Am J Physiol Cell Physiol 316: C888–C897, 2019.
First published March 13, 2019; doi:10.1152/ajpcell.00293.2018.

RESEARCH ARTICLE

Palmitate decreases migration and proliferation and increases oxidative stress and inflammation in smooth muscle cells: role of the Nrf2 signaling pathway

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Submitted 24 July 2018; accepted in final form 13 March 2019

Girona J, Rosales R, Saavedra P, Masana L, Vallvé JC. Palmitate decreases migration and proliferation and increases oxidative stress and inflammation in smooth muscle cells: role of the Nrf2 signaling pathway. *Am J Physiol Cell Physiol* 316: C888–C897, 2019. First published March 13, 2019; doi:10.1152/ajpcell.00293.2018.— Fatty acids are essential to cell functionality and may exert diverging vascular effects including migration, proliferation, oxidative stress, and inflammation. This study examined the effect of palmitate on human coronary artery smooth muscle cell (HCASMC) function. An in vitro wound-healing assay indicated that palmitate decreased HCASMC migration in dose- and time-dependent manners. Furthermore, bromodeoxyuridine incorporation assays indicated that palmitate decreased HCASMC proliferation in a dose-response manner. Palmitate also increased reactive oxygen species formation, malondialdehyde content, and intracellular lipid droplets accompanied with increased fatty acid binding protein 4 expression. Moreover, palmitate induced gene expression (monocyte chemoattractant protein 1, matrix metalloproteinase-2, IL-1β, IL-6, IL-8, and TNF-α) and intracellular protein content (plasminogen activator inhibitor-1 and urokinase plasminogen activator) of inflammatory mediators. Finally, we showed that palmitate activates the transcription factor Nrf2 and the upstream kinases ERK1/2 and Akt in HCASMCs. The inhibitor of Nrf2, trigonelline, significantly attenuated palmitate-induced HCASMC expression of the Nrf2 target gene NQO1. These findings indicate that palmitate might be critically related to HCASMC function by slowing cell migration and proliferation and inducing lipid-laden cells, oxidative stress, and inflammation in part by activation of the Nrf2 transcription factor. Palmitate's activation of proinflammatory Nrf2 signaling may represent a novel mechanism mediating the proatherogenic actions of saturated fatty acids.

human artery coronary smooth muscle cells; inflammation; lipid-laden cells; migration; oxidative stress; proliferation

INTRODUCTION

The accumulation of vascular smooth muscle cells (VSMCs) in the intima is a major feature of atherosclerotic plaque development and is a consequence of their migration from the media to the intima where they proliferate, events that are amplified through the release of inflammatory mediators and reactive oxygen species (ROS) (4, 15). The proliferation and phenotypic changes of VSMCs from quiescent and contractile to a synthetic form are critical in atherosclerosis. In addition, strong layers of VSMCs as top atherosclerotic lesions are regarded as beneficial because they cover the macrophage/foam cell/lipid-laden atheromatous core with a protective fibrous cap and thereby inhibit its breakdown.

Palmitate is a major circulating saturated free fatty acid (FFA) that accounts for ≈30 to 40% of serum FFAs. Additionally, it is known that FFA levels are increased in obese patients and are associated with metabolic syndromes and diabetes and that they predict cardiovascular events (22). Different studies have described proinflammatory effects of palmitate on VSMCs, although they have reported different biomarkers involved in this effect. In particular, palmitate is able to exert a proinflammatory effect on rat VSMCs by stimulating C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), and inducible nitric oxide synthase (iNOS) expression (29) and interleukin-8 (IL-8) expression via the Toll-like receptor (TLR)/nuclear factor-κB (NF-κB) pathway (23). In addition, in cultured VSMCs from different sources, palmitate alters the production of the extracellular matrix favoring lipoprotein deposition and atherogenesis (24); increases the levels of ROS leading to extracellular signal-regulated kinase (ERK)1/2 activation (3, 10); promotes neointima formation by inducing inflammatory phenotypes, which is in part mediated by the TLR4/MyD88/NF-κB/NADPH oxidase (NOX)/ROS pathway (25); and also promotes apoptosis (2). Fatty acids have different effects on the migration and proliferation of VSMCs. While polyunsaturated fatty acids inhibit the migration of human aortic SMCs via mitogen-activated protein kinase (MEK)/ERK-dependent mechanisms (18, 28), it is well established that oleic acid induces human and rat VSMC proliferation (12, 16, 31) and migration (9, 31). However, the effect of palmitate on VSMCs is not fully understood. Some studies have shown that palmitate has no effect on the migration and proliferation of human coronary SMCs (12, 13). Others showed that palmitate induces downregulation of human bladder SMC proliferation through NF-κB and the formation of reactive intermediates (21). Chung et al. (5) showed that palmitate-conditioned media from macrophages increased rat aortic VSMC proliferation and migration possibly mediated by bone morphogenetic proteins from macrophages. Thus, although the effects of palmitate on VSMCs from different sources have been evaluated in some studies, their direct effect on migration, proliferation, and inflammation in human coronary artery smooth muscle cells (HCASMCs) needs further evaluation.

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In the present study, we show that palmitate increases lipid-laden cells, cellular oxidative stress and inflammation but also attenuates the migration and proliferation of HCASMCs. Furthermore, activation of nuclear transcription factor Nrf2 is involved. Collectively, these findings demonstrate the important role of the FFA palmitate in the mechanisms regulating VSMC function.

MATERIALS AND METHODS

Cell culture and reagents. Primary HCASMCs were obtained from Cascade Biologics (Invitrogen, Life Technologies, Paisley, UK). After thawing, the cells were seeded into 75-cm^2 flasks and cultured according to the supplier's recommendations in Medium 231. Medium 231 was supplemented with smooth muscle growth supplement and 1% gentamicin/amphotericin solution (Invitrogen, Life Technologies). The supplemented medium contained fetal bovine serum (4.9% vol/vol final concentration), 2 ng/ml human basic fibroblast growth factor, 0.5 ng/ml human epidermal growth factor, 5 ng/ml heparin, 5 μg/ml insulin, and 0.2 μg/ml bovine serum albumin. The cells were placed in a humidified incubator at 37°C and 5% CO_2 until reaching 70–80% confluence. In the current study, the HCASMCs were used at passage 6. Before the initiation of the assays, HCASMCs that were in the exponential growth phase were transferred to Medium 231 supplemented with 0.1% fetal bovine serum in the absence of growth factors for 24 h to achieve cell quiescence. Palmitate was obtained from Nu-Check (Elysian, MN). Palmitate was dissolved in ultrapure water at 70°C for 10 min in a water bath. The palmitate aliquots were stored at −80°C until further use. To analyze the effects of inhibitors, HCASMCs were pretreated with 10 mM of the antioxidant *N*-acetyl-L-cysteine (NAC), 10 μM of a c-Jun inhibitor (SP600125), and 0.5 μM of the Nrf2 inhibitor trigonelline or DMSO (Sigma-Aldrich, St. Louis, MO) for 1 h before the addition of palmitate. Intracellular glutathione peroxidase (GPX) concentrations were assessed using an ELISA kit from R&D Systems (Minneapolis, MN), and GPX activity was determined using commercially available assays according to the manufacturer's instructions (Cayman Chemical).

