

Palmitic acid increases HCK gene and protein expression levels in vascular smooth muscle cells

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Research Article

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Abstract

Background and Aims: Saturated fatty acids are known to involve in atherosclerosis through different biologic pathways. The aim of this study was to investigate the effects of palmitic acid (PA) on the tyrosine-protein kinase (HCK) gene and protein expression levels in vascular smooth muscle cells (VSMCs).

Methods: The human Vascular Smooth Muscle Cells (VSMC) were treated with palmitic acid (0.5 mM, 24 hours) based on the cellular viability studies. The HCK gene and protein expression levels were measured by real-time qRT-PCR and western blotting techniques, respectively. Oil Red O staining method was used to determine the intracellular lipid values.

Results: The HCK gene expression level was increased significantly in the PA-treated VSMCs ($p=0.02$). The total and phosphorylated HCK (p-HCK) protein expression levels increased in VSMCs. There was a significant increase in p-HCK value ($P=0.001$).

Conclusion: The results showed that the palmitic acid increases intracellular p-HCK value so that it may affect the HCK-mediated pathways in VSMCs.

Introduction

Over the past decades, obesity was considered as a potential risk factor for cardiovascular diseases through different mechanisms involved in adipokines, inflammation, oxidative stress, and vessel cellular dysfunction (1, 2). On considering the lifestyle, the atherogenic effects may improve due to the intake of fatty acids. Some reports have shown that palmitic acid develops the risk of cardiovascular diseases (3-5). In another study, the serum palmitic acid-related phospholipid fraction was reported to involve with myocardial infarction (6). Many studies reported that cardiovascular events relate to the cellular biological pathways involved with lipid homeostasis (1, 7). It is well known that endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are involved in the formation of atherosclerotic plaques. Regarding some hypotheses, the contractile VSMCs are engaged through phenotypic alteration in order to organize the extracellular matrix (ECM) and to stabilize atherosclerotic plaques (8). Furthermore, VSMC proliferation plays an important role in vessel stenosis and restenosis (9).

The SRC family (SRC, YES, FYN, LCK, FGR, BLK, LYN, YRK, and HCK), known as non-receptor protein tyrosine kinase (SFK), expresses predominantly in mammalian cells. Considering expression patterns, the SRC, YES, and FYN have ubiquitously expressed in all tissues and cells. Furthermore, the HCK is reported to relate to many cancers such as leukemia, pancreatic, breast, ovarian, prostate, colon, and kidney. This protein triggers cellular proliferation and has a key role in cellular migration via the remodeling of the extracellular matrix. Given its role, HCK, therefore, can be a therapeutic target (10-13).

Based on the known roles of palmitic acid in the development of cardiovascular diseases (CVD) (14), the aim of this study was to investigate the effects of palmitic acid on the HCK gene and protein expression

levels in vascular smooth muscle cells.

Materials And Methods

2.1. Cell culture

The human Vascular Smooth Muscle Cells (VSMC C591) were purchased from Pasteur Institute (Tehran, Iran). This cell line had been established from the aorta of an 11 months old child. The cells were cultured in DMEM F-12 enriched with fetal bovine serum (FBS) 10% and Penicillin-Streptomycin 1% using humidified incubator (CO₂, 5%; T, 37°C). In this study, the cultured cells were divided into two groups including the control and treated groups which were grown in DMEM-F12 containing ethanol 0.1% (as the palmitic acid vehicle) and DMEM-F12 containing palmitic acid, respectively.

2.2. Cell viability assay

The effect of palmitic acid on the cell viability was carried out by microculture tetrazolium (MTT) colorimetric assay. Initially, the cells were grown in 96-well plate and were treated with different concentrations of palmitate (0, 0.1, 0.5, 1, and 5 mM). After 24 and 48 hours, the cellular media were removed and the cells were re-incubated for 2 hours in the presence of MTT solution (200 µl, 0.5 mg/ml). Then, the produced formazan crystals were dissolved in DMSO (150 µl for 4 hours) and light absorbance was measured at 570 nm using a microplate reader.

