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Palmitate induces C-reactive protein expression in human aortic endothelial cells. Relevance to fatty acid–induced endothelial dysfunction

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Abstract

Circulating levels of free fatty acids are commonly elevated in patients with the metabolic syndrome and exert, through activating proinflammatory pathways, harmful effects of the vascular endothelium. In this study, we examined the effect of palmitate (PA) on endothelial C-reactive protein (CRP) expression and the role of CRP in PA-induced nitric oxide (NO) inhibition. Palmitate increased, in a dose-dependent manner, CRP protein expression and production in human aortic endothelial cells (HAECs). Induction of CRP protein was mimicked by ceramide, whereas bromopalmitate and other common free fatty acids such as oleate or linoleate were ineffective. Palmitate also elicited reactive oxygen species production in HAECs, an effect prevented by protein kinase C (PKC) inhibition and adenosine monophosphate–activated kinase (AMPK) activation. Palmitate-treated HAECs showed increased CRP messenger RNA expression and nuclear factor (NF)–κB activation. Induction of CRP expression by PA was prevented by antioxidants and normalized by PKC and mitogen-activated protein kinase inhibitors. Disrupting NF-κB and Janus kinase/signal transducers and activators of transcription pathways or inducing AMPK activation also suppressed the stimulatory effect of PA on CRP messenger RNA expression. Finally, in HAECs, PA reduced NO release, an effect reversed by anti-CRP antibody. These data demonstrate that PA-induced endothelial CRP expression involves PKC-driven oxidative stress, possibly through AMPK inhibition, and activation of downstream redox-sensitive signaling pathways, including NF-κB. They further support a role for endothelial cell–derived CRP as mediator of the suppressive effect of PA on NO production.

1. Introduction

Endothelial dysfunction, the earliest event in atherogenesis, is an important component of the metabolic syndrome. In insulin-resistant (IR) states such as diabetes and obesity, endothelial dysfunction has been linked to the damaging effects of high circulating levels of free fatty acids (FFAs) on the vascular endothelium [1], resulting in impaired insulin signaling and reduced endothelial nitric oxide synthase (eNOS) activity [2-4]. Recent data have shown that activation of proinflammatory signaling pathways plays a critical role in FFA-mediated vascular insulin resistance. Indeed, it has been demonstrated that IκB-kinase β (IKKβ) mediates the negative effect of palmitic acid (PA) on endothelial nitric oxide (NO) production, thus establishing this inflammatory pathway as a critical mediator of the deleterious effect of this FFA on endothelial function [5,6].

Diabetes, obesity, and other IR states are associated with a systemic inflammatory response characterized by increased levels of circulating markers of inflammation. Among these, the prototypic marker of inflammation C-reactive protein (CRP) has been implicated as an important etiologic factor in the development of cardiovascular disease, insulin resistance, and type 2 diabetes mellitus [7]; and adding high-sensitivity CRP to the definition of the metabolic syndrome has been shown to improve the prediction of cardiovascular disease among apparently healthy women [8]. In addition to being a cardiovascular risk marker, CRP might have a causal role in atherogenesis and contribute, through direct effects on vascular cells, to endothelial dysfunction. Supporting this possibility, an inverse relationship between CRP levels and endothelial

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function has been documented in patients with diabetes and coronary artery disease [9,10]; and an inhibitory effect of CRP on endothelial NO expression and vasoreactivity has been documented both in vitro and in vivo [11-15].

Vascular endothelial cells are an important component of the atherosclerotic lesion and do produce CRP in response to inflammatory and metabolic factors associated with diabetes and obesity, including proinflammatory cytokines [16], high glucose [17], and adipocyte-derived secretory products [17,18]. Thus, high local CRP concentrations may occur in the atherosclerotic lesion of patients with the metabolic syndrome and promote endothelial dysfunction. To further characterize the regulation of endothelial cell-derived CRP in IR states, we examined the effect of PA, the most abundant saturated FFA in plasma [19], on CRP expression by cultured human aortic endothelial cells (HAECs) and investigated the signaling pathways involved in this effect. In view of the striking similarity in vascular changes induced by PA and CRP, the role of CRP as mediator of the inhibitory effect of FFA on endothelial NO production was further evaluated.

2. Material and methods

2.1. Reagents

Fetal bovine serum was purchased from Wisent (St Bruno, Quebec, Canada). Penicillin-streptomycin, sodium dodecyl sulfate, glycine, and TRIzol reagent were obtained from Invitrogen Life Technologies (Burlington, Ontario, Canada). The antioxidant compounds *N*-acetyl-L-cysteine (NAC) and apocynin, the 5′-adenosine monophosphate–activated kinase (AMPK) activator AICAR, the protein kinase C (PKC) inhibitors calphostin C and GF109203X, the extracellular signal–regulated kinases (ERK) inhibitors U0126 and PD98059, the c-jun N-terminal kinase (JNK) inhibitor SP600125, the IκBα phosphorylation inhibitor BAY11-7085, and the Janus kinase (Jak)/signal transducers and activators of transcription (STAT) inhibitor AG490 were purchased from Calbiochem (La Jolla, CA). The specific PKCβ inhibitor LY379196 was kindly provided by Eli Lilly (Indianapolis, IN). Affinity-purified polyclonal antibody against IκBα and anti-CRP and PKCβ antibodies were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphorylated and unphosphorylated AMPK and STAT-3 antibodies were kindly provided by Dr Marc Prentki, CRCHUM, University of Montreal, Canada. E-TOXATE kit, sodium azide (NaN3), endotoxin- and FFA-free bovine serum albumin (BSA) fraction V, and 2′,7′-dichlorofluorescin diacetate (DCF-DA) were purchased from Sigma (St Louis, MO). END-X B15 endotoxin removal affinity resin kit was obtained from Seikagaku America (Falmouth, MA). Highly purified (99%) recombinant human native CRP was purchased from Calbiochem. CRP was free of endotoxin, as assessed by the Limulus assay. NaN3 was removed from the commercial CRP preparation by dialysis against 500 mL 20 mmol/L Tris-HCl, pH 7.5, 140 mmol/L NaCl, and 2 mmol CaCl2 at 4°C.

2.2. Preparation of PA-albumin complexes

PA was added to the culture medium as PA-BSA complex. Briefly, sodium salt PA was dissolved in 10% FFA-free BSA fraction V at 5 mmol/L. This stock solution was filter-sterilized and stored at −20°C. PA was added to the culture medium at final concentrations of 0.05 to 0.3 mmol/L.

