Oxidized low-density lipoprotein increases superoxide production by endothelial nitric oxide synthase by inhibiting PKCα

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Abstract

Objective: Oxidized low-density lipoprotein (ox-LDL) increases superoxide anion (O₂⁻) production by the endothelial nitric oxide (NO) synthase (eNOS). We assessed whether the uncoupling of eNOS was associated with alterations in eNOS phosphorylation and/or the assembly of the eNOS signaling complex.

Methods and results: In unstimulated human endothelial cells, eNOS Thr495 was constitutively phosphorylated. ox-LDL, but not native LDL, enhanced the production of O₂⁻ by endothelial cells, an effect that was partially sensitive to NOS inhibition. ox-LDL, but not native LDL, induced a time- and concentration-dependent decrease in the phosphorylation of eNOS on Thr495. Protein kinase C (PKC) has been reported to phosphorylate this residue, and the increase in the phosphorylation of Thr495 induced by phorbol 12-myristate 13-acetate was attenuated in cells pretreated with ox-LDL. Moreover, the phosphorylation and activity of PKCα was attenuated by ox-LDL and paralleled the changes in eNOS phosphorylation. ox-LDL also induced the dissociation of eNOS from the plasma and Golgi membranes. In COS-7 cells, a T495A eNOS mutant generated significantly more O₂⁻ than a T495D mutant did, indicating that the dephosphorylation of Thr495 alone can increase O₂⁻ production by eNOS. However, although the dephosphorylation of Thr495 in histamine-stimulated endothelial cells enhanced the binding of calmodulin to eNOS, calmodulin no longer bound to eNOS from ox-LDL-treated endothelial cells.

Conclusions: These results indicate that a decrease in the activity of PKCα in ox-LDL-treated endothelial cells is associated with the dephosphorylation of eNOS, dissociation of the eNOS signaling complex, and the enhanced production of eNOS-derived O₂⁻.

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1. Introduction

Oxidative stress is generally accepted to play a central role in the genesis of endothelial dysfunction and coronary artery disease. Over the last 5 years, evidence has accumulated to suggest that a significant proportion of the oxygen-derived free radicals that contribute to this phenomenon in vivo are derived from the endothelial nitric oxide (NO) synthase (eNOS). All of the NOS isoforms are reported to generate superoxide anions (O₂⁻) and hydrogen peroxide (H₂O₂) under specific conditions, i.e., lower than optimal concentrations of the essential cofactor tetrahydobipterin (H₄B) or the substrate L-arginine (for review, see Ref. [1]). The lack of H₄B results in the uncoupling of NOS, which basically means that the transport of electrons to the ferrous-heme-O₂ species generated during the stepwise activation of O₂ by NOS does not occur fast enough to prevent their decay, the result being the...
generation of reactive oxygen species. Although eNOS uncoupling has been successfully reversed by enhancing the cellular levels of H4B, either using sepiapterin [2] or by preventing the oxidation of H4B [3], circumstantial evidence indicates that the association of Hsp90 with eNOS [4], as well as eNOS phosphorylation [5], can also affect the degree of coupling.

Oxidized low-density lipoprotein (ox-LDL) is reported to increase \( \text{O}_2^- \) production in endothelial cells and to decrease the bioavailability of NO via a process involving the lectin-like ox-LDL receptor-1 [6,7]. Since we have previously shown that the dephosphorylation of eNOS on Thr495 is associated with the enhanced production of \( \text{O}_2^- \) [5], we set out to determine whether the ox-LDL-induced uncoupling of eNOS can be linked to changes in its phosphorylation.

2. Materials and methods

2.1. Materials

The spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrroloidine-HCl (CMH) was from Alexis Biochemicals (Grünberg, Germany). \( N^e \text{nito-L-arginine (L-NA)}, N^e \text{nito-L-arginine methyl ester (L-NAME)}, \) lysophosphatidylcholine (LPC), lucigenin, diethylenetriamine-pentaacetic acid (DTPA), and all other substances were obtained from Sigma (Deisenhofen, Germany).

2.2. Cell culture

The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki. Human umbilical vein endothelial cells and porcine aortic endothelial cells were isolated by ultracentrifugation, it was not possible to assess the effects of ox-LDL by ESR.

