Food Sci. Biotechnol. 26(2): 501-505 (2017)
DOI 10.1007/s10068-017-0069-3

Food Science
and
Biotechnology

Research Note

Comparison of trans-fatty acids on proliferation and migration of vascular smooth muscle cells

Haewon Kim, Chang Hee Jeong, Hong Gu Lee[1], and Sung Gu Han*

Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea
[1]Department of Animal Science and Technology, Konkuk University, Seoul 05029, Korea

Received April 12, 2016
Revised August 26, 2016
Accepted August 30, 2016
Published online April 30, 2017

*Corresponding Author
Tel: +82-2-450-0526
Fax: +82-2-455-1044
E-mail: hansg@konkuk.ac.kr

pISSN 1226-7708
eISSN 2092-6456

Abstract Consumption of trans-fatty acids has been linked to an increased risk of cardiovascular diseases, such as atherosclerosis. Milk and dairy products contain trans-fatty acids, such as trans-vaccenic acid (TVA) and conjugated linoleic acid (CLA). Although artificially hydrogenated trans-fatty acids (e.g., elaidic acid (EA)) are known to induce atherosclerosis, it is unclear whether ruminant trans-fats, such as TVA, are associated with such diseases. Therefore, we investigated the effects of TVA on vascular smooth muscle cells (VSMCs). VSMCs were treated with TVA, CLA, and EA at 0-100 μM for 24 h. Cell proliferation and migration increased upon treatment with EA, not with TVA and CLA. EA increased protein expression of proliferation-associated proteins (cyclin-dependent kinase 4 (CDK4) and cyclin D1), while TVA and CLA decreased CDK4 expression. These results suggest that TVA is not as risky as other trans-fatty acids such as EA in the vascular system.

Keywords: trans-vaccenic acid, elaidic acid, vascular smooth muscle cell, proliferation, migration

Introduction

Cardiovascular diseases, such as atherosclerosis, are the leading cause of death in developed countries. Atherosclerosis is an inflammatory process which involves several stages such as the accumulation of cholesterol ester-filled foam cells, smooth muscle cell migration into the intima, formation of necrotic core and calcification (1). Among these stages of atherosclerosis development, migration and proliferation of vascular smooth muscle cells (VSMCs) is important events occurred during intimal hyperplasia (2). In the intima, macrophages uptake and accumulate oxidized low-density lipoprotein and that results in converting the macrophage to foam cells. Then, migration of VSMC from the media into the intima changes the relatively simple fatty streak to more complex lesion (3). Intimal VSMC can contribute to form fibrous plaques through cell proliferation, absorption of modified lipoproteins and secretion of extracellular matrix proteins (3). In the healthy vasculature, migration and proliferation of VSMCs are tightly regulated by humoral mediators, cell cycle regulators, and cytokines (2). However, in the diseased state, proliferation and migration of VSMCs from the media into the intima of the vessel wall contribute to the pathogenesis of atherosclerosis, such as plaque development and reduction of the vessel lumen (2,4).

Cell cycle is an important factor in cell proliferation and migration. Cell cycle regulators, such as cyclin-dependent kinases (CDKs), are a family of proteins involved in cell cycle regulation; activated CDKs control progression through the cell cycle checkpoints (5). The increased expression of CDKs and cyclin D1 accelerates cell cycle progression (6). Upon stimulation, VSMCs can undergo the G1/S phase transition as they progress through the cell cycle. Cyclin D1/CDK4 and cyclin E/CDK2 complexes can promote the transition from G0/G1 to S phase and maintain cell cycle progression (7).

Dietary or plasma fatty acid (FA) compositions are known to be associated with the occurrence of cardiovascular disease (8). Trans-fatty acids such as *trans*-9 elaidic acid (C18:1 *trans*-9; EA) and *trans*-11 vaccenic acid (C18:1 *trans*-11; TVA) are frequently found in hydrogenated vegetable oils and dairy products, respectively. Intake of trans-fatty acids has been suggested to promote atherosclerotic lesion formation (9); specifically, EA has been associated with increased atheroma lesion development in LDL receptor-deficient mice (9). However, the role of TVA in the vasculature has not been studied extensively. TVA is predominantly found in dairy products (accounts for 60-80% of the total natural ruminant trans-fats), and it is the precursor of conjugated linoleic acid (CLA) (10). It has been suggested that TVA can exert beneficial effects, such as protecting the arteries against atherosclerosis development, in the vascular system of LDL receptor-deficient mice (11). In addition, in contrast to EA, inflammatory responses in VSMCs following treatment with TVA have not been observed (12).

