

# Palmitate impairs angiogenesis via suppression of cathepsin activity

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**Abstract.** Angiogenesis requires the interaction of multiple variable factors to promote endothelial cell adhesion, migration and survival. Palmitate, a free fatty acid, exhibits an anti-angiogenic effect via interference with endothelial cell function, whereas cysteine proteases are important in protein turnover and are termed positive modulators of neovascularization. However, the association between these two factors regarding the regulation of human endothelial cell function remains to be elucidated. By using cell counting kit-8, the Transwell method and an annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit, the present study reported that high levels of palmitate result in a significant decrease in endothelial cell proliferation and invasion, and induced cell apoptosis; cathepsin L and S inhibitors may suppress palmitate-induced apoptosis. Conversely, the results of the cathepsin L and S activity assay and reverse-transcription-quantitative polymerase chain reaction indicated that palmitate inhibited cathepsin-induced endothelial cell invasion, partially via suppressing the expression and activity of cathepsin L and S. The findings of the present study suggested that the potent anti-angiogenic properties of palmitate may be mediated by cysteine proteases.

## Introduction

Angiogenesis is a complex multi-step process that occurs in response to ischemic stimuli. These steps include degradation of the basement membrane, proliferation, apoptosis and invasion of endothelial cells, tube formation, and anastomosis (1,2). Various proteases including the family of matrix metalloproteinases (MMPs), and cysteine and serine proteases contribute to the complex processes of new vessel formation and remodeling. Urbich *et al* (3) demonstrated that cysteine

protease cathepsin L-deficient mice presented impaired wound recovery, indicating an important role for cathepsin L in neovascularization. Furthermore, the study validated the ability of cathepsin L to induce angiogenesis, as transferred cathepsin L-deficient progenitor cells did not migrate to ischemic areas or augment vasculogenesis. Conversely, forced expression of cathepsin L in mature endothelial cells markedly enhanced cell invasion. Furthermore, Shi *et al* (4) demonstrated that cathepsin S was required for microvessel formation. Cysteine proteases have previously been demonstrated to be important in apoptosis and cell survival, separate from their role in proteolysis of extracellular matrix in vascular remodeling (5-7); however, the underlying mechanism of this process remains to be fully elucidated.

Angiogenesis is associated with atherosclerosis, and numerous risk factors of atherosclerosis, including diabetes and insulin resistance, are accompanied by high levels of free fatty acids (FFA) (8,9). However, the specific role of FFAs and their association with angiogenesis remains to be elucidated. FFAs have been demonstrated to exert effects on endothelial cells via enhancing reactive oxygen species levels or impairing nitric oxide production (10); therefore, increased levels of FFA will inhibit angiogenesis. Palmitate, which is the most frequently occurring form of saturated FFA present in human serum, contributes to lipotoxicity (11). In addition to the aforementioned characteristics, palmitate has been detected to induce apoptosis in a variety of tissues (10,12,13) and decrease cardioprotein synthesis, resulting in the release of cytochrome c (14).

Cathepsin L and S have therefore been confirmed to be important in endothelial cell angiogenesis; however, it remains to be elucidated as to whether FFA levels may influence cathepsin-mediated angiogenesis. The present study examined the proliferation, apoptosis and invasion of human umbilical vein endothelial cells (HUVECs) following exposure to palmitate in the presence or absence of selective cathepsin inhibitors, and observed that palmitate impaired cathepsin protein expression levels and activity.