2.3. Human aortic endothelial cells

Primary HAECs (2×10^6 cells) were plated in 24-well cell culture plates (Costar, Corning, NY) and grown in endothelial growth medium (EGM) under recommended conditions. The EGM was supplemented with 2% fetal bovine serum containing 0.2 μg/mL cupric sulfate, 0.01 μg/mL human epidermal growth factor, 0.1% gentamicin sulfate amphotericin B, 1 μg/mL hydrocortisone, and 12 μg/mL bovine brain extract protein content. At 80% confluence, cells were incubated in serum- and growth factor–free media in the presence of PA bound to BSA. Control cells were incubated with serum-free EGM containing equal concentrations of BSA as present in PA-treated cells. HAECs were used in the experiments at passages 3 to 5.

2.4. Determination of CRP protein expression

HAECs were treated for 24 hours with increasing concentrations of PA (0.05-0.3 mmol/L) or were stimulated for 24 hours with PA (0.2 mmol/), bromopalmitate (0.2 mmol/L), oleate (0.2 mmol/L), linoleate (0.2 mmol/L), or C_2-ceramide (15 μmol/L). In some experiments, HAECs were pretreated for 1 hour with calphostin C (CAL C) (0.1 μg/mL), NAC (10 mmol/L), or apocynin (10 μmol/L) and then exposed to PA (0.2 mmol/L) for 24 hours.

At the end of the incubation period, cells were lysed; and CRP protein expression was determined by Western blot analysis. Briefly, cell protein extracts (15 μg) were applied to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using a Bio-Rad (Mississauga, Ontario, Canada) transfer blotting system at 100 V for 1 hour. Nonspecific binding was blocked with 5% milk for 1 hour at room temperature. After washing with phosphate-buffered saline–Tween 0.1%, blots were incubated overnight at 4°C with anti-CRP (1/1000) or anti-β-actin antibodies (1/5000). After further washing, membranes were incubated for 1 hour at room temperature with a horseradish peroxidase–conjugated donkey anti-mouse immunoglobulin (Ig) G (1/5000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

2.5. Determination of CRP secretion

The amounts of CRP secreted by the cells exposed to increasing PA concentrations (0.05-0.3 mmol/L) for 24 hours were measured in 20-fold concentrated supernatants using a commercially available enzyme-linked immunosorbent assay kit specific for human CRP (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The minimum detectable concentration with this assay is 78 pg/mL. Levels of CRP in the cell supernatants were normalized to the levels of total cell proteins.

2.6. Measurement of intracellular reactive oxygen species generation

Intracellular reactive oxygen species (ROS) generation was determined according to the method of Royall and Ischiropoulos [20]. HAECs (1×10^6 cells) were plated in 24-well cell culture cluster plates (Costar) in EGM under recommended conditions. At confluence, cells were incubated in serum- and growth factor–free media and pretreated or not for 1 hour with appropriate agents before exposure to PA for a further 3-hour period at 37°C, with addition of the cell-permeable fluorogenic probe DCF-DA (20 μg/mL) during the last 20 minutes of incubation. After washing, cells were trypsinized; and intracellular ROS production was monitored by measuring fluorescence in an LS50B luminescence spectrophotometer (Perkin Elmer, Boston, MA) using excitation and emission wavelengths of 498 and 522 nm, respectively.

2.7. Measurement of CRP gene expression

CRP gene expression was measured by semiquantitative polymerase chain reaction (PCR) in HAECs incubated with the combination of interleukin (IL)-1β (25 ng/mL) and IL-6 (10 ng/mL), lipopolysaccharide (LPS) (1 μg/mL), CRP (10 μg/mL), or PA (0.2 mmol/L) for 6 or 24 hours. In some experiments, CRP messenger RNA (mRNA) levels were determined in HAECs pretreated for 1 hour with CAL C (0.1 μg/mL), LY379196 (20 nmol/L), GF109203X (20 nmol/L), PD98059 (10 μmol/L), U0126 (20 μmol/L), SP600125 (50 μmol/L), NAC (10 mmol/L), apocynin (10 μmol/L), AICAR (0.5 μmol/L), BAY11-7085 (10 μmol/L), or AG490 (20 μmol/L) before incubation with PA (0.2 mmol/L) for 24 hours. Total RNA for use in the PCR reaction was extracted from cells by an improvement of the acid-phenol technique of Chomczynski. Briefly, cells were lysed with TRIzol reagent; and chloroform was added to the solution. After centrifugation, the RNA present in the aqueous phase was precipitated and resuspended in diethyl pyrocarbonate water. Complementary DNA (cDNA) was synthesized from RNA by incubating total cellular RNA (2 μg per reaction) with 0.1 μg oligodT (Pharmacia, Piscataway, NJ) for 5 minutes at 98°C and then by incubating the mixture with reverse transcription buffer for 1 hour at 37°C. The cDNA obtained was amplified by using 0.8 μmol/L of 2 synthetic primers specific for human CRP (5′-TCGTATGCCACCAAGAGACAAGACA-3′ and 5′-AACACTTCGCCTTGCACTTCATACT-3′) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′-CCCTTCATTGACCTCAACTACATGG-3′ and 5′-AGTCTTCTGGGTGGCAGTGATGG-3′) used as internal standard in the PCR reaction mixture. A 440–base pair human CRP cDNA fragment and a 456–base pair human GAPDH cDNA fragment were amplified enzymatically by 35 and 20 repeated cycles, respectively. An aliquot of each reaction mixture was then subjected to electrophoresis on 1% agarose gel containing ethidium bromide. The intensity of the bands was measured by an image analysis scanning system (Alpha Imager 2000; Packard Instrument, Meriden, CT). Titrating the cDNA samples ensured that the signal lies on the exponential part of the standard curve. Results obtained by semiquantitative PCR were confirmed by quantitative real-time PCR as previously described [21] using specific primers for human CRP (5′-GTGTTTCCCAAAGAGTCGGATACT-3′ and 5′-CCACGGGTCGAGGACAGTT-3′).

2.8. Western blots for PKC, AMPK, STAT-3, and IκBα protein expression

Western blotting for PKCβ, phosphorylated AMPK, phosphorylated STAT-3, and IκBα was performed using isozyme-specific monoclonal antibody against PKCβ (1/1000), anti-phosphorylated AMPK (1/1000) and STAT-3 (1/1000) antibodies, and affinity-purified polyclonal antibody against IκBα (1/1000). β-Actin or unphosphorylated total antibodies were used as internal controls.

