Linoleic acid-induced endothelial activation: role of calcium and peroxynitrite signaling

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Abstract Hypertriglyceridemia, an important risk factor of atherosclerosis, is associated with increased circulating free fatty acids. Research to date indicates that linoleic acid (LA), the major fatty acid in the American diet, may be atherogenic by activating vascular endothelial cells. However, the exact signaling mechanisms involved in LA-mediated proinflammatory events in endothelial cells still remain unclear. We previously reported increased superoxide formation after LA exposure in endothelial cells. The objective of the present investigation is to determine the role of calcium and peroxynitrite in mediating the proinflammatory effect of LA in vascular endothelial cells. LA exposure increased intracellular calcium, nitric oxide, and tetrahydrodiopterin levels as well as the expression of E-selectin. Inhibiting calcium signaling using 1,2-bis(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid and heparin decreased the expression of E-selectin. Also, LA-mediated nuclear factor kappa B activation and E-selectin gene expression were suppressed by Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (a superoxide scavenger), *N*[G]-mono-methyl-ʟ-arginine (an endothelial nitric oxide synthase inhibitor), and 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) chloride (a peroxynitrite scavenger). LA exposure resulted in increased nitrotyrosine levels, as observed by Western blotting and immunofluorescence. Our data suggest that the proinflammatory effects of LA can be mediated through calcium and peroxynitrite signaling.—Saraswathi, V., G. Wu, M. Toborek, and B. Hennig. **Linoleic acid-induced endothelial activation: role of calcium and peroxynitrite signaling.** *J. Lipid Res.* 2004. **45:** 794–804.

Supplementary key words fatty acid • endothelial nitric oxide synthase • nitric oxide • tetrahydrobiopterin • nuclear factor kappa B • adhesion molecules • nitrotyrosine • oxidative stress

Factors implicated in the pathogenesis of atherosclerosis are numerous and include certain dietary lipids such as omega-6 fatty acids (1, 2). Increased circulating free fatty acid levels are associated with hypertriglyceridemia and obesity, which are important risk factors leading to atherosclerosis (3–5). Dietary balance of long-chain fatty acids may influence processes involving leukocyte-endothelium interactions, such as atherogenesis and inflammation (6). In contrast to earlier epidemiological studies showing the reduced risk of coronary heart disease attributable to high PUFA intake (7), current data on dietary fats indicate that it is not just the presence of PUFAs but the type of PUFAs that is important. A high n-6 PUFA content and n-6/n-3 ratio in dietary fats are considered to be atherogenic and diabetogenic (2).

The parent omega-6 fatty acid, linoleic acid (LA), is abundant in the Western diet and is the major fatty acid in safflower, sunflower, corn, soybean, and cotton seed oils, accounting for greater than 50% of the total fatty acid content in these oils (8). LA is known to elicit an inflammatory response (9). It has been reported that LA is proatherogenic by causing arterial smooth muscle cell proliferation (10). Furthermore, there is evidence that LA, derived from the hydrolysis of triglyceride-rich lipoproteins, may be atherogenic by causing endothelial injury or dysfunction (11), an initial event in atherosclerosis. It has an overall stimulatory effect on the endothelium by increasing the activation of transcription factors such as nuclear factor kappa B (NF-κB) and AP-1 and the expression of adhesion molecules and by triggering apoptosis (12–14). The overall effect of LA on endothelial activation is mediated mostly through oxidative stress-sensitive signaling. However, the upstream cellular signals leading to LA-mediated oxidative stress and subsequent endothelial activation are not well understood.

Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid; BH4, tetrahydrobiopterin; DAF, 4-amino-5-methyl-amino-2′,7′-difluorofluorescein diacetate; eNOS, endothelial nitric oxide synthase; FeTPPS, 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) chloride; IP3, inositol 1,4,5-triphosphate; LA, linoleic acid; L-NMMA, *N*[G]-monomethyl-ʟ-arginine; MnTMPyP, Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride; NO, nitric oxide; ROS, reactive oxygen species.

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We recently reported that LA generates superoxide anions via induction of CYP 2C9 in vascular endothelial cells (15). Furthermore, long-chain n-6 PUFAs such as LA and arachidonic acid are able to liberate calcium from internal stores and thus induce calcium entry into the cells (16, 17). However, the role of increased calcium in mediating LA-induced endothelial cell activation is not yet clear.

A perturbation of intracellular calcium is an early and critical event in the development of cytotoxicity, causing the ultimate loss of cell viability through the activation of various calcium-dependent processes (18, 19). The importance of calcium signaling in modulating endothelial functions has been studied extensively (20, 21). It is generally accepted that important cross-talk between intracellular calcium and nitric oxide (NO) occurs in the endothelium because of the calcium sensitivity of constitutive endothelial nitric oxide synthase (eNOS) (22, 23).

Thus, substances that influence endothelial cell calcium homeostasis potentially influence the production of endothelial factors such as NO, which, under oxidative stress, can lead to the formation of peroxynitrites. Peroxynitrite, which is formed by the fast reaction between NO and superoxide anion, has received increasing attention as a vascular toxin (24). Peroxynitrite can react with a wide range of biomolecules to result in lipid peroxidation, oxidation of sulfhydryl groups, and nitration of tyrosine, causing damage to cellular constituents including proteins, DNA, and lipids (25, 26). Therefore, the formation of peroxynitrite may result in the loss of many of the beneficial effects of NO, including vasodilation.

LA is the major polyunsaturated fatty acid found in common vegetable oils. As LA has the ability to generate superoxide anions and to increase intracellular calcium levels, we hypothesized that calcium and peroxynitrite may be involved in mediating the proinflammatory effect of LA. Our results suggest that LA may play a key role in contributing to chronic inflammation in atherogenic processes via oxidative stress and that peroxynitrite can partially mediate the observed effects of LA on endothelial activation.

MATERIALS AND METHODS

Materials

LA (>99% pure) was obtained from Nu-Chek Prep. (Elysian, MN). FLUO-3 AM and 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate (DAF) were obtained from Molecular Probes (Eugene, OR). 1,2-Bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA), heparin, A-23187, and (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (tetrahydrobiopterin, BH$_4$) were obtained from Sigma (St. Louis, MO). Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), N^G-monomethyl-L-arginine (L-NMMA), 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) chloride (FeTPPS), and mouse anti-NO synthase monoclonal antibody against bovine endothelial cell were purchased from Calbiochem (San Diego, CA).

