

Palmitic acid exerts pro-inflammatory effects on vascular smooth muscle cells by inducing the expression of C-reactive protein, inducible nitric oxide synthase and tumor necrosis factor- α

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Abstract. Atherosclerosis is a chronic inflammatory disease in the vessel, and inflammatory cytokines play an important role in the inflammatory process of atherosclerosis. A high level of free fatty acids (FFAs) produced in lipid metabolism disorders are known to participate in the formation of atherosclerosis through multiple bioactivities. As the main saturated fatty acid in FFAs, palmitic acid stimulates the expression of inflammatory cytokines in macrophages. However, it is unclear whether palmitic acid exerts a pro-inflammatory effect on vascular smooth muscle cells (VSMCs). The purpose of the present study was to observe the effect of palmitic acid on the expression of C-reactive protein (CRP), tumor necrosis factor α (TNF- α) and inducible nitric oxide synthase (iNOS) in VSMCs. Rat VSMCs were cultured, and palmitic acid was used as a stimulant for CRP, TNF- α and iNOS expression. mRNA expression was assayed with reverse transcription-polymerase chain reaction, and protein expression was detected with western blot analysis and immunocytochemistry. The results showed that palmitic acid significantly stimulated mRNA and protein expression of CRP, TNF- α and iNOS in VSMCs in time- and concentration-dependent manners, and therefore, palmitic acid is able to exert a pro-inflammatory effect on VSMCs via stimulating CRP, TNF- α and iNOS expression. The findings provide a novel explanation for the direct pro-inflammatory and atherogenic effects of palmitic acid, and for the association with metabolic syndrome, such as type 2 diabetes mellitus, obesity and atherosclerosis. Therefore, the intervention with

anti-inflammatory agents may effectively delay the formation and progression of atherosclerosis in patients with metabolic syndrome.

Introduction

Atherosclerosis is a chronic disease of the vascular wall and a primary cause of stroke, coronary artery disease and peripheral vascular diseases (1). Accumulating evidence has shown that inflammation plays a key role in pathogenesis of atherosclerosis (2). Inflammatory cytokines, such as C-reactive protein (CRP) (3), tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS) and interleukin-6 (IL-6), are involved in the process of inflammatory response (4). The expression of inflammatory cytokines and lipid accumulation promote the formation of atheromatous plaque and regulate aspects of the plaque biology that trigger the thrombotic complications of atherosclerosis (5).

Type 2 diabetes mellitus, metabolic syndrome, insulin resistance and cardiovascular diseases are characterized by the increased levels of free fatty acids (FFAs) (6). FFAs participate in the formation of atherosclerosis through impairing endothelium-dependent vasodilation, promoting adhesion of monocytes to endothelial cells and activating inflammatory responses in the vascular wall (7).

As the most abundant saturated fatty acid in FFAs (8), palmitic acid plays an important role in the development of atherosclerosis. Palmitic acid activates inflammatory signaling to produce numerous inflammatory cytokines in macrophages, including TNF- α , IL-6 and monocyte chemoattractant protein-1 (MCP-1) (9), selectively upregulates lectin-like oxidized low-density lipoprotein receptor-1 expression in macrophages, and promotes oxidized low-density lipoprotein uptake by macrophages (7). Palmitic acid also inhibits NO release by endothelial cells (10). Although the elevated palmitic acid level has been indicated to contribute to the formation and development of atherosclerosis, there is no direct evidence to demonstrate the pro-inflammatory effect of palmitic acid on the vascular cells. Growing evidence has identified that vascular smooth muscle cells (VSMCs) are a source of plasma inflammatory cytokines. Additionally, the

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Key words: palmitic acid, C-reactive protein, tumor necrosis factor- α , inducible nitric oxide synthase, vascular smooth muscle cells, inflammation, atherosclerosis

Table I. PCR amplification conditions.

Gene	PCR amplification conditions
<i>CRP</i>	1 cycle of 94°C for 3 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec; 1 cycle of 72°C for 5 min
<i>TNF-α</i>	1 cycle of 94°C for 3 min; 32 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec; 1 cycle of 72°C for 5 min
<i>iNOS</i>	1 cycle of 94°C for 3 min; 28 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec; 1 cycle of 72°C for 5 min
<i>GAPDH</i>	1 cycle of 94°C for 3 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec; 1 cycle of 72°C for 5 min

Table II. Primers used for RT-PCR analysis.