Cell cytotoxicity and viability studies. Cells were treated with palmitate at 5–100 μM for 5 and 24 h. The cell viability assay based on the quantitation of the adenosine triphosphate (ATP) present as an indicator of metabolically active cells was carried out using a Cell Titer-Glo Luminescent Cell Viability Kit according to the manufacturer's protocol (Promega, Madrid, Spain). A cytotoxicity assay was performed by analyzing lactate dehydrogenase (LDH) release into the medium using a Cytotoxicity Detection Kit (Roche Diagnostics, Basel, Switzerland).

Cell proliferation studies. Cell proliferation was analyzed by measuring DNA synthesis with a colorimetric bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics), according to the manufacturer's instructions. Briefly, 1×10^4 cells were seeded into a 96-well microplate and cultured with or without palmitate (2–200 μM) for 24 h. The cells were then labeled with BrdU-labeling reagent for 10 h. After fixation, the cells were incubated with anti-BrdU antibody for 90 min. After the cells were washed, 100 μl of substrate (tetramethylbenzidine) were added to each well, and the plates were incubated at room temperature for 30 min. The absorbance at 450 nm was measured with an ELISA reader (Synergy H4; Biotek).

In vitro wound-healing assay. Cell migration was analyzed via an in vitro scratch assay (31). The cells were cultured in 12-well plates, and after the induction of quiescence, a single scratch wound was created in the center of the cell monolayer by the gentle removal of the attached cells with a sterile plastic pipette tip. The cells were incubated with palmitate (2–100 μM) for 24 h in serum-reduced Medium 231 (containing 0.1% fetal calf serum in the absence of growth factors). Images of the cells migrating into the wound were taken at 0 h and then every 2 h until the scratch wound was closed at 24 h; the images were compared with quantify the migration rate of the cells. The closure of the wound was considered to represent 100% migration. The cell images were captured using a microscope (Olympus IX71) and analyzed using imaging software (Xcell).

Nile red staining, ROS production, and thiobarbituric acid reactive substances. To visualize the lipid droplets, cells were incubated with the lipophilic fluorescent dye Nile red (100 ng/ml; Sigma-Aldrich) diluted in PBS for 5 min at room temperature without prior fixation. Fluorescence images were captured with an Olympus IX71 inverted microscope. Fluorescence intensity was detected at 488/540 nm using a fluorescence reader (Multilector Synergy HT; Biotek). Intracellular ROS production was examined after incubation of the cells with 2′,7′-dichlorodihydrofluorescein diacetate (Sigma-Aldrich) for 30 min, and the dichlorodihydrofluorescein fluorescence intensity was detected at 485/530 nm using a fluorescence reader (Multilector Synergy HT; Biotek). Malondialdehyde (MDA) concentrations in cell lysates were analyzed by thiobarbituric acid reactive substances assay. After palmitate treatment, cells were lysed with TCA (35–70%) and then thiobarbituric acid was added to form thiobarbituric acid-MDA adducts. A standard curve of MDA (0–410 μmol/ml) was used to analyzed the content of MDA in our samples. Fluorescence was read at excitation: 530 nm/emission: 590 nm using a Synergy HT lector.

Total cellular and nuclear extracts. To obtain total cellular extracts, HCASMCs were cultured in 10-cm culture dishes until the cells reached 90% confluence. After the induction of quiescence, the cells were incubated with palmitate (50 μM) for 5, 15, or 30 min. At different time points, the cells were rinsed with ice-cold PBS and lysed in lysis buffer, which was composed of 50 mM Tris·HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate, and phosphatase inhibitors (Roche Diagnostics, Basel, Switzerland). The protein extracts were then stored at −80°C until they were processed. The total protein concentration was measured using a Bradford assay kit (Bio-Rad, Hercules, CA), and the immunoblot analysis was then performed. For the nuclear extracts, HCASMCs were cultured in 10-cm culture dishes until the cells reached 90% confluence. After the induction of quiescence, the cells were incubated with palmitate (50 μM) for 24 h. Nuclear protein extracts were prepared essentially as described below. The HCASMCs were homogenized in ice-cold hypotonic buffer (10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT, and phosphatase inhibitors) and centrifuged at 16,000 *g* for 10 min at 4°C. The homogenate was layered onto extraction buffer (20 mM HEPES, 25% glycerol, 0.42 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and phosphatase inhibitors) and centrifuged at 16,000 *g* for 60 min at 4°C. After centrifugation, the supernatant (nuclear extract) was collected, the protein concentration was measured with a Bradford assay kit (Bio-Rad), and the immunoblot analysis was performed. The extracts were used immediately or stored at −80°C for later use.

Protein analysis. Electrophoresis and immunoblot analyses were performed using a NuPAGE protein analysis system (Invitrogen, Life Technologies). The membranes were blocked with a 2% ECL Advance Blocking Reagent (Amersham Biosciences) and incubated with anti-Akt (4691, diluted 1:2,000; Cell Signaling Technology), anti-phospho-Akt (Ser^{473}) (4060; diluted 1:2,000; Cell Signaling Technology), anti-ERK1/2 (4695; diluted 1:10,000; Cell Signaling Technology), anti-phospho-p44/p42 MAPK (ERK1/2) Thr^{202}/Tyr^{204}(4370, diluted 1:10,000; Cell Signaling Technology), anti-phospho-Nrf2 (Ser^{40}) (2073; diluted 1:1,000; Epitomics; Abcam, Cambridge, UK), anti-Nrf2 (sc-722; diluted 1:1,000; Santa Cruz Biotechnology Dallas, TX), anti-NF-κBp65 (8242p; diluted 1:1,000; Cell Signaling Technology), anti-phosphor NF-κBp65 (Ser^{536}) (3033; diluted 1:1,000; Cell Signaling Technology), anti-fatty acid binding protein 4 (FABP4) (AF3150; diluted 1:1,000; R&D Systems), and anti-actin (sc-1616; diluted 1:10,000; Santa Cruz Biotechnology). The antigen-antibody complexes were detected by incubating the membrane with an horse-