Cell culture and experimental media

Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described (27). The basic culture medium consisted of M199 (GIBCO Laboratories, Grand Island, NY) containing 10% FBS (Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate (GIBCO). The experimental medium contained 5% FBS supplemented with LA (90 μM). Preparation of the experimental medium with LA was performed as described earlier (27, 28).

Measurement of intracellular calcium and NO formation

Intracellular calcium and NO were measured by fluorescent plate reader and flow cytometric methods in endothelial cells. For the measurement of calcium by the plate reader method, endothelial cells were cultured in 24-well plates, treated with LA for 3 h, and stained with the membrane-permeable calcium-sensitive fluorescent dye FLUO-3 AM (10 μM) for the last 30 min of the treatment period. To measure NO formation, cells were treated with LA and/or L-NMMA (10 μM) for 3 h and stained with the membrane-permeable NO-sensitive fluorescent probe DAF (10 μM) for the last 30 min of the treatment period. After staining, the cells were washed with HEPES buffer, pH 7.4, and postincubated in the same buffer for 15 min. Relative fluorescence intensity was measured at an excitation wavelength of 490 ± 5 nm and an emission wavelength of 520 ± 5 nm using a multiwell fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

To measure intracellular calcium and NO production by flow cytometry, endothelial cells were stained with FLUO-3 AM or DAF (2.0 μM) for 30 min. Subsequently, calcium and NO levels in the cells were determined by flow cytometric analysis (29).

Measurement of BH$_4$ in endothelial cells

BH$_4$ was analyzed by HPLC as described by Meininger and Wu (30). Endothelial cells (2×10^6) were lysed in 0.3 ml of 0.1 M phosphoric acid containing 5 mM dithioerythritol and 35 μl of 2 M TCA. One hundred microliters of the cell extract or 50 nM BH$_4$ standard was mixed with 15 μl of 0.2 M TCA and 15 μl of acidic oxidizer (1% I$_2$/2% KI in 0.2 M TCA) (acidic oxidation) or with 15 μl of 1 M NaOH and 15 μl of alkaline oxidizer (1% I$_2$/2% KI in 3 M NaOH) (alkaline oxidation). After 1 h of incubation at 25°C in the dark, excess iodine was removed by adding 25 μl of 20 mg/ml ascorbic acid. After neutralization, 50 μl of the solution was analyzed on a Phenosphere 5 ODS-1 column (4.6 mm × 25 cm, 5 μm) using isocratic elution (flow rate of 1 ml/min) and fluorescence detection (excitation at 350 nm and emission at 440 nm). The mobile phase solvent was 5% HPLC-grade ethanol, 95% HPLC-grade water, 7.5 mM sodium phosphate, pH 6.35 (running time was 15 min). The amount of BH$_4$ in cell extracts was determined by subtracting the amount of biopterin measured after alkaline oxidation from the amount of biopterin measured after acidic oxidation.

Nuclear extracts and electrophoretic mobility shift assays

Nuclear extracts from endothelial cells were prepared according to the method of Beg et al. (31). Binding reactions were performed in a 20 μl volume containing 4 μg of nuclear protein extracts, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 1 M glycerol, and 0.5 μg of poly[dI-dC] (nonspecific competitor) and incubated at room temperature for 10 min. A ^{32}P-labeled specific oligonucleotide probe (40,000 cpm) was added to the reaction and incubated for 20 min at room temperature. Double-stranded oligonucleotide for NF-κB (5′-GTT-GAGGGGACTTTCCCAGGC-3′) was purchased from Promega (Madison, WI). The oligonucleotide was end labeled with [^{32}P]ATP
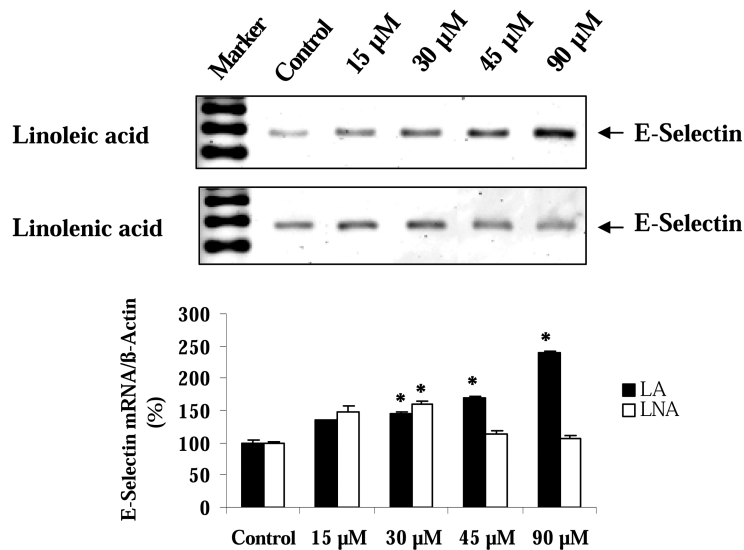
Fig. 1. Effect of increasing concentrations of linoleic acid (LA) and linolenic acid (LNA) on E-selectin gene expression in vascular endothelial cells. Cells were exposed to either LA or LNA for 6 h. Fatty acid concentrations ranged from 0 to 90 μM. RNA samples were extracted, and E-selectin mRNA was quantitated by RT-PCR. The bar graph shows the densitometric analysis of the gels. Data are presented as means ± SEM from three sets of experiments. * Significantly different from control values for each respective fatty acid.

(Amersham Pharmacia Biotech, Piscataway, NJ) using a T$_4$ polynucleotide kinase (Promega, Madison, WI). The protein-DNA complexes were then resolved on native 5% polyacrylamide gels using Tris/borate/EDTA buffer.

