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Linoleic acid induces proinflammatory events in vascular endothelial cells via activation of PI3K/Akt and ERK1/2 signaling

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Abstract

Linoleic acid (18:2n-6), is a major unsaturated fatty acid in the American diet. Linoleic acid is considered to be atherogenic because of its pro-oxidative and proinflammatory properties. There is substantial evidence that linoleic acid (LA) can activate vascular endothelial cells and contribute to an inflammatory response. To explore the mechanisms of LA-induced proinflammatory signaling pathways, the present study addresses the role of the phosphatidylinositol 3-kinase/amino kinase terminal (PI3K/Akt), extracellular signal regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) pathways during vascular endothelial cell activation. After a 3- to 6-h exposure, LA significantly activated both Akt and ERK in endothelial cells, as assessed by western blot and immunofluorescence. In contrast, LA activated p38 MAPK already at 10 min, suggesting that p38 MAPK signaling occurred upstream of the ERK1/2 pathway. Furthermore, inhibition of ERK activity by PD98059 and PI3K/Akt activity by LY294002 or wortmannin significantly reduced the LA-induced activation of nuclear factor kappa B (NF-κB). These results suggest a contribution of both the ERK1/2 and PI3K/Akt pathways to the effect of LA on NF-κB-dependent transcription. Indeed, LA-mediated gene expression of the vascular cell adhesion molecule 1 was suppressed by PD98059, wortmannin and LY294002. These data indicate that both PI3K/Akt- and ERK1/2-mediated proinflammatory signaling events are critical in LA-induced endothelial cell activation and vascular inflammation.

Keywords: Linoleic acid; Endothelial cell; Cell signaling; Phosphatidylinositol 3-kinase; Extracellular signal regulated kinase

1. Introduction

Regulation of vascular function and homeostasis is mediated by endothelial cells [1]. Endothelial activation and dysfunction are critical events during the early pathogenesis of atherosclerosis [2]. Accumulated lipids in subendothelial regions and circulating lipids (dietary fatty acids) are involved in metabolic interactions with endothelial cells and can modify an endothelial inflammatory response [3].

A number of proteins that are involved in cell signaling by unsaturated fatty acids include members of the Src-kinase family, such as G-proteins. Members of this kinase family are found to be activated by a wide variety of mitogenic and nonmitogenic agents via a cascade of kinase/effector molecules, which include extracellular signal regulated kinase 1/2 (ERK1/2) [4] and p38 mitogen-activated protein kinase (MAPK) [5]. Members of the phosphatidylinositol 3-kinase/amino kinase terminal (PI3K/Akt) are also involved in the transduction of extracellular signals [6,7]. Kinases in the PI3K/Akt and p38 MAPK pathways have been reported to act in the regulation of nuclear translocation of inflammatory transcription factor, such as nuclear factor kappa B (NF-κB) [8,9].

Abbreviations: Akt, amino kinase terminal; FBS, fetal bovine serum; NF-κB, nuclear factor kappa B; RT-PCR, reverse transcriptase-polymerase chain reaction; VCAM-1, vascular cell adhesion molecule 1; ERK, extracellular signal regulated kinase; PI3K, phosphatidylinositol 3-kinase; p38 MAPK, p38 mitogen activated protein kinase; PBS, phosphate buffer saline.

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There is evidence that the major dietary unsaturated fatty acid in the American diet, i.e., linoleic acid (LA; the parent omega 6 fatty acid), has proatherogenic and proinflammatory effects by activation of endothelial cells [10]. LA increases the activation of NF-κB and expression of cytokines and cell adhesion molecules [11,12]. Oxidative stress-sensitive signaling, including NF-κB-dependent transcriptions, appear to be a significant denominator during LA-induced endothelial cell activation. However, not much is known about the upstream signaling cascade leading to LA-mediated endothelial cell activation.

We propose that the MAPK and PI3K/Akt signaling pathways participate in the LA-mediated endothelial cell activation. Results from our study suggest that both PI3K/Akt and ERK1/2 pathways can regulate the effects of LA on NF-κB-dependent transcription. We also demonstrate that these signaling pathways may be involved in activation of LA-mediated gene expression of vascular cell adhesion molecule 1 (VCAM-1). These data suggest that LA can induce complex inflammatory processes via activation of the PI3K/Akt and ERK1/2 signaling.