2.9. Determination of NO production

NO production by HAECs was determined in the supernatants of cells pretreated for 1 hour with anti-CRP antibody (10 μg/mL), anti-IgG antibody (10 μg/mL), AICAR (0.5 μmol/L), or BAY11-7085 (10 μmol/L) before exposure to PA (0.2 mmol/L) for 24 hours. NO production by HAECs treated with CRP (10 μg/mL) for 24 hours was also evaluated. The measure of NO released by HAECs was performed according to the method of Green et al [22]. Results were expressed as nitrite production per total cell protein content.

2.10. Determination of endotoxin levels

The endotoxin content of the PA preparations (0.2 mmol/L) was determined by the limulus amebocyte lysate assay and was consistently found to be lower than 3 pg/mL.

2.11. Determination of protein concentrations

Total protein content was measured according to the Bradford method using a colorimetric assay (Bio-Rad).

2.12. Determination of cell viability

To exclude the possibility that PA, at maximal concentrations used in this study, may exert cytotoxic effect, cell viability after treatment with PA was determined by trypan blue exclusion. It was consistently found to be equal to or higher than 80% (data not shown).

2.13. Statistical analysis

Statistical analysis of the results was performed by 1-way analysis of variance followed by the Student-Newman-Keuls test. Differences were considered to be of statistical significance at $P < .05$. Results are expressed as the mean ± SEM.

3. Results

3.1. PA induces CRP protein expression and production by HAECs

Incubation of HAECs with PA enhanced, in a dose-dependent manner, CRP protein expression (Fig. 1A). Maximal effect of PA on CRP protein expression was observed at 0.2 mmol/L, with no further increase at higher concentrations (data not shown). In contrast to PA, the nonmetabolizable palmitate analog 2-bromopalmitate and the unsatured FFAs oleate and linoleate showed no inductive effect (Fig. 1B). Exposure of HAECs to C_2-ceramide, a cell permeable ceramide analog, mimicked the effect of PA (Fig. 1B). Recovery of enhanced amounts of CRP from the culture media reflected the dose-dependent increase in intracellular CRP protein expression in response to PA (CRP [picograms per milligram cell protein per milliliter]: medium: 65.83 ± 5.9; BSA: 88.63 ± 7.7; PA [0.05 mmol/L]: 81.12 ± 9.5, $P > .05$ vs BSA; PA [0.1 mmol/L]: 194.8 ± 16.8, $P < .05$ vs BSA; PA [0.2 mmol/L]: 683.1 ±32.6, $P < .001$ vs BSA; PA [0.3 mmol/L]: 751.4 ± 26.1, $P < .001$ vs BSA).

3.2. Oxidative stress mediates PA-induced endothelial CRP expression: role of PKC and AMPK

In HAECs, PA induced a significant increase in ROS production; and C_2-ceramide recapitulated this effect (Fig. 2A). The increase in ROS production elicited by PA was prevented by the panspecific and selective classic PKC inhibitors calphostin C and GF109203X, respectively, and by the selective PKCβ inhibitor LY379196 (Fig. 2A), whereas mitogen-activated protein kinase (MAPK) inhibitors were ineffective (data not shown). Similar inhibitory effect was observed after treatment of the cells with the cell permeable AMPK activator AICAR (Fig. 2A). Treatment of HAECs with the antioxidants NAC and apocynin or with the PKC inhibitor calphostin C inhibited the stimulatory effect of PA on CRP protein expression (Fig. 2B).

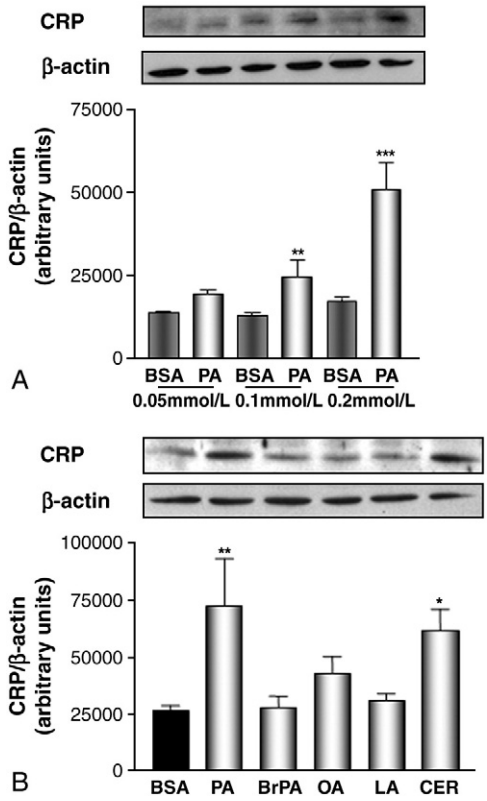

Fig. 1. Effect of PA on endothelial CRP protein expression. Role of ceramide. Human aortic endothelial cells were treated for 24 hours with (A) increasing concentrations of PA (0.05-0.2 mmol/L) or with (B) PA (0.2 mmol/), bromopalmitate (0.2 mmol/L), oleate (0.2 mmol/L), linoleate (0.2 mmol/L), or C_2-ceramide (15 μmol/L). At the end of the incubation period, cells were lysed; and CRP protein expression was determined by Western blot analysis. CRP level was normalized to the level of β-actin protein. Data represent the means ± SEM of 5 independent experiments. *$P < .05$, **$P < .01$, and ***$P < .001$ vs BSA. BrPA indicates bromopalmitate; OA, oleate; LA, linoleate; CER, C_2-ceramide.

3.3. PA up-regulates CRP mRNA expression in HAECs

Barely detectable under basal conditions, levels of CRP mRNA significantly increased in PA-treated HAECs, as assessed by semiquantitative reverse transcriptase (RT)–PCR. Induction of CRP gene by PA was time dependent, with maximal effect being observed after 6 hours of incubation (Fig. 3A). Extent of CRP mRNA induction by PA was greater than that observed with the combination of IL-1 and IL-6 or with LPS at 6 hours, whereas it was comparable to that elicited by these CRP stimulatory agents at 24 hours (Fig. 3A). Quantitative determination of the induction of CRP mRNA levels by PA using real-time PCR is presented in Fig. 3B. Preincubation of HAECs with PKC inhibitors (calphostin C, GF109203X, LY379196), ERK and JNK MAPK inhibitors (PD98059, U0126, SP600125), or antioxidants (NAC, apocynin) decreased PA-induced CRP gene expression, as assessed by both quantitative (Fig. 3B) and semiquantitative (Fig. 3C) RT-PCR. A similar effect was observed when the cells