Gene	Accession number	Primer sequence	Expected size (bp)
<i>CRP</i>	NM_017096	5'-CATCTGTGCCACCTGGGAGTC-3' 5'-AAGCCACCGCCATACGAGTC-3'	153
<i>TNF-α</i>	NM_012675	5'-GCTGGTAGGTTCTGTGTTTTC-3' 5'-CACCACGCTCTTCTGTCTACTG-3'	381
<i>iNOS</i>	NM_012611	5'-GAGATTTTTCACGACACCCTTC-3' 5'-GAGATTTTTCACGACACCCTTC-3'	262
<i>GAPDH</i>	NM_017008	5'-GCAAGTTCAACGGCACAGTCAAG-3' 5'-ACATACTCAGCACCAGCATCACC-5'	124

RT-PCR, reverse transcription polymerase chain reaction; bp, basepair.

locally synthesized CRP by VSMCs in the arterial wall may play an essential role in the whole inflammatory process of atherosclerosis. Therefore, the present study observed the effect of palmitic acid on CRP, TNF- α and iNOS expression in rat VSMCs.

Materials and methods

Reagents. Palmitic acid was purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was from Genview (Glenview, IL, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from HyClone (Logan, UT, USA). Penicillin and streptomycin were produced by Gibco-BRL (Grand Island, NY, USA). Anti-CRP and anti-iNOS antibodies were provided by Abcam (Cambridge, UK). The antibody against rat TNF- α was purchased from Abnova (Taipei, Taiwan).

Cell culture. Sprague-Dawley (SD) rats were obtained from the Laboratory Animal Center of Xi'an Jiaotong University School of Medicine (Xi'an, China). All the experimental procedures carried out were performed in accordance with the International, National and Institutional rules, and approved by the Institutional Animal Care Committee of Xi'an Jiaotong University. Primary VSMCs were isolated from the thoracic aorta of 1-2-month-old male SD rats as previously described (11), and grown in DMEM (containing 4500 mg/l glucose) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μ g/ml) in a 5% CO₂ atmosphere at 37°C. Finally, VSMCs were identified by morphological examination, and showed 99% purity as estimated with the immunocytochemical staining for α -actin (Beijing Biosynthesis Biotechnology, Co.,

Ltd., Beijing, China). The cells between passages 3 and 7 were used for the subsequent experiments.

Preparation of palmitic acid. As the formation of BSA complexes is important for reducing the possible cell toxicity of fatty acid, palmitic acid complexed with BSA was prepared according to the method previously described (6,12). In brief, palmitic acid powder was dissolved in ethanol to 200 mmol/l at 70°C. Subsequently, palmitic acid solution was diluted with a 10% solution of fatty acid-free BSA to 5 mmol/l at 55°C for 10 min. The complexed solution was cooled to room temperature, and filtrated through a 0.45- μ m pore membrane filter. Finally, the solution was sterilized and stored at -20°C for use. In palmitic acid-stimulated experiments, the stock solution (5 mmol/l) was warmed prior to use, and added to the culture medium at the final concentrations of 25-200 μ mol/l.

RNA isolation and analysis. Total RNA was isolated from VSMCs following treatment using RNAfast200 (Fastagen Biotechnology Co., Ltd., Shanghai, China). RNA (1-2 μ g) was reverse transcribed into cDNA using PrimeScript™ RT Master Mix (Takara Biotechnology, Inc., Shiga, Japan) according to the manufacturer's instruction. PCR amplification conditions are shown in Table I. Sequence-specific primers for rat *CRP*, *TNF- α* , *iNOS* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed and synthesized by Sangon Biotech Co., Ltd., (Shanghai, China) (see Table II for the sequences). *GAPDH* was used as an internal control. The samples were run in triplicate. An equal volume of the reaction mixture from each sample was loaded on a 2% TAE agarose gel containing ethidium bromide, and resolved by electrophoresis. Images were digitally captured, and the band intensity was analyzed using

Gel Pro Analyzer software, version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). The relative amount of each mRNA was normalized to the reference gene, *GADPH* mRNA.

