, we show that PA triggers autophagy, which did not counteract but in contrast promoted endothelial cell death. The PA-induced cell death was predominantly necrotic as indicated by annexinV and propidium iodide (PI) staining, absence of caspase activity, low levels of DNA hypoploidy and an early ATP depletion. In addition PA induced a strong elevation of mRNA levels of ubiquitin carboxyl-terminal hydrolase (CYLD), a known mediator of necroptosis. Moreover, siRNA-mediated knock-down of CYLD significantly antagonized PA induced necrosis of endothelial cells. In contrast, inhibition and knock down of receptor interacting protein kinase 1 (RIPK1) had no effect on PA-induced necrosis, indicating the induction of a CYLD dependent but RIPK1 independent cell death pathway. PA was recognized as a strong and early inducer of autophagy. The inhibition of autophagy by both pharmacological inhibitors and genetic knock-down of the autophagy specific genes, vacuolar protein sorting 34 (VPS34) and autophagy-related protein 7 (ATG7), could rescue the PA-induced death of endothelial cells. Moreover, the initiation of autophagy and cell death by PA was reduced in endothelial cells loaded with the Ca2+ chelator 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid aetra(acetoxymethyl) ester (BAPTA-am), indicating that Ca2+ triggers the fatal signaling of PA. In summary, we introduce an unexpected mechanism of lipotoxicity in endothelial cells and provide several novel strategies to counteract the lipotoxic signaling of PA.
Lipotoxic signaling of palmitic acid in endothelial cells

Palmitic acid (PA) is the most abundant saturated free fatty acid in the bloodstream and is known to induce cellular dysfunction and cell death in a number of cell types. The lipotoxic effect of PA appears predominantly when cells from non-adipose tissues are chronically exposed to high levels of this saturated fatty acid. This leads to lipid overload, which is particularly relevant in the pathology of obesity and type 2 diabetes (1). Numerous studies using cultured cells such as pancreatic beta-cells (2), cardiomyocytes (3), hepatocytes (4), and other cell lines (5), improved our understanding of the potential molecular mechanisms behind PA-induced cytotoxic signaling and cell damage. However, little is known about respective processes in endothelial cells although this cell type is particularly known to suffer from hyperlipidemia leading to vascular dysfunction a main cause for morbidity and mortality in the industrial countries (6). Notably, many studies point to mitochondria as initial targets of the lipotoxic effect of PA or other fatty acids (7), whereupon disturbed synthesis of cardiolipin, a mitochondria-specific phospholipid, and mitochondrial generation of reactive oxygen species (ROS) is believed to facilitate cell death by apoptosis (1). Interestingly, there are some reports in which a role of PA in the initiation of autophagy (8) and lysosomal dysfunction is emphasized (9). Macroautophagy commonly known as autophagy is an evolutionarily conserved mechanism by which cells sequester and transport damaged organelles and macromolecules for degradation in lysosomes. This process is known to serve as a survival mechanism in states of starvation (10), oxidative stress (11) and other harmful conditions where it helps to replenish the cell with nutrients. The process may sometimes become detrimental by turning into type II programmed cell death referred to as autophagic cell death (12,13) which is thought to be achieved by the same autophagy machinery but with a different outcome (14). Autophagy is known to be associated with organelle dysfunction and cellular damage and may lead to cell death either by extensive self eating (15), or by the activation of apoptosis (16) or necrosis (14,17). This study was designed to investigate a putative role of autophagy in the lipotoxic signaling of PA in endothelial cells. Here, we demonstrate that PA induces programmed necrotic death (necroptosis) of endothelial cells via the initiation of enhanced autophagy. This is an unexpected finding, which points to new possibilities in antagonizing lipotoxicity.

EXPERIMENTAL PROCEDURES

Materials: Cell culture materials were obtained from Invitrogen (Vienne, Austria) and fetal calf serum and media supplements were from PAA laboratories (Pasching, Austria). Palmitic acid, oleic acid, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), histamine, 2, 5-di-tert-butylhydroquinone (BHQ), wortmannin and 3-methyadenine (3-MA) were purchased from Sigma-Aldrich (Vienne, Austria) and Fura-2/AM and BAPTA/AM were obtained from Molecular probes, Invitrogen (Vienne, Austria). All other chemicals were from Roth (Karlsruhe, Germany), if not mentioned otherwise. Antibody against LC3 was obtained from Cell Signalling (New England Biolabs, Germany), and β-actin from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell culture: Endothelial cells from the human umbilical vein endothelial cell-derived cell line EA.hy926 (18) at passages 45 to 85 were used in this study. Cells were grown in DMEM containing 10% FCS, 1% HAT (5 mM hypoxanthin, 20 μM aminopterin and 0.8 mM thymidine), 50 units/ml penicillin, 50 μg/ml streptomycin, and were maintained at 37 °C in 5% CO₂ atmosphere.

Free Fatty Acid Treatment: Cells were treated with the free fatty acids (FFA); palmitic acid (PA) or oleic acid (OA) complexed to bovine serum albumin (BSA) in a ratio of 6:1 (FFA:BSA) in full culture medium (19). As a control BSA alone was added to cells. Cells were pretreated with the mentioned chemicals 20 min before the addition of FFA or BSA.

