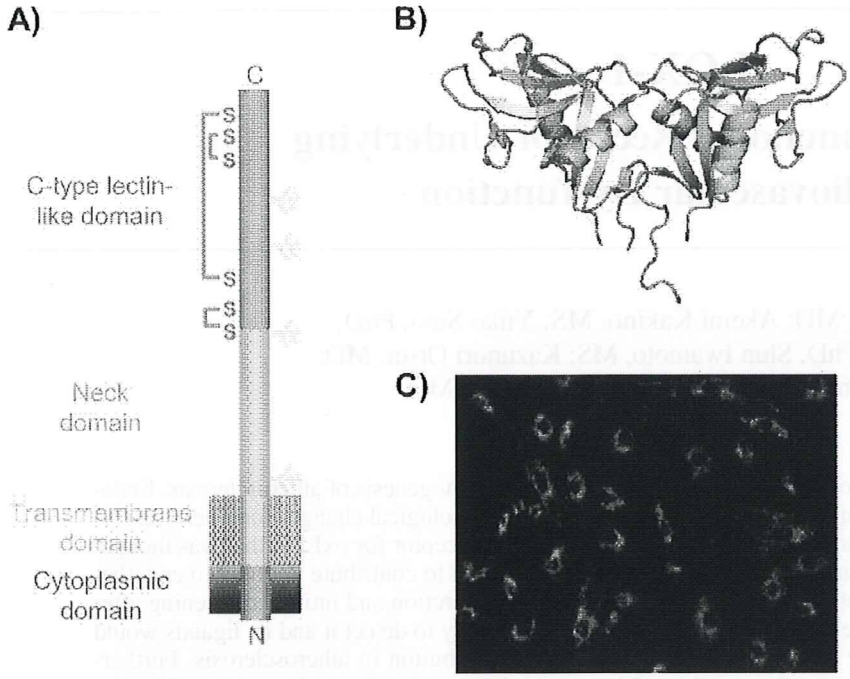

Figure 1. (**A**) Schematic model of the structure of human lectin-like oxidized LDL receptor-1 (LOX-1). LOX-1 has 4 domains: cytoplasmic, transmembrane, neck and C-lectin-like. (**B**) LOX-1 forms homodimers under physiological conditions. (**C**) DiI-oxLDL uptake in human umbilical vein endothelial cells (HUVECs).

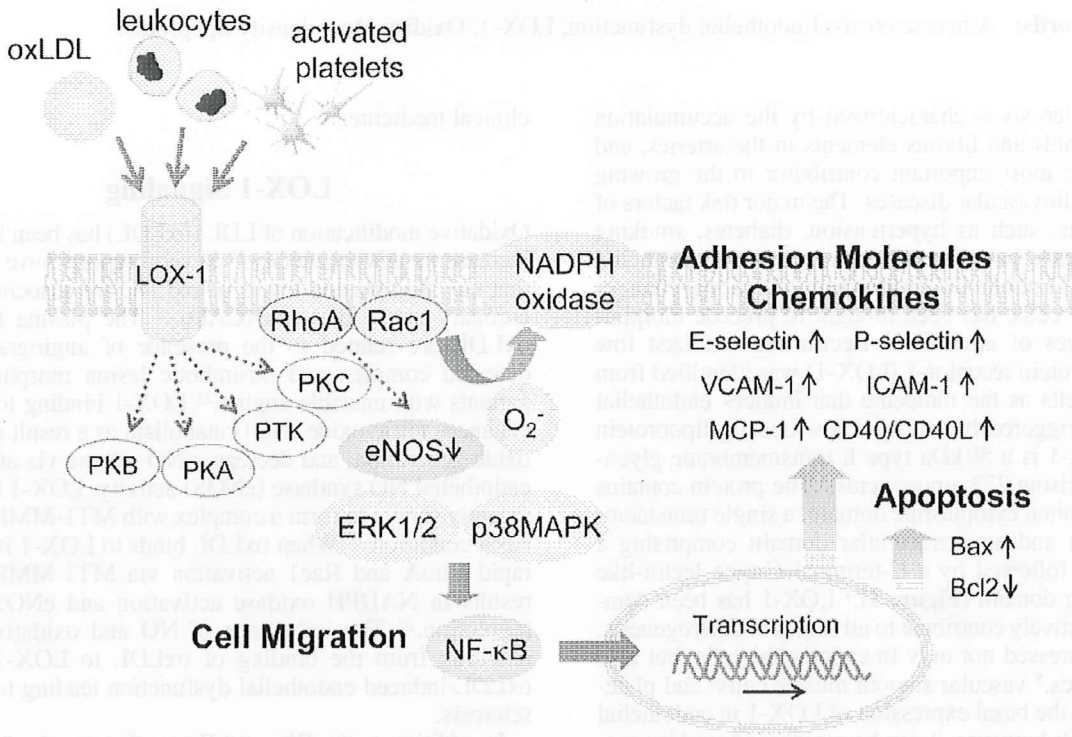

Figure 2. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) signaling pathway. MAPK, mitogen-activated protein kinase C; PKC, protein kinase C; PKB, protein kinase B; PTK, protein tyrosine kinase; eNOS, endothelial nitric oxide synthase; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemotactic protein-1.

sion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1),[25,29,30] which brings proinflammatory changes to the vessel wall. Additionally, LOX-1 activation changes endothelial cells and smooth muscle cells prone to apoptosis by increasing the Bcl-2-associated X protein (Bax)/Bcl-2 ratio (**Figure 2**).[6]

LOX-1 and the Vascular System

Atherosclerosis is characterized by chronic local inflammation of the vascular wall.[1] To date, multiple lines of evidence have implicated LOX-1 in atherosclerosis (**Figure 3**). We created mice over-expressing bovine LOX-1 (LOXtg) and crossed them with atherosclerosis-prone apolipoprotein