Although TVA is abundant in milk and dairy products, its association

with cardiovascular disease is not completely understood. Therefore, in this study, we examined the effects of TVA on the proliferation and migration of VSMCs, an initial event during atherosclerosis progression. In addition, we investigated the expression of cell cycle regulators, such as cyclin D1, CDK4, and CDK6, to identify the underlying mechanisms.

Materials and Methods

Cell culture and treatments Rat VSMCs, isolated from the aortas of Sprague-Dawley rats, were cultured using DMEM medium (HyClone, Rockford, IL, USA) with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Stock solutions of bovine serum albumin (BSA)-fatty acid complex were prepared by dissolving fatty acids and fatty acid-free BSA (1 mM) in phosphate-buffered saline (PBS), followed by filtration. Cells were treated with TVA (C18:1 *trans*-11), EA (C18:1 *trans*-9) or CLA (C18:2 *cis*-9, *trans*-11) (Sigma-Aldrich, St. Louis, MO, USA) at the final concentrations of 0-100 μM for 24 h. The concentrations of trans-fatty acids were determined based on previous literatures where cells were treated with fatty acids up to 100 μM in cell culture conditions (12). In addition, according to a recent report, plasma concentrations of these trans-fatty acids were 3.7-4.4 μg/mL (13.1-15.6 μM) (13).

Cell proliferation and migration (scratch) assay Cell proliferation was determined using trypan blue dye exclusion test. Cells were seeded in 12-well plates followed by treatment with fatty acids (0, 10, 50, and 100 μM) for 24 h. The number of viable cells was determined by trypan blue dye exclusion and counted manually using a hemocytometer. In addition, cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at concentrations up to 200 μM for 24 h. For cell migration (scratch) assay, cells were grown in 12-well plates up to 90% confluence and treated with fatty acids (0, 10, 50, and 100 μM); three perpendicular wounds were inflicted on the cells using a yellow pipette tip after which they were monitored for 18 h and the widths of the wounds were photographed using an inverted light microscope (40X magnification). To evaluate the scratch widths, four different sites per scratch were monitored.

Cell lysate preparation, SDS-PAGE, and Western blot analysis After the treatments, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor mixture. The cell lysates were centrifuged at 21,000×*g* for 15 min at 4°C. The supernatants were collected and protein concentrations were determined using Bradford assay (Sigma-Aldrich). Protein samples were stored at −80°C until use. For SDS-PAGE, 30 μg of protein was loaded into each well of the gel and electrophoresed for 2 h. After separation, proteins were transferred onto a nitrocellulose membrane using the semi-dry gel transfer system (Bio-Rad, Hercules, CA, USA). The membranes were blocked in buffer containing 2% BSA and incubated overnight at 4°C with primary antibodies. Primary antibodies (CDK4, CDK6, and cyclin D1; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used at a dilution of 1:2,000. After washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, at a dilution of 1:4,000, and visualized using ECL detection reagents (Lugen, Seoul, Korea). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology Inc.), a housekeeping protein, was used as the loading control. Bands were quantified using ImageJ (National Institute of Health, Bethesda, MD, USA) software and normalized to GAPDH.

Statistical analysis Data were expressed as the mean±standard error of the mean (SEM). Statistical significance was determined by one-way or two-way ANOVA, followed by Student-Newman-Keuls method using Sigma Stat 3.1 software (Systat Software Inc., San Jose, CA, USA). A probability value (p-value) of less than 0.05 ($p<0.05$) was considered statistically significant.