Measurement of mRNA levels of E-selectin by RT-PCR

E-selectin gene expression was analyzed by RT-PCR. Total RNA was isolated from cells using RNA-STAT-60 (TEL-TEST, Friendswood, TX) according to the manufacturer's instructions. The RNA was then reverse-transcribed to cDNA and amplified by PCR. The oligonucleotide primers used to amplify the porcine E-selectin and the housekeeping gene β-actin were as described earlier (32). The sequences of the primer pairs in this experiment were as follows: porcine E-selectin (sense, 5′-GACTCGGGCAAGTGGAATGATGAG-3′; antisense, 5′-CATCACCATTCTGAGGATGGCCGAC-3′); porcine β-actin, used as an internal control (sense, 5′-ATGTTTGAGACCTTCAACACGCCGG-3′; antisense, 5′-GCAGGGACTCCATGCCCAGGAAGGAG-3′). PCR products were run by electrophoresis on a 2% agarose gel, stained with SYBR-gold, and analyzed using phosphorimaging technology (FLA-2000; Fujifilm Medical Systems, Stamford, CT).
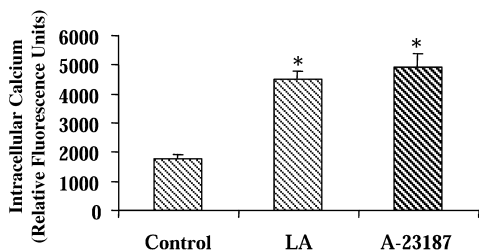
Immunoprecipitation and Western blotting

Immunoprecipitation of nitrotyrosine was carried out using anti-nitrotyrosine antibody (Cayman, Ann Arbor, MI) as described previously (33). Protein precipitates were separated by 7.5% SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked overnight at 4°C with 5% milk powder in Tris-buffered saline, pH 7.6, containing 0.05% Tween 20. After accurate washing, the membrane was incubated for 2 h in a 1:1,000 dilution of mouse monoclonal anti-nitrotyrosine antibody. The membrane was then incubated with goat anti-mouse secondary antibody conjugated to horseradish peroxidase. Signals of the immunoreactive bands were measured using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).
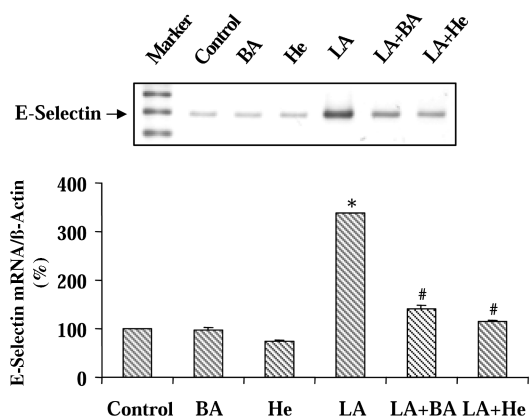

Fig. 2. Effect of LA (90 μM) and A23187 (10 μM) on intracellular calcium concentration in endothelial cells. To examine the fatty acid response, cells were exposed to 90 μM LA for 3 h. FLUO-3 AM was loaded for the last 30 min of the treatment period. To examine the response with a positive agonist, cells were loaded with the calcium-sensitive dye FLUO-3 AM (10 μM) before being challenged with A-23187 for 15 min. Data are presented as means ± SEM from three sets of experiments. * Significantly different from control values.

Fig. 3. Effect of 1,2-bis(2-aminophenoxy)-ethane-$N,N,N′,N′$-tetraacetic acid [BAPTA (BA); an intracellular calcium chelator] or heparin (He; an inositol 1,4,5-triphosphate antagonist) on LA-induced E-selectin gene expression in endothelial cells. Cells were pretreated with BAPTA (100 μM) or heparin (50 μg/ml) for 2 h followed by LA exposure for 6 h. RNA samples were extracted and E-selectin mRNA was quantitated by RT-PCR. The bar graph shows the densitometric analysis of the gels. Data are presented as means ± SEM from three sets of experiments. * Significantly different from control values. # Significantly different from LA-treated cells.
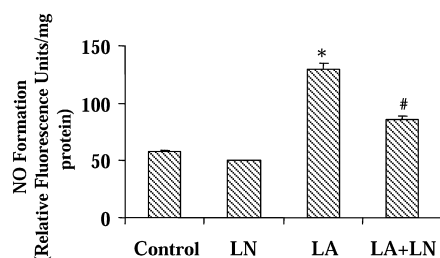
Fig. 4. Effect of LA (90 μM) and/or N^G-monomethyl-L-arginine [L-NMMA (LN); 10 μM] on nitric oxide (NO) formation in endothelial cells. To study the fatty acid response, cells were treated with LA and/or L-NMMA for 3 h and 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate was loaded for the last 30 min of the treatment period. Data are presented as means ± SEM from three sets of experiments. * Significantly different from control values. # Significantly different from the LA group.

Immunofluorescence

Cells grown in four-well slide chambers were incubated for 6 h in the presence or absence of LA. After two washes with PBS, cells were fixed in 100% ethanol for 30 min. After 30 min of blocking of nonspecific binding with PBS containing 3% BSA, cells were incubated for 2 h at room temperature with a 1:50 dilution of the mouse monoclonal anti-nitrotyrosine antibody. Negative controls were prepared by incubation of the cells with anti-IgG antibody. After accurate washings, cells were further incubated for 1 h at room temperature with anti-mouse IgG conjugated to FITC (diluted 1:100; Chemicon, Temecula, CA). After washing with PBS, cells were mounted in aqueous mounting medium and covered with coverslips. Specimens were evaluated with an epifluorescence Nikon Eclipse E600 microscope, and the images were captured using a Spot charge coupled device camera system.