2. Materials and methods

2.1. Materials

LA (>99% pure) was obtained from Nu-Chek Prep (Elysian, MN, USA). Wortmannin and LY294002 were obtained from Cell Signaling Technology (Beverly, MA, USA). PD98059 and SB 203580 were purchased from Calbiochem (La Jolla, CA, USA). Antibodies used for western blotting were anti-Akt and anti-phospho-Akt (both from Cell Signaling Technology). Anti-ERK2, anti-phospho ERK1/2, anti-p38 MAPK, anti-phospho p38 MAPK, along with antirabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protease inhibitor cocktail was purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and experimental media

Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described [13]. The basic culture medium consisted of M199 (GIBCO Laboratories, Grant Island, NY, USA), containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA). The experimental media contained 5% FBS and was supplemented with LA at the final concentration of 0–90 μM. Preparation of experimental media with LA was performed as described earlier [13].

2.3. Analysis of Akt, ERK1/2 and p38 MAPK activation by western blotting

Whole-cell lysates were prepared with a lysis buffer containing Tris-HCl 50 mM (pH 8.0), NaCl 200 mM, EDTA 20 mM, 1% SDS, 0.5% Na-deoxycholic acid, 0.01% NP-40, sodium orthovanadate 200 mM and phenylmethylsulphonyl fluoride 100 mM. Equal amounts of protein (50 μg) were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membrane was blocked overnight at 4°C with 5% nonfat milk in Tris-buffered saline (TBS, pH 7.6) containing 0.05% Tween 20, and then washed with TBS-Tween. Bands were visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies followed by ECL immunoblotting detection reagents (Amersham Biosciences, Buckinghamshire, England).

2.4. Analysis of Akt and ERK1/2 activation by immunofluorescence

Confluent endothelial cells were grown on glass culture slides and incubated with or without LA (90 μM) for 3 h. After three washes in phosphate-buffered saline (PBS), cells were fixed by 70% ethyl alcohol for 10 min and air-dried. After 30 min of blocking of nonspecific binding with PBS containing 3% bovine serum albumin (BSA), cells were incubated with p-Akt or p-ERK1/2 primary antibodies (for 1 h on ice, dilution of 1:50) and were washed twice in PBS then incubated simultaneously with fluorescein isothiocyanate-labeled secondary antibody (1 h at room temperature, dilution 1:5; Chemicon, CA, USA) Negative controls were prepared by incubation of the cells with anti-IgG antibody. The cells were washed twice in PBS and mounted in aqueous mounting medium before being observed using an epifluorescence Nikon Eclipse E600 microscope, and the images were captured using a Spot charge coupled device camera system (Nikon, Melville, NY, USA).

2.5. Transcription factor (NF-κB) activation studies: electrophoretic mobility shift assay

Nuclear extracts from endothelial cells were prepared as described earlier [14]. The ^{32}P-labeled specific oligonucleotide probe (40,000 cpm^2) contained the enhancer DNA element for the NF-κB site 5′AGTTGAGGGGACTTTCCCAGGC 3′ (Promega, Madison, WI, USA). Control reactions using 200-fold molar excess of unlabeled oligonucleotide probes or a supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF-κB.

2.6. Measurement of mRNA levels of VCAM-1 by reverse transcriptase-polymerase chain reaction

Total RNA was extracted from cells using RNA-STAT-60 (TEL-TEST, Friendswood, TX, USA), according to the manufacturer's instructions. VCAM-1 gene expression was determined through reverse transcriptase-polymerase chain reaction (RT-PCR) as described earlier [12]. The oligonucleotide primers used to amplify the porcine VCAM-1 and the house keeping gene β-actin were as described earlier [15]. The sequences of the primer pairs in this experiment were as follows: porcine VCAM-1 (sense, 5′-ATGACATGCTTGAGCCAGG-3′; antisense, 5′-GTGTCTCCTTCTTTGACACT-3′); porcine β-actin, was used as an internal control

(sense, 5′-ATGTTTGAGACCTTCAACACGCCGG3′; antisense, 5′-GCAGGACTCCATGCCCAGGAAGGAG-3′). The PCR mixture consisted of a Taq PCR Master Mix (Qiagen, Valencia, CA, USA). PCR products were run by electrophoresis on a 2% agarose gel, stained with SYBR-gold (Molecular Probes, Eugene, OR, USA) and analyzed using phosphoimaging technology (FLA-5000; Fujifilm Medical Systems, Stanford, CT, USA).