Western blot analysis. VSMCs were washed with phosphate-buffered saline (PBS), lysed and homogenized in 10 mmol/l Tris-HCl (pH 7.4) containing 0.1% sodium dodecylsulfate and a protease-inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration was measured by the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts (20 μ g) of protein were resolved on 10% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Subsequently, the membranes were blocked with 5% skimmed dry milk in Tris-buffered saline containing 0.1% Tween-20, and incubated with specific anti-CRP (1:100), anti-TNF- α (1:200), anti-iNOS (1:300) and anti-GAPDH (1:500) antibodies overnight at 4°C. GAPDH was used as loading control. The immunostaining was visualized by the enhanced chemiluminescence (Pierce). Data were normalized against those of the corresponding GAPDH. Results are expressed relative to control.

Immunocytochemical staining. VSMCs were seeded on coverslips (24x24 mm) in 6-well plates until confluency, and stimulated with the increasing concentrations of palmitic acid for 24 h. Subsequently, the cells were fixed with 4% formaldehyde-PBS for 15 min. The cell membrane was permeabilized with 0.2% Triton X-100 PBS for 20 min, and the non-specific binding sites were blocked with 10% goat serum. Following pretreatment with specific anti-CRP (1:100), anti-TNF- α (1:100) or anti-iNOS (1:100) antibodies overnight at 4°C, the cells were washed three times with PBS and incubated with the secondary antibody conjugated to fluorescein isothiocyanate for 15 min at room temperature (13). Finally, the cells were imaged with a light microscope (BX51; Olympus, Tokyo, Japan). The optical density of the experimental field was measured and analyzed with Image-Pro Plus software (Media Cybernetics, Inc.). The relative optical density was taken as the average of the values from three repeated experiments. Data are expressed relative to control.

Statistical analysis. All the results are expressed as means \pm standard error of the mean. The experiments were repeated three times and statistical significance between groups was assessed by one-way analysis of variance, followed by Fisher's exact test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Palmitic acid induces mRNA and protein expression of CRP in VSMCs. As shown in Figs. 1 and 2, palmitic acid significantly induced mRNA and protein expression of CRP in VSMCs in time- and concentration-dependent manners compared to the control. The maximal protein expression of CRP was detected after treatment of the cells with 100 μ mol/l palmitic acid for 24 h, which was 9.09 and 9.88 times higher than that of the control and BSA, respectively.

The immunocytochemical staining identified that there was weak staining of CRP in the control VSMCs. However, CRP expression in VSMCs showed a sustained increase to

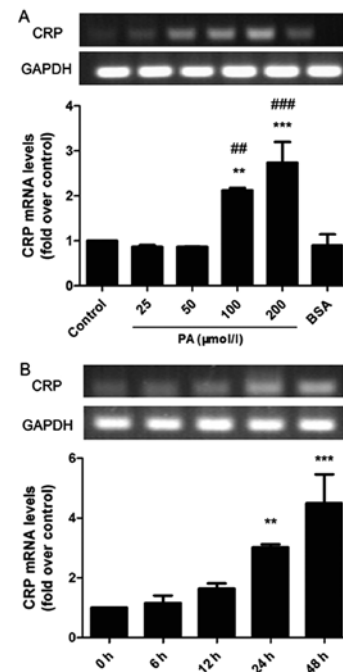


Figure 1. Palmitic acid (PA) induces *CRP* mRNA expression in VSMCs. The cells were stimulated with increasing concentrations of PA for 24 h or with 100 μ mol/l PA for the indicated time. Subsequently, *CRP* mRNA expression was assayed by RT-PCR. (A) Concentration-dependent effect of PA-induced *CRP* mRNA expression. (B) Time-dependent effect of PA-induced *CRP* mRNA expression. Data represent the mean \pm standard error of the mean of three independent experiments. ** $P < 0.01$, *** $P < 0.001$ vs. control; ** $P < 0.01$, *** $P < 0.001$ vs. BSA. CRP, C-reactive protein; VSMCs, vascular smooth muscle cells; RT-PCR, reverse transcription polymerase chain reaction; BSA, bovine serum albumin.