Statistical analysis

Statistical analysis of data was completed using SYSTAT 7.0 (SPSS, Chicago, IL). One-way ANOVA was used to compare mean responses among the treatments. The treatment means were compared using the Bonferroni least significant difference procedure. Statistical probability of $P < 0.05$ was considered significant.
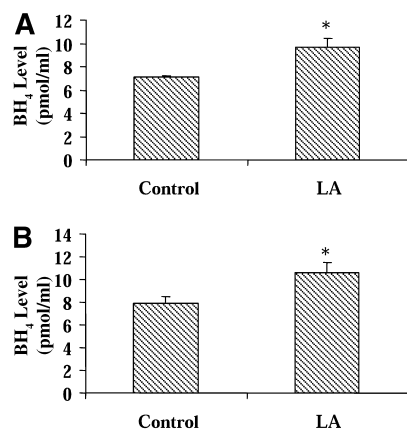
RESULTS

LA selectively increases E-selectin gene expression

LA (the parent omega-6 fatty acid) increased expression of E-selectin mRNA in a concentration-dependent manner, with an apparent maximum effect at 90 μM (**Fig. 1**). This phenomenon was not observed with linolenic acid (the parent omega-3 fatty acid). In addition, we observed that decreasing the omega-6/omega-3 fatty acid ratio reduced the activation of NF-κB in endothelial cells (unpublished data).

LA increases intracellular calcium levels in endothelial cells

The intracellular calcium level was first measured by the fluorescent plate reader method, which allows these assays to be performed in intact cells. Treatment of endothelial cells with LA for 3 h caused a highly significant increase in intracellular calcium, suggesting that this fatty acid acts through calcium signaling in endothelial cells (**Fig. 2**). The calcium ionophore A-23187, used as a positive control, also induced a significant increase in FLUO-3 AM fluorescence at a concentration of 10 μM.

We also validated the use of FLUO-3 AM to measure cellular calcium by flow cytometry and observed a significant increase in FLUO-3 AM fluorescence after treating the cells with LA (data not shown).

LA-induced endothelial cell activation is mediated through calcium signaling

The proinflammatory effect of LA in endothelial cells was studied by examining the mRNA levels of E-selectin using RT-PCR. To determine whether the proinflammatory effect of LA might be attributable to alterations in intracellular calcium levels, we pretreated the cells for 2 h with BAPTA, an intracellular calcium chelator (100 μM), or heparin, an antagonist of inositol 3-phosphate (50 μg/ml), followed by LA exposure for 6 h. E-selectin gene expression was markedly in-
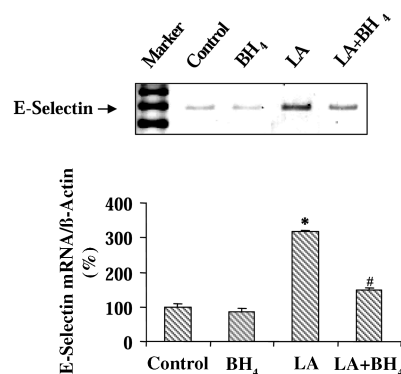

Fig. 5. Effect of LA after 6 h (90 μM; A) and 24 h (60 μM; B) of exposure on tetrahydrobiopterin (BH₄) levels in endothelial cells. Data are presented as means ± SEM from three sets of experiments. * Significantly different from control values.

Fig. 6. Effect of BH₄ on LA-mediated E-selectin gene expression in endothelial cells. Cells were either treated with 90 μM LA or cotreated with LA and BH₄ (10 μM) for 6 h. The bar graph shows the densitometric analysis of the gels. Data are presented as means ± SEM from three sets of experiments. * Significantly different from control values. # Significantly different from LA-treated cells.

creased upon LA treatment. However, pretreatment of endothelial cells with BAPTA or heparin greatly attenuated the expression of E-selectin mRNA, suggesting that LA acts via calcium signaling in endothelial cells (**Fig. 3**).

LA increases NO formation in endothelial cells

Because of the known calcium sensitivity of constitutive eNOS, and to determine if the observed increase in intracellular calcium was associated with increased NO formation, endothelial cells were treated with LA for 3 h and stained with DAF. It should be noted that the reaction between DAF and NO is irreversible (34); therefore, the accumulated level of DAF fluorescence corresponds to the total amount of cellular NO production. Similar to its effects on calcium, LA treatment led to a significant increase in DAF fluorescence. The fatty acid-induced increase in DAF fluorescence was suppressed in cells cotreated with 10 μM L-NMMA (an eNOS inhibitor), further proving that DAF fluorescence is linked to cellular NO production (**Fig. 4**).

A-23187, used as a positive control, also induced a significant increase in DAF fluorescence (data not shown). To confirm the results obtained with the plate reader method, flow cytometry analysis was also carried out. We observed a significant increase in DAF fluorescence upon LA treatment by flow cytometry as well (data not shown).