## Materials and methods

**Cell culture and incubation with fatty acids.** HUVECs were purchased from the American Type Culture Collection (Manassas, VA, USA; PCS-100-010) and cultured in M199 medium (HyClone; GE Healthcare Life Sciences,

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Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences) at 37°C in an atmosphere containing under 5% CO<sub>2</sub>. Cells were passaged every 2-3 days once they reached maximum confluence. Cells were incubated in M199/10% FBS medium supplemented with 0.05, 0.1, 0.2, 0.4 or 0.6 mM palmitate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) pre-conjugated with FFA-free bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) at a 1:1 molar ratio. Control cells were grown with the same medium containing FFA-free BSA. If not stated otherwise, cells were incubated for 1 h with 10 μM cathepsin L inhibitor (z-FF-FMK; cat. no. 219421; Calbiochem; EMD Millipore, Billerica, MA, USA) and cathepsin S inhibitor (z-FL-COCHO. H<sub>2</sub>O; cat. no. 219393; Calbiochem; EMD Millipore) at 37°C, which was followed by incubation with palmitate or FFA-free BSA for 24 h at 37°C.

**Immunofluorescence staining.** HUVECs were fixed in 4% paraformaldehyde for 20 min and incubated at 37°C in blocking buffer (PBS containing 5% BSA). Cells were incubated in the presence of mouse anti-Cluster of Differentiation (CD) 31 antibody (1:200; cat. no. SC-81158; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at 37°C and washed three times in PBS. Cells were subsequently incubated with rhodamine-conjugated goat anti-mouse IgG (H+L) secondary antibody (1:1,000; cat. no. 31660; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at 37°C. Nuclei were stained with DAPI (1:10,000; Invitrogen; Thermo Fisher Scientific, Inc.) and were examined with an Olympus IX70 inverted fluorescence microscope.

**Cell proliferation assay.** The effect of elevated palmitate concentration on HUVEC proliferation was analyzed utilizing Cell Counting kit (CCK)-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. The amount of the formazan dye generated in cells was directly proportional to the number of living cells. Absorbance of the samples was measured at a wavelength of 450 nm.

**Cell apoptosis assay.** HUVECs were grown in M199 medium and pretreated for 1 h at 37°C with indicated protease inhibitors prior to the addition of 0, 0.05, 0.1, 0.2, 0.4 or 0.6 mM palmitate. Following palmitate treatment for 24 h at 37°C, cells were harvested. A total of 1x10<sup>6</sup> cells were suspended in 200 μl Annexin V binding buffer (BD Biosciences, Franklin Lakes, NJ, USA) and incubated with 2.5 μl annexin V-fluorescein isothiocyanate (FITC) and 2.5 μl propidium iodide (PI; BD Biosciences) for 15 min at room temperature in the dark. Unstained cells and those stained with Annexin V-FITC or PI alone were used as controls to set up compensation and quadrants. Cell apoptosis was investigated by flow cytometry within 30 min after fluorescent labeling by using FlowJo<sup>®</sup> software (version 8.8.7; Tree Star, Inc., Ashland, OR, USA). Results were determined as the percentage of cells that were early (Annexin V<sup>+</sup>PI<sup>-</sup>) or late (Annexin V<sup>+</sup>PI<sup>+</sup>) apoptotic.