2.4. Measurement of superoxide anion (\( \text{O}_2^- \)) production

2.4.1. Electron spin resonance (ESR) spectroscopy

\( \text{O}_2^- \) generation in intact cells was assessed using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrroloidine (CMH) as previously described [11], with minor modifications. Briefly, COS-7 cells (\( \approx 2 \times 10^5 \) cells) expressing recombinant eNOS protein were washed with HEPES-Tyrode (ESR-HT) solution, which was pre-treated with Chelax-100 (5 g/L, overnight) and contained diethylenetriamine-pentaacetic acid (DTPA; 100 \( \mu \text{mol/L} \)). Cells were incubated with ESR-HT in the absence and presence of L-NNAME (300 \( \mu \text{mol/L} \)) for 45 min prior to the addition of CMH dissolved in argon-purged saline (2 \( \text{mmol/L} \)). After an additional 5 min, 400 \( \mu \text{L} \) of the solution was withdrawn into an insulin syringe and immediately frozen in liquid nitrogen. The formation of the stable spin label 3-methoxycarbonyl-proxyl (CMF) was determined at 77 K in a liquid nitrogen cooled dewar using a EPR EMX spectrometer (Bruker). The instrument settings were as follows: microwave frequency, 9.463 GHz; power, 20.02 mW; receiver gain, 1 \times 10^5; modulation amplitude, 2.010 G; modulation frequency, 100 KHz; conversion time, 81.92 ms; time constant, 327.680 ms; sweep time, 41.943 s; sweep width, 400 G; resolution, 512 points and 3 scans.

Trace amounts of copper ions and lipid peroxides present in the dialyzed ox-LDL interfered with the ESR signal. As this problem could not be overcome with further dialysis harvested by scraping and ion chelators, it was not possible to determine the L-NA-sensitive accumulation of cyclic GMP under resting conditions and following stimulation using a specific radioimmunoassay, as described [9].

COS-7 cells (American Type Culture Collection) were cultured in Dulbecco's modified eagle medium: nutrient mixture-F12 (1:1, DMEM-F12; GibCO BRL-Life Technology) with 10% fetal calf serum (FCS) and supplemented with penicillin (50 U/mL) and streptomycin (50 \( \mu \text{g/mL} \)).

2.3. Isolation and oxidation of LDL

Pooled human LDL was obtained from healthy subjects from the local blood bank, isolated by ultracentrifugation, oxidized by CuSO\(_4\), and analyzed as described [10]. The mobility of ox-LDL on agarose gel electrophoresis as an index for lipoprotein oxidation was 2.5- to 3.0-fold increased compared with native LDL. In total, six different preparations were used in the present study.

Cells were lysed in buffer containing Tris/HCl (pH 7.5; 50 \( \text{mmol/L} \)), NaCl (150 \( \text{mmol/L} \)), NaF (100 \( \text{mmol/L} \)), Na\(_2\)P\(_2\)O\(_7\) (15 \( \text{mmol/L} \)), Na\(_3\)VO\(_4\) (2 \( \text{mmol/L} \)), leupeptin (2 \( \mu \text{g/mL} \)), pepstatin A (2 \( \mu \text{g/mL} \)), trypsin inhibitor (10 \( \mu \text{g/mL} \)), phenylmethylsulfonyl fluoride (PMSF; 44 \( \mu \text{g/mL} \)) and Triton X-100 (1% v/v), left on ice for 10 min and centrifuged at 10,000 \( \times \)g for 10 min. Proteins in the cell supernatant or immunoprecipitates were heated with SDS-PAGE sample buffer and separated by SDS-PAGE, as
Proteins were detected using their respective antibodies and visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Germany).

The eNOS polyclonal antibody used for immunoprecipitation was generated from the keyhole limpet hemocyanin conjugated peptide sequence VPWTFDPGPDTPGP (positions 1191 to 1205 in eNOS protein) by Eurogentec (Seraing, Belgium), and the phospho-specific antibodies recognizing Thr^{495} and Ser^{1177} eNOS were purchased from Upstate (Milton Keynes, England). The monoclonal eNOS antibody used for immunoblotting was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Antibodies against Akt, as well as the phospho-specific antibodies recognizing p-Akt (Ser^{473}), phospho-protein kinase C (PKC) and the phosphorylated PKCα/β, were from Cell Signaling Technology (Frankfurt am Main, Germany).