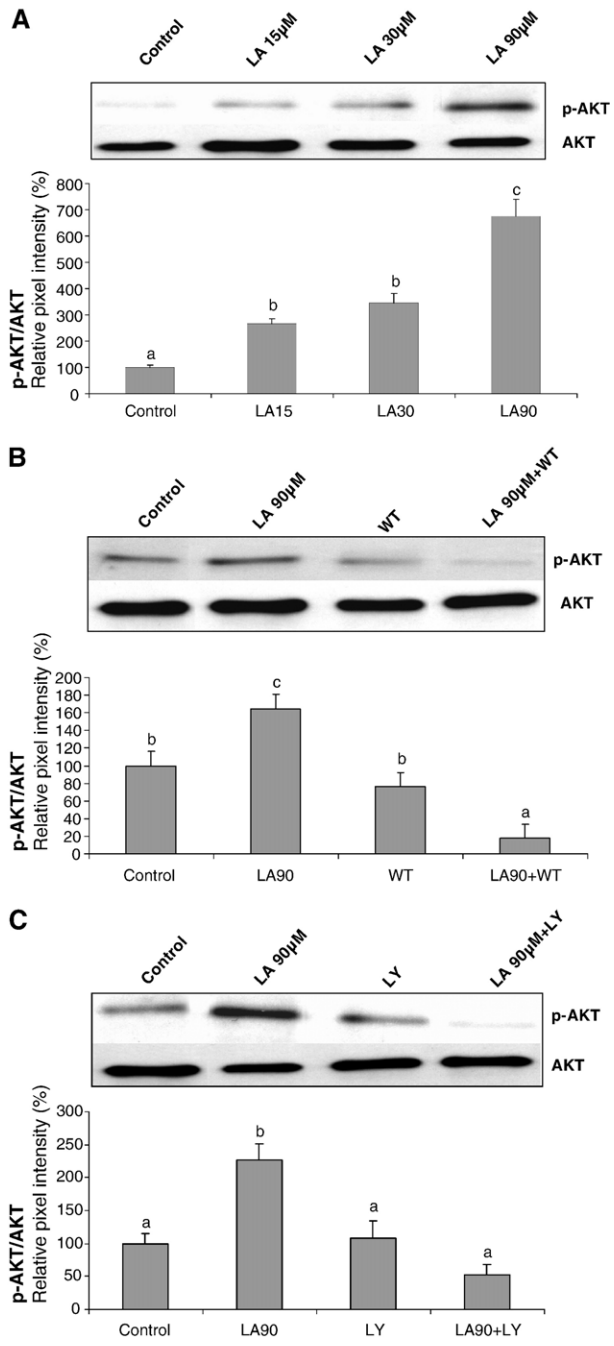

Fig. 1. LA activates the PI3K/Akt signaling in vascular endothelial cells. Total Akt and phosphorylated Akt (p-Akt) were detected by western blot using specific antibodies. (A) Cells exposed to increasing concentrations of LA (15 to 90 μM) for 6 h. (B) p-Akt in endothelial cells were pretreated with inhibitors of the PI3K/Akt pathways: wortmannin (WT; 2.5 μM for 30 min) or (C) LY294002 (10 μM for 30 min) followed by exposure to LA (90 μM) for 6 h. Blots shown represent one of three experiments. Bar graph represents the mean±S.E.M. Different letters correspond to significant difference ($P<.05$) compared to different treatment groups.