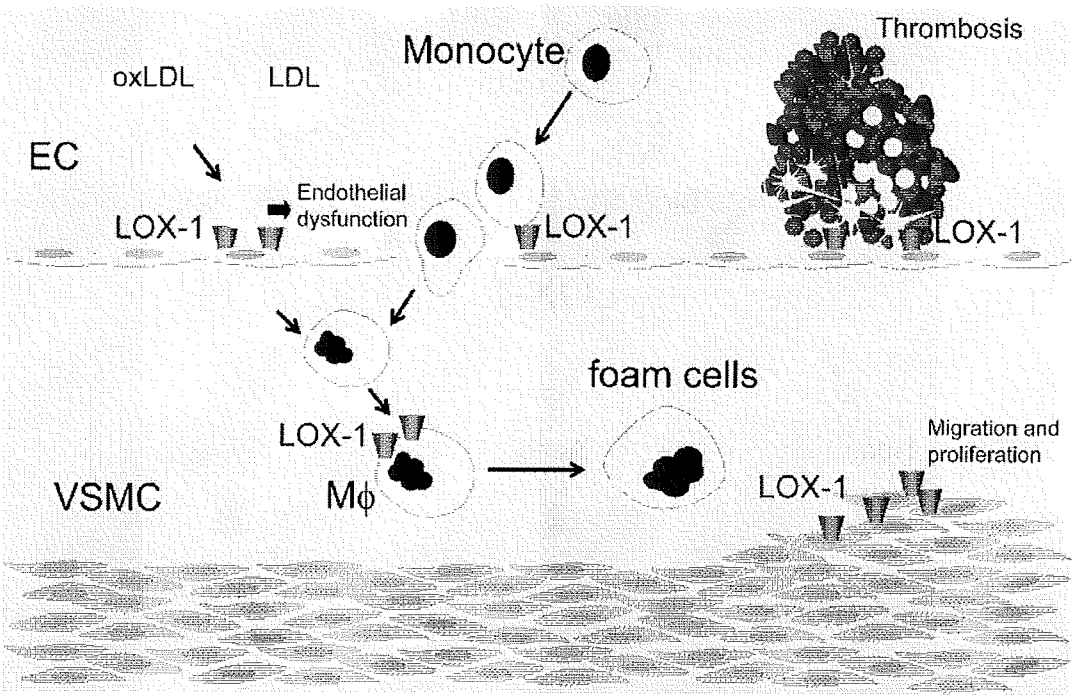

Figure 3. Role of lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) in the initiation, progression and destabilization of atherosclerosis. Oxidized LDL binds to LOX-1 and stimulates endothelial cells to produce adhesion molecules and the recruitment of leukocytes. Endothelial dysfunction leads to enhanced permeability allowing adherent monocytes to penetrate the endothelial layer. Macrophages in the sub-intimal space accumulate oxidized LDL and transform into foam cells. Intensified inflammatory activation results in the stimulation, migration and proliferation of vascular smooth muscle cells.

E knockout (ApoEKO) mice.[31] On a high-fat diet, compared with ApoEKO mice, LOXtg/ApoEKO mice showed more pronounced oxLDL accumulation, oxidative stress detected by 8-hydroxy-2'-deoxyguanosine (8-OH-dG), increased expression of ICAM-1 and VCAM-1, and infiltration of macrophages in the heart vessels. Furthermore, atheroma-like lesions in the intramyocardial vessels increased 10-fold in LOXtg/ApoEKO mice compared with ApoEKO mice. We also generated LOX-1-deficient mice by deleting a part of the lectin-like domain that is essential for ligand binding.[32] LOX-1-deficient mice were resistant to oxLDL-induced impairment of endothelium-dependent vasorelaxation.[32] When crossed with atherosclerosis-prone LDL receptor (LDLR) KO mice, the formation of atherosclerotic lesions was significantly reduced in the aorta of LOX-1/LDLR double KO mice. The serum cholesterol levels were comparable between the mice. The expression of NF-κB and infiltration of CD68-positive cells were decreased whereas anti-inflammatory cytokine IL-10 expression and superoxide dismutase activity were increased in the double KO mice.[32] LOX-1 gene deletion also resulted in less aterial collagen accumulation in LDL KO mice. These gain-of-function and loss-of-function approaches clearly demonstrate that LOX-1 plays a role in the development of atherosclerosis (**Figure 4**).

LOX-1 is likely to mediate inappropriate arterial remodeling, which is one of the causes of atherosclerosis and restenosis. In a balloon-injured carotid artery, strong LOX-1 expression was observed at first in injured medial smooth muscle cells, then in proliferating intimal smooth muscle cells, and finally in the regenerated endothelial cells. The reactive intimal thickening, ROS generation and leukocyte infiltration were attenuated by the administration of anti-LOX-1 antibody.[33] Gene silencer pyrrole-imidazole (PI) polyamide targeting the rat LOX-1 gene promoter (PI polyamide to LOX-1) inhibits neointima thickening and preserves the re-endothelialization after balloon injury.[34] Thus, LOX-1 is involved in multiple phases of vascular dysfunction, including endothelial dysfunction, atherogenesis and restenosis.

LOX-1 and C-Reactive Protein (CRP)

Based on epidemiologic studies, CRP has been long considered as a predictor of cardiovascular diseases. The results of The Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) suggest that CRP could also be, or related to, a causal factor for cardiovascular disease.[35] Several studies have shown an inverse relationship between CRP levels and endothelial dysfunction.[36–38] Of particular interest, accumulating evidence has shown that CRP and LOX-1 share a range of biological functions. LOX-1 mRNA and proteins are induced by CRP, resulting in increased monocyte adhesion to endothelial cells and oxLDL uptake.[9] Furthermore, it has been shown that CRP in vivo impairs endothelial vasoreactivity.[39,40] It has been shown that CRP-LOX-1 binding enhances vascular permeability in vivo in SHR-SP rats.[41] LOX-1 exhibits binding activity for multiple ligands, apoptic cells,[42] bacteria[43] and phoshatidylserine,[44] all of which are also recognized by CRP.[45] CRP binding to LOX-1 enhances the binding affinity of oxLDL to LOX-1.[46] CRP induces the expressions of ICAM-1, VCAM-1, E-selectin and chemokine MCP-1 as well as the activation of RhoA and p38 (MAPK).[47,48] CRP-mediated expressions of ICAM-1 and VCAM-1 genes are inhibited by LOX-1 knockdown.[46]

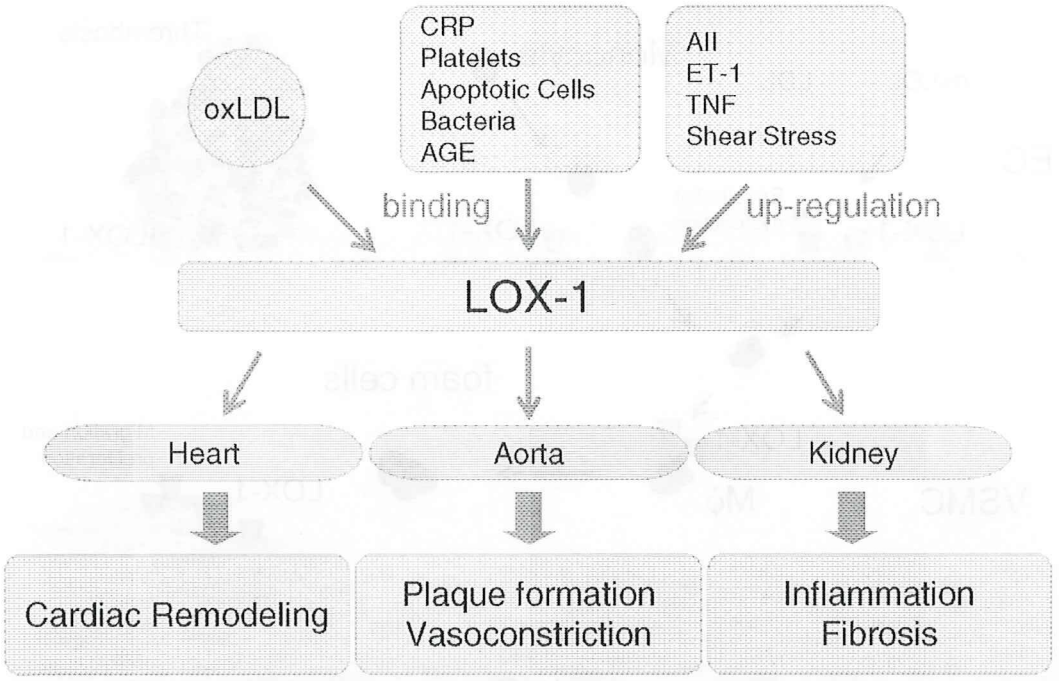

Figure 4. LOX-1 is bound and/or upregulated by the various factors indicate. The functions of LOX-1 contribute to cardiac remodeling, plaque formation, vasoconstriction and renal injury. The coordination of these triggers the onset of multiple organ damage.