Results and Discussion

TVA does not affect the proliferation of VSMCs Proliferation of VSMCs is a major event in the development of necrotic core after immigration of cells from the media to the intima (3). To determine whether TVA can modulate cellular proliferation, VSMCs were treated with fatty acids (TVA, EA, or CLA) at concentrations of 10, 50, or 100 μM for 24 h. For controls, cells were treated with fatty acid-free BSA. Cell proliferation was determined by the trypan blue dye exclusion test. Treatment of cells with TVA and CLA (10-100 μM) did not affect cell proliferation, compared to controls (Fig. 1A). In contrast, treatment of cells with EA (50-100 μM) significantly increased the number of cells, compared to controls (Fig. 1A). Increased proliferation of VSMCs is an important pathological process during the development of atherosclerosis (2). Fatty acids, such as docosahexaenoic acid, can suppress the proliferation of human VSMCs by inhibiting the nuclear translocation of nuclear factor-κB (NF-κB)-p65 in VSMCs (14). On the other hand, increased VSMC proliferation has been observed, following treatment with oleic acid, the most abundant unsaturated fatty acid in plasma (15). However, the proliferative effects of EA on VSMCs have not been reported yet. Thus, our results demonstrated that EA can induce VSMC proliferation, however, this effect was not observed in cells treated with TVA and CLA. In addition, cytotoxicity was determined using MTT assay. Results showed that treatment of fatty acids (TVA, EA, and CLA) did not affect the viability of VSMCs up to 200 μM, suggesting that these fatty acids are not important contributors for cell viability in VSMCs (Fig. 1B). In this study. both trypan blue dye exclusion and MTT tests were used in measuring viability of cells. Our data showed increase of cell proliferation by EA treatments only in trypan blue dye

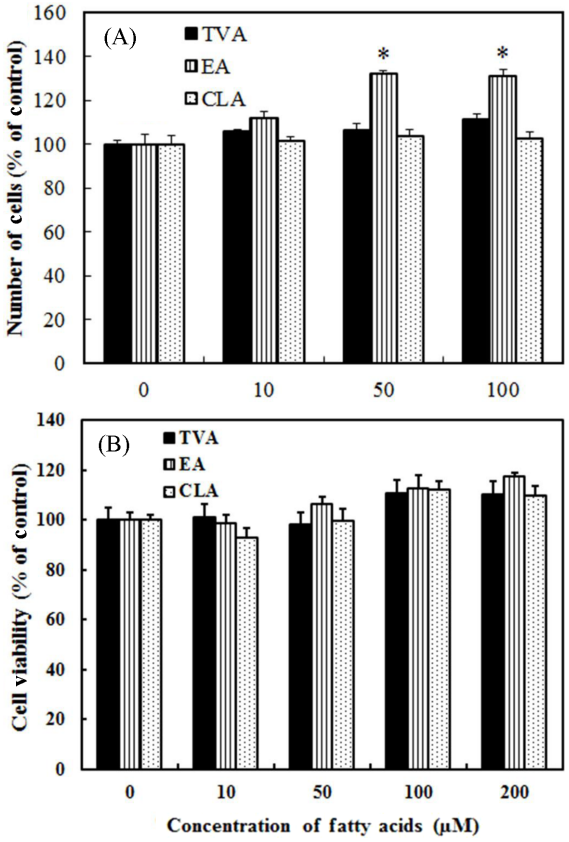

Fig. 1. Effects of fatty acids on proliferation and cytotoxicity in VSMC. (A) Cells were treated with TVA, CLA, and EA at the final concentrations of 0, 10, 50, and 100 μM for 24 h. At the end of the treatment, cells were trypsinized and trypan blue solution was added, to identify live cells. The number of live cells was counted using a hemocytometer. Asterisk indicates a significant difference compared to corresponding control (p<0.05). (B) Cytotoxicity of fatty acids were determined using MTT assay. Cells were treated with TVA, CLA, and EA at the final concentrations of 0, 10, 50, 100, and 200 μM for 24 h. Experiments were repeated three times. Data represent the mean±SEM.

exclusion test. This is probably because live cells are manually counted in trypan blue dye exclusion test while living cell's mitochondrial activity is measured in MTT assay. Thus, it is generally considered that trypan blue dye exclusion test is more accurate in measuring live cells. According to a recent report, some chemicals can react with MTT or succinate dehydrogenase which may result in inaccuracy of measuring live cells (16).