radish peroxidase (HRP)-conjugated anti-IgG antibody (Goat Anti-Rabbit Immunoglobulins/HRP, P044901-2, and Rabbit Anti-Goat Immunoglobulins/HRP, P044801-2; diluted: 1:10,000; Dako, Glostrup, Denmark). The bands were visualized using ECL reagents (Amersham Pharmacia) with a ChemiDoc image system and quantified with Image Lab software, version 5.2 (Bio-Rad). The relative levels of the phosphorylated forms of Akt and ERK1/2 were quantified after normalization to the total protein levels, and the levels of the activated forms of the transcription factors (NF-κB and Nrf2) were expressed relative to the levels of the respective factors in the nonstimulated cells at each time point; all of the values were expressed in arbitrary units. The cellular protein content of angiopoietin-2, soluble cluster of differentiation 40 ligand, heparin-binding EGF-like growth factor (HB-EGF), endoglin, soluble FAS ligand, insulin-like growth factor binding protein, interleukin 6 (IL-6), IL-8, IL-18, plasminogen activator inhibitor-1 (PAI-1), placental growth factor, transforming growth factor-α, TNF-α, urokinase plasminogen activator (uPA), vascular endothelial growth factor A (VEGF-A), VEGF-C, and VEGF-D was determined using a Bio-Plex Pro Kit (Bio-Rad).

Quantitative real-time RT-PCR. To investigate the effect of palmitate on proinflammatory genes, HCASMCs were incubated with palmitate (2–100 μM) for 24 h. The total RNA was isolated from the cells using a ABI PRISM 6100 Nucleic Acid PrepStation (Applied

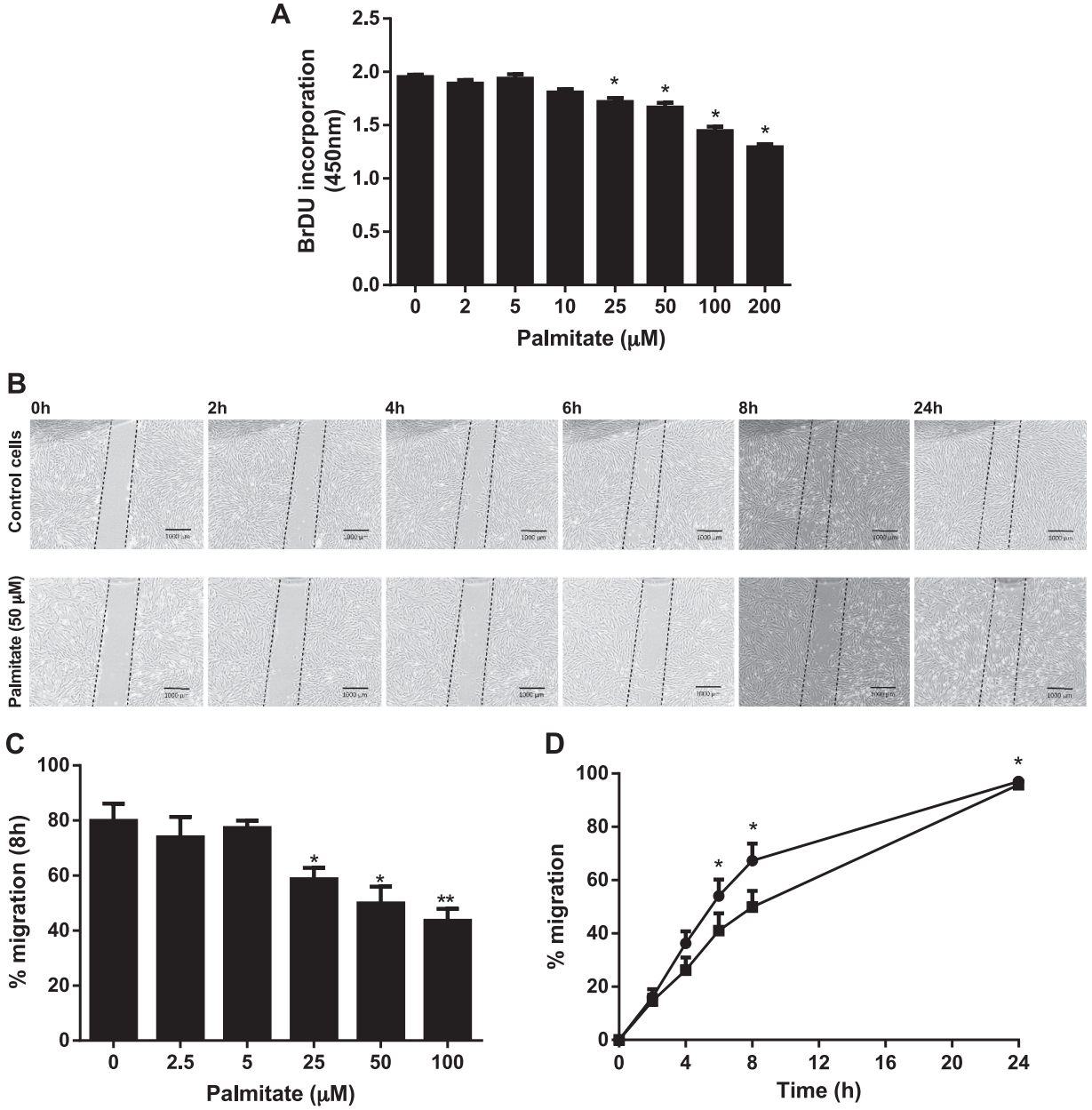

Fig. 1. The effects of palmitate on human coronary artery smooth muscle cell (HCASMC) migration and proliferation. *A*: the effects of palmitate (2–200 μM) on the proliferation of HCASMCs at 24 h were assessed by 5′-bromo-2′-deoxyuridine (BrdU) incorporation assays. *B*: an in vitro wound-healing assay indicated that palmitate at 50 μM decreased HCASMC migration. Confluent cells were scratch wounded and allowed to migrate for 24 h. Representative cells after 0, 2, 4, 6, 8, and 24 h of migration are shown. Photomicrographs of the cell images were obtained at ×40 magnification using a phase-contrast microscope. *C*: the effects of palmitate (2.5–100 μM) on HCASMC migration. *D*: a time course (0–24 h) of the effect of 50 μM palmitate on HCASMC migration. The results are shown as the percentage of the migration rate. The results are expressed as the means ± SE of 3 experiments performed in sextuplicate. *$P < 0.05$, **$P < 0.01$ vs. control (nontreated cells).

Biosystems). The absorbance at 260 nm was used to measure the RNA concentration, and an absorbance ratio of 260/280 nm was used to analyze the quality of the RNA. Total RNA (0.5 μg) was reverse transcribed to cDNA using random hexamers and SuperScript II (Invitrogen, Life Technologies) following the manufacturer's protocol. TaqMan primers and probes for FABP4, monocyte chemoattractant protein 1 (MCP1), matrix metalloproteinase-2 (MMP2), IL-1β, IL-6, IL-8, TNF-α, and NAD(P)H quinone oxidoreductase 1 (NQO1) were obtained from validated and pre-designed Assays-on-Demand products (Applied Biosystems) and were used in the real-time PCR amplifications. The mRNA expression for each gene and sample was calculated using the recommended $2-\Delta\Delta Ct$ method. The control group (untreated cells) was used for calibration in this experiment. GAPDH was used as a housekeeping gene to normalize the results of the genes of interest.