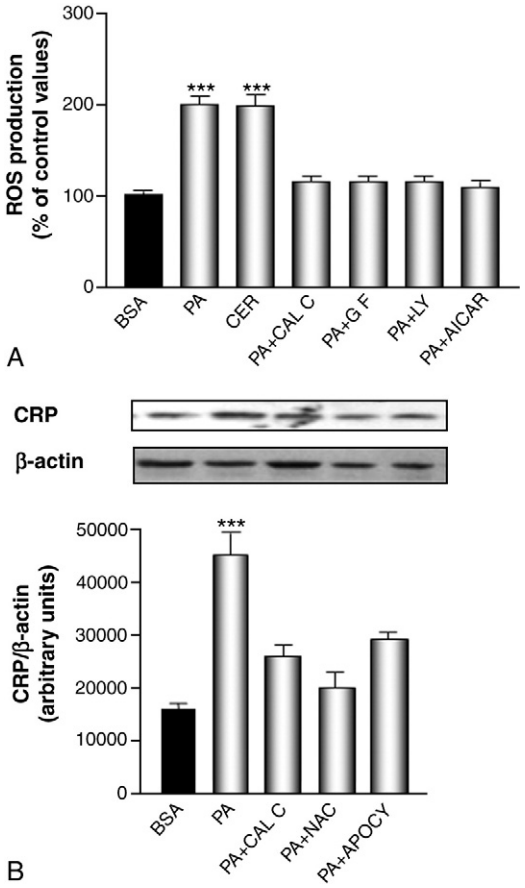

Fig. 2. Role of oxidative stress and PKC in PA-induced endothelial CRP protein expression. A, Human aortic endothelial cells were treated with PA (0.2 mmol/L) or C2-ceramide (CER) CER (15 μmol/L) alone for 3 hours or were preincubated for 1 hour with CAL C (0.1 μg/mL), GF109203X (20 nmol/L), LY379196 (20 nmol/L), or AICAR (0.5 μmol/L) before being treated with PA. During the final 20-minute incubation period, 20 μg/mL DCF-DA was added to the culture medium. Intracellular ROS generation was quickly monitored by measuring fluorescence. B, Human aortic endothelial cells were pretreated for 1 hour with CAL C (0.1 μg/mL), NAC (10 mmol/L), or apocynin (10 μmol/L) and then exposed to PA (0.2 mmol/L) for 24 hours. At the end of the incubation period, cells were lysed; and CRP protein expression was determined by Western blot analysis. CRP level was normalized to the level of β-actin protein. Data represent the means ± SEM of 3 independent experiments (A) and 4 independent experiments (B). ***P < .001 vs BSA. GF indicates GF109203X; LY, LY379196; Apocy, apocynin.

were incubated with the AMPK activator AICAR (Fig. 3B, C). In light of the inhibitory effect of LY379196, levels of PKCβ expression were further determined in PA-treated HAECs using Western blot analysis. As expected, a significant increase in PKCβ protein expression was observed in PA-treated HAECs (data not shown).

3.4. Role of the nuclear factor–κB and Jak/STAT pathways in PA-induced endothelial CRP mRNA expression

Transcription factors involved in the regulation of the CRP gene include the nuclear factor (NF)–κB subunits p50 and p65 and STAT-3. Incubation of HAECs with BAY11-7085, an inhibitor of IκBα phosphorylation, or with AG490, an inhibitor of the Jak/STAT pathway, prevented the stimulatory effect of PA on CRP mRNA expression (Fig. 4A). Reflecting the IκBα-dependent NF-κB activation induced by PA, decrease in IκBα protein expression was documented in PA-treated HAECs (Fig. 4B). PA induced NF-κB activation was prevented by the antioxidant NAC, the PKCβ inhibitor LY379196, the AMPK activator AICAR, and the MAPK inhibitors SP600125 and U0126 (Fig. 4B).

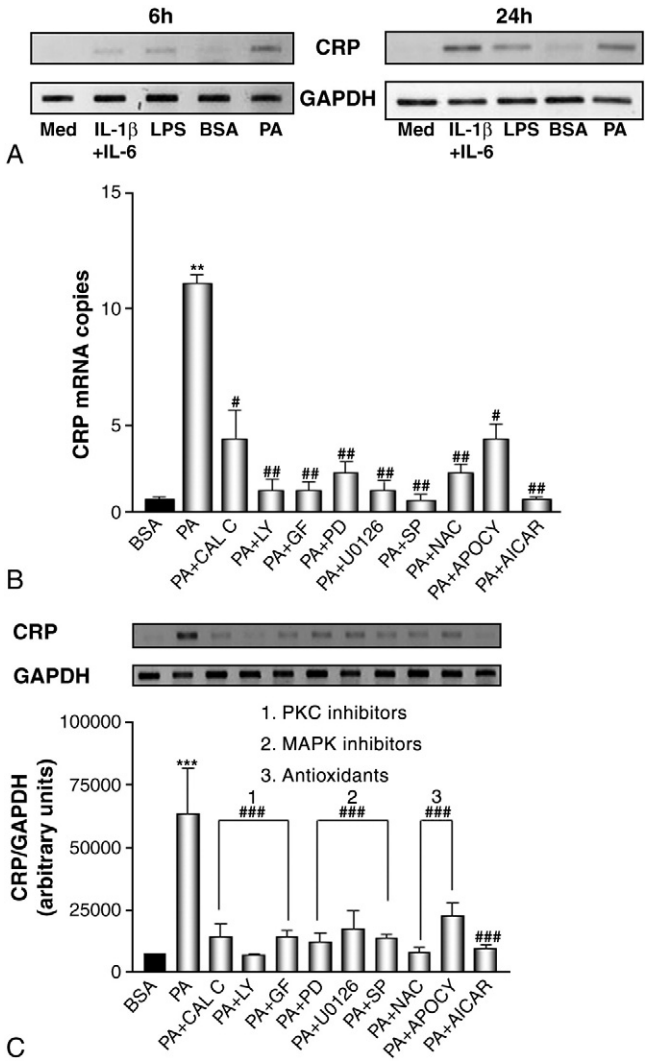

Fig. 3. Effect of PA on endothelial CRP mRNA expression. A, Semiquantitative RT-PCR. Human aortic endothelial cells were incubated with the combination of IL-1β (25 ng/mL) and IL-6 (10 ng/mL), LPS (1 μg/mL), or PA (0.2 mmol/L) for 6 or 24 hours. B and C, Human aortic endothelial cells were pretreated or not for 1 hour with CAL C (0.1 μg/mL), LY379196 (20 nmol/L), GF109203X (20 nmol/L), PD98059 (10 μmol/L), U0126 (20 μmol/L), SP600125 (50 μmol/L), NAC (10 mmol/L), apocynin (10 μmol/L), or AICAR (0.5 μmol/L) before incubation with PA (0.2 mmol/L). At the end of the incubation period, cells were lysed; and CRP mRNA expression was determined by quantitative (B) and semiquantitative (C) RT-PCR analysis. Data represent the means ± SEM of 3 independent experiments. **P < .01 and ***P < .001 vs BSA. $^{\#}P$ < .05, $^{\#\#}P$ < .01, and $^{\#\#\#}P$ < .001 vs PA. GF indicates GF109203X; LY, LY379196;PD, PD98059; SP, SP600125; apocy, apocynin.