TVA does not affect the migration of VSMCs Migration of VSMCs is an early and critical event in the development of atherosclerosis (4). To determine whether TVA can modulate VSMC migration, cells were treated with fatty acids (TVA, EA, or CLA) at the concentrations of 10, 50, or 100 μM for 24 h. Figure 2 is a representative image of VSMC migration. Treating VSMCs with TVA and CLA (10-100 μM) did not affect their migration, whereas treating them with EA (10-100 μM) markedly increased their migration, compared to that of controls (Fig. 2). Little is known about the effects of fatty acids on VSMC migration. Docosahexaenoic acid significantly reduces VSMC migration induced by interleukin-1β (17); in contrast, oleic acid stimulates VSMC migration (18). These results indicate that the effects on VSMC migration is dependent on the type of fatty acids, and our results demonstrate that EA, not TVA and CLA, can stimulate VSMC migration, which may contribute to the development of intimal hyperplasia and atherosclerosis following consumption of hydrogenated vegetable oil.

Protein expression of CDK4 and cyclin D1 is increased with EA but not with TVA In order to investigate the underlying intracellular mechanisms responsible for the observed proliferation and migration due to the trans-fatty acids treatments, VSMCs were treated with the three types of trans-fatty acids. Proliferation and migration of VSMCs is regulated by several different factors, including cell cycle-related molecules. For example, a cell cycle inhibitor, p21WAF1 is known to

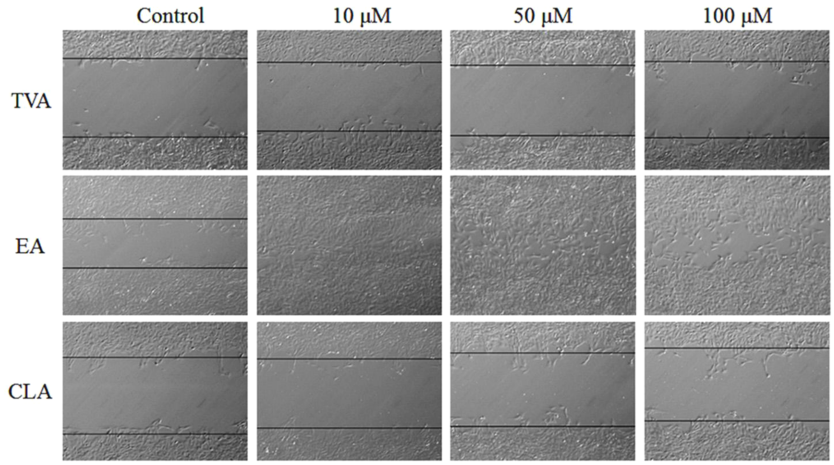

Fig. 2. Effects of fatty acids on VSMC migration. Cells were treated with TVA, CLA, and EA (0, 10, 50, and 100 μM), and three perpendicular wounds were inflicted on them using a pipette tip, following which they were grown for 18 h. The widths of the wounds were photographed using an inverted microscope (40X magnification).