These lines of evidence support the hypothesis that the CRP interaction with LOX-1 might play a role in atherogenetic inflammation, which is relevant to endothelial dysfunction.

LOX-1 and Myocardial Infarction (MI)

LOX-1 is also readily detectable in cardiomyocytes under prooxidative conditions (**Figure 4**). In vitro, a blockade of LOX-1 inhibits NF-κB activations and diminishes apoptosis, which suggests that LOX-1 plays a role in oxidative stress in cardiomyocytes.[49,50] In vivo, the expression of LOX-1 in cardiomyocytes is prominently induced by ischemia–reperfusion.[49,50] Treatment with anti-LOX-1 antibody prevents cardiac remodeling in a rat model of myocardial ischemia–reperfusion. It also reduces the size of myocardial infarct and improves left ventricular function by inhibiting apoptosis and lipid oxidation in cardiomyocytes.[49,50] Deletion of the LOX-1 gene in mice reduces myocardial remodeling following ischemia–reperfusion, attenuated NADPH oxidase expression and decreased collagen deposition.[51] LOX-1 KO mice further showed a significantly smaller decline in –dP/dt max and a smaller increase in left ventricle end-diastolic pressure after ischemia–reperfusion compared with the control group.[51]

LOX-1 and Kidney Injury

Chronic kidney disease is a risk factor for cardiovascular diseases and the enhanced progression of renal dysfunction has been reported in patients with obesity and hypertension. Several reports showed the association between LOX-1 and renal dysfunction (**Figure 4**). LOX-1 expression was increased in the renal tubules of obese–diabetic Zuker Spontaneous hypertensive heart failure (ZS) rats.[52] The anti-LOX-1 antibody injection reduced renal inflammation and fibrosis in ZS rats.[53] Kidneys from ZS rats treated with anti-LOX-1 antibody lacked neutrophil infiltration, whereas kidneys from untreated and normal IgG-injected ZS rats had abundant clusters of neutrophils along the capillary lumens. Although anti-LOX-1 antibody did not prevent albuminuria, it increased glomerular vascular endothelial growth factor levels to help preserve renal microvascular beds.[53] It was also shown that the expression of the AT1 receptor in LOX-1 KO mice was lower than wild-type mice both before and after infusion of angiotensin II (Ang II).[54] Furthermore, Ang II-induced, but not norepinephrine-induced, blood pressure increase and renal injury were attenuated in LOX-1 KO mice compared with wild-type mice.[54] LOX-1 KO mice infused with Ang II exhibited less glomerulosclerosis, arteriolar sclerosis and tubulointerstitial damage. These findings highlight the fact that the Ang II-AT1R-LOX-1 loop is an important regulator of blood pressure and renal injury. Thus, LOX-1 plays a pathological role in the heart and kidney as well as in blood vessels.

Genetics

The human LOX-1 gene spans more than 7,000 base pairs and consists of 6 exons and 5 introns. LOX-1 is within a C-type lectin gene cluster on chromosome 12p12-13.[4] Several groups have analyzed the association of LOX-1 gene polymorphisms with CAD. The 3'-untranslated region (UTR) (T allele), a C-to-T change 188 nucleotides from the stop codon (+188C-T), was associated with a higher risk of acute MI.[55] Furthermore, 7 different single nucleotide polymorphisms (SNPs), 6 of them located within introns 4, 5 and 3' UTR comprised in a linkage disequilibrium block, exhibited a significant association with an elevated risk of developing MI.[56] The SNPs give rise to a splicing variant lacking exon 5, named LOXIN, which lacks two-thirds of the ligand binding domain of LOX-1. The LOX-1/LOXIN mRNA ratio is 33% higher in monocyte-derived macro-

phages of the subjects homozygous for the risk allele compared with those homozygous for the non-risk allele.[57] LOXIN probably works as a dominant negative form dimerizing with the native form of LOX-1 to protect cells from the damage by oxLDL. Another SNP leading to the missense mutation of Lys to Asn at the 167th amino acid residue (K167N) was identified and reported to be associated with an increased risk of MI.[58] However, this association could not be replicated in a later study using a larger population. In the Atherosclerotic Disease VAscular FuNction & GenetiC Epidemiology (ADVANCE) study, an association of K167N with a decreased risk of CAD was observed, whereas in the Atherosclerosis Risk in Communities (ARIC) study using a larger population, the association was reported to be insignificant.[59] Recently, biochemical analysis showed that oxLDL binding and ERK activation were decreased by 30% in 167N-LOX-1 compared with 167K-LOX-1.[60] The structural analysis suggests that the reduced binding of oxLDL could be ascribed to the decrease of the electrostatic interaction between LOX-1 and oxLDL in 167N-LOX-1. Thus, it might be plausible that people carrying the 167N-LOX-1 allele would be at lower risk of CAD. Presently, we do not have enough evidence to determine whether the SNPs in the LOX-1 gene are useful to assess the risk and prognosis of CAD.

LOX-1 as a Clinical Tool

Soluble LOX-1

A number of membrane proteins are cleaved into soluble molecules by proteolysis at the membrane-proximal site of the extracellular domain, which is known as ectodomain shedding. LOX-1 is also released in a soluble form from the cell surface into culture media.[61] There are 2 cleavage sites that have been identified to yield soluble LOX-1 (sLOX-1; ie, Arg^{86}-Ser^{87} and Lys^{89}-Ser^{90} bonds of bovine LOX-1).[61] A member of the A Disintegrin And Metalloproteinase (ADAM) family, ADAM10, contributes to the proteolysis of LOX-1.[62] Recently, it has been reported that the serum sLOX-1 level is specifically elevated in acute coronary syndrome.[63] Peak values of sLOX-1 are observed earlier than those of Troponin T in acute coronary syndrome. Furthermore, the sensitivity and specificity of sLOX-1 in acute coronary syndrome are significantly better than high sensitivity CRP.

LOX-1 Ligand Assay

A receptor-based assay to determine the levels of modified LDL as LOX-1 ligands has been reported.[64–66] Although clinical assessment is yet to be carried out, the LOX-1 ligand level is elevated in hyperlipidemic animals in association with the extent of atheroma-formation. The circulating amount of oxidation-related moiety on LDL has been shown to be effective in predicting or diagnosing metabolic syndrome and cardiovascular diseases.[67–69] Considering that oxLDL interacts with cells via its receptors, the receptor binding activity is likely to reflect the biological activity of oxLDL better than the absolute amount of oxidatively modified moiety.

Imaging

Taking an advantage of the relatively selective expression of LOX-1 in atheroma, the methodology to obtain images of atheroma utilizing the anti-LOX-1 antibody was reported.[70] ^{99m}Tc-labeled anti-LOX-1 antibody administered into MI-prone Watanabe heritable hyperlipidemic rabbits accumulated in each aortic segment, which was a significantly higher level than that in control rabbits. High density accumulation is also observed at collagen-rich and neointimal lesions. The macrophage density and vulnerability index correlated with ^{99m}Tc-LOX-antibody accumulation, but not with ^{99m}Tc-IgG accumulation. This nuclear imaging technique might be useful for the diagnosis of plaque vulnerability.