**In vitro invasion.** HUVEC invasion across a polycarbonate membrane containing 8-μm pores was performed with

Transwell 24-well plates according to the manufacturer's protocol. Briefly, the membrane was pre-coated with type I collagen (1 mg/ml; Sigma-Aldrich; Merck KGaA). Detached cells (1x10<sup>5</sup> cells per 100 μl in FBS-free M199 containing 0, 0.05, 0.2, 0.4 and 0.6 mM palmitate) were placed in the upper chambers. The lower chamber contained 600 μl of M199 supplemented with 20% FBS and cells were incubated for 16 h at 37°C. Following incubation, cells that remained on the upper surface of the membrane were removed by wiping with a cotton swab, while cells that crossed onto the lower side of the membrane were fixed with 4% paraformaldehyde for 30 min at room temperature and subsequently stained with crystal violet for 20 min at room temperature. The number of cells was counted manually in six random microscope fields by two independent investigators. The experiments were repeated in the presence of 10 μM cathepsin inhibitors for 1 h prior to the addition of palmitate, as described above for cell culture.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted using TRIzol<sup>®</sup> reagent (Sigma-Aldrich; Merck KGaA). cDNA was synthesized from 2 μg total RNA using ReverTra Ace<sup>®</sup> qPCR Reverse Transcriptase kit (cat. no. FSQ-101; Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. qPCR was performed using the SYBR-Green Master mix (cat. no. QPK-212; Toyobo Co., Ltd.) in an ABI 7500HT Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions for PCR were 95°C for 60 sec, followed by 40 cycles at 95°C for 15 sec, 61°C for 15 sec and 72°C for 45 sec. The relative expression level was determined by the 2<sup>-ΔΔC<sub>q</sub></sup> method and normalized against GAPDH (15). The primer sequences used were as follows: Forward, 5'-CGAATCATTGAAGATCCGAGTG-3' and reverse, 5'-ATGGCTTAGAGCCCAATTATGT-3' for cathepsin L; forward, 5-TATTGCCTGATTCTGTGGACT G-3 and reverse, 5-TGATGTACTGGAAAGCCGTTGT-3 for cathepsin S; forward, 5'-GCAGATCGTAGCTGGGGTGAA CT-3' and reverse, 5'-AAGCAAGAAGGAAGGAGGGAG GG-3' for cystatin C; and forward, 5'-TGCACCACCAAC TGCTTAGC-3' and reverse, 5'-GGCATGGACTGTGGTCAT GAG-3' for GAPDH.

**Western blot analysis.** Cells were lysed in ice-cold radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) supplemented with 1 mM phenomethylsulfonyl fluoride (Thermo Fisher Scientific, Inc.). The proteins (20 μg per lane) were separated on a 12% SDS-PAGE gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and electrotransferred onto a polyvinylidene difluoride membrane. Following transfer, blocking was performed with 5% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Membranes were probed with specific primary antibodies at 4°C overnight and horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse immunoglobulin secondary antibodies (1:5,000; cat. nos. SC-2004 and SC-2005; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The primary antibodies used were mouse anti-cathepsin L (1:1,000; cat. no. ab6314; Abcam, Cambridge, UK), rabbit anti-cathepsin S (1:5,000; cat. no. ab50400; Abcam), rabbit anti-cystatin C (1:500; cat.