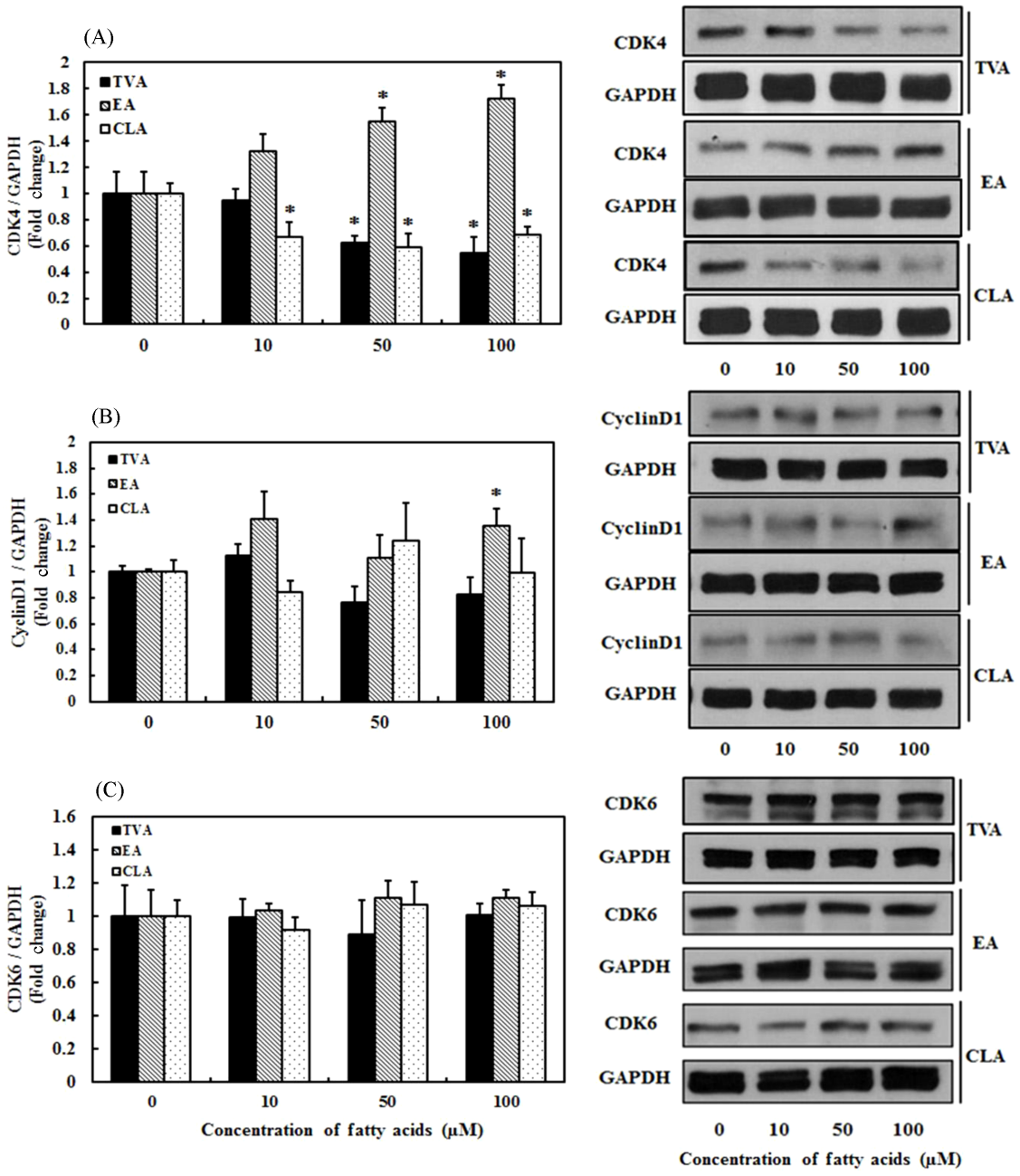

Fig. 3. Effects of fatty acids on the expression of CDK4, Cyclin D1 and CDK6. Protein expression of (A) CDK4, (B) Cyclin D1, and (C) CDK6 was measured in VSMCs treated with fatty acids (TVA, EA, and CLA; 0, 10, 50, and 100 μM) for 24 h. Whole cell lysates were used to measure the expression of proteins. Densitometry results were normalized to GAPDH. Results represent the mean±SE, with n=3. The Western blot picture shown is a representative of three independent experiments. Asterisk indicates a significant difference compared to corresponding control (p<0.05).

be associated with the inhibition of VSMC proliferation (19). Normal cell proliferation depends on the tightly regulated activation of cyclin/cyclin-dependent kinase complexes. Cyclin D1, CDK4, and CDK6 are among the central mediators of the G1/S phase transition (20). In this study, since EA affected the proliferation and migration of VSMCs, we evaluated the protein expression of cyclin D1, CDK4, and CDK6 using Western blot analysis. Protein expression levels of CDKs has been measured as a marker of cell cycle progression in smooth muscle cells (21). The protein expression of CDK4 was significantly decreased in cells treated with TVA at 50-100 μM, compared to that in the controls (Fig. 3A). CLA also significantly decreased the expression of CDK4 at the concentrations of 10-100 μM (Fig. 3A). However, CDK4 expression was markedly increased in cells treated with EA at the concentrations of 50-100 μM (Fig. 3A). Expression of cyclin D1 protein was significantly increased EA at 100 μM, but both TVA and CLA did not affect the protein expression at all the