Thus, effective tools utilizing LOX-1 and its antibody have been developed. Clinical application of LOX-1 would be diagnostic rather than therapeutic, while it warrants further investigations.

Concluding Remarks

Since it has been known that the modification of LDL increases its atherogenicity, people have been seeking what underlies "oxidation hypothesis". Identification and series of studies on the oxidized LDL receptors, including LOX-1, seems to prove the concept. Starting from epidemiology, a branch of the study of atherosclerosis proceeded in a sequence of cholesterol-LDL-oxLDL-oxLDL receptors. LOX-1 showed unexpectedly diverse ligand specificity with versatile functions, which points to a new avenue of research.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labour and Welfare of Japan; the National Institute of Biomedical Innovation; Japan Science and Technology Agency; and the New Energy and Industrial Technology Development Organization.

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Journal of Cellular Physiology

Role of LOX-1 in Monocyte Adhesion-Triggered Redox, Akt/eNOS and Ca^{2+} Signaling Pathways in Endothelial Cells

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This study was conducted to examine the role of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in monocyte adhesion-induced redox-sensitive, Akt/eNOS and Ca^{2+} signaling pathways in endothelial cells (ECs). LOX-1 was blocked by an antibody-neutralizing LOX-1 TS92 or small interfering RNA. In cultured human aortic ECs, monocyte adhesion activated Rac1 and $p47^{phox}$, and increased NADPH oxidase activity and reactive oxygen species (ROS) generation within 30 min and NF-κB phosphorylation within 1 h, resulting in redox-sensitive gene expression. Akt and eNOS phosphorylation was induced 15 min after adding monocytes and returned to control level after 30 min, whereas NO production was not altered by monocyte adhesion. Blockade of LOX-1 blunted the monocyte adhesion-triggered redox-sensitive signaling pathway and Akt/eNOS phosphorylation in ECs. Both endothelial intracellular Ca^{2+} mobilization and Ca^{2+} influx caused by monocyte attachment were markedly attenuated by pretreatment of ECs with TS92. This suggests that LOX-1 is involved in redox-sensitive, Akt/eNOS and Ca^{2+} signaling pathways in monocyte adhesion to ECs independent of oxidized low-density lipoprotein (ox-LDL). Furthermore, blockade of Ca^{2+} inhibited monocyte adhesion-triggered Rac1 and $p47^{phox}$ activation and ROS generation in ECs, whereas Ca^{2+} signaling was suppressed by blockade of NADPH oxidase and ROS generation. Finally, TS92 blocked the monocyte adhesion to ECs stimulated with or without tumor necrosis factor-α or ox-LDL. We provide evidence that LOX-1 plays a role in redox-sensitive, Akt/eNOS and Ca^{2+} signaling pathways in monocyte adhesion to ECs independent of the ox-LDL–LOX-1 axis.

J. Cell. Physiol. 220: 706–715, 2009.

Monocyte adhesion to endothelial cells (ECs) of the vascular wall is a priming event in the progression of atherosclerosis and the redox-sensitive gene expression in ECs including monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), tissue factor (TF), and plasminogen activator inhibitor-1 (PAI-1) (Ross, 1999; Libby, 2002; Ishibashi et al., 2003). We have recently reported the RhoA-mediated NF-κB and Ca^{2+}-dependent pathways in monocyte adhesion-induced TF and PAI-1 expression in ECs, as well as the crucial role of RhoA activation in endothelial dysfunction (Yokoyama et al., 2002; Ishibashi et al., 2003; Ohkawara et al., 2005; Sakamoto et al., 2007). However, the detailed mechanism(s) by which monocyte adhesion to ECs induces redox-sensitive gene expression and Ca^{2+} signaling is not well understood.

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) was identified as a major receptor for oxidized low-density lipoprotein (LDL) which is potent for inflammation and reactive oxygen species (ROS) generation, and this receptor is uniquely expressed in the ECs of large arteries (Sawamura et al., 1997). Oxidized LDL-triggered intracellular ROS generation has been shown to be mediated via LOX-1, indicating that LOX-1 activation is responsible for oxidized LDL-induced redox-sensitive gene expression including ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) which are associated with monocyte adhesion to ECs (Cominacini et al., 2000; Min et al., 2005). In addition, ROS downregulate endothelium-derived nitric oxide (NO) which regulate vascular homeostasis, and Akt/eNOS pathway is crucial for NO production.

In the present study, we hypothesized that endothelial LOX-1 plays a role in the redox-sensitive, Akt/eNOS and Ca^{2+} signaling pathways in monocyte adhesion to ECs independent of the oxidized LDL–LOX-1 axis.

Contract grant sponsor: Japan Society for the Promotion of Science;
Contract grant numbers: 14370232, 19590804, 1979056.
Contract grant sponsor: Takeda Science Foundation.
Contract grant sponsor: The Uehara Memorial Foundation.
Contract grant sponsor: Fukushima Medical University Research Project.

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Received 12 March 2009; Accepted 6 April 2009

Published online in Wiley InterScience (www.interscience.wiley.com.), 18 May 2009.
DOI: 10.1002/jcp.21818

Materials and Methods

Materials

The sources of most of the conventional reagents for the present study were described previously (Ishibashi et al., 2003; Sakamoto et al., 2005, 2007). TS92, a neutralizing antibody to LOX-1, was used for inhibition of LOX-1 and a neutralizing antibody to human PECAM-1 was purchased from BioLegend (San Diego, CA) (Sawamura et al., 1997). Fluorescent probes, 2′,7′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and fura-2/acetoxymethyl ester were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), a ROS-sensitive fluorescent probe 2′,7′-dichlorodihydro-fluorescein-diacetate (H_2DCF-DA) from Molecular Probes (Eugene, OR), and diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase and *N*-acetylcystein (NAC), an antioxidant, were from Sigma–Aldrich Co. (St. Louis, MO).

Isolation of human peripheral blood monocytes

Human peripheral blood monocytes were isolated from normal volunteers and the purity of isolated monocytes was greater than 95% as determined by flow cytometry and cytohistochemistry as described previously (Nagata et al., 2001; Ishibashi et al., 2003). Ethical approval was obtained from Fukushima Medical University for the study.

Cell culture

Human aortic ECs (Clonetics, Inc., Walkersville, MD and Sanko Junyaku Co., Ltd., Tokyo, Japan) were cultured as described previously and used for all experiments after 5–10 passages (Yokoyama et al., 2002; Ohkawara et al., 2005). We added monocytes (5×10^5 or 1×10^6 cells) to near-confluent ECs (Cominacini et al., 2000) for the below-described purposes. We recently reported the expression of relatively monocyte-specific antigen CD14 to clarify the potential contamination of ECs with residual monocytes by Western blotting after the cultures were washed extensively to remove the monocytes, indicating the negligible effect of residual monocytes on CD14 expression in ECs (Sakamoto et al., 2005).