Plasmid construction of Venus-LC3: Venus-LC3 was cloned in multiple cloning site of pcDNA 3.1 cloning vector (Invitrogen, Austria) using the BamHI and EcoRI restriction sites followed by sub-cloning of venus using restriction sites KpnI and BamHI.

siRNA design and validation: siRNA against ATG7 (5’- CAGUGGAAUUAUUCUAAACUGAU-3’ ) was designed as described previously (20) while siRNA against VPS34 (5’- GUGUGAUGUAAGGAUAU-3) was
designed as described by Gao et al. (21), CYLD siRNA (5'-CGAAGAGGCTGAATCATAA-3') was designed as described by Stegmeier et al. (22), while RIPK1 (CTGGGCGATATTTGCAAATAACC) was designed Microsynth siRNA designing tool (Microsynth, Balgach, Switzerland). Knock down efficiency of individual siRNA was validated by real time quantitative PCR (RTq-PCR) by using sequence specific primer for VPS34 (VPS34-F 5'-GGGATTAGTGACTGAGTCCATG-3' and VPS34-R 5'-AGTCTATGTGGAAGAGTTTGCC-3'), CYLD (CYLD-F: 5'-TGGGATGGAAGATTTGATGGAG-3' and CYLD-R 5'-CATAAAGGCAAGTTTGGGAGG-3'), RIPK1 (RIPK1-F 5'-CATGGAAAAGGCGTGATACAC-3' and RIPK1-R 5'-ACTTCCCTCAGCTCATTGTG-3') and ATG7 (ATG7-F 5'-TTTTGCTATCCTGCCCTCTG-3' and ATG7-R 5'-GCTGTGACTCCTTCTGTGGAC-3') versus the control siRNA. All SiRNA's were obtained from Microsynth (Switzerland).

Transfection of siRNA and plasmid:
Cells were grown on 30 mm glass cover slips to 80% confluence and transfected with either siRNA or plasmid by using TransFast™ transfection reagent from Promega (Madison, USA). 50 pmoles of the respective siRNA(s) were mixed with the transfection reagent in 0.5 ml DMEM without FCS and incubated at room temperature for 15 minutes. The mixture was applied to the cells under normal culture conditions and incubated with 0.5 ml DMEM after 1 hour. Cells were incubated overnight and the medium was exchanged with complete culture medium after 18-20 h. For overexpression of Venus-LC3 cells were transfected with 1ml serum-free DMEM containing 2 μg plasmid DNA and 4μl of TransFast™. The medium was complemented after 1 hour with 1 ml of full culture medium. Cells were incubated for 4 h and the medium was replaced by complete culture medium. All experiments were performed 48-72 h after transfection.

MTT Assay: Cellular viability was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). For MTT assay, endothelial cells were plated in a 24 well plate. After each treatment cells were washed with warm PBS and incubated for 3 h with normal cell culture medium containing 0.5 mg/ml MTT (Sigma-Aldrich, Vienna, Austria). Cells of each well were washed twice with ice cold PBS and lysed with 200 μl of a lysis buffer composed of 0.04 M HCl in absolute isopropanol. 24 well plate was then continuously shaken at room temperature for 15 minutes on a microplate shaker. The absorbance was subsequently measured at 530 nm on a Wallace Perkin Elmer Victor 1420-004 multi-label plate reader. Data were normalized to respective controls and represented as percent viability of the controls.

Annexin V and propidium iodide (PI) staining: Cells were washed with warm PBS prior to the usage of the Annexin-V-Fluos staining kit® from Roche Biodiagnostics (Roche Diagnostics GmbH, Vienna, Austria). According to the manufacture's protocol 20 μl of Annexin-V-Fluos® were diluted in 1ml incubation buffer and 20 μl of propidium iodide were added. 100 μl of this mixture were added directly to the cells. After 20 minutes of incubation cells were analyzed on an array confocal laser scanning microscope described below.

ATP measurement: Separation of adenine nucleotides was performed on a Hypersil ODS column (5 μm, 250 x 4 mm ID), using a L2200 autosampler, two L-2130 HTA pumps, and a L2450 diode array detector (all from VWR Hitachi). The wavelength for detection of adenine nucleotides was set at 254 nm. EZchrom Elite (VWR) was used for data acquisition and analysis. After trypsinisation and mild centrifugation (supernatant discarded) cellular proteins of EA.hy926 cells were precipitated with 250 μL of perchloric acid (0.4 mol/L). After centrifugation (12,000 x g), 100 μL of the supernatant were neutralized with 10-12 μL of potassium carbonate (2 mol/L, 4°C). The supernatant obtained after centrifugation was used for HPLC analysis (injection volume: 40 μL). The pellets of the acid extract were dissolved in 0.5 mL of sodium hydroxide (0.1mol/L) and used for protein determination (BCA Assay; Pierce).

Western blotting: Endothelial cells were washed twice with ice cold PBS and total cellular protein was isolated by lysing the cells with RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was measured using Thermo Scientific Pierce BCA protein assay kit (Thermo Fisher Scientific Inc, IL, USA). 30 μg of protein were separated by
SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with the primary antibody at 4°C overnight and primary antigen-antibody complex was detected by incubating the blot with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The membrane was further developed with ECL plus western blotting detection system (GE Healthcare, Buckinghamshire, UK). To control the equal amount of protein loading all detected proteins were densitometrically normalized to β-actin.

**Cytosolic Ca\(^{2+}\) measurement:** For cytosolic Ca\(^{2+}\) measurements the Fura-2 technique was used as described previously (23). Cells were loaded with Fura-2/AM and rested prior to experiments in a Hepes-buffered solution containing (in mM): 135 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 Hepes acid, 2.6 NaHCO\(_3\), 0.44 KH\(_2\)PO\(_4\), 0.34 Na\(_2\)HPO\(_4\), 10 D-glucose, 0.1% vitamins, 0.2% essential amino acids and 1% penicillin/streptomycin; pH was adjusted to 7.4 with NaOH. For experiments in intact cells Ca\(^{2+}\) containing experimental buffer (EB) was composed of (in mM): 138 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 D-glucose and 10 Hepes acid; pH was adjusted to 7.4 with 1 M NaOH. For experiments in Ca\(^{2+}\) free solution, EB containing 1 mM EGTA instead of CaCl\(_2\) was used.