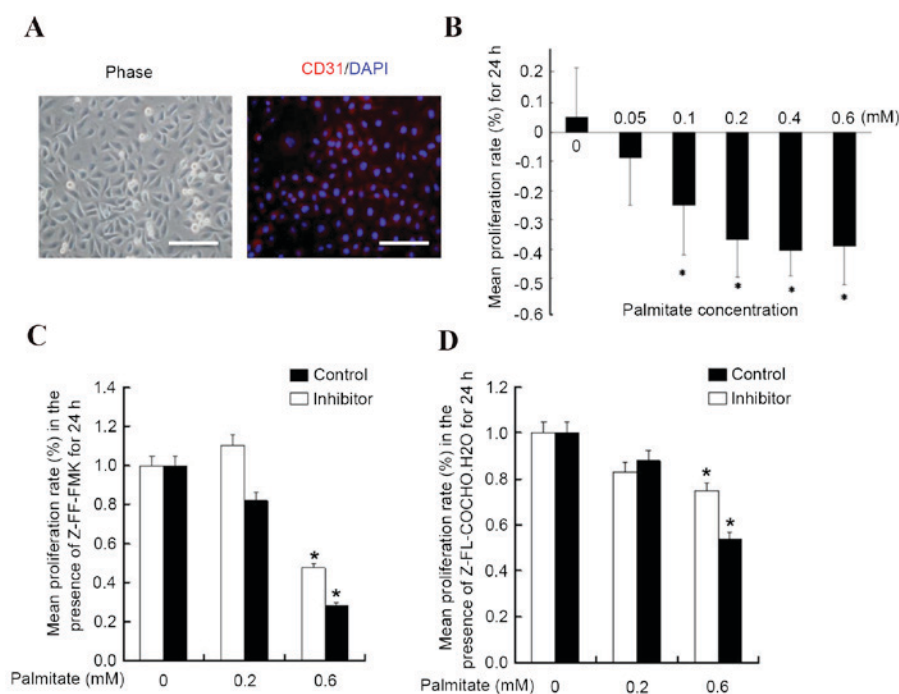


Figure 1. Effect of palmitate and cathepsin inhibitors on cell proliferation. (A) Identification of human umbilical vein endothelial cells using fluorescence microscopy. Cells were treated with an anti-CD31 antibody for 2 h followed by a rhodamine-conjugated secondary antibody, indicated in red. Nuclei were stained with DAPI, indicated in blue. Scale bar=100  $\mu$ M. (B) Cells were suspended in M199/fetal bovine serum medium supplemented with Cell Counting kit-8 solution. Absorbance values were determined at a wavelength of 450 nm. Cells were treated with specific cathepsin inhibitors against (C) cathepsin L and (D) cathepsin S for 1 h prior to exposure to palmitate for 24 h. Data are expressed as the mean  $\pm$  standard deviation from triplicate experiments. \* $P$ <0.001 vs. 0 mM. CD, cluster of differentiation.

no. ab33487; Abcam) and mouse anti-tubulin (1:1,000; cat. no. AT819; Beyotime Institute of Biotechnology, Haimen, China). Enhanced chemiluminescence was performed using the Pro-light HRP Chemiluminescence kit according to the instructions of the manufacturer (Tiangen Biotech Co., Ltd., Beijing, China). Membranes were scanned and visualized by an LAS3000 system (LAS3000 mini ImageReader, Fuji Photo Film Co., Ltd., Japan).

**Cathepsins L and S activity assay.** Cells were lysed and treated with reaction buffer that was included in the kits used for cathepsin L (cat. no. ab65306; Abcam) or cathepsin S (cat. no. ab65307; Abcam), and 10 mM fluorogenic Ac-FR-amino-4-trifluoromethyl coumarin (AFC) substrate, which is the preferred cathepsin L substrate (cat. no. ab65306; Abcam) or 10 mM Ac-VVR-AFC, which is the preferred cathepsin S substrate (cat. no. ab65307; Abcam) according to the manufacturer's protocol. Fluorescence was measured using SpectraMax M5 fluorometer, at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation of at least three independent experiments. Multiple group comparisons were performed by one-way analysis of variance followed by the least significant difference post hoc test. All tests analyzed were two-sided.  $P$ <0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using SPSS software version 11.5 (SPSS, Inc., Chicago, IL, USA).

## Results

**Effect of palmitate and cysteine protease on HUVEC proliferation.** HUVECs express the endothelial marker protein CD31 as previously described (16), and as presented in Fig. 1A. To examine the effect of palmitate on HUVEC proliferation, HUVECs were incubated with different concentrations of palmitate and it was observed that palmitate decreased HUVEC proliferation in a dose-dependent manner (Fig. 1B). High levels of palmitate (0.6 mM) inhibited the proliferation rate significantly by  $44\pm 7\%$  ( $P$ <0.05 vs. 0 mM), whereas the lowest concentration (0.05 mM) had no significant effect ( $P$ =0.09 vs. 0 mM). These results are consistent with a previous report (17). To determine whether cathepsin is associated with HUVEC proliferation in the presence of a high concentration of FFA, cysteine protease inhibitors were added to the concentrations of palmitate. It was observed that treatment with the cathepsin L inhibitor Z-FF-FMK (Fig. 1C) and the cathepsin S inhibitor Z-FL-COCHO (Fig. 1D) did not result in significant alterations of the growth rate ( $P$ >0.05), suggesting palmitate reduced HUVEC proliferation via a cathepsin-independent pathway.