2.3. Treatment

Palmitic acid was dissolved in ethanol (0.1%), and was added to cultured cells (Confluency, 70%; Concentration, 0.5 mM) for 24 hours. Then, the treated cells were harvested to evaluate the gene and protein expression levels.

2.4. Gene Selection

Based on the inflammatory events, a protein complex (containing FGR, HCK, and LYN, SRC) was considered to have a central role in the chemokine signaling pathway (KEGG, hsa04062). The gene data related to this protein complex were improved on the reactome server (<https://reactome.org>) and were the subjects on the primary network in string (<https://string-db.org>). Then, the protein network was trimmed on the string score (more than 0.9) and was enriched with gene ontology and pathway data. The HCK gene was considered as a high-edge node with Phosphotyrosine residue binding function involved in the CB2 pathway (Figure 1).

2.5. RNA extraction, cDNA synthesis, and RT-qPCR techniques

Total RNA was isolated from VSMCs using the GeneAll-Hybrid-R purification kit (Cat. No. 305-101, GeneAll Biotechnology, Seoul, Korea). The quality and quantity of extracted RNA were evaluated using electrophoresis and Nanodrop 2000, respectively. The cDNA was synthesized using Kit (Cat. No. BR631-

050, BioFACT 2 Step 2X RT-PCR Pre-Mix (Taq), Seoul, Korea). Gene expression quantitative analysis was performed by Applied Biosystems 7500 (Foster City, CA, USA) using Power SYBR Green PCR Master Mix Kit (Cat. No. A190303, Amplicon Denmark). The HCK primers were designed by primer blast (5'-CTCTTTGTCCGTGCGAGACT-3', 5'-CCGTCGTTCCCCTTCTTGTA-3'). GAPDH, as a housekeeping gene, was used to normalize the HCK gene expression level (Forward Primer: 5'-CATGAGAAGTATGACAACAGCCT-3', Reverse Primer: 5'-AGTCCTTCCACGATACCAAAGT-3').

2.6. Western Blotting technique

Total protein was isolated using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. Total protein concentration was measured by micro-lowry Kit (TP0200-1KT, Sigma, USA). The protein fractions were separated on 10% SDS-polyacrylamide gel (samples (11 µl), were loaded and run on the stacking gel (80 V, 30 minutes) followed by higher voltage (110 V) on separating gel (60 minutes) and, then were transferred on a polyvinylidene difluoride (PVDF) membrane (IPVH00010, Merck Millipore, Darmstadt, Germany; 80 V, 60 minutes). After blocking the PVDF membrane using milk blocking solution (5% milk, 60 minutes, room temperature), it was incubated separately with primary antibodies of HCK (E-AB-10359, Elabscience, China), p-Hck (phospho Y522; ab192578, abcam, United Kingdom), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-32233, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. Then, it was followed by the incubation with horseradish peroxidase-conjugated secondary antibody (sc-516102, Santa Cruz Biotechnology, CA, USA; 60 minutes, room temperature). Finally, the protein bands were observed by enhanced chemiluminescence (ECL) reagent (RPN2235, Amersham, Italy) and were analyzed by ImageJ software (version 1.51, NIH).

2.7. Oil Red O staining technique

The intracellular lipid was evaluated using the Oil Red O staining method. Briefly, the cells were washed in phosphate-buffered saline (PBS) and were fixed in formalin 4% at room temperature for 45 minutes. Then, the cells were washed with isopropanol 60% for 3 minutes. After adding the Oil Red solution (0.1 %, Cat. No. 1320-06-5, Sigma-Aldrich, USA), the cells were incubated at room temperature for 30 minutes. Finally, the cells were rinsed with water (3 times) and were observed by model IX71 microscope.

2.8. Statistical Analysis

Graph Pad Prism statistical software (v 8.3.0.538, Graphpad, USA) was applied for the data analyses. The differences between groups were determined by using independent samples t-student and Mann-Whitney tests. The IC50 was calculated in a dose-response way. A p-value less than 5% was considered to be significant.