Statistical analysis. The results are represented as the means ± SE of at least three separate experiments. Differences between the means were determined using a *t*-test or a one-way ANOVA, which was followed by a Dunnett's post hoc test for multiple comparisons. The differences were considered to be significant at $P < 0.05$ GraphPad Prism 5.0 software (GraphPad Software) was used for the statistical analyses.

RESULTS

Palmitate decreases proliferation and migration in HCASMCs. To assess the effect of palmitate on cell viability, LDH and ATP assays were assayed. None of the palmitate concentrations tested (5–100 μM) increased LDH leakage from HCASMC compared with nontreated cells at either 5 or 24 h of incubation (Supplemental Fig. S1*A*; Supplemental Material for this article can be found at https://www.doi.org/10.5281/zenodo.2607392). In addition, 5–50 μM palmitate did not decrease intracellular ATP content, although 100 μM palmitate did lower ATP content by 12.6% after 24 h of incubation. (Supplemental Fig. S1*B*).

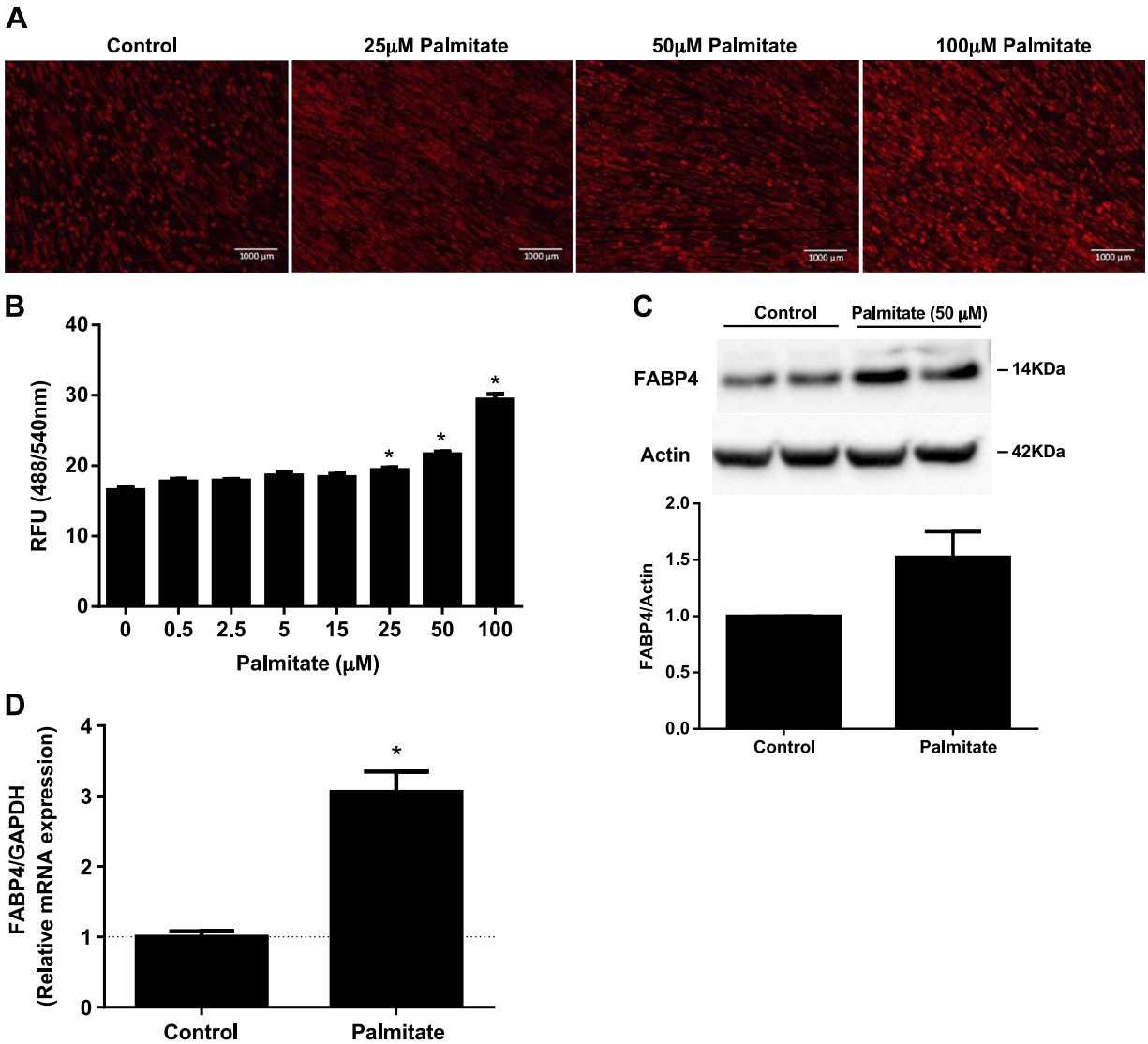

Fig. 2. Palmitate induces intracellular lipid droplets in human coronary artery smooth muscle cells (HCASMCs). *A*: fluorescence images of Nile red-stained cells incubated with and without 25, 50, and 100 μM of palmitate for 24 h. *B*: the Nile red fluorescence intensity of HCASMCs incubated with 0.5–100 μM palmitate for 24 h. RFU, relative fluorescence units. *C*: the effect of palmitate (50 μM) on fatty acid binding protein 4 (FABP4) protein expression in HCASMCs. *D*: the effect of palmitate on FABP4 mRNA expression in HCASMCs. Representative Western blots and relative densitometric analysis are shown. Actin was used as a loading control. The results are expressed as the means ± SE of at least 3 separate experiments. *$P < 0.05$ vs. control (nontreated cells).

To assess the effect of palmitate on HCASMC proliferation, we performed concentration-response experiments using BrdU uptake as a marker of DNA synthesis. When the HCASMCs were stimulated with increasing concentrations of palmitate (2–200 μM), a concentration-dependent decrease in cell proliferation was observed (Fig. 1*A*). Palmitate had no effect on cell proliferation at 10 μM, while a significant decrease in proliferation was observed at 25 μM to up to 200 μM (−10.5 and −31.6% vs. unstimulated cells, respectively, $P < 0.05$).