LA increases BH$_4$ level in endothelial cells

BH$_4$ is an essential cofactor for eNOS activity (35). Therefore, an increase in BH$_4$ level can be attributed to an increase in eNOS activity. In the present study, the level of BH$_4$ was significantly increased upon LA treatment for 6 h in endothelial cells (**Fig. 5A**), with no significant changes at 3 h (control, 2.69 ± 0.35 pmol/ml; LA treatment, 2.89 ± 0.15 pmol/ml). We also observed that LA treatment at 60 μM for 24 h increased BH$_4$ level in endothelial cells (Fig. 5B). These observations suggest that LA might increase eNOS activity and thus NO formation, in part, by increasing intracellular BH$_4$.
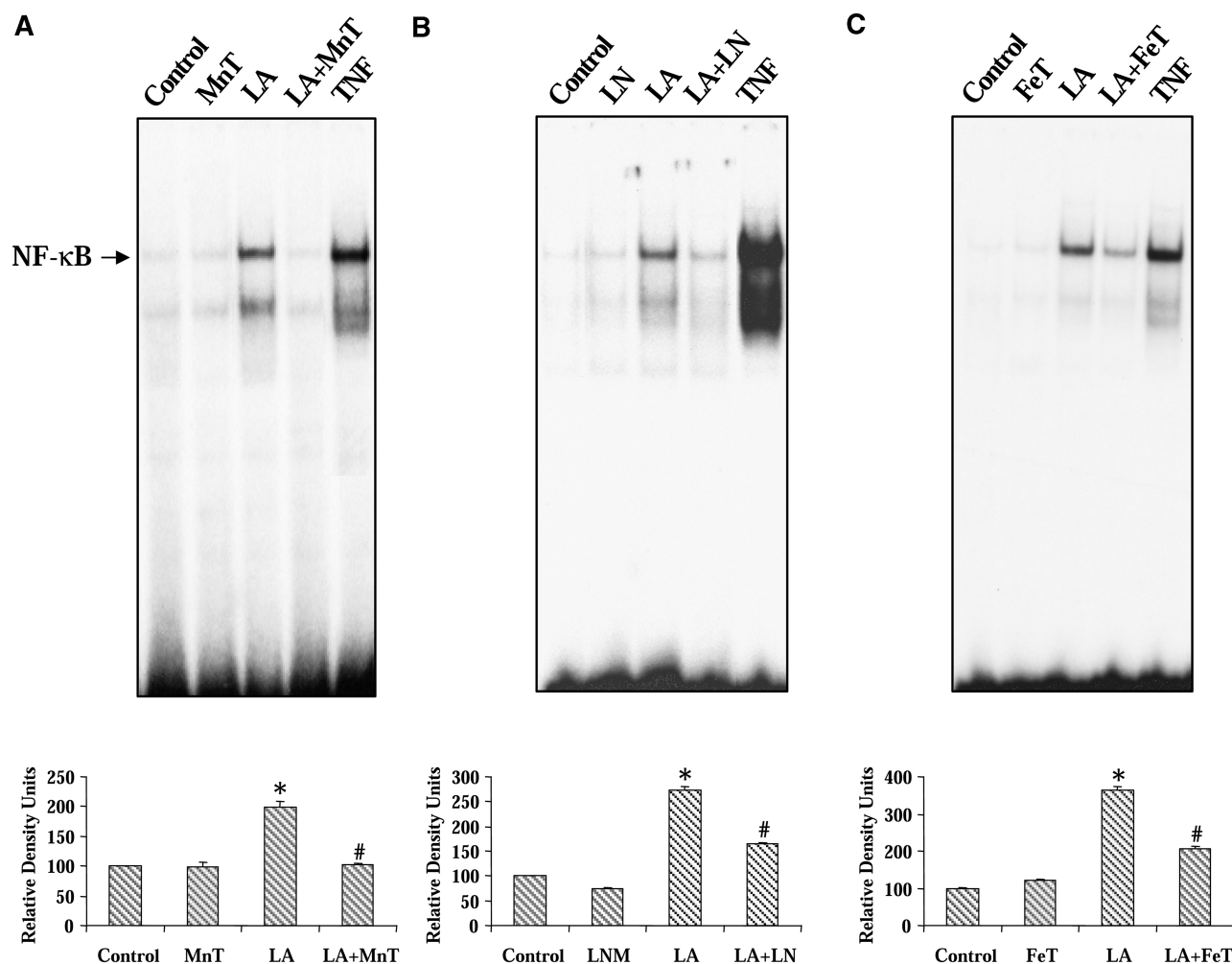

Fig. 7. Effect of 5 μM Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride [MnTMPyP (MnT); A], 10 μM L-NMMA (LN; B), and 5 μM 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) chloride [FeTPPS (FeT); C] on LA-mediated nuclear factor kappa B (NF-κB) nuclear translocation in endothelial cells. Confluent monolayers were treated with 90 μM LA in the presence or absence of the respective scavengers/inhibitors for 6 h. Tumor necrosis factor-α (TNF) was used as a positive control for NF-κB activation. Experiments were repeated three times, and the gels shown are representative of one of the experiments. The bar graphs show the densitometric analyses of the gels. Bars represent means ± SEM from three sets of experiments. * Significantly different from control values. # Significantly different from the LA group.
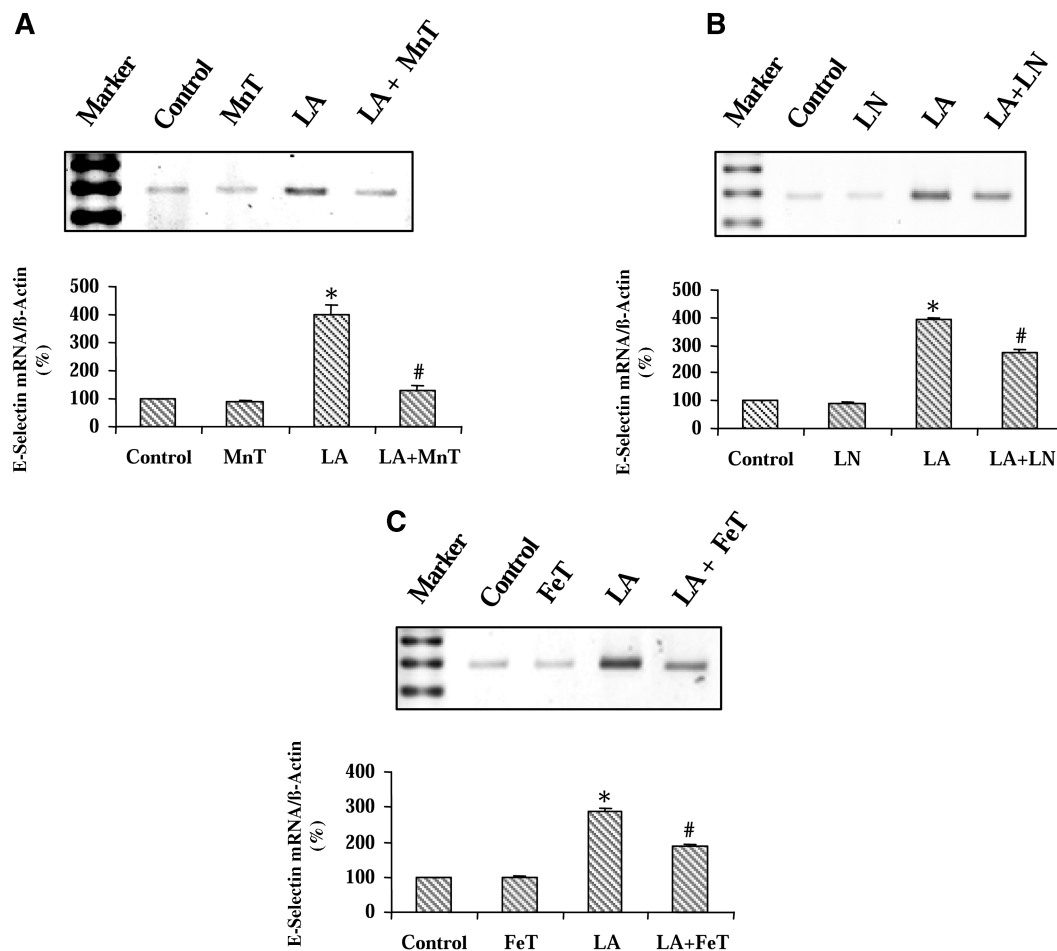
A