Results

3.1. Viability and Cytotoxicity

The results showed that the IC50 values are estimated at 0.32 mM and 0.52 mM in VSMCs treated with palmitate in the periods of 48 and 24 hours, respectively (Figure 2).

3.2. HCK gene expression level

The HCK gene expression level increased significantly up to 3.2 times in the treated cells with palmitate as compared with the control group ($p=0.0286$) (Figure 3, B).

3.3. HCK protein expression level

The total HCK protein expression level was not significant ($p= 0.8139$) (Figure 3. A, C) but the phosphorylated HCK value increased significantly in the treated VSMCs with palmitate ($p= 0.0012$) (Figure 3. A, D).

3.4. p-HCK/total HCK ratio

The p-HCK/HCK ratio in the palmitate-treated cell group increased significantly as compared to the control ($P=0.001$) (Figure 3, E).

3.5. Intracellular lipid Intensity

As shown in Figure 4, intracellular lipid intensity (red color) was highly observed in the palmitate-treated VSMCs which were (0.5mM, 24 hours) as compared with control.

Discussion

Palmitic acid has endogenous and exogenous sources in the body. This saturated fatty acid makes up about 44% of palm oil, 65% of butter, 53% of tallow, 15% of soybean, 13% of corn oil, and 17% of olive oil (9). It is also known as an atherogenic agent in the improvement of cardiovascular diseases (15). Thus, the role of palmitic acid in the function of VSMCs (16) may be interesting.

In this study, the HCK gene and protein expression levels were studied in the palmitate-treated VSMCs since the HCK is contributed in central complex involved in some cellular signaling pathways. Some studies reported that free fatty acids can proliferate VSMCs (17, 18) resulted in the development of atherosclerosis process in the rat and human (19, 20). It is reported that PA causes cardiomyocyte hypertrophy (21) and develops the cellular apoptosis (22-25). Furthermore, it developed melanoma by involving cellular proliferative pathways via the phosphorylation of Akt (26). The promotion of cancer invasiveness was also suggested by the activation of TLR4/ROS/NF κ B/MMP9-related signaling pathways (27). It is suggested that PA modifies the protein sequences by phosphorylation of tyrosine residues (28). On the confirmation of the above studies, this study showed that PA increases the HCK

gene expression level improving the cellular HCK-mediated pathways. Furthermore, the p-HCK value increased in PA-treated VSMCs on the role of PA in the modification of amino acid residues. However, the HCK protein expression level increased in PA-treated VSMCs but it was not significant as compared to control. We suggested that the activation of HCK phosphorylation (p-HCK) may diminish the total HCK synthesis. On the other hand, PA may affect the HCK-mediated signaling pathways by the phosphorylation of HCK in a fast-response way. Since other studies reported that HCK elevates via the function of BCR/ABL, TEL/ABL, EGFR /PDGFR, and PI3K/Akt (29) thus it is an impotent factor in the activation of the cellular proliferation and adhesive pathways. Moreover, HCK may co-expresses with other genes in cellular pathways such as apoptosis (30).

Conclusion

This study showed that palmitic acid increases the total p-HCK/HCK ratio in VSMCs. Since HCK contributes to the intracellular SRC complex thus it mediates some cellular signaling pathways via the inflammatory stimulators and down-regulatory factors. We suggested that the role of HCK in PA-treated VSMCs can more be evaluated by the combined effects with chemokines and the changes of downstream proteins in HCK-mediated pathways.

Declarations

Ethics approval and consent to participate: It was approved by Committee on the Ethics of IUMS.

Consent for publication: No

Availability of data and material: It is presented on the request from the corresponding author.

Competing interests: No

Funding: This work was supported by IUMS (No. 14467).

Authors' contributions: M.N and G.G designed the study; G,G, F.Z.G and A.M evaluated the gene and protein expression levels. M.N and G.G analyzed the data.