To address the effect of palmitate on HCASMC migration, a wound-healing assay was performed. We analyzed cell migration every 2 h for 24 h. As shown in Fig. 1*B*, palmitate-treated (50 μM) cells migrated later than the untreated cells and completely closed the denuded area after 24 h of treatment. As shown in Fig. 1*C*, dose-response studies (2.5–100 μM) revealed that palmitate significantly decreased cell migration at 25 μM, 50 μM and 100 μM with respect to the untreated cells at 8 h (−26.6, −37.7, and −45.5%, respectively, $P < 0.05$). As shown in Fig. 1*D*, the time-course studies (0 h-24 h) revealed that palmitate (50 μM) significantly decreased the migration of HCASMCs compared with the untreated cells, which was significant at 4 h (−27.8%, $P < 0.05$) until 10 h of treatment (−26.0%, $P < 0.05$). Both treated and untreated cells reached 100% migration at the 24-h time point.

Palmitate increases intracellular lipid droplets in HCASMC. To address the effect of palmitate on intracellular lipid droplet formation, we stained the cells with Nile red. The photographs presented in Fig. 2*A* show the increased fluorescence of Nile red-stained cells treated with palmitate at 25, 50, and 100 μM. As shown in Fig. 2*B*, dose-response studies (0.5–100 μM) revealed that palmitate significantly

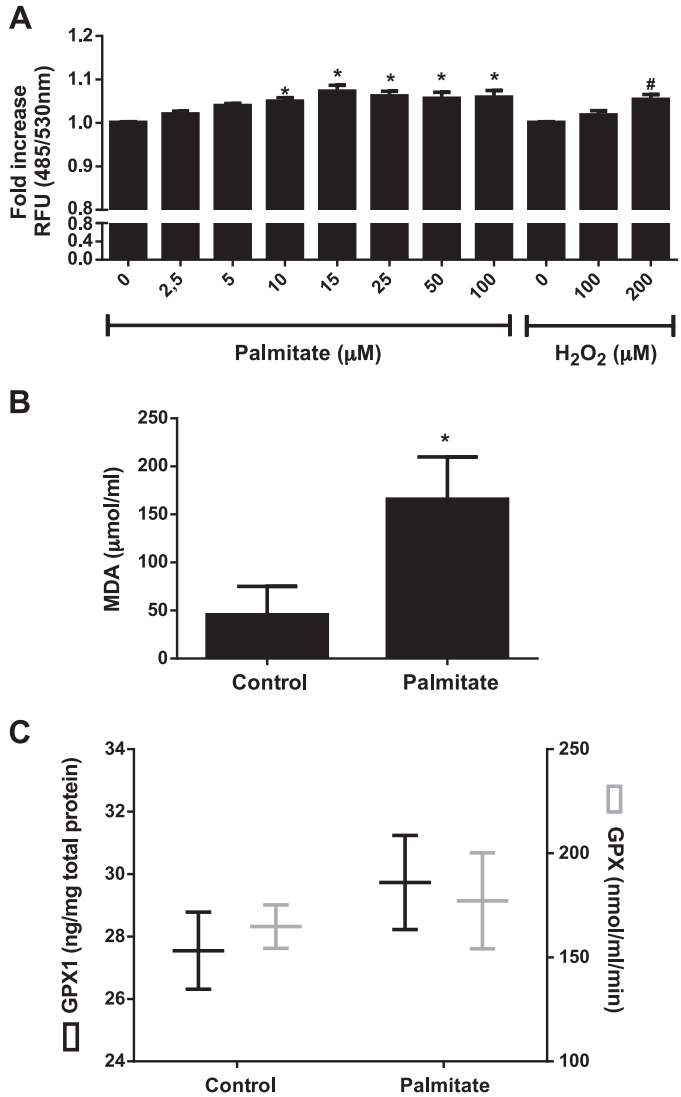

Fig. 3. Palmitate induced intracellular reactive oxygen species (ROS) production in human coronary artery smooth muscle cells (HCASMCs). *A*: intracellular ROS were examined after incubation with 2′,7′-dichlorodihydrofluororescein diacetate for 30 min, and dichlorodihydrofluorescein fluorescence intensity was detected using a fluorescence reader. Previously, HCASMCs were treated with and without palmitate (2.5–100 μM) for 24 h. H_2O_2 (100 and 200 μM) was used as a positive control of intracellular ROS production. RFU, relative fluorescence units. *B*: malondialdehyde (MDA) levels after incubation with palmitate (50 μM) for 24 h. *C*: the concentration of intracellular glutathione peroxidase (GPX) protein and activity after incubation with palmitate (50 μM) for 24 h. The results are expressed as the means ± SE of 3 experiments run at least in triplicate. *$P < 0.05$ vs. control (nontreated cells); #$P < 0.001$ vs. control (nontreated cells).

increased intracellular lipid droplets measured as the fluorescence intensity of the HCASMCs at 25, 50, and 100 μM with respect to untreated cells (17.6, 30.9, and 78.2%, respectively, $P < 0.05$). As FABP4 is one of the most well-characterized intracellular lipid transport proteins (27), we analyzed the effect of palmitate at both protein and mRNA expression. The results showed a not significant increase in FABP4 protein expression after incubation with palmitate at 50 μM for 24 h (52 ± 16% increase, $P = 0.08$) (Fig. 2*C*). In addition, palmitate led to a robust elevation in FABP4 expression at mRNA levels (3.06 ± 0.03-fold increase, $P < 0.05$) (Fig. 2*D*).

Palmitate induces intracellular ROS production in HCASMCs. To examine whether palmitate promotes the generation of ROS in HCASMCs, we measured intracellular ROS levels following treatment of the cells with palmitate (0.5 to 100 μM) for 24 h. As shown in Fig. 3*A*, 10 and 100 μM palmitate significantly increased ROS formation (5.0 and 5.9%, respectively; $P < 0.05$), reaching a maximum effect at 15 μM palmitate (7.3%; $P < 0.05$). Exposure to 200 μM H_2O_2, a positive control for ROS production, produced a similar, 5.4% increase ($P < 0.05$) in intracellular ROS. In addition, Fig. 3*B* showed that palmitate significantly increased MDA levels (72.9%, $P < 0.05$). GPX1 is an important intracellular antioxidant enzyme that protects cells from oxidative damage (17). Next, we investigated the effect of palmitate on intracellular GPX1 concentrations and activity. Palmitate at 50 μM resulted in a moderate but not significant increase in both GPX1 intracellular protein and activity at 24 h (7.3%, $P = 0.370$ and 7.5%, $P = 0.670$, respectively; Fig. 3*C*).

Palmitate induces the expression of proinflammatory genes in HCASMCs. To determine if the increase in ROS production and lipid droplet accumulation induced by palmitate are associated with an inflammatory cellular state, HCASMCs were treated with 50 μM palmitate for 24 h, and the expression levels of 18 proteins were analyzed using a Bio-Plex system. HCASMCs expressed 10 of the 18 proteins tested (IL-6, endoglin, soluble cluster of differentiation 40 ligand, VEGF-A, VEGF-C, VEGF-D, IL-8, HB-EGF, PAI-1, and uPA) (Fig. 4*A*). The main effects were obtained for PAI-1 (229.6%, $P < 0.05$) and uPA (138%, $P < 0.05$) (Fig. 4*A*).