2.6. PKC activity assay

Endothelial cells were stimulated as described in the Results section, washed once with Tyrode’s solution and frozen in liquid nitrogen. Cells were lysed by three cycles of freeze–thawing, and the phosphorylation of acetylated myelin basic protein (Ac-MBP 4-14; Sigma) was assessed in the absence and presence of the PKC inhibitor RO 31-8220 (300 nmol/l) as described previously [13].

2.7. Measurement of H_{2}B levels

Cell lysates for the assay of H_{2}B content were oxidized with MnO_{2} as described [14], and H_{2}B was determined using a high-performance liquid chromatography-based fluorimetric method [15], with a detection limit of 10–15 fmol (excitation 350 nm and emission 450 nm).

2.8. Generation of eNOS adenoviruses

eNOS mutants, based on the human eNOS wild-type sequence, were generated using a PCR-based mutagenesis kit (Stratagene), and the mutation of Ser^{1177} or Thr^{495} to alanine or aspartate was verified by sequencing, as described [12].

The full-length, myc/his-tagged eNOS wild-type and mutant cDNA inserts were excised from pcDNA3.1 myc/his with Pmel/HindIII and purified by gel purification using the gene extraction kit from Genomed (Loehne, Germany). The overhangs of the inserts were blunted by the large fragment of DNA polymerase I (Klenow fragment) and ligated into linearized and dephosphorylated RV adenoviral shuttle vectors, pAdTrack-CMV and pAdShuttle-CMV (kindly provided by Bert Vogelstein, Howard Hughes Medical Institute, Baltimore, MD; [16]). The plasmids were recombined into the adenovirus 5 genome by homologous recombination in E. coli. Briefly, the shuttle vectors carrying the eNOS cDNAs were digested with Pmel, and the linear DNA was purified by phenol/chloroform extraction and ethanol precipitation. These vectors were used to transform the E. coli strain B35183 carrying the plasmid pAdEasy-1. The resulting recombinant plasmids conferred resistance to kanamycin (70 μg/ml), and plasmids from antibiotic-resistant colonies were isolated and analyzed for eNOS by restriction digestion with KpnI/HindIII. Positive plasmids were transformed into E. coli JM109 for large-scale isolation. Following linearization with PacI and extraction with phenol/chloroform and ethanol precipitation, plasmids were transfected into HEK 293 cells (American Type Culture Collection). Transfected cells were incubated for up to 2 weeks and monitored for GFP expression (for inserts in pAdTrack-CMV) and plaque appearance. Viruses were obtained from these cells and infected into fresh HEK 293 cells for amplification and to confirm expression of mRNA and protein. Constructs that expressed eNOS mRNA (detected by Northern blotting) were screened for eNOS protein. Only one orientation (sense) resulted in increased protein expression. For large-scale amplification and purification, five T175 flasks of 90% confluent HEK cells were infected with the viruses. The virus particles were purified using the BD Adeno-X Virus purification kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions. The resulting titre of each preparation was approximately 10^{8} pfU/ml.

2.9. Adenoviral infection of COS-7 cells

COS-7 cells were incubated with recombinant adenoviruses expressing human wild-type eNOS or the TA, TD or TAD eNOS mutants (2 pfU/cell, in 1 mL medium containing 0.1% BSA) for 4 h. Thereafter, 2 mL of growth medium was added, and the cells were cultured for a further 48 h.

2.10. Immunofluorescence

Endothelial cells were grown on glass slides and fixed with formaldehyde (2% in PBS), permeabilized with Triton X-100 (0.05%) and incubated in PBS containing glycine (2%) for 10 min. Cells were coincubated with a specific eNOS monoclonal (Transduction Labs) and β-catenin (Santa Cruz Biotech) antibodies. Following incubation with Alexa dye-coupled secondary antibodies, the preparations were mounted and viewed using a confocal microscope.