B

C

Fig. 8. Effect of 5 µM MnTMPyP (MnT; a superoxide dismutase mimetic) (A), 10 µM L-NMMA (LN; an endothelial NO synthase inhibitor) (B), and 5 µM FeTPPS (FeT; a peroxynitrite scavenger) (C) on LA-induced E-selectin gene expression in vascular endothelial cells as measured by RT-PCR. Experiments were repeated three times, and the gels shown are representative of one of the experiments. The bar graphs show the densitometric analyses of the gels. Data are presented as means ± SEM from three sets of experiments. * Significantly different from control values. # Significantly different from the LA group.

Supplementation with BH$_4$ decreases LA-induced E-selectin gene expression

To examine if superoxide radicals can be produced by eNOS uncoupling, BH$_4$ supplementation studies were carried out. Endothelial cells were supplemented with BH$_4$ in the presence of LA. Compared with LA treatment alone, cotreatment with BH$_4$ downregulated E-selectin mRNA expression (**Fig. 6**).

Role of peroxynitrite in LA-mediated NF-κB activation

We reported earlier that LA causes oxidative stress by generating superoxide anions in endothelial cells (15). In the present study, we provide evidence that LA treatment leads to increased NO formation. Because oxidative stress and NO formation are increased upon LA treatment, we hypothesized that LA-induced endothelial cell activation can be mediated through peroxynitrite formation. To confirm this notion, we studied the effect of cotreatment with LA and/or respective inhibitors/scavengers on NF-κB activation. For example, to examine the role of superox-

ide anions, a superoxide dismutase mimetic, MnTMPyP, was used. L-NMMA, an inhibitor of eNOS, was used to study the involvement of NO, and FeTPPS, a potent peroxynitrite scavenger, was used to examine the contribution of peroxynitrites in LA-mediated NF-κB activation. NF-κB is a transcription factor that is regulated by the redox state of the cell and implicated in the inducible expression of a variety of genes, including those encoding adhesion molecules such as E-selectin, thereby leading to endothelial cell activation. A significant increase in NF-κB activation was observed upon treatment with LA. Cotreatment with any of the inhibitors/scavengers (**Fig. 7A–C**) greatly attenuated NF-κB activation in endothelial cells. It should be noted that not only FeTPPS, a peroxynitrite scavenger, but also MnTMPyP and L-NMMA exerted similar effects on NF-κB activation, suggesting that peroxynitrite formation may be facilitated only in the presence of both species, superoxide anions and NO. Quenching the superoxide radicals with MnTMPyP or inhibiting eNOS activity by L-NMMA may inhibit the formation of peroxynitrites in endothelial cells. The present results confirm this hypothesis, because

we observed a great reduction in NF-κB activation upon cotreatment of LA with MnTMPyP or L-NMMA.

Role of peroxynitrite in LA-induced E-selectin gene expression

To further confirm the role of superoxide, NO, and peroxynitrite in endothelial activation, the effects of Mn-TMPyP, L-NMMA, and FeTPPS (**Fig. 8A–C**) on LA-mediated E-selectin gene expression were studied. Our data show an inhibitory effect of these chemicals on LA-induced E-selectin mRNA expression in endothelial cells, suggesting that the peroxynitrite pathway is involved in LA-induced endothelial activation.

Nitrotyrosine formation in response to LA

To confirm that peroxynitrites are being produced in endothelial cells, we next tested the effect of LA on the formation of nitrotyrosine, a marker of superoxide anion reaction with NO, resulting in peroxynitrite formation (36). LA treatment resulted in a significant increase in the formation of nitrotyrosine-modified protein in endothelial cells, whereas oleic acid, a monounsaturated fatty acid, did not cause any difference in the degree of nitrosylation (**Fig. 9A**).

Immunofluorescence analysis was also carried out to confirm the formation of nitrotyrosine in endothelial cells (Fig. 9B). Although there was negligible staining in control cultures, immunofluorescence experiments using a specific anti-nitrotyrosine antibody revealed positive staining in LA-treated cells.

DISCUSSION

It is now well known that biologically active lipids such as fatty acids play an important role in the development of atherosclerosis. Polyunsaturated fatty acids as well as their oxidized metabolites can have potent biological effects in various cell types. However, the specific mechanisms by which they act as proinflammatory agents are still unclear. In particular, there is increased interest in determining the cellular signaling pathway that could lead to the inflammatory response by these fatty acids.

We previously demonstrated that LA can significantly increase oxidative stress by generating superoxide anions in endothelial cells (15). In the present study, we provide evidence that LA is a potent stimulus for increasing intracellular calcium in endothelial cells. Alterations in intracellular calcium are generally associated with cellular distress. The importance of calcium signaling in modulating endothelial function is supported by the observation that induction of NF-κB by various stimuli requires calcium for proper signal transduction (37, 38). Furthermore, calcium can also function as a second messenger necessary for the induction of adhesion molecules in endothelial cells (20, 39).