**Cysteine protease inhibitors attenuate palmitate-induced HUVEC apoptosis.** Palmitate may trigger apoptotic pathways in HUVECs (17); therefore, the present study examined whether cathepsin regulated palmitate-induced HUVEC apoptosis (Fig. 2A). As presented in Fig. 2B, cathepsin L and S inhibitors exhibited the ability to reduce the early apoptosis of HUVECs induced by palmitate by almost 50%, suggesting a key cathepsin-dependent pathway in

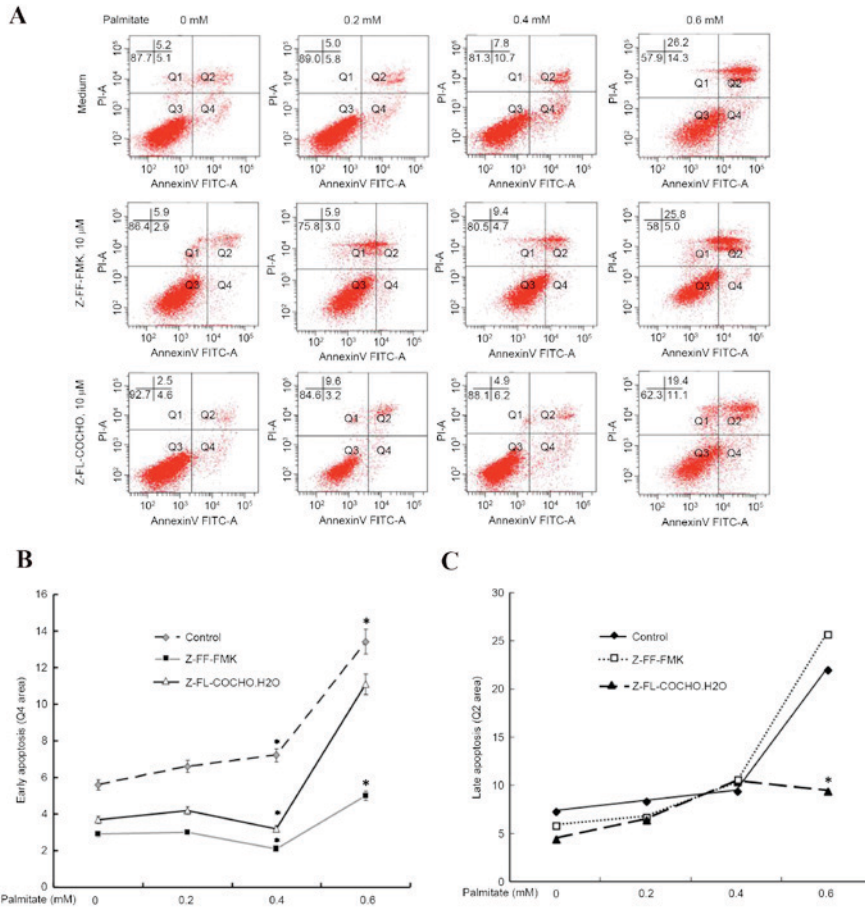


Figure 2. Effect of palmitate and cathepsin inhibitors on cell apoptosis. Cell apoptosis was analyzed by flow cytometry. (A) A total of  $1 \times 10^6$  cells suspended in  $200 \mu\text{l}$  binding buffer containing  $2.5 \mu\text{l}$  annexin V-FITC and  $2.5 \mu\text{l}$  PI were analyzed following incubation in the dark. Percentage of cells in (B) early (lower right quadrant, Annexin V<sup>+</sup>PI<sup>-</sup>) and (C) late (upper right quadrant, annexin V<sup>+</sup>PI<sup>+</sup>) apoptotic stages. Three separate experiments were performed. Data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. 0 mM. FITC, fluorescein isothiocyanate; PI, propidium iodide.