2.11. Statistical analysis

Data are expressed as mean±S.E.M., and statistical evaluation was performed using Student’s t test for unpaired data, one-way analysis of variance (ANOVA), followed by a Bonferroni t test, or ANOVA for repeated measures, where appropriate. Values of P<0.05 were considered statistically significant.
3. Results

3.1. Effect of ox-LDL on eNOS uncoupling

The bioavailability of eNOS-derived NO was assessed by monitoring the basal and bradykinin-induced increase in cyclic GMP. Under basal conditions, ox-LDL attenuated cyclic GMP production by approximately 25%, while the bradykinin-induced increase in cyclic GMP levels was reduced by 45% (Fig. 1A). In cells pretreated with superoxide dismutase (SOD), basal cyclic GMP levels were significantly (2.8±0.1-fold, \( P<0.01, n=8 \)) elevated such that there was no longer a difference between the control and ox-LDL-treated groups. SOD, however, failed to completely normalize the response to bradykinin in cells exposed to ox-LDL (Fig. 1A).

A low level of \( \text{O}_2^- \) could be detected using lucigenin-enhanced chemiluminescence in human endothelial cells treated with either solvent or native LDL. Superoxide anion levels were, however, markedly increased in cells treated with ox-LDL for 24 h (Fig. 1B). \( \text{l-NAME} \) attenuated \( \text{O}_2^- \) production in ox-LDL-treated cells but did not significantly affect radical production in either solvent- or native LDL-treated cells. Similar results were obtained in cells stimulated with ionomycin. Superoxide dismutase (200 U/mL) attenuated the chemiluminescence signal in all samples (data not shown).

![Fig. 1. Effect of ox-LDL on the generation of NO and \( \text{O}_2^- \) by human endothelial cells. Human endothelial cells were pretreated with either solvent (culture medium), nLDL or ox-LDL (30 \( \mu \)g/mL) for 24 h. Thereafter, the production of (A) cyclic GMP and (B) \( \text{O}_2^- \) (lucigenin-enhanced chemiluminescence) was assessed. Experiments were performed in the absence and presence of bradykinin (10 nmol/L, 5 min), superoxide dismutase (SOD, 150 U/mL) and \( \text{N}^\circ \text{nitro-L-arginine methyl ester (l-NAME, 300 \( \mu \)mol/L). The bar graphs summarize the data obtained in four to eight independent experiments; *\( P<0.05, **P<0.01 \) vs. CTL.](https://academic.oup.com/cardiovascres/article-abstract/65/4/897/445106)

3.2. Time course of the LDL-induced changes in eNOS phosphorylation

In cultured human endothelial cells, eNOS was phosphorylated on Thr\(^{495} \) but only weakly on Ser\(^{1177} \) (Fig. 2A).
The incubation of endothelial cells with native LDL did not affect the phosphorylation of eNOS, while ox-LDL resulted in the time-dependent dephosphorylation of eNOS on Thr495. The latter effect was also concentration-dependent, with almost complete dephosphorylation occurring after the 24-h treatment with 30 μg/ml ox-LDL (Fig. 2A and B). ox-LDL did not alter eNOS protein expression and did not affect the phosphorylation of Ser1177. Changes in the phosphorylation of eNOS on Ser114 or Ser633 were not observed at any of the concentrations used (data not shown). Identical results were obtained using porcine aortic endothelial cells.

We next compared the effects of histamine and long-term (24 h) stimulation of ox-LDL on the ability of CaM to bind to eNOS. Histamine induced the transient dephosphorylation of eNOS Thr495, which was temporally correlated with the association of CaM. However, although ox-LDL elicited the dephosphorylation of eNOS on Thr495, CaM did not associate with the enzyme (Fig. 2C).

In contrast to the cells treated with native LDL, the stimulation of ox-LDL-treated cells with bradykinin (100 nmol/L) did not further affect Thr495 phosphorylation (Fig. 3A) but did increase the phosphorylation of eNOS on Ser1177. ox-LDL treatment also attenuated the PMA-induced phosphorylation of eNOS Thr495 while slightly increasing that of Ser1177 (Fig. 3B). The ox-LDL-induced changes in eNOS phosphorylation were not associated with the activation of PKA or the AMP-activated protein kinase (data not shown).

The protein phosphatase 1 (PP1) inhibitor calyculin A increased the phosphorylation of eNOS Thr495 in ox-LDL-treated cells (Fig. 3C). However, the dephosphorylation of eNOS was not associated with an increase in the expression of the phosphatases PP1 or PP2A (Fig. 3D).