Acknowledgements: No.

Abbreviations

Phosphorylated HCK; p-HCK, Vascular smooth muscle cells; VSMCs, Endothelial cells; ECs, Extracellular matrix; ECM, Palmitic acid; PA, Cardiovascular diseases; CVD

References

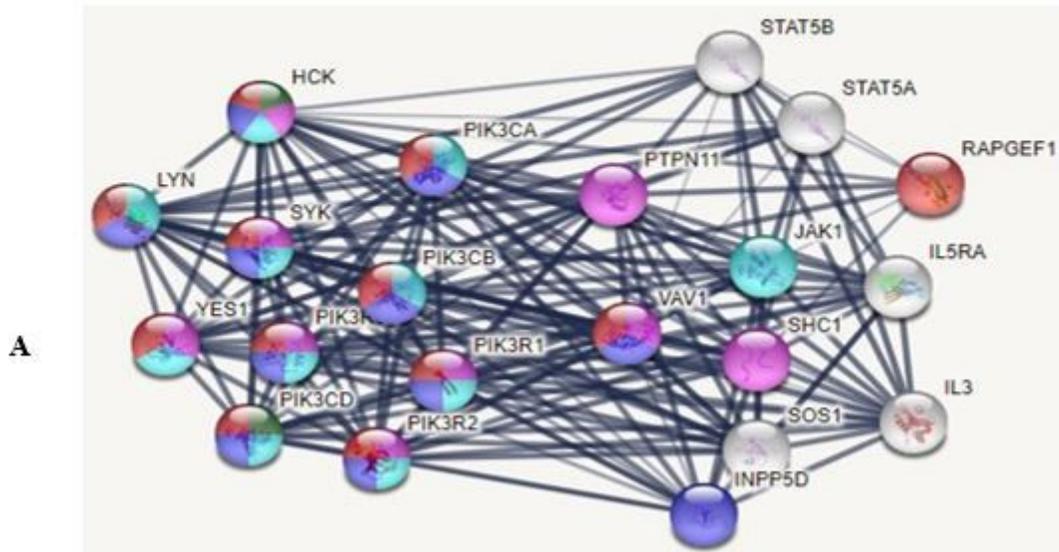
1. Lovren F, Teoh H, Verma S. Obesity and atherosclerosis: mechanistic insights. *Canadian Journal of Cardiology*. 2015;31(2):177-83.
2. Bosello O, Donataccio MP, Cuzzolaro M. Obesity or obesities? Controversies on the association between body mass index and premature mortality. *Eating and Weight Disorders-Studies on Anorexia, Bulimia and Obesity*. 2016;21(2):165-74.
3. Afonso MS, Lavrador MSF, Koike MK, Cintra DE, Ferreira FD, Nunes VS, et al. Dietary interesterified fat enriched with palmitic acid induces atherosclerosis by impairing macrophage cholesterol efflux and eliciting inflammation. *The Journal of nutritional biochemistry*. 2016;32:91-100.
4. Brown E. *Cruel Oil. How palm oil harms health, rainforest and wildlife*. Center of Science in the Public Interest; 2005.
5. Kabagambe EK, Baylin A, Ascherio A, Campos H. The type of oil used for cooking is associated with the risk of nonfatal acute myocardial infarction in Costa Rica. *The Journal of nutrition*. 2005;135(11):2674-9.
6. Siniarski A, Rostoff P, Rychlak R, Krawczyk K, Gołębiowska-Wiatrak R, Mostowik M, et al. Unsaturated fatty acid composition in serum phospholipids in patients in the acute phase of myocardial infarction. *Kardiologia polska*. 2019;77(10):935-43.
7. Jin J-L, Guo Y-L, Li J-J. Plasma free fatty acids in relation with the severity of coronary artery disease in non-diabetics: a Gensini score assessment. *IJC Metabolic & Endocrine*. 2017;14:48-52.
8. Basatemur GL, Jørgensen HF, Clarke MC, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. *Nature reviews cardiology*. 2019:1-18.
9. Fatima S, Hu X, Gong R-H, Huang C, Chen M, Wong HLX, et al. Palmitic acid is an intracellular signaling molecule involved in disease development. *Cellular and Molecular Life Sciences*. 2019:1-11.
10. Poh AR, O'Donoghue RJ, Ernst M. Hematopoietic cell kinase (HCK) as a therapeutic target in immune and cancer cells. *Oncotarget*. 2015;6(18):15752.
11. Roseweir AK, Powell AG, Horstman SL, Inthagard J, Park JH, McMillan DC, et al. Src family kinases, HCK and FGR, associate with local inflammation and tumour progression in colorectal cancer. *Cellular signalling*. 2019;56:15-22.
12. DA JDFFB, Sant'Anna CDC, Muniz JAPC, DA ROCHA CAM, Lamarao LM, NUNES CDFAM, et al. Deregulation of the SRC family tyrosine kinases in gastric carcinogenesis in non-human primates. *Anticancer research*. 2018;38(11):6317-20.
13. Ghasempour G, Mahabadi VP, Shabani M, Mohammadi A, Zamani-Garmsiri F, Amirfarhangi A, et al. miR-181b and miR-204 suppress the VSMC proliferation and migration by downregulation of HCK.