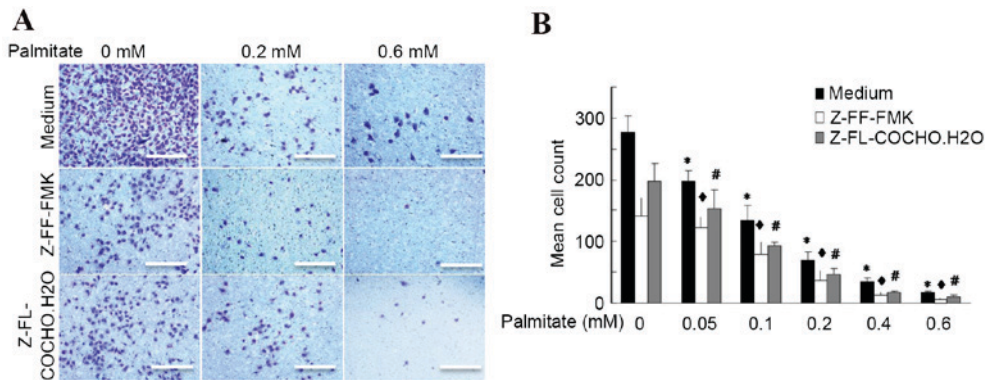


Figure 3. Effect of palmitate and cathepsin inhibitors on cell invasion. (A) Images represent alteration of cell invasion as assessed by a Transwell assay. Scale bar =  $100 \mu\text{m}$ . (B) Analysis of Transwell assay data. Data are expressed as the mean  $\pm$  standard deviation from at least three separate experiments. \* $P < 0.05$  vs. 0 mM palmitate+medium; # $P < 0.05$  vs. 0 mM palmitate+Z-FF-FMK; \* $P < 0.05$  vs. 0 mM palmitate+Z-FL-COCHO.H<sub>2</sub>O.

palmitate-induced apoptosis. Furthermore, cathepsin L inhibitor Z-FF-FMK exhibited the ability to reduce early apoptosis more effectively compared with the cathepsin S inhibitor Z-FL-COCHO. However, late apoptosis appeared to be unaffected by cathepsin inhibitors (Fig. 2C). These results suggested that cathepsin L and S are associated with cell apoptosis as the cathepsin inhibitors protected cells from high FFA level-induced apoptosis.

*Palmitate inhibits cathepsin-mediated cell invasion.* Previous reports have demonstrated that cathepsin L is required for endothelial progenitor cell invasion and neovascularization (3,18); however, whether other cathepsins may influence cell invasion and whether the effects of decreased invasion result from increased FFA levels remains to be elucidated. The present study firstly assessed the effect of palmitate on cell invasion ability using a Transwell assay. It was observed

