0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(4):1622–1628
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doi: 10.1210/en.2006-1068

p38 Mitogen-Activated Protein Kinase Mediates Palmitate-Induced Apoptosis But Not Inhibitor of Nuclear Factor-κB Degradation in Human Coronary Artery Endothelial Cells

Weidong Chai and Zhenqi Liu

Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Virginia Health System, Charlottesville, Virginia 22908-1410

Plasma free fatty acids are elevated in patients with type 2 diabetes and contribute to the pathogenesis of insulin resistance and endothelial dysfunction. The p38 MAPK mediates stress, inflammation, and apoptosis. Whether free fatty acids induce apoptosis and/or activate nuclear factor-κB inflammatory pathway in human coronary artery endothelial cells (hCAECs) and, if so, whether this involves the p38 MAPK pathway is unknown. hCAECs (passages 4–6) were grown to 70% confluence and then incubated with palmitate at concentrations of 0–300 μM for 6–48 h. Palmitate at 100, 200, or 300 μM markedly increased apoptosis after 12 h of incubation. This apoptotic effect was time ($P = 0.008$) and dose ($P = 0.006$) dependent. Palmitate (100 μM for 24 h) induced a greater than 2-fold increase in apoptosis, which was accompanied with a 4-fold increase in p38 MAPK activity ($P < 0.001$). Palmitate did not affect the phosphorylation of Akt1 or ERK1/2. SB203580 (a specific inhibitor of p38 MAPK) alone did not affect cellular apoptosis; however, it abolished palmitate-induced apoptosis and p38 MAPK activation. Palmitate significantly reduced the level of inhibitor of nuclear factor-κB (IκB). However, treatment of cells with SB203580 did not restore IκB to baseline. We conclude that palmitate induces hCAEC apoptosis via a p38 MAPK-dependent mechanism and may participate in coronary endothelial injury in diabetes. However, palmitate-mediated IκB degradation in hCAECs is independent of p38 MAPK activity. (*Endocrinology* 148: 1622–1628, 2007)

TYPE 2 DIABETES AND insulin resistance are associated with accelerated coronary atherosclerosis, which is a major cause of morbidity and mortality in these patient populations. Although the underlying mechanisms remain to be defined, many studies have implicated free fatty acids (FFAs) in the onset and development of atherosclerosis. FFAs are elevated in patients with insulin resistance and/or type 2 diabetes, and it has been repeatedly demonstrated that FFAs induce insulin resistance, inflammation, and endothelial dysfunction (1–8).

In humans, high-plasma FFAs decrease insulin receptor substrate (IRS)-1-associated phosphatidylinositol 3-kinase activity and inhibit glucose transport (9). Acute elevation of plasma FFAs via systemic infusion of intralipid/heparin induces oxidative stress, activates the nuclear factor-κB (NF-κB) pathway, impairs flow-mediated dilatation of the brachial artery (7), and blunts insulin-mediated capillary recruitment in skeletal muscle (10). In cultured bovine aortic endothelial cells, treatment with palmitate at 100 μM for 3 h significantly inhibited insulin-mediated tyrosine phosphorylation of IRS-1 and serine phosphorylation of protein kinase B (Akt) and endothelial nitric oxide synthase (eNOS), and nitric oxide (NO) production, whereas increasing inhibitor of NF-κB (IκB) kinase-β (IKKβ) activity (11). IKKβ further regulates the activation of NF-κB, a transcriptional factor associated with inflammation, and links inflammation to insulin resistance (6, 12). These findings suggest that FFAs could be a unifying contributor to the pathogenesis of insulin resistance, endothelial dysfunction, and vascular inflammation (13). Additionally, FFAs could also contribute to endothelial dysfunction and atherosclerosis by triggering endothelial cell apoptosis and inhibiting cell cycle progression (14).

The p38 MAPK belongs to the MAPK superfamily and is a stress-activated serine/threonine protein kinase. It plays a major role in apoptosis, cytokine production, transcriptional regulation, and cytoskeletal reorganization (15). Many stimuli, including UV light, irradiation, heat shock, ischemia, hypoxia, osmotic stress, proinflammatory cytokines, and certain mitogens can activate this kinase. The importance of p38 MAPK in cell death was fully demonstrated in the setting of myocardial ischemia-reperfusion injury because activation of this kinase using anisomycin preconditions the myocardium against ischemia-reperfusion injury (16, 17), and its targeted inhibition reduces the cardiac injury and cell death after ischemia-reperfusion *in vivo* (18).