Microvascular Research. 2021;136:104172.

14. Ebbesson SO, Voruganti VS, Higgins PB, Fabsitz RR, Ebbesson LO, Laston S, et al. Fatty acids linked to cardiovascular mortality are associated with risk factors. *International journal of circumpolar health*. 2015;74(1):28055.
15. Zdychova J, Cejkova S, Lesna IK, Kralova A, Maluskova J, Janousek L, et al. Co-cultivation of human aortic smooth muscle cells with epicardial adipocytes affects their proliferation rate. *Physiological research*. 2014;63:S419.
16. Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. *Nature Reviews Cardiology*. 2009;6(6):399.
17. Lamers D, Schlich R, Greulich S, Sasson S, Sell H, Eckel J. Oleic acid and adipokines synergize in inducing proliferation and inflammatory signalling in human vascular smooth muscle cells. *Journal of cellular and molecular medicine*. 2011;15(5):1177-88.
18. Lu G, Morinelli TA, Meier KE, Rosenzweig SA, Egan BM. Oleic acid–induced mitogenic signaling in vascular smooth muscle cells: a role for protein kinase C. *Circulation research*. 1996;79(3):611-9.
19. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362(6423):801-9.
20. Raman P, Madhavpeddi L, Gonzales RJ. Palmitate induces glycosylation of cyclooxygenase-2 in primary human vascular smooth muscle cells. *American Journal of Physiology-Cell Physiology*. 2018;314(5):C545-C53.
21. Chen YP, Kuo WW, Baskaran R, Day CH, Chen RJ, Wen SY, et al. Acute hypoxic preconditioning prevents palmitic acid-induced cardiomyocyte apoptosis via switching metabolic GLUT4-glucose pathway back to CD36-fatty acid dependent. *Journal of cellular biochemistry*. 2018;119(4):3363-72.
22. Chen Z, Lei L, Wen D, et al. Melatonin attenuates palmitic acid-induced mouse granulosa cells apoptosis via endoplasmic reticulum stress. *Journal of ovarian research*, 2019, 12(1): 43.
23. Ma B, Guan G, Lv Q, et al. Curcumin Ameliorates Palmitic Acid-Induced Saos-2 Cell Apoptosis Via Inhibiting Oxidative Stress and Autophagy. *Evidence-Based Complementary and Alternative Medicine*, 2021, 2021.
24. Yang L, Guan G, Lei L, et al. Oxidative and endoplasmic reticulum stresses are involved in palmitic acid-induced H9c2 cell apoptosis. *Bioscience reports*, 2019, 39(5): BSR20190225.
25. Chen Z, Wen D, Wang F, et al. Curcumin protects against palmitic acid-induced apoptosis via the inhibition of endoplasmic reticulum stress in testicular Leydig cells. *Reproductive Biology and Endocrinology*, 2019, 17(1): 1-10.