### 3.3. Effect of Thr495 on O2/ production by eNOS

To determine whether the dephosphorylation of eNOS Thr495 was sufficient to uncouple eNOS, we assessed the L-NAME-sensitive production of O2/ using ESR spectroscopy in COS-7 cells expressing either wild-type eNOS or the T495A, T495D or T495A/S1177D eNOS mutants (Fig. 4A). Ox-LDL treatment did not alter the production of O2/ in these cells (Fig. 4B). However, the dephosphorylation of eNOS Thr495 by calyculin A (10 nmol/L) increased the production of O2/ in a concentration-dependent manner (Fig. 4C). The Western blots shown are representative of data obtained in three to five independent experiments, and the bar graph summarizes data obtained in three independent experiments.
more $O_2^-$ was generated in cells expressing the non-phosphorylatable T495A mutant than in cells expressing the phospho-mimetic T495D mutant (Fig. 4A). The additional mutation of Ser$^{1177}$ to aspartate to increase electron flow through the reductase domain [17] slightly, but not significantly, enhanced $O_2$ production. The l-NAMEm-sensitive production of radicals by the T495A mutant and the wild-type enzyme were comparable, a phenomenon that can be accounted for by the fact that, unlike the situation in endothelial cells, eNOS is not basally phosphorylated on Thr$^{495}$ in COS cells (Fig. 4B).

3.4. Role of PKC in the ox-LDL-induced dephosphorylation of eNOS

Since PKC phosphorylates Thr$^{495}$, and the response to PMA was attenuated in ox-LDL-treated cells, we assessed the effects of ox-LDL on PKC phosphorylation and activity. The PKC-pan antibody used detects PKC$\alpha$, $\beta$I, $\beta$II, $\zeta$, $\epsilon$ and $\delta$ isoforms only when phosphorylated at a carboxy terminal residue.

The basal phosphorylation of a PKC isoform (approximately 77 kDa) was detected in the Triton X-100-insoluble fraction of unstimulated endothelial cells. nLDL failed to affect the phosphorylation of this enzyme while ox-LDL induced a time-dependent decrease in the phospho-PKC pan signal (Fig. 5A and B). A similar phenomenon was observed using an antibody that selectively recognizes the phosphorylated forms of PKC$\alpha/\beta$. Although a basal phosphorylation of PKC was detected in the Triton X-100-soluble cell fraction, ox-LDL affected neither the signal obtained with the PKC pan antibody nor the selective phospho PKC$\alpha/\beta$ antibody (Fig. 5C). PKC$\alpha$ was detected in the Triton X-100-insoluble fraction from unstimulated endothelial cells, and the signal was time dependently decreased by ox-LDL (Fig. 5A). Since the molecular mass of PKC$\alpha$ and the signal given by the phospho-PKC antibodies used were identical, PKC$\alpha$ appears to be the isoform that phosphorylates eNOS Thr$^{495}$ and is targeted by ox-LDL.

Global PKC activity was assessed by monitoring the phosphorylation of Ac-MBP. ox-LDL, but not nLDL, induced a time- and concentration-dependent decrease in PKC activity (Fig. 5D).

3.5. Effect of LPC on the phosphorylation of eNOS

Lysophosphatidylcholine (LPC)-treatment decreased PKC activity and led to the dephosphorylation of eNOS Thr$^{495}$. However, in contrast to the effects of ox-LDL, LPC also elicited the phosphorylation of Akt and eNOS Ser$^{1177}$ (Fig. 6). Moreover, 24 h after the application of the highest