concentrations tested (Fig. 3B). CDK4/cyclin D1 complex predominantly promotes G1/S transition of the cell cycle and their protein expression is a critical regulator of cell proliferation (6). Thus, cyclin D1 and CDK4 are often upregulated in rapidly dividing cells and decrease of CDK4 attenuates cell proliferation (22). Our results demonstrated that the increased cell proliferation due to EA treatment is associated with the increased protein expression of CDK4 and cyclin D1. The results also suggest that the unaltered proliferation and migration in cells treated with TVA and CLA are due to the decrease or unchanged expression levels of CDK4 and cyclin D1. CLA is a naturally occurring group of positional and geometric isomers of linoleic acid and TVA is a precursor of CLA (10). It has been reported that CLA decreases NF-κB activation in VSMCs (23). According to a recent report, decreased activation of NF-κB is associated with the inhibition of VSMC migration and proliferation through cell cycle arrest, ERK1/2 and MMP-9 (24). In contrast, trans-fatty acids, such as EA has been shown to increase inflammatory responses in vascular cells via NF-κB activation (25). These previous studies suggest that cellular response (e.g., cell cycle related molecules) due to trans-fatty acids is not identical but is dependent on the type of trans-fatty acids. These are probably due to the structural difference and whether it is produced naturally or artificially. CDK6 protein expression in cells treated with TVA, EA, or CLA remained unaltered and this implies that CDK6 is not a major factor for cell proliferation when cells are treated with TVA, EA and CLA in VSMCs (Fig. 3C).

Taken together, our data suggest that, unlike EA, ruminant trans-fatty acids TVA and CLA had no atherogenic effects, such as cell proliferation and migration in VSMCs. However, further studies will be useful to identify the detailed cardiovascular effects of ruminant trans-fatty acids, using *in vivo* and *in vitro* experiments.

Acknowledgments This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2014R1A1A 2057110).

Disclosure The authors declare no conflict of interest.