26. Röhrig F, Schulze A. The multifaceted roles of fatty acid synthesis in cancer. *Nature Reviews Cancer*. 2016;16(11):732.
27. Binker-Cosen MJ, Richards D, Oliver B, Gaisano HY, Binker MG, Cosen-Binker LI. Palmitic acid increases invasiveness of pancreatic cancer cells AsPC-1 through TLR4/ROS/NF- κ B/MMP-9 signaling pathway. *Biochemical and biophysical research communications*. 2017;484(1):152-8.
28. Dittmann A, Kennedy NJ, Soltero NL, Morshed N, Mana MD, Yilmaz ÖH, et al. High-fat diet in a mouse insulin-resistant model induces widespread rewiring of the phosphotyrosine signaling network. *Molecular Systems Biology*. 2019;15(8).
29. Suh H-S, Kim M-O, Lee SC. Inhibition of granulocyte-macrophage colony-stimulating factor signaling and microglial proliferation by anti-CD45RO: role of Hck tyrosine kinase and phosphatidylinositol 3-kinase/Akt. *The Journal of Immunology*. 2005;174(5):2712-9.
30. Shivakrupa R, Radha V, Sudhakar Ch, Swarup G. Physical and functional interaction between Hck tyrosine kinase and guanine nucleotide exchange factor C3G results in apoptosis, which is independent of C3G catalytic domain. *J Biol Chem*. 2003; 26;278(52):52188-94.

Figures



B

Process	Annotation/Enrichment	Code	Description	P-Value
Gene Ontology	● Molecular Function	GO: 00001784	Phosphotyrosine residue binding	1.63e-17
Gene Ontology	● Biological Process	GO: 00002679	Respiratory burst involved in defense response	0.00011
Gene Ontology	● Environment	GO: 00019898	Extrinsic component of membrane	9.12e-14
Pathway	● reactome	HSA912631	Regulation of signaling by CB2	7.76e-28
Pathway	● KEGG	Hsa04466	Fc gamma R-mediated phagocytosis	2.54e-19

Figure 1

Gene Selection. A, Gene network is categorized on the enrichment of nodes with B, Gene ontology and pathway data. The gene data improved on the reactome. Then, the gene network was made and trimmed using string, and was subject for enrichment with gene ontology and pathway data. The HCK considered as a high-edge node and involved in some pathways.