Moreover, we tested the effects of palmitate on the gene expression of genes that encode inflammatory markers MCP1, MMP2, IL-1β, IL-6, IL-8, and TNF-α. HCASMCs were treated with palmitate (100 μM) for 24 h, and the expression levels of MCP1, MMP2, IL-1β, IL-6, IL-8, and TNF-α were analyzed (Fig. 4*B*). The results showed that palmitate at 100 μM significantly increased the mRNA levels of the genes studied ($P < 0.05$); the greatest effect was observed for TNF-α (30.17 ± 12.43-fold increase, $P < 0.05$).

Palmitate activates the transcription factor Nrf2 and the upstream kinases ERK1/2 and Akt in HCASMCs. We next studied two transcription factors associated with a ROS and inflammatory/proatherogenic state, NF-κB and Nrf2. HCASMCs were treated with 50 μM palmitate for 24 h, and pNF-κB and pNrf2 in the nucleus were analyzed. The results revealed a significant increase in pNrf2 (1.61 ± 0.07-fold change, $P < 0.05$) without a significant effect on pNF-κB (1.89 ± 0.67-fold change, $P = 0.256$) (Fig. 5, *A* and *B*, respectively). To confirm the involvement of the transcription factor Nrf2 in the palmitate-induced oxidative stress response, HCASMCs were preincubated with NAC (10 mM), an antioxidant drug, before the addition of palmitate (100 μM). We found that NAC significantly attenuated palmitate-induced HCASMC activation of pNrf2 (0.53 ± 0.02-fold change, $P < 0.05$) (Fig. 5*A*). SP600125, a JNK inhibitor, did not show an effect on palmitate-induced HCASMC activation of pNrf2.

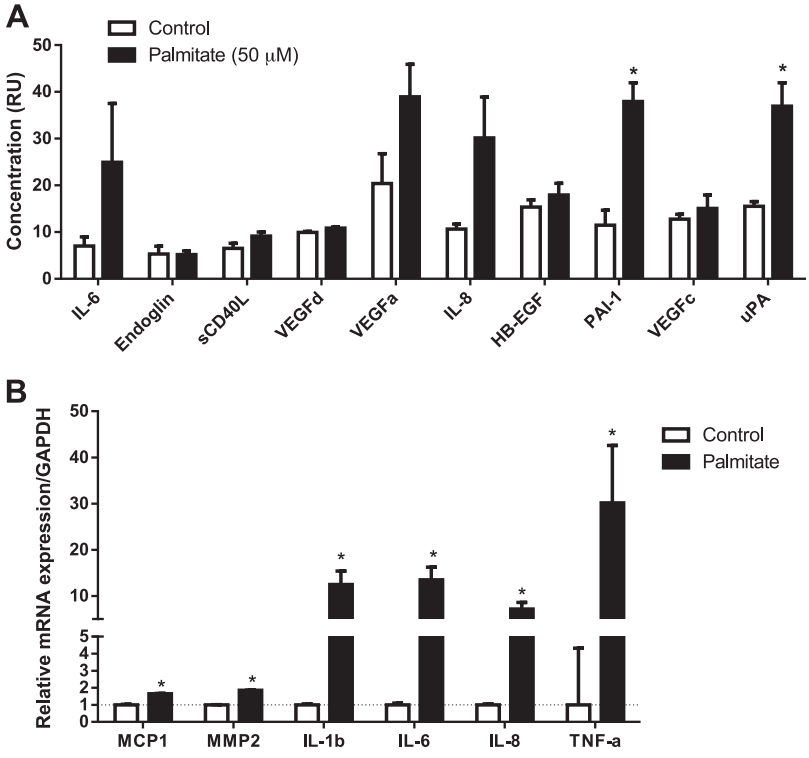

Fig. 4. The effect of palmitate on the expression of proinflammatory genes in human coronary artery smooth muscle cells (HCASMCs). *A*: cellular protein content of soluble cluster of differentiation 40 ligand (sCD40L), endoglin, heparin-binding EGF-like growth factor (HB-EGF), IL-6, IL-8, plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator (uPA), vascular endothelial growth factor (VEGF)-A, VEGF-C, and VEGF-D after 24 h of incubation with palmitate at 50 μM. The analytes were analyzed via a Luminex multiplex immunoassay. RU, relative units. *B*: HCASMCs were incubated with 50 μM of palmitate for 24 h, the total RNA was extracted from cells, and the expression levels of matrix metalloproteinase-2 (MMP2), monocyte chemoattractant protein-1 (MCP1), IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) were measured by quantitative RT-PCR using TaqMan primers and the $2-\Delta\Delta Ct$ method. The data are the means ± SE of 3 experiments performed at least in triplicate. *$P < 0.05$ vs. control (nontreated cells).