Experiments were performed on a Nikon inverted microscope (Eclipse 300TE, Nikon, Vienna) equipped with CFI Plan Fluor \(\times 40\) oil immersion objective lens (NA 1.3, Nikon, Vienna, Austria), an epifluorescence system (150 W XBO; Optiquip, Highland Mills, NY, USA), a computer controlled z-stage (Ludl Electronic Products, Haawthrone, NY, USA) and a liquid-cooled CCD camera (−30 °C; Quantix KAF 1400G2, Roper Scientific, Acton, MA, USA). All devices were controlled by the MetaFluor4.0 software (Visitron Systems). Emission was acquired with a cooled-coupled device (CCD) camera (CoolSNAP-HQ, Photometrics, Tucson, USA). All devices were controlled by VisiView Premier acquisition software (Visitron Systems).

**Detection of caspase-3 activity using Casper3-GR:** Cells expressing the Förster resonance energy transfer (FRET) based sensor Casper3-GR (24), (Casper3-GR vector DNA was purchased from BioCat GmbH, Heidelberg, Germany) for caspase-3 activity were imaged on the ACLSM using a 40x objective (Zeiss). The following 3 channels for imaging the red fluorescence protein (RFP), the green fluorescence protein (GFP) and the FRET signal between GFP and RFP were used to determine the cleavage of the sensor by caspase-3: RFP-channel: excitation 561 nm laser line, emission was collected at 630/75 nm; GFP-channel: excitation 488 nm laser line, emitted light was measured at 480/40 nm; FRET between GFP and RFP: excitation 488 nm laser line, emitted light was imaged at 630/75 nm. The 3 channels were imaged consecutively using the same exposure times (500 ms). Caspase-3 activity was expressed as the ratio of GFP/FRET. 24 h after transfection cells were treated with BSA or PA for 16-18 h. Staurosporin was used as a positive control. For more details see Supplementary Fig. 2.
Quantification of DNA hypoploidy by flow cytometry: Endothelial cells were grown to 90% confluence and were treated with BSA, 0.5 mM palmitic acid (PA) and 300 nM staurosporine (STS) for 16 h. Cells were trypsinized followed by washing with PBS, and pelleted by centrifugation. Cells were fixed by incubation with 70% ethanol on ice for 15 min. Cells were pelleted and resuspended in 500 µl propidium iodide (PI) solution (in PBS: 50 µg/ml PI, 0.1 mg/ml RNase A, 0.05% Triton X-100) and incubated at 37 °C for 40 minutes. The PI solution was diluted by adding 3 ml of PBS and cells were pelleted by centrifugation. Cells were resuspended in 500 µl PBS and were analysed by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA). Individual cells were distinguished from cell debris and aggregated cells by means of forward-scatter/side-scatter gating, and PI fluorescence intensity was measured in 10,000 cells (25).

Statistical analysis: Data shown represent the mean ± SEM. Statistical analyses were performed with unpaired Student’s t-test. n represents the number of independent experiments and P<0.05 was considered to be significant.

RESULTS

PA induces necrotic cell death in endothelial cells. First we tested the susceptibility of the endothelial cell line, EA.hy926, to PA-induced cell death. For this purpose cells were treated with a complex of PA and bovine serum albumin (BSA) and cell viability was measured with the 3-(4,5-Dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay at different times of incubation (Fig. 1A). After 12 h of incubation with PA, cell viability was found to be already significantly reduced and further decreased with time. The cell viability declined more than 80 % after 28 h of incubation with PA (Fig. 1A), pointing to a strong cytotoxic effect of this saturated fatty acid on endothelial cells. The PA-induced cell death was further examined using long-term phase contrast imaging, which showed an impaired cell proliferation and an accumulation of detached, dying cells upon incubation with PA (Supplementary Fig. 1 and Movie 01). In contrast, cell treatment with oleic acid (OA) did not affect the viability of the cells (Fig. 1A). These findings are in line with earlier studies using other cell types (26) and approved EA.hy926 cells as a suitable model to explore the molecular mechanisms of PA-mediated lipotoxicity.

Next, the mode of PA-induced cell death was investigated by annexin V and propidium iodide (PI) staining. A major proportion of the cells exposed to PA for 12 h were found to be positive both for annexin V and PI (Fig. 1B and 1C), which is indicative of necrosis. Moreover, the annexinV/PI positive cells contained round and intact nuclei (Fig. 1C), pointing to necrotic cell death. To further exclude the role of apoptosis in the process of PA-induced death of the endothelial cells, cells were treated with benzylxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk), a cell permeable, irreversible pan caspase inhibitor that hinders apoptosis (27). zVAD-fmk failed to exhibit any effect on PA-induced endothelial cell death (Fig. 1D), thus, further confirming that the mode of PA-induced death of the endothelial cells was necrotic and not apoptotic. The absence of caspase activation in PA-induced cell death was further documented using the caspase-3 apoptosis sensor Casper3-GR (24). Casper3-GR is a FRET based sensor for measuring the caspase-3 activity, consisting of a green (GFP) and a red fluorescence protein (RFP) that are connected by the caspase-3 cleavage sequence DEVD (Supplementary Fig. 2A). As a positive control cells expressing Casper3-GR were treated with staurosporine, which revealed a strong increase of the GFP/FRET ratio after 80 min, indicating the cleavage of the sensor by the activation of caspase-3 (Supplementary Fig. 2B and 2C). In contrast, cell treatment with PA for 16 h failed to increase the basal GFP/FRET ratio of Casper3-GR, indicating the absence of caspase-3 activation (Fig. 1D and Supplementary Fig. 2D). Moreover, the quantification of DNA hypoploidy by FACS showed only a small proportion of cells with reduced DNA content when cells were treated with PA for 18 h (Fig. 1F and 1G). In line with these findings PA did not induce DNA laddering even if cells were treated for 24 h with the fatty acid (Supplementary Fig. 2E), while under these conditions 70% of the cells were found dead with the MTT assay. In summary these data confirm that the PA induced cell death is predominantly necrotic.