Measurement of intracellular ROS generation

Detection of intracellular ROS generation was performed by a previously established method using H2DCF-DA (Cominacini et al., 2000). Monocytes (5×10^5 cells) were added to nearly confluent ECs cultured on 60-mm dishes and incubated at 37°C for 15, 30, and 60 min, respectively. Fluorescence was measured using a confocal microscope (Olympus Co. Ltd., Tokyo, Japan) at excitation and emission wavelength of 475 and 525 nm, respectively. Images were stored in a computer and the intensity of fluorescence was quantitatively analyzed by the NIH Image Program (ImageJ).

Membrane translocation of Rac1 and p47^{phox}

The activities of Rac1 and p47^{phox}, components of the NADPH oxidase, were determined by membrane translocation, followed by Western blotting as described previously (Yokoyama et al., 2002; Ishibashi et al., 2003). For immunoblotting, we used a mouse monoclonal antibody to Rac1 (Upstate Biotechnology, Lake Placid, NY) diluted 1:1,000 and a rabbit polyclonal antibody to p47^{phox} (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:200.

Determination of NF-κB activation

NF-κB phosphorylation was determined by Western blotting using an anti-phospho-NF-κB p65 antibody (Ser-536, dilution 1:1,000, Cell Signaling Technology, Inc., Beverly, MA) as described previously (Ishibashi et al., 2003; Sakamoto et al., 2005).

Measurement of NADPH oxidase activity

The enzymatic activities of NADPH oxidase of homogenates of the ECs were assessed by lucigenin-enhanced chemiluminescence (L-CL) as previously described (Ejiri et al., 2005). The assay solution contained 50 mmol/L HEPES (pH 7.4), 1.0 mmol/L EDTA, 6.5 mmol/L $MgCl_2$, 5.0 μmol/L lucigenin as an electron acceptor, and 1 mmol/L NADPH as a substrate. After preincubation at 37°C for 10 min, the reaction was started by adding 50 μg of homogenate. Final volume of the reaction solution was 1.0 ml. Photon emission was continuously recorded for 15 min with a CL reader (ALOKA, BLR-201). The chemiluminescent signals observed in the absence of homogenates were subtracted from the chemiluminescence signals of the samples. The chemiluminescence signal was corrected for the protein concentration of each cell homogenate and expressed as counts per minute (cpm) per milligram protein for a 15-min period. In some experiments, the homogenates were preincubated with 10 μmol/L DPI, a selective NADPH oxidase inhibitor, for 20 min before L-CL measurement.

Small interfering RNA (siRNA)

LOX-1 expression was inhibited by siRNA 5′-GAGGCCAUUCCGAAUCAAGA-3′ (sense strand) (RNAi Co., Ltd., Tokyo, Japan) (Naito et al., 2004). ECs were transfected with double-stranded siRNA in serum-free medium mixed with oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Four hours after transfection, ECs were incubated in a medium containing 2% fetal bovine serum (FBS) for 48 h. Alternatively, ECs were treated with an irrelevant siRNA 5′-GUACCGCACGUCAUUCGCAUC-3′ (sense strand) as a negative control. LOX-1 protein suppression by siRNA was evaluated by fluorescent immunohistochemical staining using a primary antibody against LOX-1 (termed TS20) and anti-mouse Alexa 488 (Molecular Probes) as a secondary antibody. The fluorescent intensity of LOX-1 expression was quantitatively analyzed by the ImageJ.

Western blotting

ECs were pretreated with 10 μmol/L DPI and 10 mmol/L NAC for 30 min, and 5 μg/ml TS92 for 1 h. ECs treated with TS92 were washed three times before adding monocytes. Monocytes were then added to ECs and the cells were cultured for 18 h, followed by Western blotting after washing out monocytes. After scanning immunoblots onto a computer (EPSON GT5500ART, Suwa, Japan), individual bands were analyzed for optical density using NIH image as described previously (Ohkawara et al., 2005; Sakamoto et al., 2005, 2007).

Determination of EC surface TF activity

TF activity was determined as factor X activation by factor VIIa/TF complex on ECs as described previously (Ishibashi et al., 2003).

Determination of Akt and eNOS phosphorylation

Akt and eNOS phosphorylation in response to monocyte adhesion in ECs was determined by Western blotting at various time intervals after adding monocytes. Anti-phospho-Akt (Ser-473, dilution 1:1,000, Cell Signaling Technology, Inc.) and anti-phospho-eNOS (Ser-1177; dilution 1:1,000, Cell Signaling Technology, Inc.) antibodies were used as a primary antibody.

NO detection

Measurement of NO in cell culture medium was electrochemically performed using NO-sensitive electrodes (ISO-NOPF, 100)

manufactured by WPI (Sarasota, FL) as described previously (Shibuki and Okada, 1991; Tsukahara et al., 1993). Briefly, the NO probe consists of a working argentum anode and a reference/counter electrode. A gas-permeable membrane covers the tip of the electrode. NO diffusing across the electron membrane is oxidized on the surface of prepolarized working electrode, resulting in electrical current. The magnitude of the current, being proportional to the activity of NO in a sample, was detected by an amplifier and registered on a chart recorder.

Measurement of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$)

ECs were cultured in 35-mm glass bottom dishes (MatTek Co., Ashland, MA), and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured in response to monocyte attachment in the absence or presence of external Ca^{2+} as described previously (Yokoyama et al., 2002; Sakamoto et al., 2005). Intracellular Ca^{2+} mobilization was the value in the absence of external Ca^{2+}, whereas the Ca^{2+} influx was defined as the value when the Ca^{2+} value in the absence of external Ca^{2+} was subtracted from the value in the presence of external Ca^{2+}.

Monocyte adhesion assay

Monocyte adhesion to ECs was determined by a previously established method (Fischer et al., 2005). ECs cultured on 12-well plates were pretreated with or without 10 ng/ml TNF-α or 100 μg/ml oxidized LDL overnight and then treated with 5 μg/ml TS92 for inhibition of LOX-1 for 1 h. In parallel, monocytes were labeled with 3 μg/ml BCECF-AM at 37°C for 30 min. Labeled monocytes (2.5×10^5 cells) were added to ECs after washing out the antibodies and incubated at 37°C for 30 min. After aspiration of the medium, nonadherent monocytes were removed by three washing steps. Adherent monocytes were lysed in 0.1 mol/L Tris/0.1% (w/v) Triton X-100, and fluorescence was measured by a SPECTRA microplate reader (Molecular Devices Co., Sunnyvale, CA) at an excitation of 492 nm and emission of 535 nm.

Preparation of oxidized LDL

Oxidized LDL was prepared as described previously (Yokoyama et al., 1998). Human native LDL (density 1.019–1.063 g/ml) was isolated from serum of fasting normolipidemic volunteers by sequential ultracentrifugation and was stored at 4°C in 0.3 mmol/L EDTA. Oxidized LDL was prepared by incubating native LDL for 24 h at 4°C in phosphate-buffered saline (PBS) containing 5 μmol/L $CuSO_4$, and then extensively dialyzed against PBS and sterilized by filtration.

Statistical analysis

Statistical analyses were performed using ANOVA with Scheffé's post hoc test if appropriate. Data are expressed as means ± SD. A value of $P < 0.05$ was considered significant.