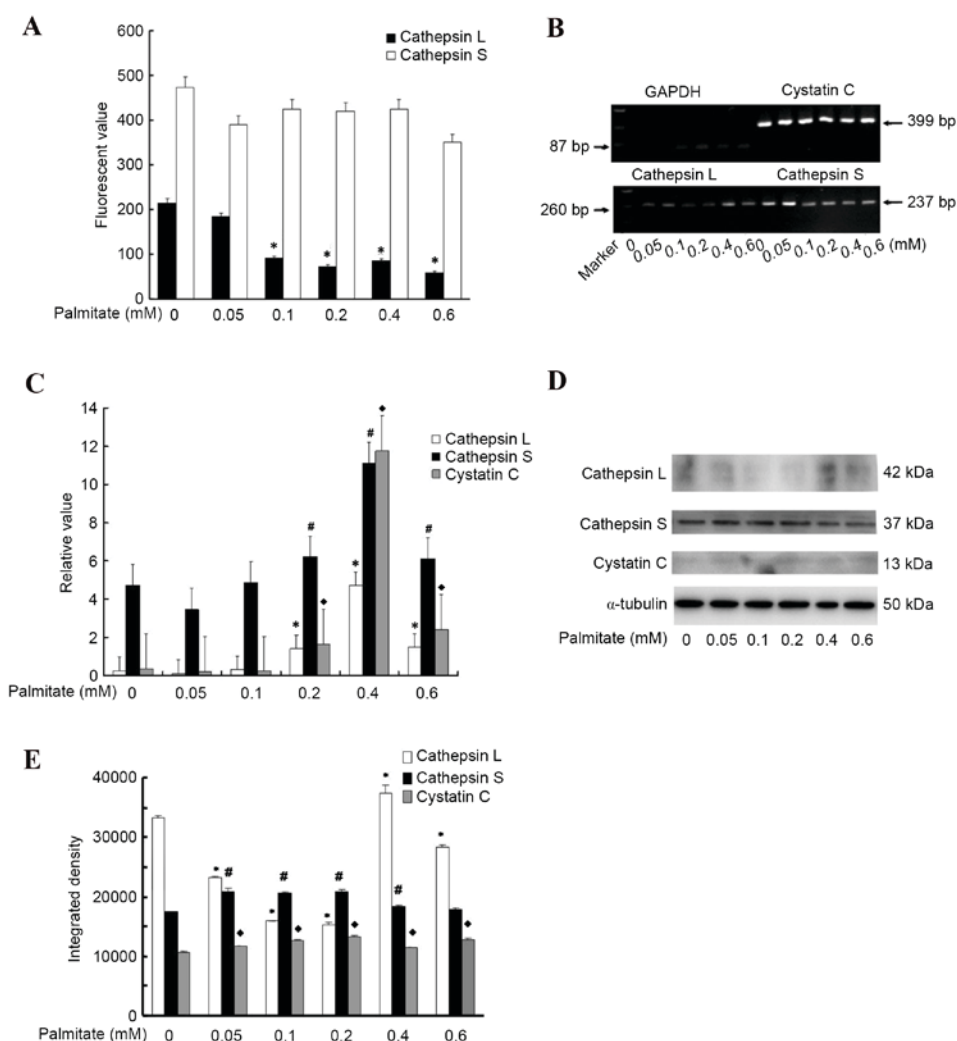


Figure 4. Inhibition of cathepsins S and L by palmitate. (A) Cell lysates were incubated with the fluorogenic substrate detecting cathepsin L or S activity. Fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. \* $P < 0.05$  vs. 0 mM. Cathepsin L and S and cystatin C mRNA expression levels were (B) detected and (C) quantified using reverse transcription-quantitative polymerase chain reaction. Cathepsin L and S and cystatin C protein expression levels were (D) detected and (E) quantified using western blot. Tubulin served as loading control. Blots are representative of at least 3 independent experiments. Data are presented as the mean  $\pm$  standard deviation from triplicate experiments. \* $P < 0.05$  vs. 0 mM palmitate, cathepsin L; # $P < 0.05$  vs. 0 mM palmitate, cathepsin S; \* $P < 0.05$  vs. 0 mM palmitate, cystatin C.

that 0.4 and 0.6 mM palmitate reduced endothelial cell invasion by 88 and 94%, respectively, compared with untreated cells (Fig. 3), indicating that palmitate inhibits cell invasion in a dose-dependent manner. Furthermore, cathepsin S and L inhibitors significantly reduced the cell migration by almost 2-fold, even in the presence of a high level of palmitate (Fig. 3). These findings indicated that cathepsins contribute to cell migration and invasion and palmitate is a potential factor that may reduce cathepsin-mediated cell invasion.

*Palmitate suppresses the activity of cathepsins L and S.* To investigate the underlying mechanism of cathepsins L and S reduction in response to high FFA levels, the present study measured the effect of high palmitate levels on the mRNA and protein expression levels, and activities of cathepsin L and S in HUVECs. The protease activity experiment demonstrated that cells incubated in palmitate for 24 h presented a markedly attenuated cathepsin L activity ( $P < 0.05$ ; Fig. 4A) compared with those cells cultured in medium without palmitate. The