The p38 MAPK also regulates endothelial function. Selective inhibition of p38 MAPK dose-dependently reduces TNF-α or lipopolysaccharide-induced intercellular adhesion molecule-1 expression in cultured human umbilical vein en-

First Published Online January 18, 2007

Abbreviations: DAPI, 4′,6′-Diamidino-2-phenylindole; eNOS, endothelial NO synthase; EPC, endothelial progenitor cell; FFA, free fatty acid; hCAEC, human coronary artery endothelial cell; HUVEC, human umbilical vein endothelial cell; IκB, inhibitor of NF-κB; IKKβ, IκB kinase-β; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; POD, peroxidase; RM-ANOVA, repeated-measure ANOVA; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

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dothelial cells (HUVECs) (19). Chronic suppression of p38 MAPK blunts combined high-salt/high-fat diet-induced hypertension, improves survival and restores NO-mediated endothelium-dependent relaxation in spontaneously hypertensive-stroke prone rats, in whom phosphorylated p38 MAPK is localized to the aortic endothelium and adventitia but not in aortae from normotensive rats (19). Moreover, patients with coronary artery disease or diabetes mellitus have a reduced number of endothelial progenitor cells (EPCs), which are vital in angiogenesis/vascular repair, and EPCs from coronary artery disease patients have significantly higher basal p38 MAPK phosphorylation, compared with EPCs from healthy subjects (20). Inhibition of p38 MAPK with SB203580 or transfection with a dominant-negative p38 MAPK-expressing adenovirus significantly increases the basal number of EPCs (20), whereas activation of p38 MAPK has opposing effects on the proliferation and migration of endothelial cells (21). In addition, C-reactive protein inhibits endothelium-dependent NO-mediated dilation in coronary arterioles by activating p38 MAPK and reduced nicotinamide adenine dinucleotide phosphate oxidase (22). Taken together, these results confirm that p38 MAPK plays a very important role in vascular inflammation and endothelial dysfunction/repair.

Whether p38 MAPK modulates both FFA-induced apoptosis and the activation of the NF-κB inflammatory pathway in human coronary artery endothelial cells (hCAECs) is the focus of the current study. We here report for the first time that palmitate, the most abundant fatty acid in human plasma, induces apoptosis in cultured hCAECs in a time- and dose-dependent fashion via a p38 MAPK-dependent mechanism. However, palmitate-induced IκB degradation is independent of the p38 MAPK pathway.

Materials and Methods

Culture of hCAECs

hCAECs in primary culture were purchased from Cambrex Bio Sciences (Walkersville, MD) and grown in endothelial cell basic media-2, which contained 5.3 mM glucose and was supplemented with 5% fetal bovine serum, 0.2 ml hydrocortisone, 0.5 ml human epithelial growth factor, 0.5 ml vascular endothelial growth factor, 2.0 ml human fibroblast growth factor-B, 0.5 ml R^3-IGF-I, 0.5 ml ascorbic acid, and 0.5 ml gentamicin/amphotericin-B, as specified by the manufacturer. Cells between passages 4 and 6 were used after reaching 70% confluence. We did not serum starve the cells because serum starvation itself induces apoptosis (23). Cells were exposed to palmitate 0, 20, 50, 100, 200, or 300 μM ± SB203580 20 μM (a specific inhibitor of p38 MAPK) for 6, 12, 24, or 48 h. Control cells were exposed to 0.025% ethanol and 0.02% dimethylsulfoxide, solvents used to dissolve palmitate and SB203580, respectively. The final concentration of albumin (30 μmol/liter) was the same as reported by Kim *et al.* (11). Cells were then used for either apoptosis assay or Western blotting.