![Fig. 5. Effect of nLDL and ox-LDL on the phosphorylation and activity of PKC in endothelial cells. Human endothelial cells were pretreated with either solvent (culture medium) nLDL or ox-LDL (30 μg/mL) for 8 to 24 h. The phosphorylation of PKC was determined by Western blotting with phospho-specific specific antibodies (A) and quantified densitometrically (B). (C) Comparison of the effect of ox-LDL (30 μg/mL, 18 h) on the phosphorylation of PKC in the Triton X-100 insoluble (Tx-insol) and soluble (Tx-sol) cell fractions. (D) Time- and concentration-dependent effect of ox-LDL pretreatment on the activity of PKC in endothelial cell lysates, as determined by the phosphorylation of MBP. Cells treated with PMA (1 μmol/L, 10 min) served as a positive control. The graphs summarizes data obtained in three to ten independent experiments; *P<0.05, **P<0.01, ***P<0.001 vs. time 0 or CTL.](https://academic.oup.com/cardiovascres/article-abstract/65/4/897/445106)
Fig. 6. Time- and concentration-dependent effect of lysophosphatidyl choline (LPC) on the phosphorylation of eNOS. Human endothelial cells were incubated with LPC (10 to 100 μmol/L) for 4 to 24 h. (A) The phosphorylation of eNOS on Thr495, Ser633 and Ser1177 and of Akt on Ser473 was assessed by Western blotting with phospespecific antibodies. (B) Changes in the phosphorylation of eNOS Thr495 were quantified relative to total eNOS levels, and the graph summarizes data obtained in six independent experiments; **$P<0.01$, ***$P<0.001$ vs. time 0.

Fig. 7. Effects of nLDL and ox-LDL on the intracellular localization of eNOS. Immunohistochemical staining of eNOS (red) and β-catenin (green) in human endothelial cells pretreated with either solvent (CTL), nLDL (30 μg/mL) or ox-LDL (30 μg/mL) for 24 h. The results presented are representative of data obtained in two further experiments.
concentration of LPC used (100 μmol/L), eNOS expression was attenuated (data not shown).

3.6. Effect of ox-LDL on the eNOS signalosome

As H4B is reported to influence eNOS coupling [2,3], we assessed cellular H4B levels in the different groups. H4B levels were 11.5±1.0, 7.1±1.4 and 9.8±0.3 pmol/mg protein in cells treated with solvent, nLDL and ox-LDL, respectively (30 μg/mL, n=3).

In endothelial cells treated with either solvent of native LDL, eNOS was localized to the plasma membrane, as well as to the perinuclear Golgi apparatus. ox-LDL (30 μg/mL, 24 h) resulted in the redistribution of the enzyme within the cytosol that the Golgi signal disintegrated and eNOS was no longer detectable at the cell membrane (Fig. 7).

4. Discussion

The results of the present investigation indicate that the uncoupling of eNOS (i.e., the increase in O2 production by the enzyme) in ox-LDL-treated cells can be attributed to the inactivation and down-regulation of PKC, which results in the dephosphorylation of the enzyme on Thr495. That alterations in the phosphorylation of this residue can affect O2 production by eNOS was evidenced by the fact that a T495A eNOS mutant generates significantly more O2 than a phospho-mimetic T495D eNOS mutant does.

ox-LDL has previously been reported to both stimulate and attenuate the phosphorylation of eNOS on Ser1177. A transient enhanced phosphorylation of Ser1177 has been reported in endothelial cells exposed to concentrations of ox-LDL much higher (150 μg/mL) than those employed in the present study (maximal 30 μg/mL; [18]), while similar levels of ox-LDL levels were found to attenuate eNOS Ser1177 phosphorylation in VEGF-stimulated endothelial cells and, thus, to inhibit cell migration [19]. However, we found no significant attenuation of the bradykinin-induced phosphorylation of Ser1177 in cells pretreated with ox-LDL. This latter discrepancy may be explained by the fact that the kinases activated by the two stimuli that mediate the phosphorylation of eNOS on Ser1177 are distinct; VEGF-induced phosphorylation is Akt dependent, while the phosphorylation induced by bradykinin is CaM kinase II-dependent [12]. While such observations indicate that ox-LDL differentially affects Akt-mediated cell signaling, we found that ox-LDL-treatment resulted in modest, but significant, time- and concentration-dependent activation of this kinase without a concomitant increase in eNOS Ser1177 phosphorylation, rather than the dephosphorylation and inactivation of Akt reported previously [19].

Theoretically, the ox-LDL-stimulated dephosphorylation of eNOS Thr495 could reflect either the inhibition of a kinase or the activation of a phosphatase. The phosphatase that dephosphorylates Thr495 is thought to be PP1 [12,20], but although the PP1 inhibitor, calyculin A, was able to increase Thr495 phosphorylation in ox-LDL-treated cells, we found no evidence to suggest that its activity or expression was increased by ox-LDL. The serine/threonine phosphatase, calcineurin (PP2B), has also been suggested to dephosphorylate eNOS Thr495 [21], but this is not a universally observed phenomenon [12,20]. Moreover, since this phosphatase can be inhibited by O2 [22], ox-LDL would be expected to result in a decrease rather than an increase in its activity. Therefore, our data indicate that ox-LDL-induced dephosphorylation of eNOS Thr495 can be attributed to the down-regulation/inactivation of a kinase.