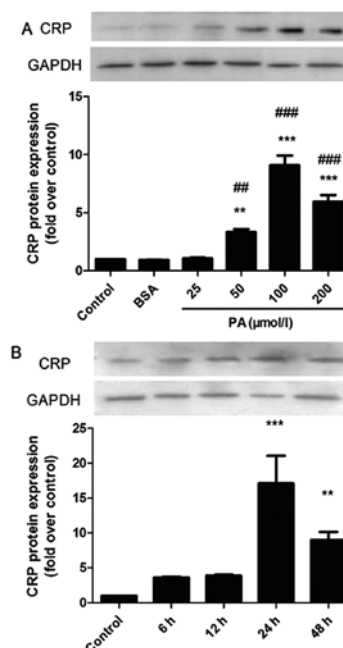


Figure 2. Palmitic acid (PA) upregulates CRP protein expression in VSMCs. The cells were stimulated with increasing concentrations of PA for 24 h or with 100 μ mol/l PA for the indicated time. Subsequently, CRP protein expression was determined by western blot analysis, and normalized to GAPDH. (A) Concentration-dependent effect of PA-induced CRP protein expression. (B) Time-dependent effect of PA-induced CRP protein expression. Data represent the mean \pm standard error of the mean of three independent experiments. ** $P < 0.01$, *** $P < 0.001$ vs. control; ** $P < 0.01$, *** $P < 0.001$ vs. BSA. CRP, C-reactive protein; VSMCs, vascular smooth muscle cells; BSA, bovine serum albumin.

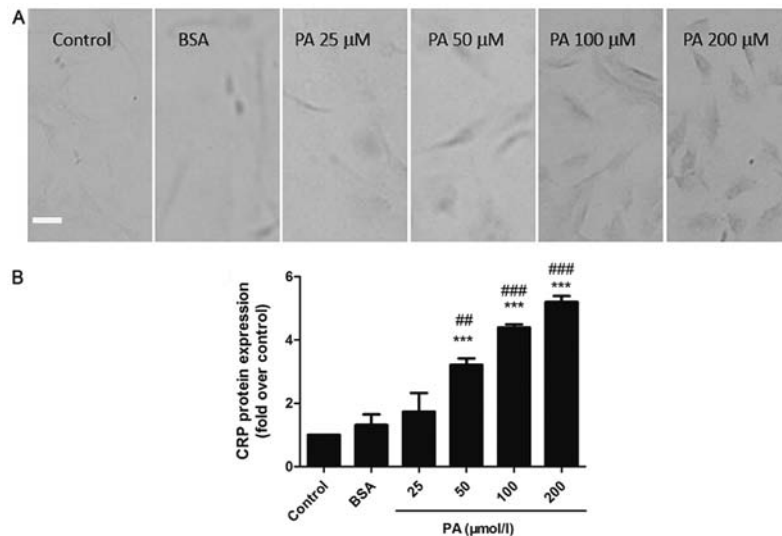


Figure 3. Palmitic acid (PA) stimulates CRP generation in VSMCs. The cells were stimulated with increasing concentrations of PA for 24 h. Subsequently, CRP generation was identified with the immunocytochemical staining. (A) Representative PA-induced CRP generation. (B) Statistical result of PA-induced CRP generation. Data represent the mean \pm standard error of the mean of three independent experiments. *** P <0.001 vs. control; ** P <0.01, ### P <0.001 vs. BSA. Scale bar, 100 μ m. CRP, C-reactive protein; VSMCs, vascular smooth muscle cells; BSA, bovine serum albumin.