Apoptosis assay

Cell apoptosis was quantitated using cell death detection ELISA[PLUS] kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instruction, which measures cytoplasmic DNA-histone nucleosome complexes generated during apoptotic DNA fragmentation. Briefly, cells were plated in 24-well cluster plates and grown to 70% confluence and then incubated with different concentrations of palmitate (0–300 μM) for 6, 12, 24, or 48 h. Cells were then lysed using the lysis buffer supplied in the kit. The lysate supernatant was incubated with antihistone-biotin and anti-DNA-peroxidase (POD) antibodies in a streptavidin-coated microplate for 2 h. The biotin-labeled antihistone antibody binds to the histone component of the nucleosomes and the streptavidin-coated microplate, whereas the POD-labeled DNA-specific antibody binds to the DNA component of the nucleosomes. After removing the unbound antibodies, 2, 2′-azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt was added, and POD activity (apoptosis) was quantitated photometrically at 405 nm.

Due to inherent limitations with individual available apoptosis assay, we used a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay to confirm selected findings and to assess nuclear morphology of the cultured cells. hCAECs were plated on coverslips and grown to 70% confluence. Cells were then treated with 100 or 200 μM palmitate with or without SB203580 (20 μM) for 24 h. The slides were fixed in 4% paraformaldehyde in PBS. TUNEL assays were performed using the DeadEnd fluorometric TUNEL system kit (Promega, Madison, WI), and the slides were counterstained for 5 min with 4′,6′-diamidino-2-phenylindole (DAPI; 5 μg/ml). Images (×400) were captured under a fluorescence microscope using fluorescein isothiocyanate (TUNEL-positive cells) and DAPI (total cells) filter sets. For each experiment, a total of around 500 cells were counted and the percent of TUNEL-positive cells were calculated.

Western blotting and quantitation of protein phosphorylation

After growing to 70% confluence, hCAECs were incubated with or without palmitate at 100 μM for 24 h and then lysed in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were centrifuged for 10 min at 4 C (12,000 × *g*) and the supernatants used for Western blotting. Aliquots of supernatant containing approximately 100 μg protein were diluted with an equal volume of sodium dodecyl sulfate sample buffer and electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and blocked with 5% low-fat milk in Tris-buffered saline plus Tween 20. Membranes were subsequently probed with antibodies against phospho-ERK1/2 (Thr^{202}/Tyr^{204}), ERK1/2, phospho-p38 MAPK (Thr^{180}/Tyr^{182}), p38 MAPK, IκB-α (New England BioLabs, Beverly, MA), phospho-HSP27 (Ser^{82}), HSP27, phospho-stress-activated protein kinase/c-Jun N-terminal kinase (JNK) (Thy^{183}/Tyr^{185}), stress-activated protein kinase/JNK, phospho-Akt1 (Ser^{473}), or Akt1 (Upstate Cell Signaling, Lake Placid, NY). After incubating with a donkey antirabbit IgG coupled to horseradish peroxidase, the blots were developed using enhanced chemiluminescence (Amersham Life Sciences, Piscataway, NJ). Autoradiographic films were scanned densitometrically (Molecular Dynamics, Piscataway, NJ) and quantitated using ImageQuant 3.3 (Molecular Dynamics). Both the total and phospho-specific densities were quantitated and the ratios of phosphospecific density to total density calculated.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using Student's t test or repeated-measure ANOVA (RM-ANOVA) as appropriate. $P \leq 0.05$ was considered statistically significant.

Results

Time course and dose response of palmitate-induced apoptosis in cultured hCAECs

To examine the time course and dose response of palmitate-induced apoptosis in cultured hCAECs, cells were incubated with palmitate at various concentrations (0–300 μM) for 6, 12, 24, or 48 h. As shown in Table 1, palmitate-induced apoptosis in hCAECs in a time ($P = 0.008$) and dose ($P = 0.006$, RM-ANOVA on ranks using Student-Newman-Keuls method for *post hoc* testing) dependent fashion. Palmitate's proapototic action required more than 6 h but was marked

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TABLE 1. Time course and dose response of palmitate-induced apoptosis in cultured hCAECs

Dose (μM)	6 h	12 h	24 h	48 h
20	−0.12 ± 0.02	−0.20 ± 0.02	−0.11 ± 0.01	0.42 ± 0.02
50	−0.08 ± 0.01	−0.19 ± 0.03	−0.10 ± 0.02	1.20 ± 0.02
100	0.10 ± 0.01	0.91 ± 0.05	2.35 ± 0.09	13.94 ± 0.10
200	−0.02 ± 0.01	2.72 ± 0.03	4.99 ± 0.10	23.40 ± 0.18
300	0.07 ± 0.02	2.86 ± 0.07	5.61 ± 0.09	29.10 ± 0.20