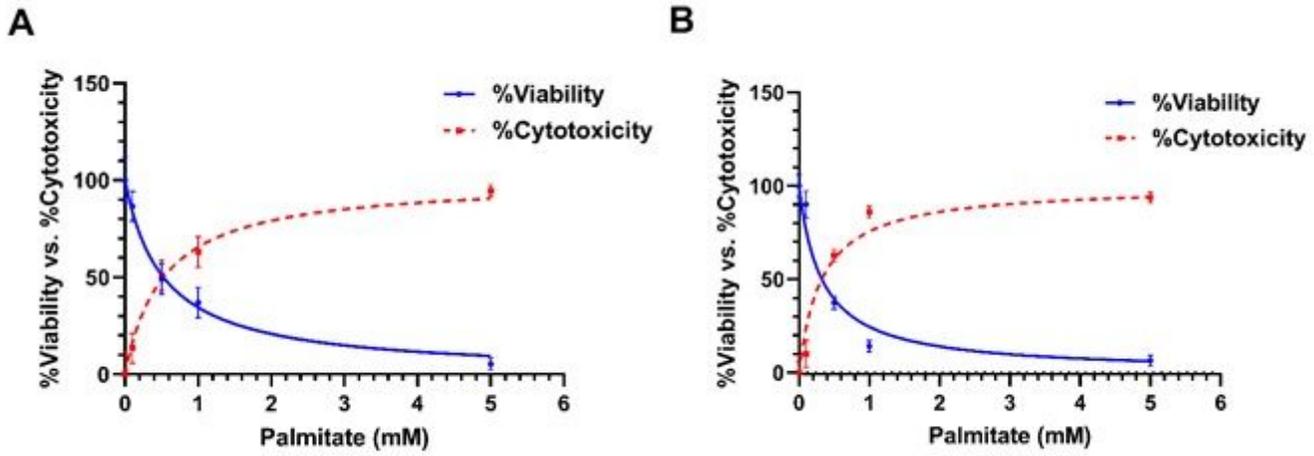


Figure 2

IC50 values in VSMCs treated with palmitic acid. A, 24 hours. B, 48 hours. The cells treated with different concentrations of palmitate (0-5 mM) after 24 and 48 hours. Then, the cell counts evaluated on the basis of MTT technique.