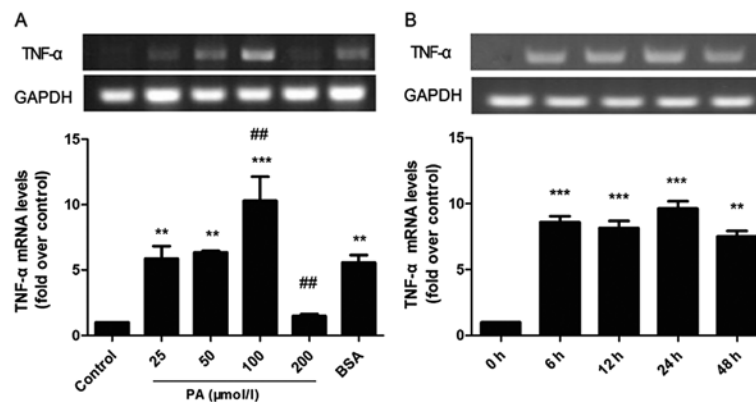


Figure 4. Palmitic acid (PA) induces *TNF- α* mRNA expression in VSMCs. The cells were stimulated with increasing concentrations of PA for 24 h or with 100 μ mol/l PA for the indicated time. Subsequently, *TNF- α* mRNA expression was assayed by RT-PCR, and normalized to *GAPDH*. (A) Concentration-dependent effect of PA-induced *TNF- α* mRNA expression. (B) Time-dependent effect of PA-induced *TNF- α* mRNA expression. Data represent the mean \pm standard error of the mean of three independent experiments. ** P <0.01, *** P <0.001 vs. control; ## P <0.01 vs. BSA. *TNF- α* , tumor necrosis factor- α ; VSMCs, vascular smooth muscle cells; RT-PCR, reverse transcription polymerase chain reaction; BSA, bovine serum albumin.

reach a maximum in the observed concentrations after stimulation of the cells for 24 h with palmitic acid (Fig. 3).

Due to the use of BSA in preparing the palmitic acid complex, BSA alone was also used for the control. The BSA concentration for the control corresponded to the BSA contained in the maximal concentration of palmitic acid (200 μ mol/l). The results showed that BSA at the concentration used in the present experiment did not significantly affect mRNA and protein expression of CRP in VSMCs.

Palmitic acid stimulates mRNA and protein expression of TNF- α in VSMCs. As observed in Figs. 4 and 5, concentrations of palmitic acid from 25 to 100 μ mol/l evidently produced a time- and concentration-dependent increase in mRNA and protein expression of TNF- α in VSMCs. The maximal expression of TNF- α protein was 5.45-fold and 2.98-fold higher than that of the control and BSA, respectively,

following stimulation of the cells with 100 μ mol/l palmitic acid for 24 h.

Fig. 6A shows that the unstimulated VSMCs exhibited a basic expression (control) of TNF- α . Compared to the control, incubation of the cells with palmitic acid for 24 h induced TNF- α expression in a concentration-dependent manner (Fig. 6B).

Although mRNA and protein expression of TNF- α in VSMCs manifested an increasing tendency in the BSA control, only *TNF- α* mRNA expression showed a significant difference in comparison to the control.

Palmitic acid increases mRNA and protein expression of iNOS in VSMCs. The results in Figs. 7 and 8 indicated that palmitic acid at 25 to 100 μ mol/l increased mRNA and protein expression of iNOS in VSMCs in time- and concentration-dependent fashions. The maximal response of protein expression was reached after incubation of the cells with 100 μ mol/l palmitic

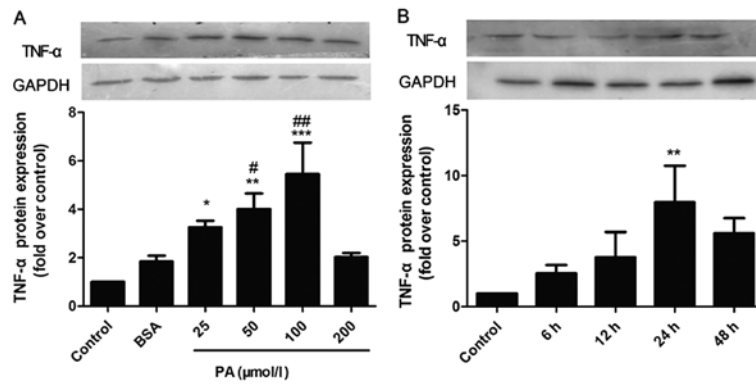


Figure 5. Palmitic acid (PA) upregulates TNF- α protein expression in VSMCs. The cells were stimulated with increasing concentrations of PA for 24 h or with 100 μ mol/l PA for the indicated time. Subsequently, TNF- α protein expression was determined by western blot analysis, and normalized to GAPDH. (A) Concentration-dependent effect of PA-induced TNF- α protein expression. (B) Time-dependent effect of PA-induced TNF- α protein expression. Data represent the mean \pm standard error of the mean of three independent experiments. * P <0.05, ** P <0.01, *** P <0.001 vs. control; # P <0.05, ## P <0.01 vs. BSA. TNF- α , tumor necrosis factor- α ; VSMCs, vascular smooth muscle cells; BSA, bovine serum albumin.