Cells were incubated with 0–300 μM palmitate for 6–48 h. Results are expressed as fold increase over control (solvent-treated) cells and are the average of four experiments. Palmitate induced time- and dose-dependent apoptosis ($P = 0.008$ and 0.006, respectively, RM-ANOVA on ranks using Student-Newman-Keuls Method as *post hoc* testing).

at 12 h ($P < 0.001$), 24 h ($P < 0.001$), and 48 h ($P < 0.001$). Palmitate at 100, 200, or 300 μM induced a 0.9-, 2.7-, and 2.9-fold increase in apoptosis at 12 h; 2.4-, 5-, and 5.6-fold increase at 24 h; and 14-, 23- and 29-fold increase at 48 h, respectively. Palmitate at 20 and 50 μM did not induce cellular apoptosis in the first 24 h but did increase the apoptotic rates by 40 and 120% at 48 h, respectively.

Because each apoptosis assay has its limitations, we performed additional experiments using the TUNEL assay (n = 3 for each group). Again, palmitate at 100 μM markedly increased apoptosis seen as the percentage of TUNEL-positive cells from 4.2 ± 1.7% (control) to 12.4 ± 2%. Palmitate at 200 μM further increased the rate of apoptosis (Fig. 1, A and B).

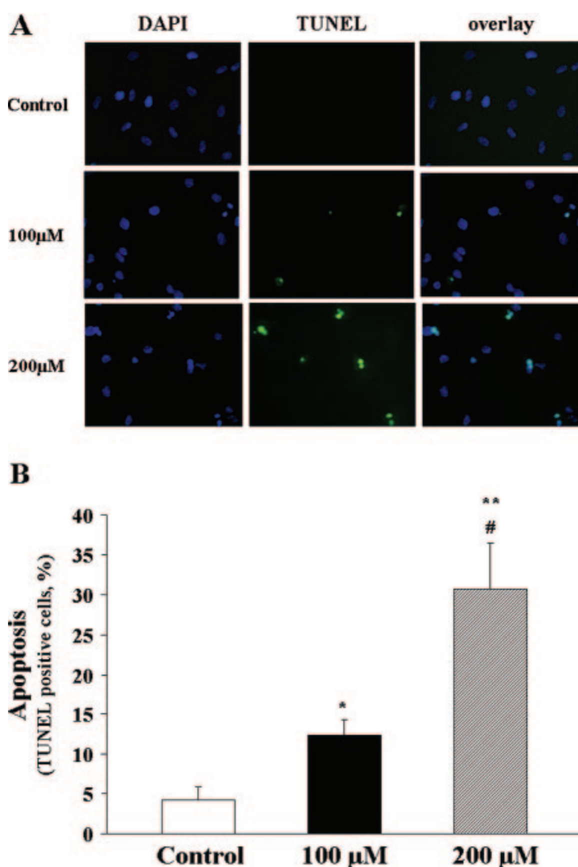

FIG. 1. Palmitate induces apoptosis in cultured hCAECs demonstrated with TUNEL staining. Cells were incubated with 100 or 200 μM palmitate for 24 h. A, Representative TUNEL staining images. B, Quantitative analysis of TUNEL-positive cells (n = 3 for each). Compared with control, *, $P < 0.008$, **, $P < 0.04$; compared with 100 μM, #, $P < 0.05$.

Effect of palmitate at 100 μM on MAPKs and Akt1 phosphorylation

To probe the signaling pathways underlying palmitate-induced apoptosis, we examined the effects of palmitate on all three major MAPKs (p38, JNK, and ERK1/2) and Akt/PKB because all are involved in the regulation of cell survival and apoptosis. Based on the results presented in Table 1, all subsequent experiments were conducted using 100 μM palmitate and a 24-h incubation time. As shown in Fig. 2, palmitate significantly increased the phosphorylation of p38 MAPK (from 0.92 ± 0.21 to 2.06 ± 0.38, $P < 0.03$) and JNK (from 0.23 ± 0.03 to 0.36 ± 0.04, $P < 0.05$) but did not significantly alter the phosphorylation of ERK1/2 or Akt1. We did not observe apparent change in cell or nuclear morphology (as shown in Fig. 1A using DAPI staining) in cells treated with palmitate at this concentration.