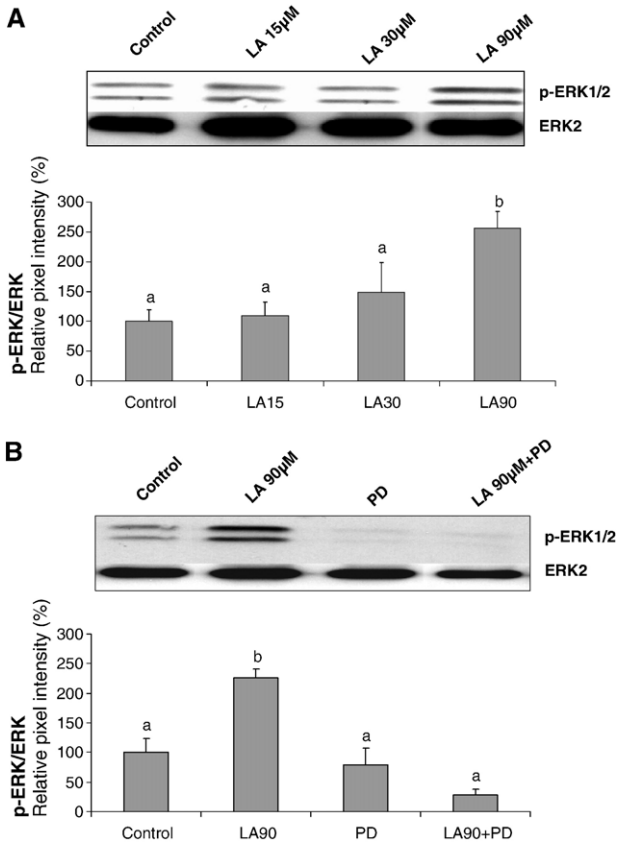

Fig. 2. LA activates the ERK1/2 signaling in vascular endothelial cells. Total ERK1/2 and p-ERK1/2 were detected by western blot using specific antibodies. (A) Cells were exposed to increasing concentrations of LA (15–90 μM) for 6 h. (B) To indicate the specificity of the reaction, endothelial cells were pretreated with the ERK1/2 inhibitor PD98059 (20 μM for 1 h), followed by exposure to LA (90 μM) for 6 h. Blots shown represent one of three experiments. Bar graph represents the mean± S.E.M. Different letters correspond to significant difference ($P<.05$) compared to different treatment groups.

2.7. Statistical analysis

Values are reported as percentage of controls ± S.E.M. Square root transformation of the data was performed before statistical analysis. Comparisons between treatments were made by one-way analysis of variance followed by Tukey's pairwise multiple comparison procedure using SYSTAT 10.0 (Systat Software, Point Richmond, CA, USA) or SigmaStat 2.0 software (Jandel Corp., San Rafael, CA, USA). Statistical probability of $P<.05$ was considered significant.

3. Results

3.1. Exposure to LA activates the Akt and ERK signaling pathways in vascular endothelial cells

To assess the effects of LA on activation of Akt or ERK1/2, endothelial cells were exposed to increasing concentrations

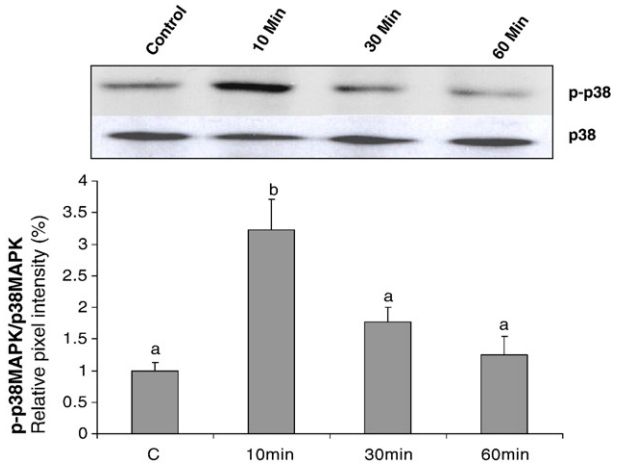

Fig. 3. LA treatment activates p38 MAPK. Cells were exposed to 90 μM of LA from 10 min up to 1 h. Phosphorylated p38 MAPK (p-p38 MAPK) and total p38 MAPK were detected by western blot using specific antibodies. Blots shown represent one of three experiments. Bar graph represents the mean±S.E.M. Different letters correspond to significant difference ($P<.05$) compared to different treatment groups.

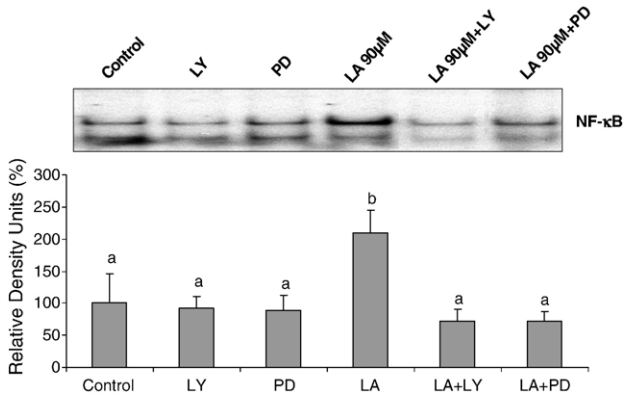

Fig. 5. The PI3K/Akt and ERK1/2 pathways are involved in LA-mediated NF-κB activation. Endothelial cells were pretreated with or without the PI3K/Akt inhibitor LY294002 (LY, 10 μM for 30 min) or the ERK inhibitor PD98059 (PD, 20 μM for 1 h), followed by exposure to LA (90 μM) for 6 h. Activation of NF-κB was determined by EMSA. Autoradiograph shown represents one of three experiments. Bar graph represents the mean±S.E.M. Different letters correspond to significant difference ($P<.05$) compared to different treatment groups.