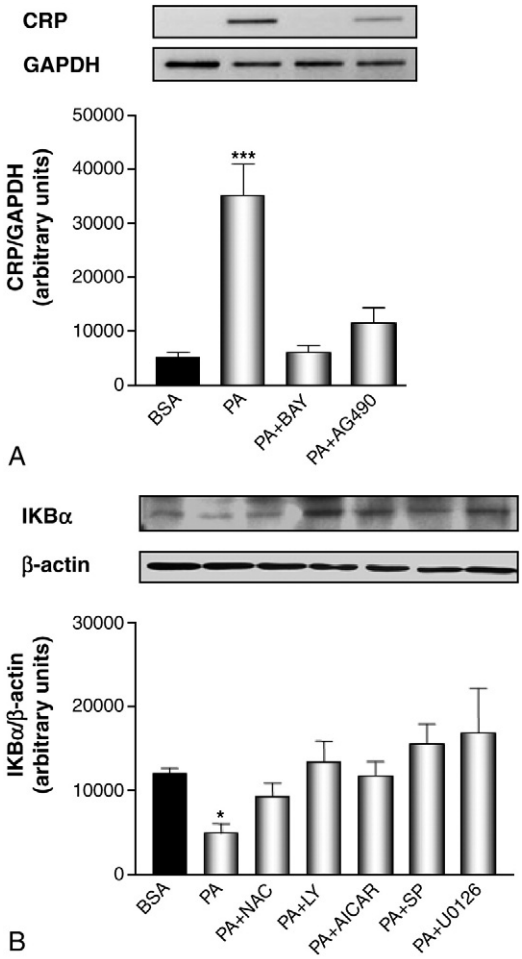

Fig. 4. Role of the NF-κB and Jak/STAT pathways in PA-induced endothelial CRP mRNA expression. A, Human aortic endothelial cells were preincubated for 1 hour with BAY11-7085 (10 μmol/L) or AG490 (20 μmol/L) before being treated with PA (0.2 mmol/L) for 24 hours. B, Human aortic endothelial cells were preincubated for 1 hour with NAC (10 mmol/L), LY379196 (20 nmol/L), AICAR (0.5 μmol/L), SP600125 (50 μmol/L), or U0126 (20 μmol/L) before exposure to PA (0.2 mmol/L) for 3 hours. At the end of the incubation period, cells were lysed; and CRP gene (A) and IκBα protein (B) expressions were determined by RT-PCR and Western blot analysis, respectively. CRP mRNA and IκBα protein levels were normalized to the levels of GAPDH mRNA and β-actin protein. Data represent the means ± SEM of 5 independent experiments. *$P < .05$ and ***$P < .001$ vs BSA. BAY indicates BAY11-7085; LY, LY379196; SP, SP600125.

To further evaluate the role of JAK/STAT in PA-induced CRP gene expression, the effect of PA on STAT-3 phosphorylation was further examined. As expected, PA significantly increased STAT-3 activity in HAECs, as reflected by increased levels of STAT-3 phosphorylation in these cells (data not shown).

3.5. CRP mediates PA-induced decrease in endothelial NO production

Exposure of HAECs to PA for 24 hours dramatically decreased the production of NO by these cells. This effect was abolished by an anti-CRP antibody, whereas an irrelevant IgG antibody was without effect (Fig. 5A). The inhibitory effect of PA on NO release was mimicked by CRP (Fig. 5A). Anti-CRP treatment significantly decreased the amount of CRP in the conditioned media, thus demonstrating the efficacy of immunoneutralization of PA-induced CRP release with anti-CRP antibody (CRP [picograms per milligram cell protein per milliliter]: BSA: 91.5 ± 40.3; PA: 1121.0 ± 54.9, $P < .001$ vs BSA; PA + anti-CRP: 109.7 ± 20.9).

3.6. Role of AMPK and NF-κB in PA-induced inhibition of NO

To evaluate the role of AMPK and NF-κB in the suppressive effect of PA on NO production, HAECs were preincubated with AICAR and BAY11-7085 before exposure to PA. As shown in Fig. 5B, treatment of HAECs with these agents reversed the inhibitory effect of PA on NO production. Although neither AICAR nor BAY11-7085 had an effect on basal NO production on their own, both compounds increased NO production over basal values in PA-treated cells (Fig. 5B).

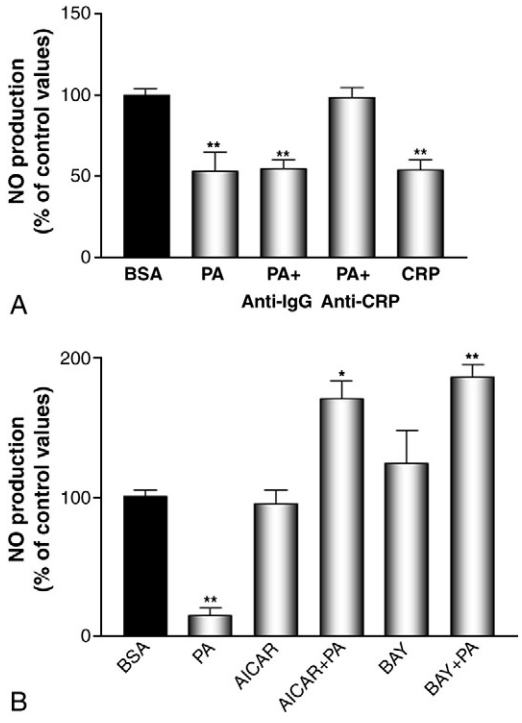

Fig. 5. Role of CRP in PA-induced decrease in endothelial NO production. Involvement of AMPK and NF-κB pathways. Human aortic endothelial cells were pretreated for 1 hour with (A) anti-CRP antibody (10 μg/mL), anti-IgG antibody (10 μg/mL), or CRP (10 μg/mL) or (B) AICAR (0.5 μmol/L) or BAY11-7085 (10 μmol/L) and then exposed to PA (0.2 mmol/L) for 24 hours. At the end of this incubation period, the amounts of NO in the cell supernatants were determined. Data represent the means ± SEM of 4 to 5 independent experiments. *$P < .05$ and **$P < .01$ vs BSA. BAY indicates BAY11-7085.