Palmitate stimulates p38 MAPK activity in cultured hCAECs

Because p38 MAPK mediates cell apoptosis and endothelial dysfunction and because we have here demonstrated that palmitate markedly (2.7 ± 0.8-fold) increases p38 MAPK phosphorylation, we next examined whether palmitate also increased p38 MAPK activity as measured by Ser^{82} phosphorylation of its downstream substrate HSP27. hCAECs were incubated with 100 μM palmitate for 24 h in the presence or absence of 20 μM SB203580, a specific inhibitor of p38 MAPK. Palmitate increased p38 MAPK activity by 4-fold (Fig. 3, from 0.37 ± 0.07 to 1.48 ± 0.26, $P < 0.001$). SB203580 decreased HSP27 phosphorylation by approximately 70% (to 0.72 ± 0.06). Palmitate also significantly ($P = 0.002$) enhanced the phosphorylation of ATF-2 (data not shown), a signaling molecule downstream of both p38 MAPK and JNK. Taken together, these data confirm that palmitate both phosphorylates and activates p38 MAPK.

Palmitate induces apoptosis in cultured hCAECs via a p38 MAPK-dependent mechanism

We next examined whether blocking p38 MAPK activation affected palmitate-induced apoptosis of cultured hCAECs. Cells were incubated ± 100 μM palmitate for 24 h in the presence or absence of 10 or 20 μM SB203580, and the extent of apoptosis was analyzed (Fig. 4). Palmitate induced a greater than 2-fold increase in apoptosis (2.35 ± 0.05, $P = 0.009$). SB203580 alone did not affect cellular apoptosis; however, SB203580 at 10 μM nearly completely and at 20 μM completely abolished palmitate-induced apoptosis. This was

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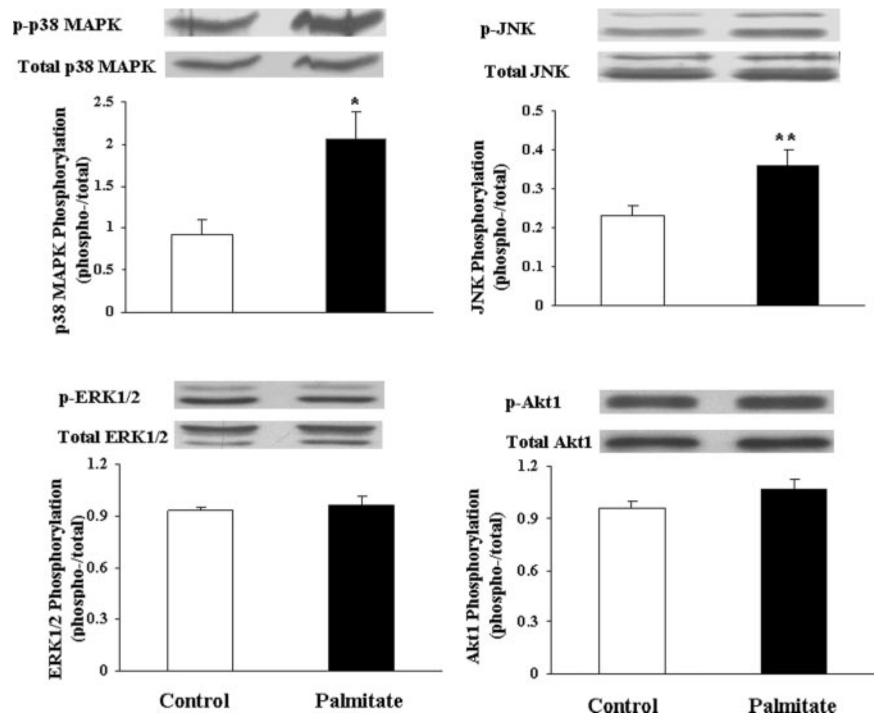

FIG. 2. Effects of palmitate on the phosphorylation of MAPKs and Akt1. hCAECs were incubated with 100 μM palmitate for 24 h. The results are the average of five to 10 experiments. Compared with respective control, *, $P < 0.03$ and **, $P < 0.05$.

confirmed using the TUNEL assay. SB203580 at 20 μM alone did not affect the percentage of TUNEL-positive cells (2.9 ± 1.1%), but it prevented palmitate-induced increase (3.7 ± 0.7%).