In line with these findings, HPLC analysis revealed significantly reduced ATP levels in cells that have been exposed to PA already at 8 h and further decreased tremendously until 16 h (Fig. 1H), emphasizing necrosis as the terminal point of PA-induced cell damage. As the onset of cell
death is between 8 h and 16 h time point the drop in ATP levels are earlier than the onset of cell death and more than 40% of the cells were already found dead after 16 h as indicated in Fig 1A. These data challenge the dogma that lipotoxicity is exclusively apoptotic and align with our earlier findings that free fatty acids induce necrosis and not apoptosis in yeast (7).

**PA induces RIPK3 and CYLD-dependent but RIPK1 independent programmed necrosis (necroptosis).** Evidence accumulated that the necrotic pathway can also occur in a highly regulated manner (28). Particularly the kinases receptor-interacting protein kinase-1 (RIPK1), RIPK3 and the receptor interacting protein (RIP)1 de-ubiquitin enzyme CYLD are known initiators of programmed necrosis, a cell death pathway, which is also referred to as necroptosis (29,30). Accordingly, we next investigated the molecular mechanism of PA-induced necrosis in endothelial cells. For this purpose we first used necrostatin-1, a specific inhibitor for RIPK1. As represented in figure 2A necrostatin-1 did not affect the PA-induced cell death. In line with this finding siRNA-mediated knock-down of RIPK1 (Fig. 2B) could not inhibit PA induced cell death (Fig. 2C). On the other hand a knock down of CYLD (Fig. 2D) could inhibit the PA induced cell death significantly (Fig 2E). In line with these results we unveiled a strong elevation of the mRNA level of CYLD after 16 h of incubation with PA, while no change in the expression pattern of RIPK1 could be observed under these conditions (Fig. 2F). We failed to perform respective experiments for RIPK3 expression levels most likely because of too low mRNA levels coding for this protein in the endothelial cells used (Supplementary Fig. 4A and 4B). Nevertheless, cells treated with a validated siRNA against RIPK3 (29), showed a significant increased viability if treated with PA for 24 h in four sets of independent experiments (Supplementary Fig 4C). Although we could not quantify the RIPK3 knock down efficiency these findings suggest that PA induces a CYLD and RIPK3 dependent but RIPK1 independent programmed necrosis.

**PA triggers autophagy in endothelial cells.** Recent data demonstrated the induction of autophagy by PA in beta cells (8). Accordingly, a possible role of autophagy in PA-induced cell death was investigated. For this purpose cleavage of the microtubule-associated protein 1 light chain 3 (LC3), a marker for autophagy (31), was blotted. Considerable cleavage and lipidation of LC3 to LC3-I (not lipidated) and the phosphatidylethanolamine (PE)-conjugated LC3-II was detected in cells that were treated with PA, while OA did not induce LC3-II formation (Fig. 3A and 3B). In order to gauge the magnitude of the induction of autophagy by PA cells were also treated with 50 nM rapamycin, a well-characterized inducer of autophagy (32). As shown in Fig. 3A and 3B PA was even more effective than rapamycin to stimulate LC3-II formation under these conditions. The PA induced conversion of LC3-I to LC3-II depended on both the time of PA incubation (Fig. 3C and 3D) and the concentration of the fatty acid (Fig. 3E and 3F). The induction of autophagy by PA was further examined by imaging the cellular distribution of venus-LC3, a fusion construct of the yellow fluorescent protein venus with LC3. In control cells that were treated with BSA alone venus-LC3 was mainly distributed in the cytosol and only a small number of autophagosomes were visible, indicating a low level of autophagy under these conditions (Fig. 3G, left panel). In contrast, cells incubated with PA for 8 h exhibited a large number of autophagosomes with accumulated venus-LC3 (presumably as venus-LC3-II). This confirms the induction of autophagy by PA in the EA.hy926 cells (Fig. 3G, right panel).

In order to verify whether or not the PA-induced autophagy goes to completion, we measured LC3-II also in the presence of bafilomycin A (50 nM), a known inhibitor of the autophagic flux (32). As shown in Fig. 3H and 3I LC3-II was further increased in the presence of bafilomycin A under control conditions and in cells pretreated with PA until 8 h. These findings indicated that PA is a genuine and strong inducer of autophagy in endothelial cells. However, at 16 h of incubation with PA the accumulation of LC3-II was hampered, indicating the onset of incomplete autophagy. This finding was further supported by the degradation pattern of sequestome 1 (p62), a frequently used marker for the measurement of the autophagic flux (32). Corresponding to the pattern of LC3-II accumulation (Fig. 3H and 3I), the levels of p62 gradually decreased in cell treated with PA until 8 h, while a significant accumulation of this protein was observed at 16 h (Figure 3J and 3 K).

**Inhibition of autophagy rescues endothelial cells from PA-induced cell death.** In order to investigate the role of autophagy induction on cell death we inhibited autophagy with wortmannin or 3-methyladenine (3-MA) and measured the effect on lipotoxic cell death.
Wortmannin and 3-MA are known to prevent autophagy by their inhibitory effect on VPS34 (33, 34). As expected, inhibition of VPS34 by either wortmannin prevented PA-induced autophagy indicated by a lack of LC3 cleavage in cells pretreated with this inhibitor (Fig. 4A, 4B, 4C and 4D).