References

1. Aluganti Narasimhulu C, Fernandez-Ruiz I, Selvarajan K, Jiang X, Sengupta B, Riad A, Parthasarathy S. Atherosclerosis -- do we know enough already to prevent it? Curr. Opin. Pharmacol. 27: 92-102 (2016)
2. Lacolley P, Regnault V, Nicoletti A, Li Z, Michel JB. The vascular smooth muscle cell in arterial pathology: A cell that can take on multiple roles. Cardiovasc. Res. 95: 194-204 (2012)
3. Glass CK, Witztum JL. Atherosclerosis: The road ahead. Cell 104: 503-516 (2001)
4. Gomez D, Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis. Cardiovasc. Res. 95: 156-164 (2012)
5. Tanner FC, Greutert H, Barandier C, Frischknecht K, Luscher TF. Different cell cycle regulation of vascular smooth muscle in genetic hypertension. Hypertension 42: 184-188 (2003)
6. Lim S, Kaldis P. Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. Development 140: 3079-3093 (2013)
7. Kim TJ, Lim Y, Kim DW, Kwon JS, Son JH, Jin YR, Son DJ, Jung JC, Avery MA, Hong JT, Yun YP. Epothilone D, a microtubule-stabilizing compound, inhibits neointimal hyperplasia after rat carotid artery injury by cell cycle arrest via regulation of G1-checkpoint proteins. Vasc. Pharmacol. 47: 229-237 (2007)
8. De Caterina R. n-3 fatty acids in cardiovascular disease. New Engl. J. Med. 364: 2439-2450 (2011)
9. Gebauer SK, Destaillats F, Mouloungui Z, Candy L, Bezelgues JB, Dionisi F, Baer DJ. Effect of trans fatty acid isomers from ruminant sources on risk factors of cardiovascular disease: Study design and rationale. Contemp. Clin. Trials 32: 569-576 (2011)
10. Wang T, Lee HG. Advances in research on cis-9, trans-11 conjugated linoleic acid: A major functional conjugated linoleic acid isomer. Crit. Rev. Food Sci. 55: 720-731 (2015)
11. Bassett CM, Edel AL, Patenaude AF, McCullough RS, Blackwood DP, Chouinard PY, Paquin P, Lamarche B, Pierce GN. Dietary vaccenic acid has antiatherogenic effects in $LDLr^{-/-}$ mice. J. Nutr. 140: 18-24 (2010)
12. Iwata NG, Pham M, Rizzo NO, Cheng AM, Maloney E, Kim F. Trans fatty acids induce vascular inflammation and reduce vascular nitric oxide production in endothelial cells. PLoS ONE 6: e29600 (2011)
13. Abdelmagid SA, Clarke SE, Wong J, Roke K, Nielsen D, Badawi A, El-Sohemy A, Mutch DM, Ma DW. Plasma concentration of cis9trans11 CLA in males and females is influenced by SCD1 genetic variations and hormonal contraceptives: A cross-sectional study. Nutr. Metabolism 10: 50 (2013)
14. Song J, Kwon N, Lee MH, Ko YG, Lee JH, Kim OY. Association of serum phospholipid PUFAs with cardiometabolic risk: Beneficial effect of DHA on the suppression of vascular proliferation/inflammation. Clin. Biochem. 47: 361-368 (2014)
15. Yun MR, Lee JY, Park HS, Heo HJ, Park JY, Bae SS, Hong KW, Sung SM, Kim CD. Oleic acid enhances vascular smooth muscle cell proliferation via phosphatidylinositol 3-kinase/Akt signaling pathway. Pharmacol. Res. 54: 97-102 (2006)
16. Wang P, Henning SM, Heber D. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. PLoS ONE 5: e10202 (2010)
17. Delbosc S, Glorian M, Le Port AS, Bereziat G, Andreani M, Limon I. The benefit of docosahexanoic acid on the migration of vascular smooth muscle cells is partially dependent on Notch regulation of MMP-2/-9. Am. J. Pathol. 172: 1430-1440 (2008)
18. Zhang Y, Liu C, Zhu L, Jiang X, Chen X, Qi X, Liang X, Jin S, Zhang P, Li Q, Wang D, Liu X, Zeng K, Zhang J, Xiang Y, Zhang CY. PGC-1alpha inhibits oleic acid induced proliferation and migration of rat vascular smooth muscle cells. PLoS ONE 2: e1137 (2007)
19. Ferguson JE, 3rd, Patterson C. Break the cycle: The role of cell-cycle modulation in the prevention of vasculoproliferative diseases. Cell Cycle 2: 211-219 (2003)
20. Casimiro MC, Velasco-Velazquez M, Aguirre-Alvarado C, Pestell RG. Overview of cyclins D1 function in cancer and the CDK inhibitor landscape: Past and present. Expert Opin. Inv. Drug. 23: 295-304 (2014)
21. Park SL, Won SY, Song JH, Kambe T, Nagao M, Kim WJ, Moon SK. EPO gene expression promotes proliferation, migration and invasion via the p38MAPK/AP-1/MMP-9 pathway by p21WAF1 expression in vascular smooth muscle cells. Cell. Signal. 27: 470-478 (2015)
22. Park ES, Lee KP, Jung SH, Lee DY, Won KJ, Yun YP, Kim B. Compound K, an intestinal metabolite of ginsenosides, inhibits PDGF-BB-induced VSMC proliferation and migration through G1 arrest and attenuates neointimal hyperplasia after arterial injury. Atherosclerosis 228: 53-60 (2013)
23. Ringseis R, Gahler S, Eder K. Conjugated linoleic acid isomers inhibit platelet-derived growth factor-induced NF-kappaB transactivation and collagen formation in human vascular smooth muscle cells. Eur. J. Nutr. 47: 59-67 (2008)
24. Suh SJ, Jin UH, Kim SH, Chang HW, Son JK, Lee SH, Son KH, Kim CH. Ochnaflavone inhibits TNF-alpha-induced human VSMC proliferation via regulation of cell cycle, ERK1/2, and MMP-9. J. Cell. Biochem. 99: 1298-1307 (2006)
25. Bryk D, Zapolska-Downar D, Malecki M, Hajdukiewicz K, Sitkiewicz D. Trans fatty acids induce a proinflammatory response in endothelial cells through ROS-dependent nuclear factor-kappaB activation. J. Physiol. Pharmacol. 62: 229-238 (2011)