Inasmuch as JNK has been shown to mediate cellular apoptosis and we have in the current study demonstrated that palmitate at 100 μM significantly increased the phosphorylation of JNK in cultured hCAECs (Fig. 2), we examined whether SB203580 may have also inhibited palmitate-induced JNK phosphorylation. Our result indicates that SB203580 is quite specific in inhibiting p38 MAPK as JNK phosphorylation remained elevated in palmitate- and SB203580-treated cells (0.37 ± 0.02, $P < 0.002$, compared with control). This confirms that it is indeed p38 MAPK that mediated palmitate-induced apoptosis.

Palmitate decreases IκB level in cultured hCAECs independent of p38 MAPK

Because previous evidence suggests that palmitate activates the IKKβ/NFκB pathway, which mediates inflammatory processes, we tested whether palmitate-induced IκB degradation is also p38 MAPK dependent. As shown in Fig. 5, incubation with palmitate significantly decreased the level of IκB in hCAECs (from 1.02 ± 0.01 to 0.71 ± 0.02, $P < 0.0001$). However, despite blocking apoptosis, SB203580 did not restore IκB levels back to baseline, suggesting that palmitate-induced decrease in IκB level was independent of p38 MAPK

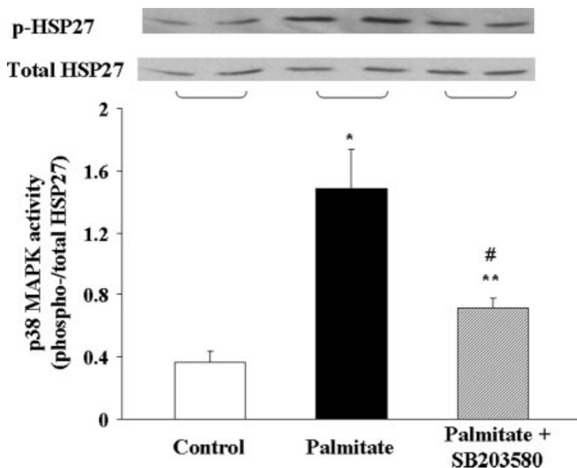

FIG. 3. Palmitate increases p38 MAPK activity in cultured hCAECs. Cells were incubated with palmitate at 100 μM for 24 h with or without 20 μM SB203580, and the phosphorylation of HSP27 was assessed. Results are averages of 12 experiments. Compared with control, *, $P < 0.001$ and **, $P < 0.004$; compared with palmitate, #, $P < 0.02$.

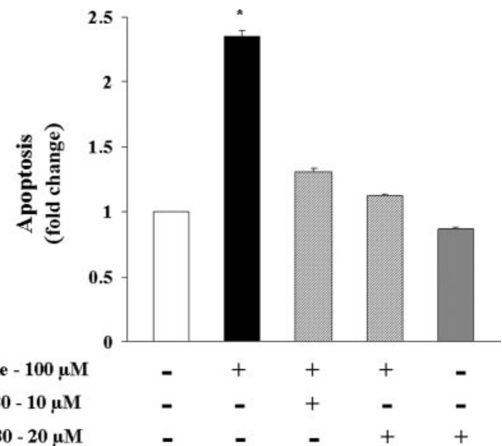

FIG. 4. Palmitate increases hCAEC apoptosis via a p38 MAPK-dependent pathway. Cells were incubated with 100 μM palmitate with or without 10 or 20 μM SB203580. Results are averages of four experiments. Compared with control, *, $P < 0.01$.

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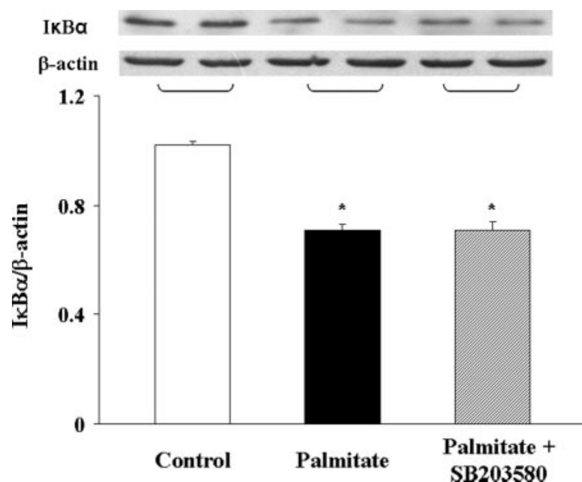