Results

Redox-sensitive signaling pathway in ECs by monocyte adhesion

Monocyte adhesion generated intracellular ROS in ECs in a time-dependent manner. Figure 1A shows the time course of the levels of intracellular ROS generation in ECs in response to monocyte adhesion from 15 to 60 min after adding monocytes. To determine the source of ROS generation induced in ECs by monocyte adhesion, we treated the ECs with 10 μmol/L DPI and 10 mmol/L NAC for 30 min before adding monocytes. DPI, as well as NAC, prevented the ROS generation caused by monocyte adhesion in ECs 60 min after adding monocytes (Fig. 1B).

We next determined whether Rac1 and $p47^{phox}$ are activated by monocyte adhesion in ECs. Membrane translocation assays showed that the levels of Rac1 and $p47^{phox}$ in membrane fractions of ECs were increased 30 min after adding monocytes and that monocyte adhesion decreased the levels of Rac1 and $p47^{phox}$ in cytosol fractions (Fig. 1C). NADPH oxidase activity in ECs was enhanced by monocyte adhesion 15 min after adding monocytes as described later in Figure 2C.

Monocyte adhesion increased the levels of NF-κB phosphorylation in ECs 1 h after adding monocytes, which was prevented by the pretreatment with 10 μmol/L DPI and 10 mmol/L NAC for 30 min before adding monocytes (Fig. 1D).

We then examined the effect of monocyte adhesion on redox-sensitive gene expression in ECs. Western blotting revealed that monocyte adhesion to ECs induced an increase in the expression of MCP-1, ICAM-1, TF, and PAI-1 in ECs after 18 h of coculture as described later in Figure 3A, which was prevented by pretreatment of ECs with DPI and NAC (data not shown). DPI and NAC also suppressed the increased EC surface TF activity caused by monocyte adhesion (data not shown). These results suggest the redox-sensitive signaling pathway of NADPH oxidase/ROS/NF-κB in monocyte adhesion-triggered gene expression in ECs.

Role of LOX-1 in monocyte adhesion-triggered redox signaling pathway and gene expression

Next, we analyzed the effect of inhibition of LOX-1 on the redox-sensitive signaling pathway and gene expression induced in ECs by monocyte adhesion. The increased ROS generation in ECs induced by monocyte adhesion was prevented by pretreatment of ECs with antibodies to LOX-1, ICAM-1, and VCAM-1 for 1 h before adding monocytes to ECs, whereas pretreatment with an antibody against PECAM-1, which was not involved in monocyte adhesion, did not affect monocyte adhesion-triggered endothelial ROS generation (Fig. 2A). We then determined the effect of inhibition of LOX-1 by TS92 on the activation of Rac1 and $p47^{phox}$ in ECs when monocytes adhere to ECs. Figure 2B demonstrated that inhibition of LOX-1 markedly attenuated the membrane translocation of Rac1 and $p47^{phox}$ in ECs caused by monocyte adhesion. The enhanced NADPH oxidase activity induced in ECs by monocyte adhesion was markedly reduced by pretreatment of ECs with TS92 (Fig. 2C). The phosphorylation of NF-κB in ECs induced by monocyte adhesion was also suppressed by preincubation of ECs with 5 μg/ml TS92 for 1 h before adding monocytes (Fig. 2D).

Western blotting demonstrated that pretreatment of ECs with TS92 suppressed the increased expression of MCP-1, ICAM-1, TF, and PAI-1 in ECs caused by monocyte adhesion (Fig. 3A). To confirm this finding, we silenced LOX-1 by siRNA. Fluorescent immunohistochemistry demonstrated that the siRNA treatment for knockdown of LOX-1 decreased LOX-1 protein expression by approximately 75% compared with scrambled siRNA (Fig. 3B). Similar results regarding molecular expression were obtained with silencing of LOX-1 by siRNA to the treatment with TS92 (Fig. 3C).

These results indicated the integral role of LOX-1 in monocyte adhesion-triggered NADPH oxidase-dependent signaling pathways.

Effect of inhibition of LOX-1 on Akt/eNOS phosphorylation

Figure 4A,B shows the time course of Akt and eNOS phosphorylation induced in ECs by monocyte adhesion, indicating that monocyte adhesion enhanced Akt and eNOS phosphorylation 15 min after adding monocytes to ECs and the levels of Akt and eNOS phosphorylation returned to control