Hence, we investigated the putative role of autophagy on lipotoxic cell death. We found that the cells pretreated with wortmannin were resistant to PA-induced cell death even after 36 h of incubation with the fatty acid (Fig. 4E). As wortmannin inhibits all different classes of PI3Ks, the effect of 3-MA, a highly specific inhibitor of PI3K class III (vesicular protein sorting 34; VPS34) was tested. As shown in Fig. 4F 3-MA was also highly effective in preventing PA-induced cell death. The effect of PI3K (class III) inhibitors on PA-induced cell death was further tested using long-term phase-contrast microscopy, which confirmed their potency to rescue endothelial cells from PA-mediated lipotoxicity (Supplementary Fig. 3 and Movie02).

Genetic knock-down of autophagy related genes rescues PA-induced cell death. To further confirm the role of autophagy in the cell death process we knocked-down VPS34 and ATG7, two autophagy specific proteins and the effects thereof on PA-induced cell death were investigated. The siRNAs against both VPS34 and ATG7 were efficient in decreasing respective mRNA levels (Fig. 5A). Knock down of either VPS34 or ATG7 strongly reduced PA-induced endothelial cell death (Fig. 5B and 5C).

PA-induced Ca\(^{2+}\) elevation triggers autophagy in endothelial cells. Recently there are some reports about the role of Ca\(^{2+}\) in autophagy (35). We chelated cytosolic Ca\(^{2+}\) with BAPTA-am and measured the cleavage of LC3 by western blotting. It was found that autophagy was inhibited by BAPTA-am treatment (Fig. 6A and 6B). This indicated that the induction of autophagy by PA was dependent on cytosolic Ca\(^{2+}\) elevation.

To verify whether or not cell treatment with PA impacts Ca\(^{2+}\) signaling of endothelial cells we measured cytosolic Ca\(^{2+}\) under the conditions of fatty acid exposure. Basal [Ca\(^{2+}\)]\(_{\text{cyto}}\) was assessed first in the presence and then in the absence of extracellular Ca\(^{2+}\) using Fura-2/AM loaded cells. Subsequently, Ca\(^{2+}\) was released from the endoplasmic reticulum (ER) in a Ca\(^{2+}\)-free medium by cell stimulation with the IP\(_3\)-generating agonist histamine and the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibitor 2,5-di-t-butyl-1,4-benzohydroquinone (BHQ) in order to assess the ER Ca\(^{2+}\) content (Fig. 6C). In a Ca\(^{2+}\)-containing buffer the treatment with PA caused a significant increase of basal levels of [Ca\(^{2+}\)]\(_{\text{cyto}}\) already after 4 h of incubation, and [Ca\(^{2+}\)]\(_{\text{cyto}}\) remained elevated until 8 and 16 h of incubation (Fig. 6D and 6E). Removal of extracellular Ca\(^{2+}\) led to a decrease in basal [Ca\(^{2+}\)]\(_{\text{cyto}}\) in cells that were pretreated with PA, indicating that store operated Ca\(^{2+}\) entry (SOCE) was activated (Fig. 6C). In contrast, cells incubated with OA did not show significant changes of basal Ca\(^{2+}\) levels in the cytosol (Fig. 6C and 6D). The amount of releasable Ca\(^{2+}\) from the ER was clearly smaller in cells pretreated with PA (Fig. 6C and 6E). Notably, the effect on ER Ca\(^{2+}\) mobilization was seen already after 2 h incubation with PA and was more pronounced with time (Fig. 6E). Cells pretreated with OA, however, revealed normal Ca\(^{2+}\) signals upon ER Ca\(^{2+}\) depletion (Fig. 6C and 6E). These data indicate that the accumulation of PA within endothelial cells is associated with a slow, time-dependent leak of Ca\(^{2+}\) from the ER. The consequence is a reduced Ca\(^{2+}\) content of the ER and elevated basal cytosolic Ca\(^{2+}\) levels, possibly due to the stimulation of Ca\(^{2+}\) entry via the SOCE pathway.

Further, we investigated the role of cytosolic Ca\(^{2+}\) on cell viability. Cells were treated with BAPTA-am for 20 min prior to treatment with PA for 24 h, and cellular viability was measured with MTT assay. It was found that cell loading with BAPTA-am inhibited the PA-induced cell death significantly (Fig. 6F).

DISCUSSION

Herein we provide evidence that exposure of endothelial cells to pathological PA concentrations generates a lipotoxic response that finally leads to necrotic cell death. The lethal effect of PA is initially triggered by the development of Ca\(^{2+}\) leakage of the ER which leads to elevated basal cytosolic Ca\(^{2+}\). Subsequently, the initiation of Ca\(^{2+}\)-dependent autophagy turns to be harmful to the cells leading to RIPK3 and CYLD dependent necrotic cell death. These data unveil a so far unknown pathway of lipotoxicity and point to ER Ca\(^{2+}\) leakage, Ca\(^{2+}\)-dependent autophagy, and programmed necrosis as a hallmark of lipotoxicity in endothelial cells.
**PA induces programmed necrosis of endothelial cells**

Although it has been reported that apoptosis is the major executor of cell death under conditions of substrate overload (26), our data clearly indicate that an overload of endothelial cells with PA causes necrosis. We used zVAD-fmk to rule out the contribution of pan caspases in the execution of PA-induced cell death. However, this set of experiment might also point to caspase independent apoptosis in the cell model used (36). Nevertheless, our findings that cells treated with PA were positive for both annexin V and PI, did not show caspase activity, and the early depletion of ATP strongly indicate necrotic cell death. Moreover, only a small fraction (11.6%) of cells treated with PA showed DNA hypoploidy, indicating that necrosis is the predominant mode of cell death. On the other hand these experiments, which were performed with the whole cell population (i.e. attached and floating cells), might point to a mixture of apoptosis and necrosis. Nevertheless, the reduced energy supply might rather explain the necrotic mode of cell death, as it has been reported that ATP levels determine the mode of cell death (37). In addition, the nuclear morphology of cells challenged with toxic concentrations of PA supported the necrotic cell death. The dying endothelial cells contained round and intact nuclei, and no nuclear fragmentation, which typically occurs during apoptosis (38), was observed.