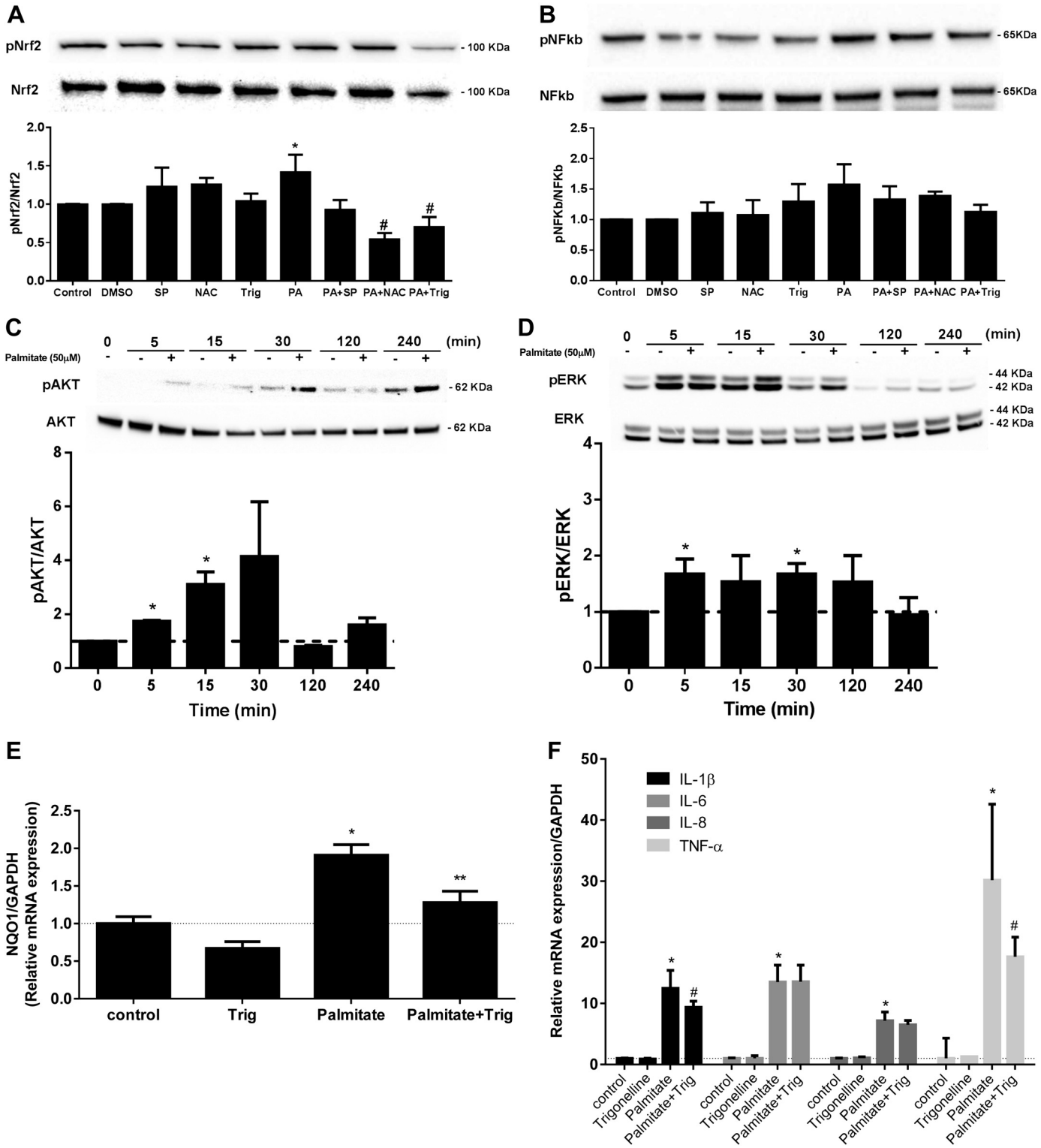

Fig. 5. The effect of palmitate (PA) on Nrf2, NF-κB, Akt, and MAPK activation. The cells were treated for 1 h with SP600125 (10 μM) and *N*-acetylcysteine (10 mM) or vehicle (DMSO), after which palmitate or vehicle was added to the medium, and the cells were incubated for an additional 24 h. *A* and *B*: the nuclear activation of Nrf2 (*A*) and NF-κB (*B*) were analyzed. *C* and *D*: the effect of palmitate (50 μM) on Akt (*C*) and MAPK (*D*) activation in the total lysates of human coronary artery smooth muscle cells (HCASMCs) at the indicate times. Representative Western blots and relative densitometric analyses are shown. *E*: effect of palmitate on NAD(P)H quinone oxidoreductase (NQO1) mRNA expression in HCASMC. The cells were treated for 1 h with trigonelline (Trig; 0.5 μM), after which palmitate was added to the medium, and the cells were incubated for an additional 24 h. *F*: effect of palmitate on interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) mRNA expression in HCASMC. The cells were treated for 1 h with trigonelline (0.5 μM), after which palmitate was added to the medium, and the cells were incubated for an additional 24 h. The data are the means ± SE of 3 experiments performed at least in triplicate. $^{*}P < 0.05$ vs. control (nontreated cells); $^{**}P < 0.05$ vs. palmitate.

The Nrf2 signaling pathway can be modulated by several upstream kinases, including phosphatidylinositol 3-kinase and mitogen-activated protein kinases. Therefore, the effects of palmitate on the activation of ERK1/2 and Akt were explored. Cells were incubated with palmitate (50 μM) for 5, 15, and 30 min and 2 and 4 h, and the total extracts were analyzed. The effects of palmitate treatments on the activation of the ERK and Akt were assessed by measuring the ratio of phosphorylated/total ERK (both ERK1 and 2) and total Akt based on Western blotting. The results indicated that palmitate rapidly and significantly activated ERK1/2 and Akt in as little as 5 min ($P < 0.05$) (Fig. 5, *C* and *D*); the phosphorylation returned to basal levels after 120 min.

We next examined the expression of the Nrf2 target gene NQO1. The results indicated that palmitate significantly increases twofold the expression of the gene. Addition of trigonelline significantly attenuated palmitate-induced HCASMC expression of mRNA NQO1 (0.63 ± 0.02-fold change, $P < 0.05$) (Fig. 5*E*). We found that trigonelline significantly attenuated palmitate-induced HCASMC expression of IL-1β and TNF-α ($P < 0.05$) (Fig. 5*F*) but did not alter palmitate-induced HCASMC expression of IL-6 and IL-8.

DISCUSSION

The present study elucidates the effects of palmitate on HCASMC function and includes three main findings. First, we demonstrated that palmitate caused a decrease in cell migration and proliferation. Second, we found that palmitate increases the intracellular content of lipid droplets and ROS. Third, we showed that palmitate increases the inflammatory responses in these cells. Together, these findings contribute to the understanding of the effects of palmitate on HCASMC function.

Serum palmitate concentrations range between 100 μM under physiological conditions to 300 μM in diabetes (6a, 27a). The palmitate concentrations in this study (0–100 μM) did not exceed the physiological serum concentration range. In addition, it should be noted that serum palmitate concentrations include the esterified fraction, the fraction bound to proteins and the unbound free fraction. Although only a small fraction of circulating fatty acids is unbound, the interstitial concentrations of this fraction, which is in direct contact with vascular smooth muscle, would be higher than those found in plasma.

We found that palmitate decreased HCASMC proliferation and migration in a time and dose effect. This effect was not associated with an increase of cell death, although a slightly decrease in the amount of ATP at 100 μM was observed, which paralleled the decreased in the proliferation and migration of HCASMC.

There are conflicting results in the literature regarding the direct effect of palmitate on proliferation and migration. Some studies have shown that palmitate has no effect on human coronary VSMC proliferation and migration (12, 13), whereas other studies have reported that palmitate decreases the proliferation in human bladder smooth muscle cells (21) and human endothelial cells (6). Furthermore, the results of other authors demonstrate that macrophage-conditioned medium in combination with palmitate increases the migration and proliferation of rat aortic VSMCs (5), but adipocyte-conditioned medium has no effect (13). This diversity of the results could be explained, at least partially, by the fact that VSMCs can be obtained from distinct vascular beds, and one cannot assume the same behavior for a common stimulus. For instance, it has been shown that 2-hydroxyestradiol more effectively inhibits cell proliferation in coronary artery VSMCs compared with aortic VSMCs (30). Our results performed in human coronary artery VSMCs support an inhibitory effect of palmitate on the migration and proliferation of these cells. These results could represent a decrease in the plaque fibrous cap formation leading to a more unstable plaque. In addition, other characteristics of VSMC function are involved in the atherosclerotic process, such as inflammation, lipid accumulation, and oxidation.