of LA (15–90 μM) for 6 h, and the levels of phosphorylated Akt (p-Akt) and ERK1/2 (p-ERK1/2), respectively, were assessed by Western blotting. As indicated in Figs. 1 and 2, LA increased activation of both p-Akt (Fig. 1A) and p-ERK1/2 (Fig. 2A) in a concentration-dependent manner, with maximum activation at 90 μM. Furthermore, levels of phosphorylated Akt and ERK1/2 were maximal at 6 h, with little expression at 3 h (data not shown).

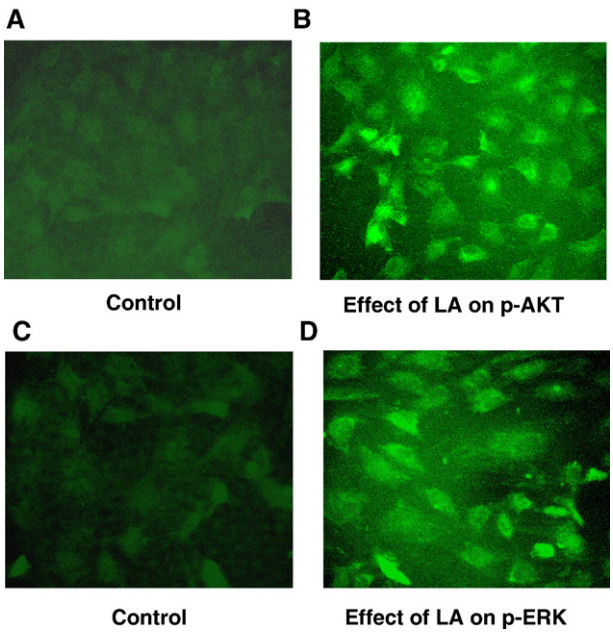

Fig. 4. LA-mediated activation of Akt and ERK1/2 as analyzed by immunofluorescence. Confluent endothelial cells were incubated with LA (90 μM) for 3 h. Cells were fixed in 70% ethyl alcohol and blocked with PBS containing 3% BSA. Cells were then incubated with respective primary antibodies (anti-p-Akt or anti-p-ERK1/2). Negative controls were prepared by incubation of the cells with anti-IgG antibody. Experiments were repeated three times and the epifluorescence micrographs shown are representative fields of one of the experiments (original magnification ×200).

To further confirm the specificity of LA-induced activation of Akt, endothelial cells were pretreated with pharmacological inhibitors of the PI3K/Akt pathway, such as wortmannin (2.5 μM for 30 min) and LY294002 (10 μM for 30 min). Subsequently, cells were washed with PBS and incubated with or without 90 μM of LA for 6 h. As indicated in Fig. 1B and C, wortmannin and LY294002, respectively, effectively blocked LA-induced activation of Akt.

Similar to Akt, the specificity of ERK1/2 activation was confirmed with a specific inhibitor, i.e., endothelial cells were pretreated for 1 h with 20 μM of the ERK inhibitor PD98059. After removing the inhibitor by washing with PBS, cells were treated with or without 90 μM of LA for 6 h. Compared to LA treatment alone, pretreatment with PD98059 inhibited LA-mediated activation of ERK1/2 (Fig. 2B).

3.2. Exposure to LA rapidly induces p38 MAPK

In addition to ERK1/2, another important member of the MAPK pathway is p38. As indicated in Fig. 3, a 10-min exposure to 90 μM of LA significantly increased the phosphorylation of p38 MAPK, which returned to baseline levels at 30 min. Further exposure to LA (1 to 6 h) did not lead to activation of p38 MAPK (data not shown).