In addition, a knock-down of the known players of programmed necrosis, RIPK3 (29) and CYLD (30), rescued the cells from PA induced cell death. Although we could not detect RIPK3 mRNA levels in control cells and cells treated with PA for 16 h we were successful in inhibiting the PA induced cell death by using a previously reported siRNA against RIPK3 (29). The rescuing effect of a siRNA-mediated knock-down of RIPK3 was less pronounced compared to that of a CYLD knock-down, but reproducible and significant. We concluded that mRNA levels coding for RIPK3 are too low to be specifically detected and quantified, possibly also because of a low stability of the respective mRNA in the preparations used. Although we cannot exclude any off target effects of the validated siRNA (29) used to knock-down RIPK3 in endothelial cells, the functional tests clearly suggested an involvement of this kinase in the toxic signaling of PA. Interestingly, our data also unveiled that PA triggers the expression of CYLD, while the molecular mechanisms involved have not been clarified so far. This finding supports the assumption that a necrotic pathway is induced by PA in endothelial cells. RIPK1 is another known player of programmed necrosis, however, siRNA-mediated knock-down of RIPK1 as well as the usage of necrostatin-1 indicated that RIPK1 does not contribute to the PA-induced necroptosis in this particular endothelial cell line. This finding is in line with recent reports, in which a RIPK1 independent but RIPK3 and CYLD dependent cell death pathway has been described (39,40). The induction of RIPK3 and CYLD dependent necroptosis by PA described here may, however, point to novel strategies for the treatment of metabolic diseases.

**PA induces autophagy, which promotes cell death**

Recently there are reports about the induction of autophagy by free fatty acid in different cell types (8,41,42). This is in line with our finding showing that PA initiates enhanced autophagy in the endothelial cells used. However, in contrast to our findings it was shown in pancreatic beta cells that the induction of autophagy was a pro-survival mechanism and inhibiting autophagy with pharmacological inhibitors enhanced the cell death (8). In contrast, our findings demonstrate that autophagy is the main trigger of PA-induced cell death, as the inhibition of autophagy with either pharmacological inhibitors or genetic knock-down could rescue the cells from PA induced cell death. At a first glance this is in line with recent reports that describe autophagy as a mechanism able to induce type II programmed cell death (43, 15). Autophagy can contribute to cell death, particularly if the process is hampered by lysosomal dysfunction (44). We did not test whether or not endothelial cells develop lysosomal dysfunction in response to PA. However, in recent reports PA was shown to induce lysosomal dysfunction in PC12 and pancreatic beta cells. Notably, PA was found to suppress autophagic turn over in beta cells leading to lower ATP levels. Our finding regarding the ATP depletion in endothelial cells treated with PA is in line with this report. Moreover, we could see clear signs of a hampered autophagic flux at 16 h of cell treatment with PA. Hence, it is tempting to speculate that PA might cause a parallel lysosomal dysfunction resulting in depletion of ATP, which ultimately leads to necrosis of the endothelial cells used.

**PA affects ER Ca\(^{2+}\) storage**
Although the detailed mechanisms that induce ER Ca\(^{2+}\) mobilization by PA leading to elevated cytosolic Ca\(^{2+}\) remain unclear our data indicate that this is an early and central process of PA-induced cell damage. We did not observe an acute effect of PA on the Ca\(^{2+}\) homeostasis of endothelial cells, indicating that the accumulation of PA, its metabolism or derivates might be responsible for developing aberrant Ca\(^{2+}\) signals in endothelial cells. The finding that cells treated with PA had a lower ER Ca\(^{2+}\) content and increased levels of [Ca\(^{2+}\)]\(_{\text{cyt}}\) is in line with a recent report that describes ER Ca\(^{2+}\) release by PA in beta-cells (45). Ca\(^{2+}\) signaling is known to be involved in all types of cell death (46) and also plays a fundamental role in the induction of ER stress (47) and autophagy (35). Our finding that cytosolic Ca\(^{2+}\) chelation by BAPTA protected the cells against PA-induced death is of particular relevance, because it points to a possibility of a therapeutic intervention against lipotoxicity.

In summary our study describes that PA overload of endothelial cells results in a Ca\(^{2+}\)-dependent development of autophagy which finally lead to programmed necrotic cell death. This molecular mechanism of lipotoxicity reported herein enlightens a new mechanism of lipotoxic cell death of endothelial cells and might guide to novel strategies in the therapy of vascular diseases that are caused by the accumulation of fatty acids in endothelial cells.