The formation of lipid-laden VSMCs has been documented in both in vivo and in vitro studies (1, 7, 8). Our results showed that palmitate-laden HCASMCs increase the intracellular content of lipid droplets and FABP4. Other studies have shown that, when aortic VSMCs are cultured in adipogenic differentiation medium, these cells are able to accumulate lipids and induce the expression of adipocyte marker genes such as adipsin, FABP4, C/EBP, peroxisome proliferator-activated receptor, and leptin (8). In addition, our results show a direct effect of palmitate on the accumulation of lipid droplets, as incubation with palmitate alone was used in our in vitro studies.

Palmitate is known to induce oxidative stress in different types of cells such as pancreatic β-cells (19), skeletal muscle cells (11), and rat aortic VSMCs (3, 25). Our results also showed that palmitate increases ROS production and MDA levels in HCASMCs, which are in turn important for the induction of a proinflammatory state in VSMCs. Although the increase in ROS levels detected with the fluorescence is small, this effect together with the 27% increase in MDA levels

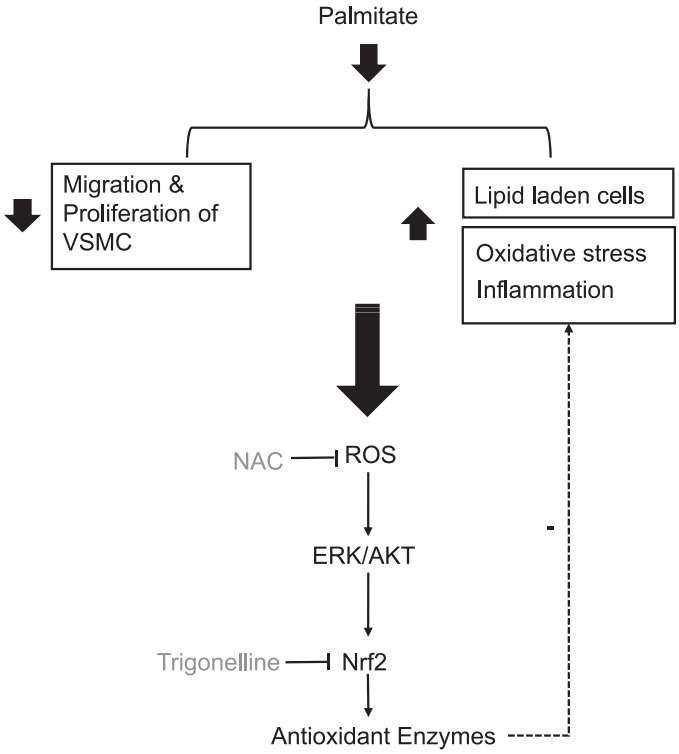

Fig. 6. Model proposed for the palmitate effect on human coronary artery smooth muscle cells. NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; VSMC, vascular smooth muscle cells.

supports that palmitate would have an impact on cell physiology. These results are in agreement with previously published studies, which have shown the same results but in different models. Shen et al. (25) showed that palmitate increased ROS levels, mediating the modulation of genes involved in inflammatory processes contributing to phenotypic modulation. In addition, rat in vitro and ex vivo experiments showed that palmitate increases calcification and ROS production (3). Furthermore, Wu et al. (29) reported that palmitate induced CRP, iNOS, and TNF-α in rat VSMCs. Moreover, Quan et al. (23) showed that palmitate induced IL-8 in human aortic VSMCs via the TLR4/NF-κB pathway. Increased ROS production was paralleled by an increase in inflammatory mediators, as indicated by increased mRNA abundances and protein contents of proinflammatory genes. In agreement with previous studies (23, 29), palmitate induced gene expression (MCP1, MMP2, IL-1β, IL-6, IL-8, and TNF-α) and intracellular protein content (PAI-1 and uPA) of inflammatory mediators. The lack of effect on GPX1, an important intracellular antioxidant enzyme that protects cells from oxidative damage (17), does not exclude expression of other antioxidant enzymes, e.g., SOD or catalase.

Different pathways are involved in palmitate ROS signaling. In this work, we found that palmitate induces ROS production and activates the transcription factor Nrf2. Inhibition of this pathway by pharmacological inhibition of ROS with NAC attenuated the activation of Nrf2 induced by palmitate in HCASMC. The Nrf2 signaling pathway can be modulated by several upstream kinases including phosphatidylinositol 3-kinase, protein kinase C, and mitogen-activated protein kinases (14). We found that palmitate rapidly activated MEK/ERK and Akt. Our results show that Nrf2 activation by palmitate could enhance ERK1/2 activation, leading to VSMC dysfunction. Upon exposure of cells to oxidative stress, Nrf2 translocates into the nucleus to bind to antioxidant-responsive elements in the genes that encode antioxidant enzymes such as NQO1 (20). We found that palmitate enhanced the expression of the Nrf2 target gene NQO1. The fact that trigonelline, an inhibitor of Nrf2, reversed the action of palmitate implicates Nrf2 in the palmitate signaling pathway.

In summary (Fig. 6), our results demonstrate that palmitate is crucially involved in vascular pathology through the induction of inflammatory phenotypes in VSMCs, which is in part mediated by the ROS/Nrf2 pathway. However, we cannot rule out that Nrf2 was activated in response to oxidative stress induced by palmitate, which would represent an attempt to minimize palmitate's inflammatory and pro-oxidant actions. These results could provide new therapeutic targets for the prevention of atherosclerosis in patients with increased circulating levels of palmitate similar to those with metabolic disorders.

The current study proposes that palmitate takes part in the deleterious vascular consequences found in patients with elevated levels of palmitate, such as in diabetic and obese patients and those with metabolic syndrome, via triggering lipid-laden cells, oxidative stress, and the inflammation of HCASMCs.

GRANTS

This work was supported by the Spanish Biomedical Research Centre in Diabetes and the Associated Metabolic Disorders (CIBERDEM) and Instituto Carlos III (FIS-PI10/02547) and Fondo Europeo de Desarrollo Regional funds.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.G., L.M., and J.-C.V. conceived and designed research; J.G., R.R., and P.S. performed experiments; J.G., P.S., L.M., and J.-C.V. analyzed data; J.G., P.S., L.M., and J.-C.V. interpreted results of experiments; J.G. and R.R. prepared figures; J.G., L.M., and J.-C.V. drafted manuscript; J.G., L.M., and J.-C.V. edited and revised manuscript; J.G., R.R., P.S., L.M., and J.-C.V. approved final version of manuscript.

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