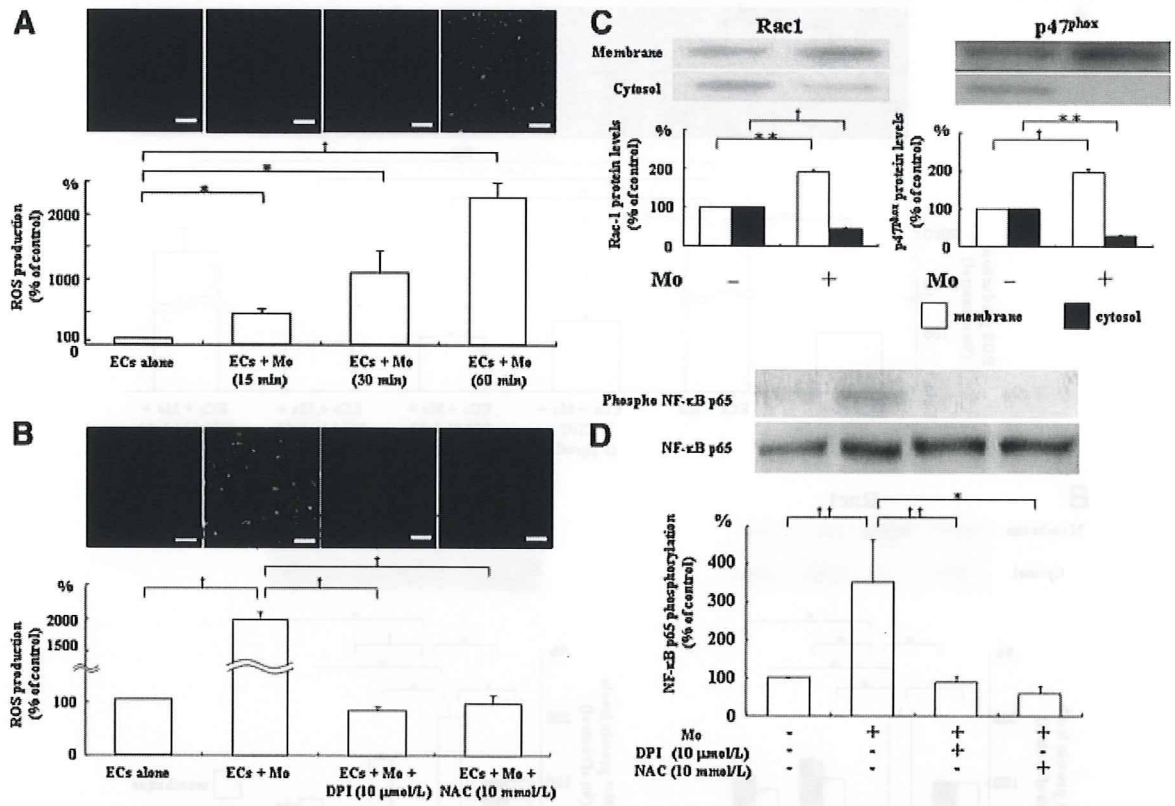

Fig. 1. Effect of monocyte adhesion on redox signaling pathway in ECs. A: Time course of intracellular ROS generation in ECs in response to monocyte adhesion (0, 15, 30, and 60 min). Bars are the means ± SD of quantitative fluorescent analyses from four separate experiments. Scale bars, 100 μm. B: Effects of 10 μmol/L DPI and 10 mmol/L NAC on ROS generation induced in ECs by monocyte adhesion. ROS generation levels were measured 60 min after adding monocytes. Bars are the means ± SD of quantitative analyses from three separate experiments. Scale bars, 100 μm. C: Effect of monocyte adhesion on membrane translocation of Rac1 and $p47^{phox}$ in ECs 30 min after adding monocytes. Data are expressed as the means ± SD of quantitative densitometric analyses from four separate experiments. Immunoblots were from an experiment representative of four similar experiments. D: Phosphorylation of NF-κB p65 in ECs 1 h after adding monocytes. Monocytes (5×10^5 cells) were added to confluent ECs pretreated with or without 10 μmol/L DPI and 10 mmol/L NAC for 30 min before adding monocytes. Representative immunoblots from three similar experiments are shown at the top. Data are the means ± SD of densitometric analyses from three separate experiments. $^{*}P < 0.01$, $^{\dagger}P < 0.0001$, $^{**}P < 0.0005$, and $^{\dagger\dagger}P < 0.05$.

with further incubation. However, NO production was not altered by monocyte adhesion 15 min after adding monocytes (data not shown). Monocyte adhesion-triggered Akt and eNOS phosphorylation in ECs was prevented by pretreatment of ECs with TS92, indicating that monocyte adhesion mediates Akt/eNOS phosphorylation via LOX-1 in ECs (Fig. 4C,D). The same concentration of antibody isotype control IgG as TS92 had a negligible effect on eNOS phosphorylation in the presence or absence of monocyte adhesion (Fig. 4D).

Effect of inhibition of LOX-1 on eNOS expression

Monocyte adhesion decreased eNOS expression 18 h after adding monocytes, which was prevented by pretreatment with TS92 (Fig. 5A), whereas DPI, a relatively specific inhibitor of NADPH oxidase, also attenuated monocyte adhesion-triggered downregulation of eNOS protein expression (Fig. 5B). This suggests significant interaction between decreased eNOS expression and LOX-1-mediated ROS generation in monocyte–EC interaction.

Role of LOX-1, ICAM-1, and VCAM-1 in Ca^{2+} signaling

To further determine the mechanism(s) by which monocyte adhesion activates redox-signaling pathway in ECs through LOX-1, we focused on monocyte attachment-induced Ca^{2+} signaling. Monocyte attachment exerted a rapid and transient increase in $[Ca^{2+}]_i$ in ECs as described previously (Ziegelstein et al., 1994; Sakamoto et al., 2005). Figure 6A shows the values of endothelial $[Ca^{2+}]_i$ in response to monocyte attachment in the presence of external Ca^{2+} when ECs were pretreated with 3, 5, and 10 μg/ml TS92 for 1 h, indicating that inhibition of LOX-1 by TS92 suppressed monocyte attachment-induced $[Ca^{2+}]_i$ increase in ECs ($P < 0.0001$). There was no significant difference in the suppressive effect on monocyte attachment-induced $[Ca^{2+}]_i$ increase among 3, 5, and 10 μg/ml TS92 treatment. Control antibody of IgG (5 μg/ml) did not change monocyte attachment-induced $[Ca^{2+}]_i$ increase in ECs (Fig. 6A). In addition, inhibition of ICAM-1 and VCAM-1 suppressed monocyte attachment-induced $[Ca^{2+}]_i$ increase in ECs similar to inhibition of LOX-1 ($P < 0.0001$, each, data not shown). Furthermore, inhibition of LOX-1 by TS92 at a concentration of 5 μg/ml blocked both intracellular Ca^{2+} mobilization and Ca^{2+} influx in response to monocyte attachment in ECs ($P < 0.0001$, each, Fig. 6B).

Role of LOX-1 in monocyte adhesion to ECs

We then examined the effect of inhibition of LOX-1 on monocyte adhesion to ECs. Pretreatment of ECs with TS92 for 1 h before adding monocytes decreased monocyte adhesion to