**FOOTNOTES**

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References

FIGURE LEGENDS

Figure 1: Palmitic acid (PA) induces necrotic cell death in endothelial cells.
A) Cells were treated with BSA alone (white columns, n=3), 0.5 mM OA (grey columns, n=3) or 0.5 mM PA (black columns, n=3) and cell viability was measured with MTT assay at the time points indicated. Fatty acids were complexed to BSA. Data were normalized to BSA as control and represented as percentage viability. *P < 0.05 vs. BSA. B) Annexin V/PI costaining for the cells exposed to 0.5 mM PA (n=4) or BSA alone (n=4) (asterisk refers to Annexin V/PI). *P < 0.05 vs. BSA. C) Representative images of Annexin V/PI stained endothelial cells treated with 0.5 mM PA for 12 h (n=4). Images were taken using a 40x objective. D) Cells were treated with the solvent (DMSO, control, -zVAD-fmk, n=3) or with 20 µM zVAD-fmk (+zVAD-fmk, n=3) prior to treatment with 0.5 mM PA or BSA alone and cellular viability was measured with MTT assay. *P < 0.05 vs. BSA. (51.3±6.7% in the zVAD-fmk treated as compared to 55.9±7.7% in controls) E) Caspase-3 activity measured with the FRET based sensor casper3-GR, expressed as ratio GFP/FRET of randomly selected cells under control conditions (BSA, white column, n=3, 81 cells) and after cell treatment with 0.5 mM PA for 16-18 h (n=3, 102 cells). F) Histograms of cell cycle analysis by flow cytometry for the DNA hypoploidy. Cells were treated with BSA or 0.5 mM PA and were analysed after 18 h of incubation. G) Statistical analysis of the histograms shown in panel F.). H) Statistical data representing nmol ATP/mg protein in the cells exposed to 0.5mM PA (black circles, dotted line, n=6 for each time point) or BSA alone (white circles, continuous line, n=6 for each time point). *P < 0.05 vs. BSA.

Figure 2: PA induces RIPK1 independent but CYLD dependent necroptosis in endothelial cells.
A) Cells were pretreated with solvent (DMSO, control, -Nec-1, n=4) or 30 µM necrostatin-1 (+Nec-1, n=4) for 20 min and were incubated for 24 h with BSA or 0.5 mM PA and cellular viability was measured with MTT assay. *P < 0.05 vs. BSA. B) Knock-down efficiency of RIPK1 (RIP1) siRNA in percentage of mRNA expression compared to scramble siRNA (n=3 for all columns presented). *P < 0.05 vs. Scrambled siRNA. C) Cell viability 48 h after cells transfection with either scrambled siRNA (left pair of columns, n=18 for both conditions) or siRNA against RIPK1 (right pair of columns, n=18 for both conditions). Cells were incubated for 24 h with BSA alone (white columns) or 0.5 mM PA complexed to BSA (black columns) and cell viability was measured using the MTT assay. Viability is expressed in percentage, whereas the average value of cells treated with scrambled siRNA and BSA alone was defined as 100 %. *P < 0.05 vs. BSA, and *P < 0.05 vs. PA with scrambled siRNA. (47.8±1.0% dead cells in RIPK1-siRNA as compared to 41.2±1.6% dead cells in scrambled siRNA). D) Knock-down efficiency of CYLD siRNA in percentage of mRNA expression compared to scramble siRNA (n=6). *P < 0.05 vs. Scrambled siRNA. At 16h more than 40% of the cells were found dead by MTT assay as shown in Fig. 1A. E) Impact of siRNA mediated knock down of CYLD on cell viability under analogous conditions described in panel C (n=18 for each column). *P < 0.05 vs. BSA, and *P < 0.05 vs. PA with scrambled siRNA. F) The relative mRNA expression of RIPK1 and CYLD in cells treated with BSA or 0.5 mM PA for 2, 4, 8 and 16 h. The relative expression is presented as a percentage of the BSA treated cells at the given time point. *P < 0.05 vs. BSA at the given time point (n=3).

Figure 3: PA induces autophagy in endothelial cells.
A) Cells were treated with BSA, OA, PA and Rapamycine (Rapa) for 8 h and extracted protein were immunoblotted against LC3 antibody. β-actin was used to normalize the data for equal protein loading. B) Columns represent the average band densities from 3 different experiments as shown in panel a. *P < 0.05 vs. BSA. C & D) Time dependency of the PA induced cleavage of LC3 expressed as the LC3-II/LC3-I ratio over time (n=3 for each time point). E &F) Concentration response curve of PA-induced cleavage of LC3 (n=3 for each concentration). PA was complexed to BSA. G) Representative images of cells expressing venus-LC3. Images show the subcellular distribution of venus-LC3 in control cells (left image, BSA alone for 8 h after cell transfection) and in cells treated for 8h with 0.5 mM PA complexed to BSA (right image). H) Cells were treated with BSA or 0.5 mM PA in the presence or absence of bafilomycin A1 (Baf-A1) and the isolated proteins at the given time point were immunoblotted for LC3. Representative image of three independent experiments is presented while statistical data of three independent experiments are shown in panel I). *P < 0.05 vs. BSA at a given time point, and *P < 0.05 vs. PA with treated with DMSO (control, -Baf-A1) at a given time point (n=3). J) A representative image of three independent experiments showing the degradation pattern of p62. Cells were treated with BSA or 0.5 mM PA and isolated proteins at the given time points were blotted for p62. Statistical data of three independent experiments are shown in panel K). *P < 0.05 vs. BSA at a given time point, *P < 0.05 vs. PA at 8h time point (n=3).
Figure 4: Inhibition of autophagy rescued endothelial cells from PA-induced death
A) Representative western blot showing LC3 cleavage of cells that were incubated for 8h with BSA alone, 0.5 mM OA, or 0.5 mM PA in the absence (-) and presence (+) of 10 µM wortmannin. B) Statistical data of LC3 cleavage from western blots shown in panel f (n=3 for all conditions). *P < 0.05 vs. Control. C) Representative western blot showing LC3 cleavage of cells that were incubated for 8h with BSA alone or 0.5 mM PA in the absence (-) and presence (+) of 10 mM 3-MA. D) Statistical data of LC3 cleavage from western blots shown in panel f (n=3 for all conditions). *P < 0.05 vs. Control. E) Columns represent average cell viabilities that were determined using the MTT assay of cells not pretreated with wortmannin (left pair of columns, -Wortmannin) that were incubated for 24 h with BSA alone (left white column, n=3) or with 0.5 mM PA complexed to BSA (left black column, n=3), and cells pretreated with 10 µM wortmannin (right pair of columns, +Wortmannin) for 20 min prior to an incubation with either BSA alone (right white column, n=3) or with 0.5 mM PA complexed to BSA (right black column, n=3). *P < 0.05 vs. BSA, and **P < 0.05 vs. PA without wortmannin. F) Cells were treated with 10 mM 3-MA, another specific inhibitor of PI3K III and autophagy, and cell death was analyzed by MTT assay (n=3 for all conditions). *P < 0.05 vs. BSA, and **P < 0.05 vs. PA without 3-MA.