In the present study, inhibition of calcium signaling using BAPTA as an intracellular calcium chelator resulted in a decrease in the expression of the soluble adhesion molecule E-selectin. The LA-induced proinflammatory response
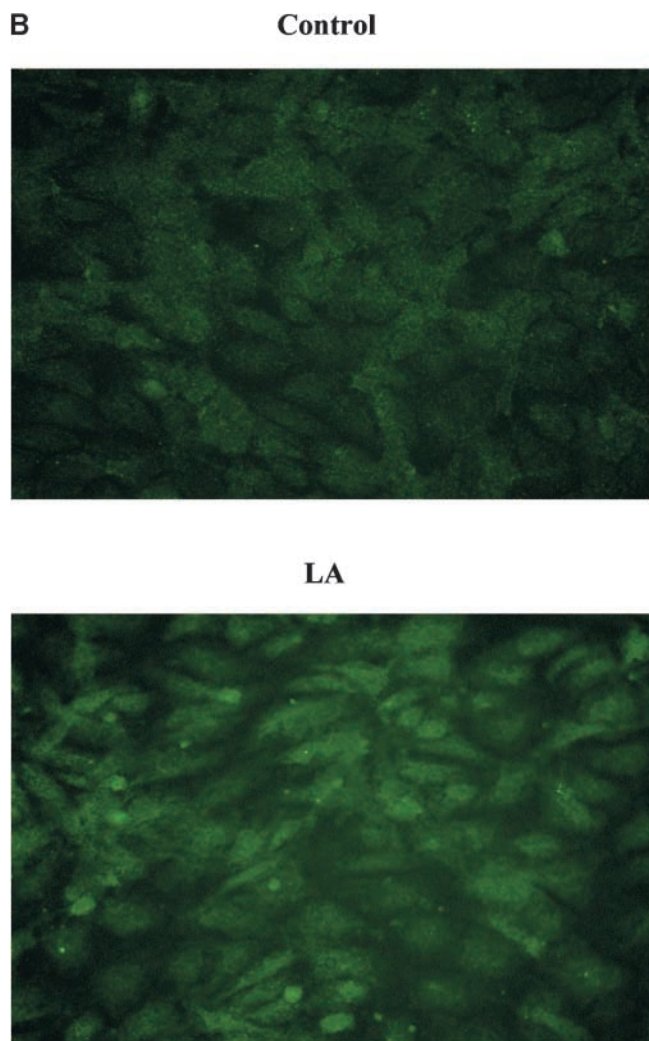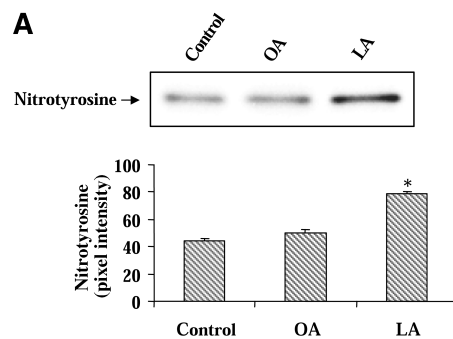

Fig. 9. A: Effect of 90 μM oleic acid (OA) and LA on nitrotyrosine formation as examined by immunoprecipitation followed by Western blotting in porcine pulmonary artery endothelial cells. Experiments were repeated three times, and the images presented are representative of one of the experiments. The bar graph shows the densitometric analysis of the gels. Data are presented as means ± SEM from three sets of experiments. * Significantly different from control values. B: Effect of LA on nitrotyrosine formation in vascular endothelial cells as analyzed by the immunofluorescence method.

in endothelial cells was also prevented by heparin, an inositol 1,4,5-triphosphate (IP3) receptor antagonist (40, 41). IP3 receptor is a tetrameric ion channel that releases calcium from intracellular stores in response to a variety

of environmental stimuli (42). The inhibitory effect of heparin on the proinflammatory response caused by LA suggests that IP3-sensitive stores in endothelial cells are critical in increasing the intracellular calcium level and subsequent endothelial cell activation upon treatment with LA.

In the present work, we showed that calcium is necessary to mediate the proinflammatory effect of LA. However, the exact process involved in transducing the calcium signal leading to an inflammatory response in vascular endothelial cells is not yet clear. Calmodulin could possibly transduce the calcium signal by activating eNOS, a calmodulin-dependent enzyme involved in the production of NO in endothelial cells (43, 44). Although the possibility of NO production in the endothelium via modification of the calcium sensitivity of NO synthase by Akt kinase has been reported (45, 46), the increase of cellular calcium is still the most common initiator of NO production. Indeed, in the present study, the increased calcium level was associated with increased NO production in endothelial cells.

NO, an important mediator of biological processes such as neurotransmission, inflammatory response, and vascular homeostasis (47), is synthesized from L-arginine by a family of NO synthases. eNOS is localized in endothelial cell caveolae, small invaginations of the plasma membrane that are abundant in the transmembrane protein caveolin (48, 49). Caveolin-bound eNOS remains inactive unless calcium-calmodulin displaces caveolin from an overlapping binding site, thereby activating the enzyme (50). Hence, the activity of this enzyme is critically dependent on increased intracellular calcium concentrations (22, 23); therefore, factors that induce calcium release can modulate NO production in endothelial cells.

NO has both beneficial and nonbeneficial effects depending on site and concentration (51). Despite the ability to maintain vascular homeostasis, excess NO production under certain conditions may enhance the cellular stress, probably via peroxynitrite formation. Peroxynitrite is a highly reactive intermediate known to cause nitration

of protein tyrosine residues and to cause cellular oxidative damage (52). Thus, calcium increase and subsequent overproduction of NO can be a novel mechanism of LA-mediated endothelial activation. Our data support this hypothesis, and we showed that LA increased NO formation in endothelial cells as observed by DAF fluorescence.