FIG. 5. Palmitate decreases IκB level independent of p38 MAPK in hCAECs. Cells were incubated with 100 μM palmitate with or without 20 μM SB203580, a specific inhibitor of p38 MAPK. Results are averages of six experiments. Compared with control, *, $P < 0.0001$.

and probably not directly related to FFA-driven increases in apoptotic activity.

Discussion

FFAs have been implicated in causing insulin resistance, inflammation, endothelial dysfunction, and atherosclerosis. However, the signaling pathways underlying these actions of FFAs remain unclear. Increased endothelial cell apoptosis may contribute to endothelial dysfunction, vascular inflammation, and atherosclerosis. Our current results provide a first indication that the p38 MAPK directly mediates FFA-induced apoptosis but not the activation of IKKβ/NF-κB pathway in hCAECs. This suggests that FFAs induce endothelial cell death and inflammation via distinct signaling pathways, which may collectively contribute to endothelial dysfunction and accelerated atherosclerosis in the coronary circulation of type 2 diabetic patients.

Many inflammatory mediators released during tissue injury/disease, including IL-1 and TNF-α, can activate p38 MAPK pathway with functional consequences of recruiting leukocytes to sites of inflammation and resultant tissue injury (24). The latter process requires a complex intercellular communication between infiltrating leukocytes and the resident cells (in the case of the arterial wall, the endothelial and smooth muscle cells). In the current study, palmitate increased both the phosphorylation and activity of p38 MAPK and apoptosis in cultured hCAECs. That inhibition of p38 MAPK activity with its specific inhibitor SB203580 completely abolished the proapoptotic effect of palmitate strongly suggests that FFAs induce apoptosis in hCAECs via a p38 MAPK-dependent pathway. This is consistent with a recent report that fatty acids liberated from low-density lipoprotein also trigger endothelial apoptosis via the p38 MAPK pathway in cultured primary endothelial cells from human aorta (25). This FFA-induced phosphorylation of p38 MAPK occurs via the apoptosis signaling kinase-1 (25), which is upstream of both MAPK-activating kinase-3 and -6, two kinases known to activate p38 MAPK (15). On the other hand, activation of p38 MAPK also leads to decreased EPCs, a cell population with pivotal role in repairing the vascular endothelium (20). Taken together, it is very likely that p38 MAPK plays a key role in orchestrating FFA-induced endothelial cell injury/dysfunction, macrophage recruitment, and atherosclerosis in human coronary artery.

Although JNK has been shown to mediate cellular apoptosis in multiple cells lines and we have in the current study demonstrated that palmitate at 100 μM also significantly increased the phosphorylation of JNK in cultured hCAECs (Fig. 2), it appears that JNK activation alone cannot account for palmitate-induced apoptosis in cultured hCAECs, at least in our experimental setting, because SB203580 completely abolished palmitate-induced apoptosis despite persistent elevation in JNK phosphorylation.

In addition to triggering endothelial cell apoptosis, FFAs also induce insulin resistance and modulate inflammatory responses in various tissues, including the vascular endothelium. It appears that FFAs mediate vascular insulin resistance and inflammation via a common effector IKKβ (11, 13), a serine kinase that controls the activation of NF-κB. IKKβ also regulates insulin sensitivity by directly phosphorylating IRS-1 at serine residues (26, 27). Inhibition of IKKβ activity by salicylate or decreased IKKβ expression decreases the IKKβ-mediated IRS-1 serine phosphorylation and improves insulin sensitivity. Kim *et al.* (11) demonstrated that treatment of vascular endothelial cells with palmitate activates IKKβ; impairs insulin-dependent IRS-1, Akt, and eNOS phosphorylation; and decreases insulin-stimulated production of NO. Whereas transfection of the endothelial cells with a dominant-negative IKKβ abrogates FFA-mediated insulin resistance, overexpression of wild-type IKKβ recapitulates the effect of FFAs (11). In the current study, we quantitated the IκB protein content because it reflects IKKβ-activated proteasomal degradation of IκB over time. Therefore, decreased levels of IκB represent enhanced IKKβ activity and subsequent nuclear translocation of NF-κB. As expected, palmitate significantly reduced the level of IκB, suggesting palmitate directly activates the IKKβ/NF-κB inflammatory pathway. However, unlike the apoptosis response, inhibition of p38 MAPK did not return IκB levels to baseline. These divergent findings suggest that palmitate-induced IKKβ/IκB/NF-κB activation is independent of the p38 MAPK pathway. Inasmuch as the IKKβ/IκB/NF-κB pathway has anti-apoptotic/prosurvival property (28), activation of this pathway may actually represent a rescue mechanism against FFA-mediated apoptosis in hCAECs.