The constitutively active kinase that phosphorylates eNOS Thr495 is most probably PKC [12,20,23], a finding that can account for the fact that protein kinase inhibitors and the down-regulation of PKC attenuate the phosphorylation of this residue. The inhibition and down-regulation were also associated with a marked increase in endothelial NO production, which is consistent with a negative regulatory role of Thr495 on CaM binding and eNOS activity [24,25]. Although, ox-LDL was previously found to acutely stimulate PKC activity [26], the results of the present investigation clearly demonstrate that a constitutively active isoform of PKC is targeted by ox-LDL and that this effect underlies the ox-LDL-stimulated Thr495 dephosphorylation of eNOS. Indeed, the dephosphorylation of Thr495 was temporally correlated with a decrease in the phosphorylation and activity of PKC in ox-LDL-treated endothelial cells. The PKC isoform targeted by ox-LDL was tentatively identified as PKCα, on the basis of its molecular mass and the fact that the same signal was obtained using three different antibodies. Whether PKCα forms part of the eNOS signalosome remains to be determined, as this analysis is hampered by the fact that the activated PKC is detergent-insoluble. However, eNOS can also be detected in the Triton-insoluble cell fraction and the bradykinin—as well as the fluid shear stress-induced increase in NO production has been linked to a change in the solubility of eNOS [9,27]. Moreover, while the activation of PKC by PMA enhances the recovery of eNOS from the Triton-insoluble cell fraction, this phenomenon was not observed in ox-LDL-treated cells (author’s unpublished observation), ox-LDL has previously been reported to acutely increase (within 15 min) the activity of PKCα in coronary artery smooth muscle cells [28] and to increase global PKC activity (over 24 h) in coronary endothelial cells by activating the LOX-1 receptor [29]. Clearly, our results are in direct contrast with the results of this study. The reasons for this apparent contradiction can most likely be attributed to the differences in the concentration and time of incubation with ox-LDL, as well as the cells used. However, it is important to note that, in the present study, we were careful to use cells after only one passage to avoid artefacts related to the gradual
loss of cell signaling pathways in multipassaged cultured cells.

It is unlikely that the effects of ox-LDL on eNOS uncoupling can be solely attributed to its effects on PKC activity, since the down-regulation of PKC by long-term treatment with PMA was previously found to enhance rather than attenuate NO production by eNOS [12]. Indeed, while the PMA-induced dephosphorylation of eNOS Thr495 was associated with an increase in the binding of CaM to the enzyme, eNOS is no longer associated with CaM after ox-LDL-induced Thr495 dephosphorylation. Thus, it appears that ox-LDL also affects additional eNOS regulatory mechanisms, including the stability of the eNOS signaling complex. ox-LDL and hypochlorite-modified LDL have been reported to alter the intracellular localization of eNOS that the enzyme is depleted from caveolae [30–32], as well as to interfere with its association with Hsp90 [4,33]. We observed that the changes in PKC activity and eNOS phosphorylation were associated with a marked change in the intracellular localization of the enzyme.

The most effective means of functionally recoupling eNOS and/or preventing its uncoupling is by the supplementation of H4B [2,3]. Levels of this essential cofactor are reported to be decreased by oxidative stimuli, in particular, by ONOO−, which oxidizes H4B [11,34], as well as by ox-LDL [35]. The H4B oxidation hypothesis also implies that eNOS uncoupling is preceded by the activation of other endothelial O2− generating enzymes [36]. Arguing against such a central role of H4B in the uncoupling of eNOS, in the present study, ox-LDL did not significantly affect endothelial H4B levels, and the mutation of eNOS Thr495 to alanine was sufficient to increase O2− production. Taken together, the results of the present investigation highlight the crucial role played by Thr495 in the regulation of NO and O2− production by eNOS and link the uncoupling of eNOS to changes in the activity of PKCα.

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