3.3. LA-induced activation of Akt and ERK1/2 in vascular endothelial cells as observed by immunofluorescence

To provide further evidence that LA can activate the Akt and ERK1/2 signaling pathways in vascular endothelial cells, immunofluorescence analyses also were performed. Fig. 4 indicates that incubation with 90 μM of LA for 3 h markedly increased p-Akt and p-ERK1/2 expression over untreated control cells.

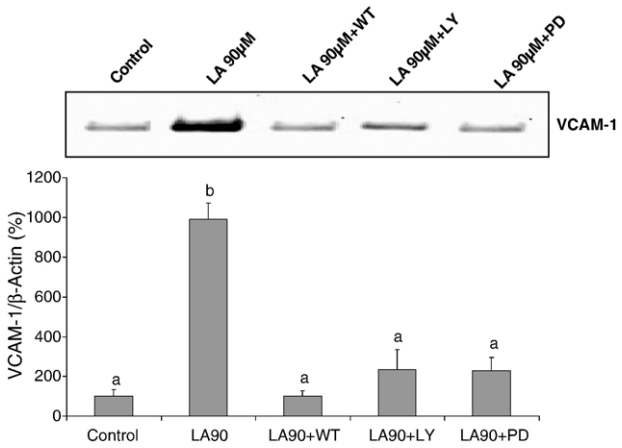

Fig. 6. The PI3K/Akt and ERK1/2 pathways are involved in LA-induced VCAM-1 gene expression. Endothelial cells were pretreated with or without the PI3K/Akt inhibitors [wortmannin (WT) 2.5 μM for 30 min or LY294002 (LY) 10 μM for 30 min] or the ERK1/2 inhibitor PD98059 (PD, 20 μM for 1 h) and then exposed to LA (90 μM) for 6 h. VCAM-1 gene expression was quantified by RT-PCR. The gel shown represents one of three experiments. Bar graph represents the mean±S.E.M. Different letters correspond to significant difference ($P<.05$) compared to different treatment groups.

3.4. The PI3K/Akt and ERK1/2 pathways regulate LA-mediated activation of NF-κB and induction of VCAM-1 expression

As previously reported [11], endothelial cell exposure to LA induces NF-κB DNA binding activity. To evaluate whether the PI3K/Akt and ERK1/2 pathways can be involved in this effect, endothelial cells were pretreated with either the PI3K/Akt inhibitor LY294002 or the ERK inhibitor PD98059. Cells were then washed with PBS and incubated with or without 90 μM of LA for 6 h, and DNA binding of NF-κB was assessed by electrophoretic mobility shift assay (EMSA). Fig. 5 illustrates that pretreatment of endothelial cells with LY294002 or PD98059 significantly reduced the LA-mediated activation of NF-κB.

NF-κB is implicated in inducible expression of a variety of genes, including those encoding for adhesion molecules, such as VCAM-1. Therefore, the influence of the PI3K/Akt and ERK1/2 signaling on LA-induced VCAM-1 expression was assessed in the present study (Fig. 6). Exposure of endothelial cells to 90 μM of LA for 6 h significantly increased VCAM-1 mRNA levels, as determined by RT-PCR. However, cellular pretreatment with inhibitors of PI3K/Akt (wortmannin or LY294002) or of ERK1/2 (PD98059) markedly attenuated these effects.

4. Discussion

Fatty acids are precursors of lipid-signaling molecules and serve as ligands for transcription factors that control cellular metabolic gene expression [16]. However, the specific mechanisms of their action as proinflammatory agents are still unclear. Different signaling pathways appear to mediate both overlapping and distinct effects in the activation of both PI3K/Akt and the MAPK cascades [17], contributing to the stimulation of NF-κB-dependent transcription in endothelial cells. PI3K/Akt, MAPK and NF-κB may play important roles in maintaining vascular integrity during a chronic inflammatory response [18].