Our data are consistent with observations that FFAs cause oxidative stress, inflammation, insulin resistance, and impaired vascular endothelial dysfunction *in vivo*. Exposing HUVECs to plasma samples containing high FFA concentrations obtained from human volunteers after infusion of intralipid or heparin induced a 1.9- to 4.2-fold increase of apoptosis in HUVECs (14). This is not surprising because raising FFAs in humans markedly increases reactive oxygen species generation by leukocytes, increases NF-κB binding activity in the monocyte nuclear extracts, and diminishes flow-mediated dilation of the brachial artery (7). FFAs also induce endothelial dysfunction and insulin resistance at the microcirculation level. Insulin at physiological concentra-

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tions activates eNOS (29) and stimulates microvascular perfusion in the skeletal muscles via a NO-dependent fashion (30–34), and infusion of intralipid/heparin blocks this action (10).

In the current study, we tested only palmitate because it is the most abundant fatty acid *in vivo*, accounting for approximately 26% of the total plasma fatty acids (35). It is likely that other fatty acids may also affect hCAECs. Incubating the primary endothelial cells from human aorta with either 100 μM linoleic acid or oleic acid also led to significant phosphorylation of p38 MAPK (25). Stearic acid, oleic acid, linoleic acid, γ-linolenic acid, and arachidonic acid all are capable of inducing apoptosis in cultured HUVECs, although the concentrations required varied significantly (14). Similar to our observation, all above-named FFAs concentration-dependently reduced the expression of NF-κB inhibitor, IκBα, and eNOS (14).

A potential limitation to the current study is the concentration of albumin (30 mmol/liter or 2.1 g/liter) used. This was done to allow our results to be compared with data obtained by other investigators (11). In addition, it is difficult *in vitro* to mimic the *in vivo* physiological milieu. Even if additional albumin was added, it would still not be physiological because palmitic acid is only one of many different fatty acids present in the plasma and various fatty acids may interact with each other to coordinate different physiological and pathological responses. Albumin *per se* also regulates various cell signaling pathways, either directly or by its interaction with various substrates. Indeed, albumin has been shown to bind to the 60-kDa cell surface albumin-binding protein, gp60, to induce Src activation in endothelial cells (36, 37) and activate ERK via epithelial growth factor receptor in cultured human renal epithelial cells (38).

In conclusion, palmitate induced dose- and time-dependent apoptosis via a p38 MAPK-dependent pathway and reduction in IκB in hCAECs independent of p38 MAPK activity. These suggest that palmitate induces apoptosis and inflammation in hCAECs via distinctly different mechanisms and p38 MAPK may have exerted key role in FFA-induced coronary endothelial injury and atherosclerosis in diabetes. However, because inflammatory cytokines are potent activators of p38 MAPK, which plays very important roles in modulating inflammation, most likely p38 MAPK is also involved in FFA-mediated inflammation and insulin resistance in the vascular endothelium.

Acknowledgments

The authors thank Eugene J. Barrett, M.D., Ph.D., for his thoughtful discussion and critical reading of this manuscript.

Received August 4, 2006. Accepted January 9, 2007.

Address all correspondence and requests for reprints to: Zhenqi Liu, M.D., Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Virginia Health System, P.O. Box 801410, Charlottesville, Virginia 22908-1410. E-mail: zl3e@virginia.edu.

This work was supported by a research grant from the American Diabetes Association (to Z.L.) and P30-DK063609 (to the University of Virginia Diabetes Endocrinology Research Center).

Disclosure: Both W.C. and Z.L. have nothing to declare.

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