greatest palmitate level decreased the activity of cathepsin L by almost 3.6 fold (Fig. 4A). In addition, the high palmitate level attenuated cathepsin S activity; however, this alteration was not significant (Fig. 4A). To further analyze the effect of high palmitate levels on protease expression, the present study detected the mRNA expression levels of cathepsin L, S and their natural inhibitor cystatin C, and observed that palmitate an increased expression levels in a dose-dependent manner (Fig. 4B and C), suggesting a regulatory interaction between palmitate and cathepsins or their endogenous inhibitor. Consistent with the mRNA expression, cathepsin L protein level was upregulated by high palmitate levels at 0.4 mM but was downregulated by 0.6 mM palmitate (Fig. 4D and E), thus, high palmitate levels ( $> 0.4$  mM) may have a role in inhibiting cathepsin L activity. However, the protein expression levels of cathepsin S and cystatin C appeared to vary only slightly with different palmitate concentrations, the differences between the mRNA and protein results may be a result of posttranscriptional modifications.

## Discussion

The present study demonstrated that exposure of cultured HUVECs to palmitate led to impairment of cell proliferation and invasion, and increased cell apoptosis, and that the function of palmitate is associated with cathepsin L and S. Further experiments demonstrated that palmitate inhibited the activity of cathepsin L and S in HUVECs.

The effects of FFA on cell proliferation, apoptosis and invasion have previously been investigated; Listenberger *et al* (10) observed that saturated fatty acids increase nicotinamide adenine dinucleotide phosphate oxidase-generated oxidant stress, nuclear factor- $\kappa$ B activation and ceramide accumulation. The latter, which may be synthesized *de novo* from saturated fatty acids and the amino acid serine, has been implicated in apoptosis and oxidative stress modulation (19-21). Consistent with these reports, high concentrations of saturated fatty acids may trigger apoptosis and inhibit cell cycle progression in cultured human endothelial cell monolayers (22-24). In addition, trophoblast cells treated with long-chain fatty acids revealed increased lipid droplet deposition, severe mitochondrial damage and significantly decreased invasion (25). The present study demonstrated that the FFA palmitate exhibits similar effects on HUVECs.

Cysteine proteases contribute to angiogenesis under pathophysiological conditions. Cathepsins were originally identified as members of the cysteine protease family localized in lysosomes. However, previous data revealed unexpected roles for cathepsins in pathological conditions including cell survival, metabolic disorder and atherosclerosis-based cardiovascular disease (26-31). However, the definite underlying molecular mechanism remains to be fully elucidated. A variety of cathepsins (B, D, L, S and K) have been demonstrated to cleave the pro-apoptotic B-cell lymphoma-2 (Bcl-2) family member BH3 interacting domain death agonist (Bid) into its potent pro-apoptotic tBid fragment *in vitro* (32,33). Translocation of these pro-apoptotic fragments to the mitochondrial outer membrane may induce the release of apoptogenic factors including cytochrome c and subsequent activation of downstream caspases. In addition to Bcl-2 family members, caspases may be appropriate candidates as cathepsin substrates (34-39). It was therefore hypothesized that cysteine proteases contribute to the regulation of apoptotic signaling mechanisms. Consistent with this, the present study observed that cathepsin inhibitors protected cells from high FFA level-induced apoptosis. In addition, it has previously been demonstrated that intracellular and extracellular cathepsin S, L and K activities were reduced in non-invasive compared with highly invasive cells (40), and downregulation of cathepsins L and S led to defective endothelial cell invasion and suppression of proliferation (41,42), indicating that cathepsins are important for angiogenesis. Therefore, cathepsins S and L are required for endothelial progenitor cell-induced neovascularization in response to ischemic stress (3,43).

In conclusion, the results of the present study, in conjunction with previous findings, support the anti-angiogenic action of FFA. The results suggested that the cathepsin-dependent effects should be considered when developing novel therapies during human endothelial cell angiogenesis. These findings

suggested a novel approach to pharmacological and genetic rescue strategies in FFA-mediated angiogenesis.

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