4. Discussion

There is now clear evidence that human endothelial cells express and produce CRP in response to various inflammatory and metabolic factors relevant to diabetes and obesity, including cytokines, high glucose, leptin, and adiponectin. The present study demonstrates for the first time that PA, at concentrations that could be reached in patients with the metabolic syndrome [23], induces CRP synthesis and secretion in cultured HAECs. It further points to a pathogenic role for CRP as mediator of the suppressive effect of PA on NO production. These observations identify endothelial cell–derived CRP as a possible determinant of the vascular damaging effects of FFA.

Data generated herein identify several important steps in PA-induced endothelial CRP expression (Fig. 6). First, there is evidence that PA should be metabolized to induce CRP because the nonmetabolizable analog of palmitate, 2-bromopalmitate, had no effect. It is well known that saturated fatty acids are preferentially converted to ceramide, and recent studies have revealed that de novo ceramide synthesis mediates several adverse effects of PA in endothelial cells, including the induction of IL-6 [23] and the inhibition of the Akt/eNOS signaling pathway [24,25]. Our data that demonstrate that C_2-ceramide mimicked the effect of PA on CRP expression, whereas oleate or linoleate, which do not cause ceramide accumulation, had no effect [24-26], strongly suggest that ceramide is the intermediate molecule mediating PA-induced CRP expression.

Second, our results with antioxidants and PKC inhibitors provide clear recognition that PKC-driven oxidative stress mediates the PA-induced increase in endothelial CRP expression. Enhanced production of ROS in endothelial cells is a central mechanism underlying vascular lipotoxicity

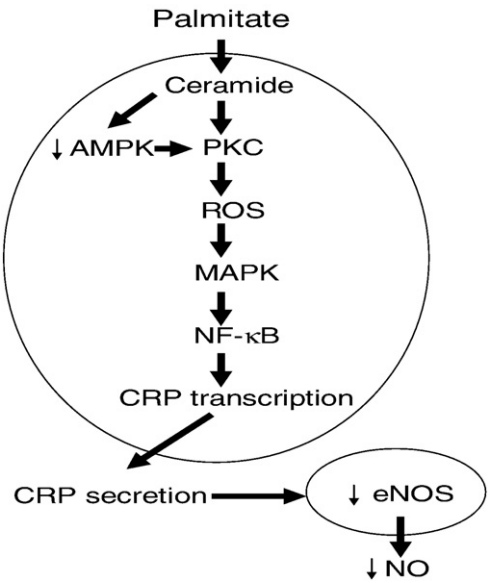

Fig. 6. Relationship among the signaling pathways potentially involved in PA-induced decrease in NO production.

[27], and previous work has shown that PA stimulates ROS production through PKC-dependent activation of nicotinamide adenine dinucleotide phosphate (reduced form) (NAD[P]H) in endothelial cells [28]. Of the PKC isoforms studied so far, activation of PKCβ appears to occur preferentially in IR states and shows close relationship with vascular oxidant stress. In agreement with a key role of PKCβ in PA-induced ROS production, our results demonstrate that the specific PKCβ inhibitor LY379196 abrogates ROS production elicited by PA in HAECs. Given the deleterious effect of excessive oxidative stress on endothelial cell function, these data support the notion that PKCβ isoform selective inhibitors may attenuate FFA-induced vascular oxidative stress and protect vascular function in IR subjects. In contrast to PKC, AMPK seems to play an important role in protecting endothelial cells against glucose and FFA-induced oxidative stress [29,30]. In agreement with a role of AMPK in the regulation of ROS production by PA, our results demonstrate that AICAR, a specific AMPK activator, prevents the oxidative effect of PA in HAECs and abrogates the inhibitory effect of PA on AMPK phosphorylation in these cells (Supplementary data). One mechanism by which AICAR may suppress PA-induced ROS production is through the inhibition of the DAG-PKC pathway. Supporting this possibility, it has been recently reported that AMPK mediates the inhibitory effect of thialozidinediones on PKC activation [30] and suppresses NAD(P)H in neutrophils [31]. If this hypothesis is true, most of the oxidative effect of PA could be determined by its ability to inhibit AMPK activation. Experiments aimed at evaluating the oxidative effects of PA when AMPK is inhibited are under way to evaluate this possibility.

Endothelial CRP induction in response to PA appears to be exerted at the transcriptional level, as reflected by the parallel increase in CRP gene and protein expression in PA-treated HAECs. Results with pharmacologic inhibitors and antioxidants demonstrate that PA-induced CRP gene expression is mediated through oxidative stress and involves PKC/AMPK- and MAPK-dependent pathways. Transcriptional activation of the CRP gene by PA may theoretically involve NF-κB. Supporting this possibility, activation of IKKβ by PA has been documented in endothelial cells [8,32,33]; and a binding site for NF-κB has been located in the regulatory sequence of the CRP gene [34]. Consistent with a critical role of the IKKβ/NF-κB pathway in the induction of CRP gene expression by PA, we found that PA reduces the level of IκBα in HAECs and that BAY11-7085, an IκBα phosphorylation inhibitor, totally prevented PA-induced CRP mRNA levels. Activation of PKC, in particular PKCβ, can lead to the activation of NF-κB through direct phosphorylation of IκBα [35] or by causing the generation of oxidative stress that can secondarily activate IKK. Our results showing that antioxidants and the PKCβ inhibitor LY379196 prevent the degradation of IκBα caused by PA support the notion that NF-κB activation in PA-treated HAECs involves PKCβ-dependent ROS production. These results are in line with those of Maloney et al [33] who

recently reported that NAD(P)H oxidase-dependent superoxide links PA to NF-κB signaling in human endothelial cells. It has been previously reported that AMPK may function as an anti-inflammatory agent and inhibit FFA-induced NF-κB activation as well as increase in NF-κB–mediated gene expression [36]. Furthermore, recent data have shown that activation of this kinase by adiponectin reduced CRP synthesis in endothelial cells [16]. In agreement with these observations, the present study demonstrates that AICAR prevents the decrease in IκBα levels induced by PA in endothelial cells and abrogates PA-induced CRP gene expression. PA is a potent inducer of IL-6 expression [6], and this cytokine participates in the transcriptional activation of the CRP gene through activating STAT3 [37]. In endothelial cells, PA may activate the JAK/STAT pathway through generating NAD(P)H oxidase-derived ROS [38]. Our data that demonstrate that AG490, a specific JAK2 inhibitor, decreases the induction of CRP gene expression by PA support a role for the JAK/STAT pathway in the transcriptional regulation of endothelial CRP by this FFA. In the whole, our results suggest that not one transcription factor alone but multiple redox-sensitive transcription factors together modulate the transcription rate of CRP in response to FFA. The potential synergistic impact of these factors on endothelial CRP transcription needs to be specifically addressed in future studies.