Furthermore, we observed that the level of BH_4, an essential cofactor for the activity of all NO synthases (35), increases in endothelial cells upon treatment with LA. The exact role of BH_4 in NO synthase catalysis remains incompletely defined, but it appears to facilitate electron transfer from the eNOS reductase domain and maintains the heme prosthetic group in its redox active form (53). Intracellular BH_4 levels are regulated by the activity of the de novo biosynthetic pathway. In the present study, treatment with LA significantly increased BH_4 level in endothelial cells. Even though the significant increase of BH_4 was only observed at 6 h of exposure to LA, the true biological significance may occur much earlier. We measured total BH_4 in endothelial cells, whereas eNOS is located primarily in membrane-associated caveolae. There may be compartmentation of BH_4 in endothelial cells, as was observed with arginine (54), such that a distinct pool of BH_4 at the site of NO synthesis increased to a greater extent than indicated by the increase in total intracellular BH_4 content. An increase in intracellular BH_4 is associated with increased eNOS activity (35). Hence, the present finding suggests that LA might increase eNOS activity in part by increasing intracellular BH_4, with subsequent increase of NO. The beneficial effect of BH_4 in vasodilation has been reported. For example, some of the cholesterol-lowering drugs, such as statins, might increase eNOS activity by increasing intracellular BH_4 (55). LA metabolites also have been reported to increase the expression of eNOS as a possible means for endothelial cells to compensate for oxidative injury in the early stages of hypercholesterolemia (56). Our observed increase in BH_4 levels after both 6 and 24 h of treatment suggests that the LA-induced increase in BH_4 might result in the production of NO at concentrations that would facilitate reaction with superox-
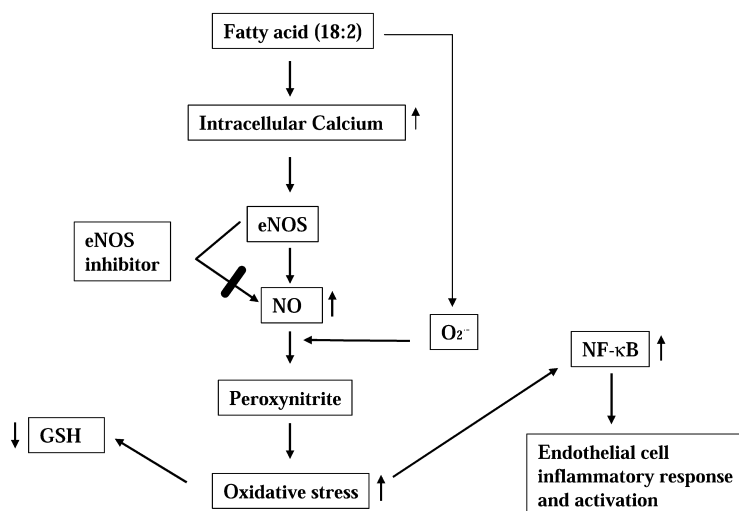

Fig. 10. Proposed model for the mechanism of LA-mediated endothelial cell activation. LA treatment results in the formation of superoxide radicals as well as the initiation of calcium signaling in endothelial cells. Endothelial NO synthase (eNOS) is activated by calcium signaling, leading to increased production of NO. Under oxidative stress, the reaction between superoxide radicals and NO is facilitated, resulting in peroxynitrite formation. Peroxynitrite, being a potent oxidative and nitrating agent, can activate NF-κB and induce the expression of adhesion molecules in endothelial cells.

ide anions to result in peroxynitrite formation. It is well documented that the balance between NO and superoxide plays a critical role in the production of peroxynitrites (57–59). This would explain to some extent why, in spite of increasing NO, a vasodilator, LA treatment led to endothelial cell activation in the present study. It is also possible that uncoupling of eNOS occurs in the endothelial cells upon LA treatment, leading to increased production of superoxides. Our experiments in which the cells were treated with LA in the presence of BH_4 (BH_4 supplementation study) suggest that supplemental BH_4 reduces uncoupling and thus overall oxidative stress. We found that supplementation with BH_4 downregulated the expression of E-selectin as mediated by LA.

Furthermore, to explore the role of peroxynitrites in endothelial activation, the effects of specific inhibitors or scavengers of superoxide anions, NO, and peroxynitrite on NF-κB activation were studied. Treatment with the superoxide radical scavenger MnTMPyP almost completely abolished NF-κB activation and suppressed E-selectin gene expression, suggesting that reactive oxygen species (ROS), and especially superoxide radicals, play a major role in LA-mediated endothelial cell activation. In addition to peroxynitrite, superoxides can also form other oxidizing species, such as H_2O_2. The involvement of ROS in fatty acid-mediated endothelial cell dysfunction is further supported by our previous observation that pretreatment with vitamin E blocked LA-mediated NF-κB activation in endothelial cells (12).

We also showed that cotreatment with L-NMMA, an eNOS inhibitor, decreased the inflammatory response in endothelial cells. These data suggest that the reaction between superoxide and NO to form peroxynitrite is important in the observed LA-induced proinflammatory effect. The fact that L-NMMA blocked NF-κB activation and the expression of E-selectin in endothelial cells suggests that not only superoxide generation, but also peroxynitrite formation via the reaction between superoxide and NO, plays a major role in mediating the proinflammatory effect of LA. This is consistent with previous studies that have demonstrated that superoxide anions react rapidly with NO to form peroxynitrite (60). Furthermore, in vivo evidence is emerging that superoxides can mediate their cytotoxicity via peroxynitrite formation (61).

To further characterize the role of peroxynitrite in the activation of NF-κB and the expression of E-selectin by LA, FeTPPS, a selective peroxynitrite scavenger, was added to the culture medium, and its effects on the LA-mediated inflammatory response were studied. FeTPPS has been used as an effective peroxynitrite scavenger in many systems (62, 63). It catalyzes the isomerization of peroxynitrite to nitrate anion and thereby decreases its decomposition to highly reactive intermediates such as nitrogen dioxide and hydroxyl radicals (63). In the present study, we successfully demonstrated that FeTPPS could block LA-mediated NF-κB activation and expression of E-selectin.

In our studies, evidence for peroxynitrite generation by LA was confirmed by immunoprecipitation followed by Western blotting using anti-nitrotyrosine antibody. Oleic acid, a monounsaturated fatty acid, was also used to compare the effect of polyunsaturated fatty acids in causing peroxynitrite formation in endothelial cells. Unlike LA, oleic acid did not induce peroxynitrite formation in endothelial cells. In contrast to other long-chain fatty acids, oleic acid is rather ineffective at inducing cellular oxidative stress (64). However, oleic acid has been reported to increase the production of NO in endothelial cells (65). Because both superoxides and NO are needed for peroxynitrite formation, it seems reasonable that the level of nitrotyrosine is unaltered upon treatment with oleic acid in endothelial cells. The immunofluorescence experiment carried out in the present study also proves that peroxynitrite radicals are produced in vascular endothelial cells upon treatment with LA.

In summary, our results provide a framework for our understanding of how cross-talk between calcium and NO signaling leads to LA-mediated proinflammatory response in vascular endothelial cells (**Fig. 10**). Our data support the role of both superoxide and peroxynitrite, and uncoupled eNOS as a potential source of these ROS, whereas calcium may exacerbate oxidative stress via the stimulation of superoxide production and peroxynitrite from eNOS.

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