Figure 5: Genetic knock-down of autophagy specific genes rescued cells from PA induced cell death.
A) Knock-down efficiency of individual siRNA in percentage of mRNA expression compared to scramble siRNA (n=3 for all columns presented). *P < 0.05 vs. Scrambled siRNA. B) 48 h after cells transfection with either scrambled siRNA (left pair of columns, n=9 for both conditions) or siRNA against VPS34 (right pair of columns, n=9 for both conditions) cells were incubated for 24 h with BSA alone (white columns) or 0.5 mM PA complexed to BSA (black columns) and cell viability was measured using the MTT assay. Viability is expressed in percentage, whereas the average value of cells treated with scrambled siRNA and BSA alone was defined as 100 %. *P < 0.05 vs. BSA, and **P < 0.05 vs. PA with scrambled siRNA. C) Impact of siRNA mediated knock down of ATG7 on cell viability under analogous conditions described in panel b (n=9 for each column). *P < 0.05 vs. BSA, and **P < 0.05 vs. PA with scrambled siRNA.

Figure 6: PA induced autophagy and cell death is a Ca$$^{2+}$$ dependent process.
A) Representative western blot showing LC3 cleavage of BAPTA-AM loaded cells that were incubated for 8h with BSA alone or 0.5 mM PA. B) Statistical data of LC3 cleavage from western blots (n=3 for all conditions). *P < 0.05 vs. Control. PA induces release of Ca$$^{2+}$$ from ER and results in cytosolic Ca$$^{2+}$$ elevation. C) Representative Ca$$^{2+}$$ signals of Fura-2/AM loaded cells 16 h after incubation with BSA alone (black continuous line), 0.5 mM OA (grey continuous line), and 0.5 mM PA (black dotted line). As indicated signals were first recorded in Ca$$^{2+}$$ containing medium (2 mM) and subsequently Ca$$^{2+}$$ was mobilized from the ER by cell stimulation with 100 µM histamine and 15 µM BHQ in a Ca$$^{2+}$$ free medium (1 mM EGTA). D) Statistics of basal ratio values of Fura-2/AM loaded cells in the presence of extracellular Ca$$^{2+}$$ (2 mM) and E) average delta values of the maximal Ca$$^{2+}$$ peaks upon cell stimulation with 100 µM histamine and 15 µM BHQ in a Ca$$^{2+}$$ free medium (1 mM EGTA). D) Statistics of basal ratio values of Fura-2/AM loaded cells in the presence of extracellular Ca$$^{2+}$$ (2 mM) and E) average delta values of the maximal Ca$$^{2+}$$ peaks upon cell stimulation with 100 µM histamine and 15 µM BHQ in a Ca$$^{2+}$$ free medium (1 mM EGTA). F) Cells were treated with 0.5 mM PA or BSA alone for 24 h. Cell viability was measured with MTT assay and data were normalized to BSA as a control and represented as mean viability (n=3, for all conditions). *P < 0.05 vs. BSA, and **P < 0.05 vs. PA without BAPTA-AM loaded cells.
Figure 1

A: Graph showing cell viability over time for BSA, OA, and PA.

B: Bar graph comparing the number of cells per field for Annexin V and PI.

C: Images of Annexin V, PI, and Merge with 10µm scale bars.

D: Graph showing cell viability with and without ZVAD-fmk for BSA and PA.

E: Graph showing the ratio of GFP/FRET for Caspase-3 activity.

F: Flow cytometry graphs for BSA and PA.

G: Bar graph showing the percentage of total count for subG1, G0/G1, S, and G2/M.

H: Line graph showing ATP/mg protein over time for BSA and PA.
Figure 2

A. Cell viability (%)

B. Relative mRNA expression

C. Cell viability (%)

D. Relative mRNA expression

E. Cell viability (%)

F. Relative mRNA expression (%)
Figure 6

A

LC3-I
LC3-II
β-actin

BSA  BSA+BAPTA  PA  PA+BAPTA

Ca2+
EGTA

Histamine+BHQ

C

Ratio F₃₄₀/F₃₈₀

Time (min)

0  1  2  3  4  5  6

D

Basal Ca²⁺ (Ratio F₃₄₀/F₃₈₀)

2h  4h  8h  16h

BSA  OA  PA

E

Releasable Ca²⁺ (Δ Ratio F₃₄₀/F₃₈₀)

2h  4h  8h  16h

BSA  OA  PA

F

Cell viability (%)

-BAPTA  + BAPTA

BSA  PA
Inhibition of autophagy rescues palmitic acid induced necroptosis of endothelial cells
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