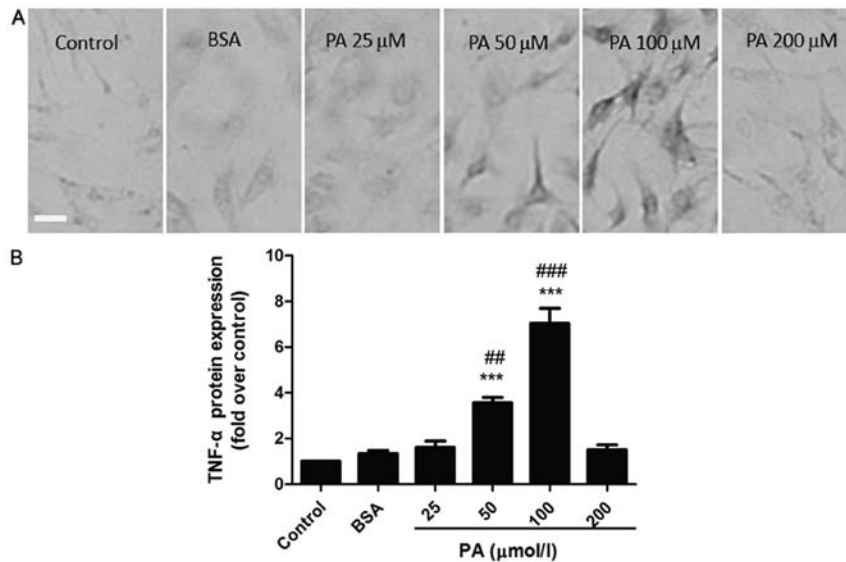


Figure 6. Palmitic acid (PA) stimulates TNF- α generation in VSMCs. The cells were stimulated with increasing concentrations of PA for 24 h. Subsequently, TNF- α generation was identified with the immunocytochemical staining. (A) Representative PA-induced TNF- α generation. (B) Statistical result of PA-induced TNF- α generation. Data represent the mean \pm standard error of the mean of three independent experiments. *** P <0.001 vs. control; # P <0.01, ### P <0.001 vs. BSA. Scale bar, 100 μ m. TNF- α , tumor necrosis factor- α ; VSMCs, vascular smooth muscle cells; BSA, bovine serum albumin.

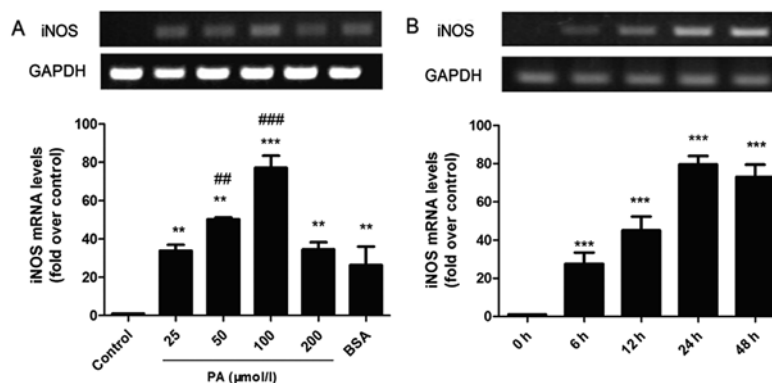


Figure 7. Palmitic acid (PA) induces *iNOS* mRNA expression in VSMCs. The cells were stimulated with increasing concentrations of PA for 24 h or with 100 μ mol/l PA for the indicated time. Subsequently, *iNOS* mRNA expression was assayed by RT-PCR and normalized to GAPDH. (A) Concentration-dependent effect of PA-induced *iNOS* mRNA expression. (B) Time-dependent effect of PA-induced *iNOS* mRNA expression. Data represent the mean \pm standard error of the mean of three independent experiments. ** P <0.01, *** P <0.001 vs. control; # P <0.01, ### P <0.001 vs. BSA. *iNOS*, inducible nitric oxide synthase; VSMCs, vascular smooth muscle cells; RT-PCR, reverse transcription polymerase chain reaction; BSA, bovine serum albumin.