In the present study, we demonstrated that exposure of cultured endothelial cells to LA can markedly and, in a dose-dependent manner, activate the PI3K/Akt pathway. Similar to activation of Akt, LA was able to elevate the level of p-ERK1/2 in a concentration-dependent manner. In addition to the ERK pathway, we also investigated whether p38 MAPK can be involved in LA-mediated cell activation. Our data indicated that LA can stimulate phosphorylation of p38 MAPK within minutes of fatty acid exposure. This suggests that p38 MAPK acts upstream of ERK1/2 signaling. Other studies also suggested a critical role of p38 MAPK in fatty acid-induced cytotoxicity. For example, lipid peroxidation and oxidative stress mediated by exposure to arachidonic acid was related to activation of p38 MAPK in hepatocytes [19]. LA also has been shown to activate ERK and Akt/protein kinase B, which may be regulated in part via G protein-coupled receptors [20]. The classical members of the MAPK family, ERK1 and ERK2, can be activated by a variety of cell growth and differentiation stimuli and play a central role in mitogenic signaling [21]. The role of p38 MAPK is more complex and includes regulation of inflammatory cytokine production, cell cycle entry, and cellular differentiation [22]. The results of the present study demonstrated that PI3K/Akt and ERK1/2 together with the p38 MAPK pathways are all involved in the LA-mediated endothelial cell activation.

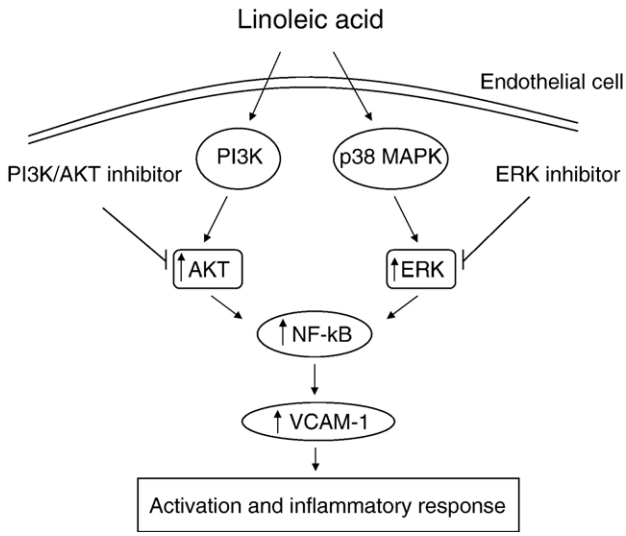

Fig. 7. Proposed mechanism for LA-mediated endothelial cell activation. LA treatment activates both PI3K/Akt and the ERK1/2 signaling in endothelial cells. These signaling pathways regulate the NF-κB-driven expression of vascular adhesion molecules in endothelial cells, subsequent endothelial cell activation and an inflammatory response.

The relationship between signaling pathways, including p38 MAPK, PI3K/Akt, ERK1/2 and NF-κB activation remains poorly understood and may differ between cell types and in response to different stimuli [23–25]. Moreover, these pathways may act sequentially and may also demonstrate cross-talk [26,27]. Our results provide evidence that LA-mediated activation of both the PI3K/Akt and p38 MAPK, and its downstream ERK1/2 signaling can trigger activation of NF-κB and expression of inflammatory mediators, such as VCAM-1. NF-κB is highly expressed during the pathology of atherosclerosis and has been associated with endothelial cell activation, survival, dysfunction as well as participating in inflammatory processes [28]. For example, NF-κB is implicated in inducing the expression of a variety of genes including vascular adhesion molecules [29]. Nuclear translocation related to protein phosphorylation is the key event in NF-κB activation [28]. It was demonstrated that PI3K/Akt can stimulate the transcriptional activity of NF-κB by targeting the basal levels of nuclear NF-κB [8]. In addition, the p38 MAPK and ERK1/2 pathways were suggested to be required for NF-κB activation [9]. In our study, we used pharmacological inhibitors of ERK1/2 and PI3K/Akt to demonstrate that ERK1/2 and PI3K/Akt signaling pathways play a critical role in the regulation of LA-induced activation of NF-κB. Indeed, LA-induced NF-κB transcriptional activity in endothelial cells was inhibited by LY294002, an inhibitor of PI3K/Akt. We also provided evidence that the ERK1/2 inhibitor PD98059 significantly attenuated the LA-induced NF-κB activity. Similarly, we demonstrated that inhibition of ERK1/2 and PI3K/Akt abolished the NF-κB-driven up-regulation of VCAM-1 mRNA levels in LA-treated endothelial cells.

In summary, our data provide evidence that LA may contribute to complex inflammatory processes in vascular endothelial cells via activation of the PI3K/Akt, p38 MAPK and ERK1/2 signaling (Fig. 7). Furthermore, it appears that both PI3K/Akt and ERK1/2-mediated pro-inflammatory signaling events are critical in LA-induced vascular endothelial cell activation, such as activation of redox-responsive transcription factors and adhesion molecules.

Acknowledgments

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