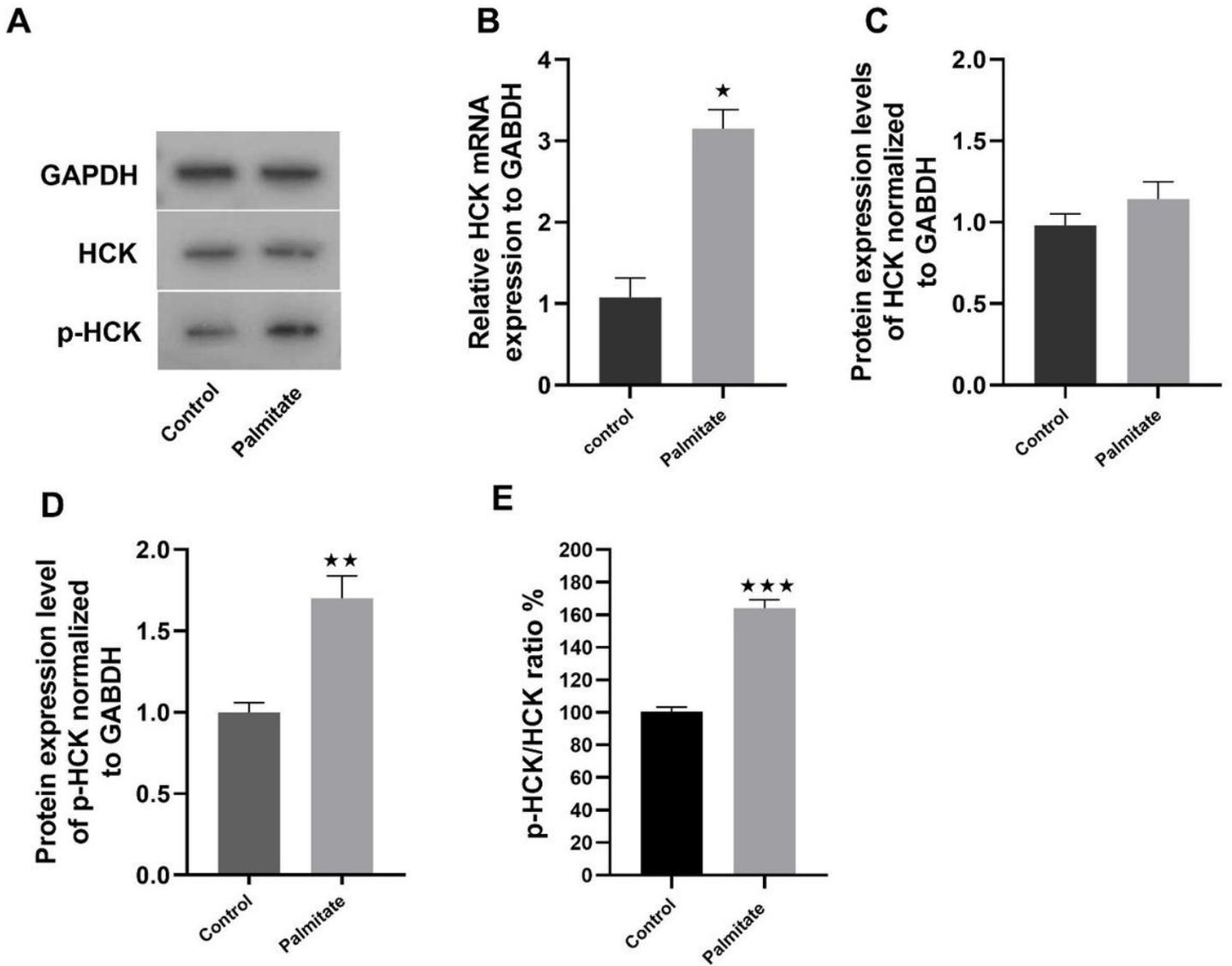


Figure 3

HCK expression assessment. A, Western blotting images. B, HCK gene expression levels. Palmitic acid increased the HCK gene expression level as compared to the control ($p=0.0286$). C, HCK protein expression levels. Although the HCK protein expression level increased in the palmitate-treated group but it was not statistically significant ($p=0.8139$). D, p-HCK gene expression levels. Palmitate increased significantly p-HCK value in the palmitate-treated group ($p=0.0012$). E, p-HCK/total HCK ratio. p-HCK/HCK ratio elevated significantly as compared to control ($p<0.001$). Control group treated with ethanol 0.1% which was used as the palmitic acid vehicle.

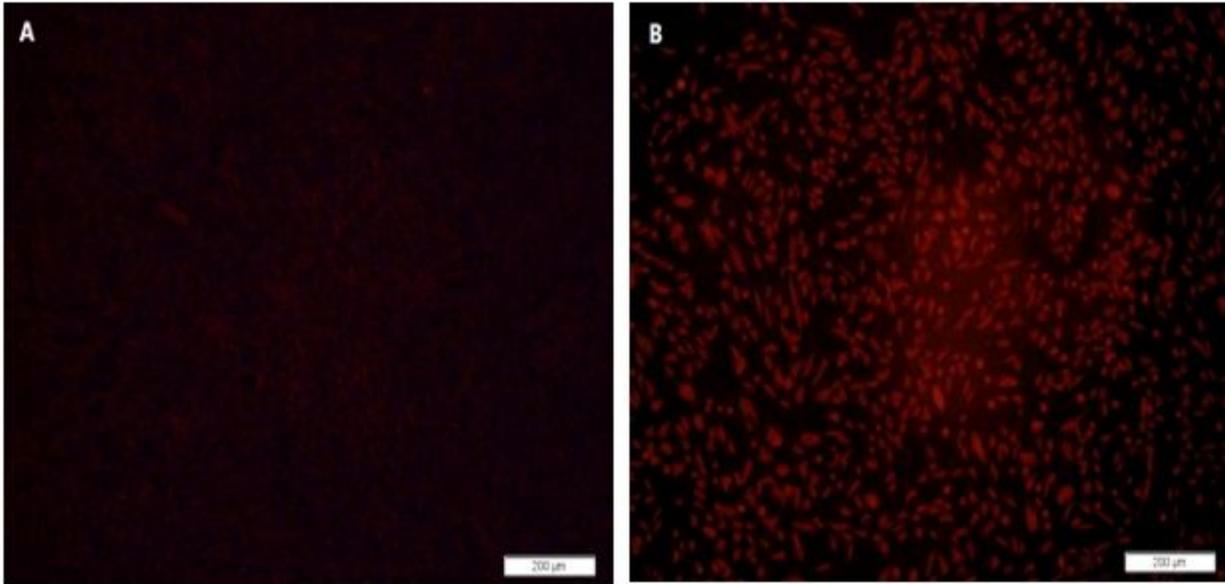


Figure 4

Cellular lipid distribution. A, Control group (treated with ethanol 0.1%). B, Palmitate-treated VSMCs. The cells fixed and incubated with Oil Red solution. Finally, the cells rinsed and observed by microscope. It is well clear that the intensity of intracellular lipid (red color) increased in the cells.