Extracellular signal–regulated kinase 1/2 and JNK MAPK are well-known downstream targets of oxidative stress and are activated by PA. Previous studies have shown that these kinases mediate PA-induced NF-κB activation [39], promote STAT3 phosphorylation [40], and contribute to the inhibitory effect of PA on eNOS [5]. Although previous studies have demonstrated the involvement of the ERK1/2-MAPK cascade in the induction of vascular CRP by endothelin and leptin [18,41], the role of the JNK pathway in endothelial CRP regulation remains unknown. Our findings that ERK1/2 and JNK inhibitors prevent both PA-induced NF-κB activation and CRP expression suggest that MAPK-dependent NF-κB activation is required for the stimulatory effect of PA on endothelial CRP expression. They further stress a potential key role for these kinases as transducers of the vascular inflammatory effects of FFAs.

Clinical studies have shown that high FFA levels are associated with impaired endothelium-dependent vasodilation as reflected by decreased basal and insulin-mediated NO production [42,43]. The cellular mechanism responsible for FFA impairment of NO production is not well understood, but activation of the IKKβ pathway seems to be involved [9]. It has been previously reported that CRP inhibits endothelial NO generation [12-14] and acts as mediator of the suppressive effect of uric acid on NO production [44] by vascular cells. Our results that demonstrate that anti-CRP antibody and endothelial CRP inhibitory agents prevent the suppressive effect of PA on NO production expand these works and support a role for CRP as paracrine regulator of NO production. This finding suggests that oxidative or inflammatory signaling pathways induced by PA are not by themselves responsible for NO inhibition and that CRP is the fundamental mechanism linking these pathways to endothelial dysfunction.

In addition to PA, several cardiometabolic factors linked to insulin resistance and endothelial dysfunction enhance CRP production by endothelial cells [17,18,45]. These results call for careful consideration of the role of endothelial cells as significant sources of vascular CRP in the setting of metabolic disorders such as obesity and type 2 diabetes mellitus and highlight endothelial CRP induction as an attractive mechanism for the proatherogenic effects of inflammatory or metabolic factors associated with the metabolic syndrome. Clearly, further studies are needed to determine whether CRP concentration is higher in diabetic atherosclerotic plaques than in nondiabetic ones and whether CRP, as a cell-based molecule, is an effector of the atherosclerotic disease and should be considered as a potential therapeutic target in diabetic vasculopathy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.metabol.2010.06.014.

References

[1] Moller DE, Kaufman KD. Metabolic syndrome: a clinical and molecular perspective. Annu Rev Med 2005;56:45-62.
[2] Davda RK, Stepniakowski KT, Lu G, Ullian ME, Goodfriend TL, Egan BM. Oleic acid inhibits endothelial nitric oxide synthase by a protein kinase C–independent mechanism. Hypertension 1995; 26:764-70.
[3] Steinberg HO, Paradisi G, Hook G, Crowder K, Cronin J, Baron AD. Free fatty elevation impairs insulin-mediated vasodilation and nitric oxide production. Diabetes 2000;49:1231-8.
[4] Vogel RA, Corretti MC, Plotnick GD. Effect of a single high-fat meal on endothelial function in healthy subjects. Am J Cardiol 1997; 79:350-4.
[5] Wang XL, Zhang L, Youker K, Zhang MX, Wang J, LeMaire SA, et al. Free fatty acids inhibit insulin signalling-stimulated endothelial nitric oxide synthase activation through upregulating PTEN or inhibiting Akt kinase. Diabetes 2006;55:2301-10.
[6] Kim F, Tysseling KA, Rice J, Pham M, Haji L, Gallis BM, et al. Free fatty acid impairment of nitric oxide production in endothelial cells is mediated by IKKβ. Arterioscler Thromb Vasc Biol 2005;25:989-94.
[7] Plutzky J. Inflammation in atherosclerosis and diabetes. Rev Endocr Metab Dis 2004;5:255-9.
[8] Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. Circulation 2003;107:391-7.
[9] Fichtlscherer S, Rosenberger G, Walter DH, Breuer S, Dimmeler S, Zeiher AM. Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. Circulation 2000;102:1000-6.