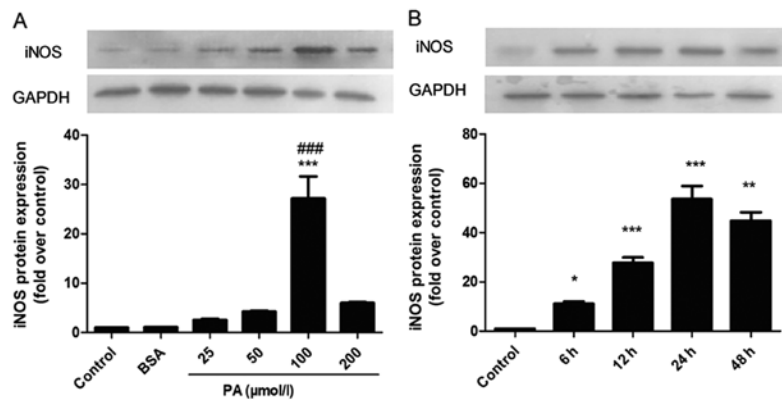


Figure 8. Palmitic acid (PA) upregulates iNOS protein expression in VSMCs. The cells were stimulated with increasing concentrations of PA for 24 h or with 100 μ mol/l PA for the indicated time. Subsequently, iNOS protein expression was determined by western blot analysis, and normalized to GAPDH. (A) Concentration-dependent effect of PA-induced iNOS protein expression. (B) Time-dependent effect of PA-induced iNOS protein expression. Data represent the mean \pm standard error of the mean of three independent experiments. * P <0.05, ** P <0.01, *** P <0.001 vs. control; ### P <0.001 vs. BSA. iNOS, inducible nitric oxide synthase; VSMCs, vascular smooth muscle cells; BSA, bovine serum albumin.

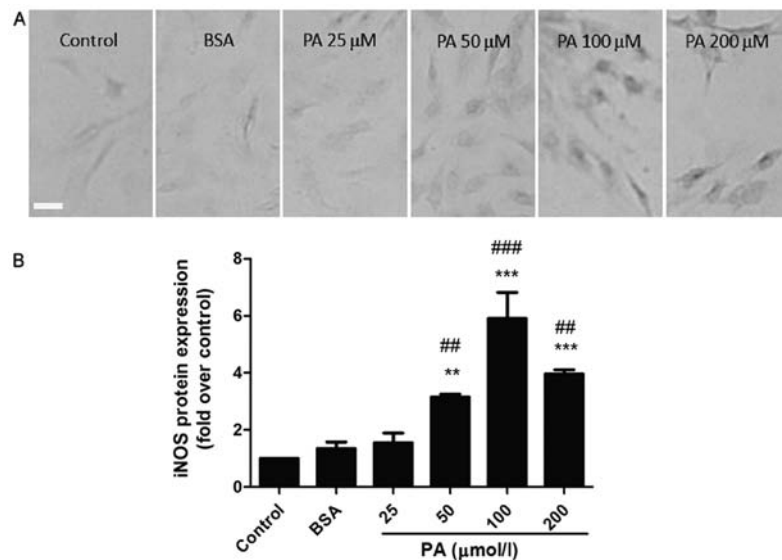


Figure 9. Palmitic acid stimulates iNOS generation in VSMCs. The cells were stimulated with increasing concentrations of palmitic acid (PA) for 24 h. Subsequently, iNOS generation was identified with the immunocytochemical staining. (A) Representative PA-induced iNOS generation. (B) Statistical result of PA-induced iNOS generation. Data represent the mean \pm standard error of the mean of three independent experiments. ** P <0.01, *** P <0.001 vs. control; ## P <0.01, ### P <0.001 vs. BSA. Scale bar, 100 μ m. iNOS, inducible nitric oxide synthase; VSMCs, vascular smooth muscle cells; BSA, bovine serum albumin.

acid for 24 h, which was 27.2 and 25.4 times higher than the control and BSA.

Identification of iNOS expression with the immunocytochemical staining revealed that there was a mild staining of iNOS in control VSMCs. However, iNOS expression in palmitic acid-stimulated VSMCs was markedly increased in a concentration-dependent manner (Fig. 9).