[10] Nyström T, Nygren A, Sjöholm A. Persistent endothelial dysfunction is related to elevated C-reactive protein (CRP) levels in type II diabetic patients after acute myocardial infarction. Clin Sci 2005;108:121-8.
[11] Venugopal SK, Devaraj S, Yuhanna I, Shaul P, Jialal I. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. Circulation 2002;106:1439-41.
[12] Singh U, Devaraj S, Vasquez-Vivar J, Jialal I. C-reactive protein decreases endothelial nitric oxide synthase activity via uncoupling. J Mol Cell Cardiol 2007;43:780-91.
[13] Schwartz R, Osborne-Lawrence S, Hahner L, Gibson LL, Gormley AK, Vongpatanasin W, et al. C-reactive protein downregulates endothelial NO synthase and attenuates reendothelialization in vivo in mice. Circ Res 2007;100:1452-9.
[14] Teoh H, Quan A, Lovren F, Wang G, Tirgari S, Szmitko PE, et al. Impaired endothelial function in C-reactive protein overexpressing mice. Atherosclerosis 2008;201:318-25.
[15] Bisoendial RJ, Kastelein JJ, Peters SL, Levels JH, Birjmohun R, Rotmans JI, et al. Effects of CRP infusion on endothelial function and coagulation in normocholesterolemic and hypercholesterolemic subjects. J Lipid Res 2007;48:952-60.
[16] Venugopal SK, Devaraj S, Jialal I. Macrophage conditioned medium induces the expression of C-reactive protein in human aortic endothelial cells. Am J Pathol 2005;166:1265-71.
[17] Devaraj S, Torok N, Dasu MR, Samols D, Jialal I. Adiponectin decreases C-reactive protein synthesis and secretion from endothelial cells. Evidence for an adipose tissue–vascular loop. Arterioscler Thromb Vasc Biol 2008;28:1368-74.
[18] Singh P, Hoffmann M, Wolk R, Shamsuzzaman ASM, Somers VK. Leptin induces C-reactive protein expression in vascular endothelial cells. Arterioscler Thromb Vasc Biol 2007;27:e302-17.
[19] Mittendorfer B, Liem O, Patterson BW, Miles JM, Klein S. What does the measurement of whole body fatty acid rate of appearance in plasma by using a fatty acid tracer really mean? Diabetes 2003;52:1641-8.
[20] Royall JA, Ischiropoulos H. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent-probes for intracellular H2O2 in cultured endothelial cells. Arch Biochem Biophys 1993;302:348-55.
[21] Pasceri V, Chang J, Willerson JT, Yeh ETH. Modulation of C-reactive protein–mediated monocyte chemoattractant protein–1 induction in human endothelial cells by anti-atherosclerotic drugs. Circulation 2001;103:2531-4.
[22] Green LC, Wagner DA, Glogowski J, Skipper PL, Whisnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and 15n-nitrate in biological fluids. Anal Biochem 1982;126:131-8.
[23] Staiger H, Staiger K, Stefan N, Wahl HG, Machicao F, Kellerer M, et al. Palmitate-induced interleukin-6 expression in human coronary endothelial cells. Diabetes 2004;53:3209-16.
[24] Wu Y, Song P, Zhang M, Zou MH. Activation of protein phosphatase 2A by palmitate inhibits AMP-activated protein kinase. J Biol Chem 2007;282:9777-88.
[25] Xiao-Yun Z, Zhuo-Xiong C, Min-Xiang L, Xingxuan H, Schuchman EH, Feng L, et al. Ceramide mediates inhibition of the AKT/eNOS signalling pathway by palmitate in human vascular endothelial cells. Med Sci Monit 2009;15:BR254-61.
[26] Lee JS, Pinnamaneni S, Eo S, Cho I, Pyo J, Kin C, et al. Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of lipid metabolites. J Appl Physiol 2006; 100:1467-74.
[27] Chinen I, Shimabukuro M, Yamakawa K, Higa N, Matsuzaki T, Noguchi K, et al. Vascular lipotoxicity: endothelial dysfunction via fatty-acid–induced reactive oxygen overproduction in obese Zucker diabetic fatty rats. Endocrinology 2007;148:160-5.
[28] Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P) H oxidase in cultured vascular cells. Diabetes 2000;49:1939-45.
[29] Li XN, Song J, Zhang L, LeMaire SA, Hou X, Zhang C, et al. Activation of the AMPK-FOX03 pathway reduces fatty acid-induced increase in intracellular reactive oxygen species by upregulating thioredoxin. Diabetes 2009;58:2246-57.
[30] Ceolotto G, Gallo A, Papparella I, Franco L, Murphy E, Iori E, et al. Rosiglitazone reduces glucose-induced oxidative stress mediated by NAD(P)H oxidase via AMPK-dependent mechanism. Arterioscler Thromb Vasc Biol 2007;27:2627-33.
[31] Alba G, Bekay R, Alvarez-Maqueda M, Chacon P, Vega A, Monteseirin J, et al. Stimulators of AMP-activated protein kinase inhibit the respiratory burst in neutrophils. FEBS Lett 2004;27:219-25.
[32] Chai W, Liu Z. p38 mitogen-activated protein kinase mediates palmitate-induced apoptosis but not inhibitor of nuclear factor–κB degradation in human coronary artery endothelial cells. Endocrinology 2007;148:1622-8.
[33] Maloney E, Sweet IR, Hockenbery DM, Pham M, Rizzo NO, Tateya S, et al. Activation of NF-kappaB by palmitate in endothelial cells: a key role for NADPH oxidase-derived superoxide in response to TLR4 activation. Arterioscler Thromb Vasc Biol 2009;29:1370-5.
[34] Kramer F, Torzewski J, Kamenz J, Veit K, Hombach V, Dedio J, et al. Interleukin-1beta stimulates acute phase response and C-reactive protein synthesis by inducing an NFkappaB- and C/EBPbeta-dependent autocrine interleukin-6 loop. Mol Immunol 2008;45:2678-89.
[35] Ghosh S, Baltimore D. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature 1990;344:678-82.
[36] Cacicedo JM, yagihashi N, Keaney JF, Ruderman NB, Ido Y. AMPK inhibits fatty acid–induced increases in NF-κB transactivation in cultured human umbilical vein endothelial cells. Biochim Biophys Res Comm 2004;324:1204-9.
[37] Zhang D, Sun M, Samols D, Kushner I. STAT-3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6. J Biol Chem 1996;271:9503-9.
[38] Schieffer B, Luchtefeld M, Braun S, Hilfiker A, Hilfiker-Kleiner D, Drexler H. Role of NAD(P)H oxidase in angiotensin II–induced JAK/STAT signalling and cytokine induction. Circ Res 2000;87:1195-201.
[39] Coll T, JOvé M, Rodriguez-Calvo R, Eyre E, Palomer X, Sanchez RM, et al. Palmitate-mediated downregulation of peroxisome proliferators–activated receptor-gamma coactivator 1 alpha in skeletal muscle cells involves MEK1/2 and nuclear factor–kappaB activation. Diabetes 2006;55:2779-89.
[40] Souza V, Escobar Mdel C, Bucio L, Hernández E, Gómez-Quiroz LE, Gutiérrez Ruiz MC. NADPH oxidase and ERK1/2 are involved in cadmium induced-STAT3 activation in HEPG2 cells. Toxicol Lett 2009;187:180-6.
[41] Wang C, Liu J, Guo F, Ji Y, Liu N. Endothelin-1 induces the expression of C-reactive protein in rat vascular smooth muscle cells. Biochem Biophys Res Commun 2009;389:537-42.
[42] Steinberg HO, Tarshoby M, Monestel R, Hook G, Cronin J, Johnson A, et al. Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. J Clin Invest 1997;100:1230-9.
[43] Watanabe S, Tagawa T, Yamakawa K, Shimabukuro M, Ueda S. Inhibition of the renin-angiotensin system prevents free fatty acid–induced acute endothelial dysfunction in humans. Arterioscler Thromb Vasc Biol 2005;25:2376-80.
[44] Kang DH, Park SK, Lee IK, Johnson RJ. Uric acid–induced C-reactive protein expression: implication on cell proliferation and nitric oxide production of human vascular cells. J Am Soc Nephrol 2005; 16:3553-62.
[45] De Rosa S, Cirillo P, Pacileo M, Di Palma V, Paglia A. Leptin stimulated C-reactive protein production by human coronary artery endothelial cells. J Vasc Res 2009;46:609-17.