The results from the BSA control showed that BSA caused an increase of *iNOS* mRNA expression, but did not affect iNOS protein generation in VSMCs.

Discussion

Type 2 diabetes mellitus is a non-infectious epidemic disease and an independent risk factor of cardiovascular disease that is often accompanied with the complication of atherosclerosis. In addition to other atherogenic mechanisms, lipid

metabolism disorders that are secondary to glucose metabolism abnormality may lead to production of excessive FFAs. As a main component in FFAs, palmitic acid participates in the formation of atherosclerosis (14). However, whether palmitic acid contributes to the progression of atherosclerosis by the pro-inflammatory effect on the vessel cells is not well defined.

At present, the majority of investigators support the concept that vascular inflammation is key for atherosclerotic lesion formation, progression and eventual plaque rupture. CRP, the best-characterized biomarker of inflammation, is not only an independent predictor of future cardiovascular events, but also a direct participant in the pathogenesis of atherosclerosis (15). The elevated CRP level contributes to the increase of cardiovascular risk (16,17). It is well known that CRP modulates the activities and expression of multiple factors implicated in atherogenesis, such as stimulating releases of endothelin-1 and IL-6 from endothelial cells (18,19), increasing the expression

of adhesion molecules and MCP-1 (20), facilitating the uptake of low-density lipoprotein by macrophages (21).

High levels of TNF- α and iNOS have been detected in VSMCs of human and/or rabbit atherosclerotic plaques (22). TNF- α is a major pleiotropic pro-inflammatory and immunomodulatory cytokine (23). As an important contributor to the development of atherosclerosis, TNF- α promotes the expression of adhesion molecules in endothelial cells, as well as the recruitment and activation of inflammatory cells, stimulates migration and proliferation of VSMCs, and initiates the inflammatory cascade inside the arterial wall (23). A high level of TNF- α in serum has been associated with a worse prognosis in patients following acute myocardial infarction (5). Therefore, TNF- α is of significance in the development of atherosclerotic lesion and the risk of acute cardiovascular events.

iNOS is a significant enzyme that mediates inflammatory processes, and the improper upregulation of iNOS has been associated with the pathophysiology of inflammatory disorders. Since inflammation is closely linked to atherosclerosis, the increased iNOS activity may contribute to the process of atherosclerosis (24). iNOS mainly exists in human atherosclerotic lesions, particularly in the lipid core surrounded by macrophages and foam cells. Several factors present in atherosclerotic plaque, such as TNF- α , IL-1 β , IL-2 and γ -interferon, are able to induce iNOS expression in VSMCs. High iNOS activity produces large quantities of NO, which may lead to cellular damage, inflammation, apoptosis and peroxynitrite (ONOO⁻) formation. Peroxynitrite has been established as a powerful oxidant causing oxidative damage of DNA or hindering DNA repair in endothelial cells and VSMCs, and therefore is important in atherosclerosis pathology (5,24).

For these aforementioned reasons, the effects of palmitic acid were observed on CRP, TNF- α and iNOS expression in rat VSMCs. The results showed that palmitic acid at the concentrations used significantly stimulated CRP, TNF- α and iNOS expression in VSMCs at the mRNA and protein levels. These are also similar to other studies in which palmitic acid can activate inflammatory signaling in adipose cells and macrophages (9). As there is a close association between inflammation and atherosclerosis, atherogenic effect of palmitic acid (FFAs) is partially attributed to its pro-inflammation.

In conclusion, the present study demonstrates that palmitic acid is able to exert a pro-inflammatory effect on VSMCs via stimulating CRP, TNF- α and iNOS expression. Although the exact molecular mechanisms underlying the effects are unknown, the findings provide a novel explanation for the direct pro-inflammatory and atherogenic effects of palmitic acid, and for the association with metabolic syndrome, such as type 2 diabetes mellitus, obesity and atherosclerosis. Therefore, the intervention with anti-inflammatory agents may effectively delay the formation and progression of atherosclerosis in patients with metabolic syndrome, particularly with type 2 diabetes mellitus. Further studies are required to characterize the mechanisms responsible for the pro-inflammatory effect of palmitic acid.

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