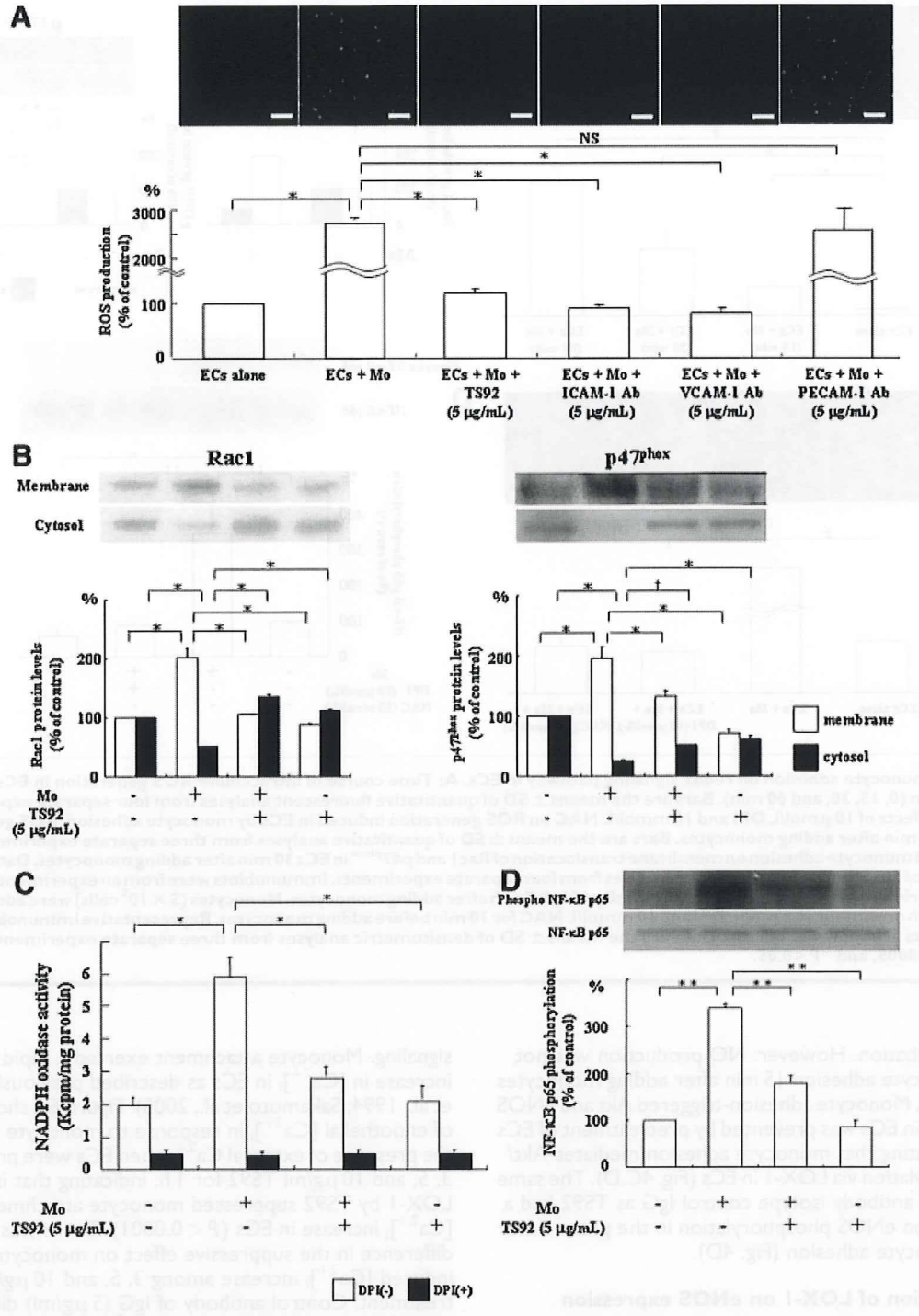

Fig. 2. Effect of inhibition of LOX-1 on ROS generation (A), Rac1 and p47^{phox} membrane translocation (B) NADPH oxidase activity (C), and NF-κB phosphorylation (D) in ECs by monocyte adhesion after 60 min. A: Near-confluent ECs in 60-mm dishes were pretreated with or without 5 μg/ml antibodies to LOX-1 (TS92), ICAM-1, VCAM-1, and PECAM-1 for 1 h and ROS generation was determined 60 min after adding monocytes. Bars are the means ± SD of quantitative analyses from four separate experiments. Scale bars, 100 μm. B: Monocytes (1 × 10^6 cells) were added to ECs in 100-mm dishes pretreated with or without TS92 for 1 h. After 30 min of coculture, Rac1 and p47^{phox} membrane translocation was determined. Data are the means ± SD of densitometric analyses of Western blotting from four separate experiments. Immunoblots were from an experiment representative of four similar experiments. C: Endothelial NADPH oxidase activity was measured with or without inhibition of LOX-1 15 min after adding monocytes. Treatment of endothelial homogenates with DPI abolished the enzymatic activity of NAPDH oxidase. Data are the means ± SD from five separate experiments. D: Monocytes (5 × 10^5 cells) were added to confluent ECs in 60-mm dishes pretreated with or without 5 μg/ml TS92 for 1 h. After 1 h of incubation with monocytes, NF-κB p65 phosphorylation in ECs was determined. Data are expressed as means ± SD of densitometric analyses of Western blotting from three separate experiments. Representative immunoblots from three similar experiments are shown. *$P < 0.0001$, †$P < 0.0005$, and **$P < 0.001$.

ECs by 47 ± 6%, compared with control (Fig. 7, $P < 0.01$). Control with nonimmune IgG had a negligible effect on monocyte adhesion. We pretreated ECs with 10 ng/ml TNF-α and 100 μg/ml oxidized LDL overnight, which enhanced monocyte adhesion to ECs (Fig. 7, $P < 0.001$, each). The TNF-α- and oxidized LDL-induced monocyte adhesion was markedly reduced by inhibition of LOX-1 by TS92 (Fig. 7, $P < 0.01$, each). Control with nonimmune IgG did not significantly affect the monocyte adhesion with or without TNF-α or oxidized LDL activation.

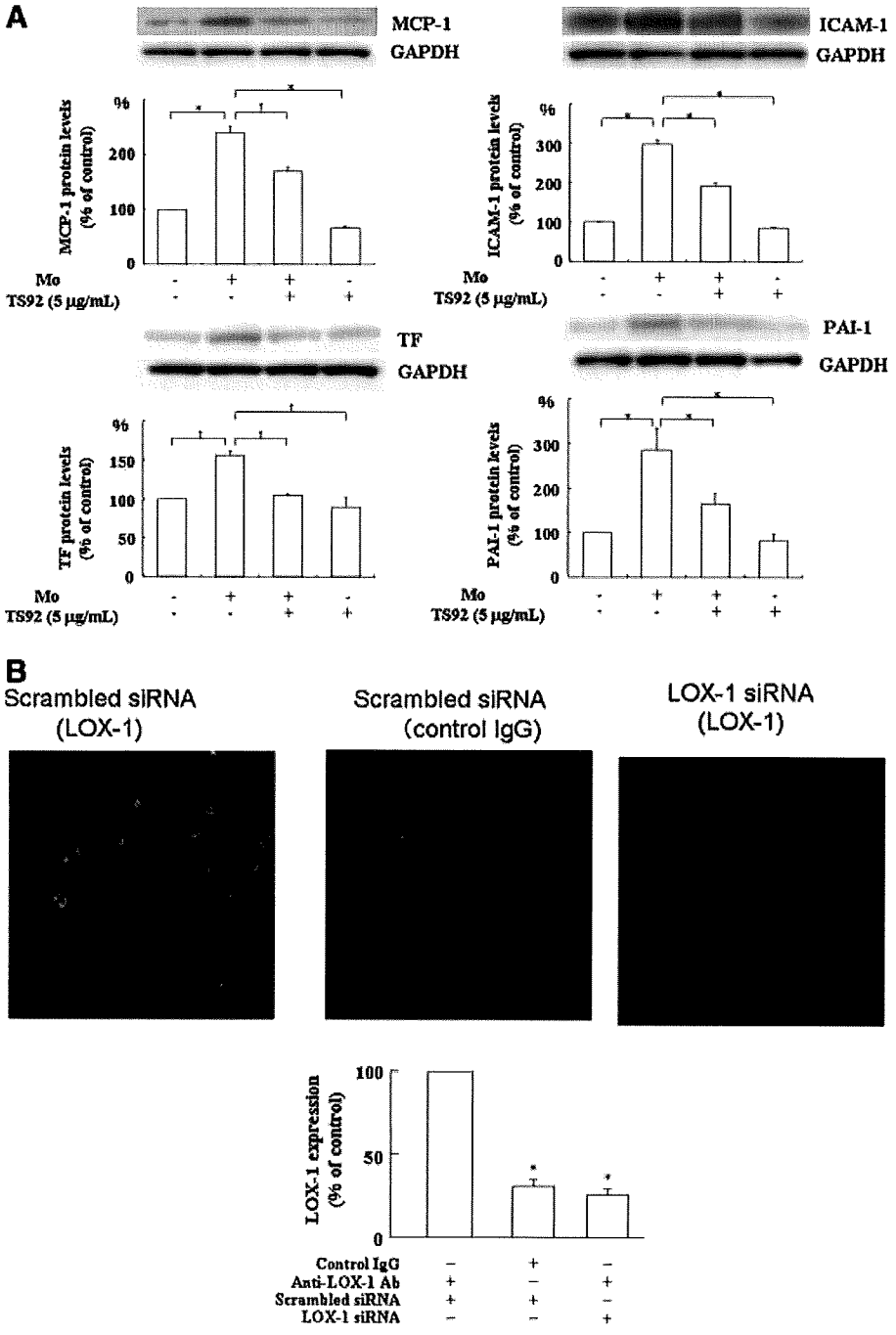

Fig. 3. Effect of inhibition of LOX-1 by TS92 (A) or siRNA (C) on the increased expression of MCP-1, ICAM-1, TF, and PAI-1 induced in ECs by monocyte adhesion. A: ECs were pretreated with TS92 for 1 h before adding monocytes. After 18 h of coculture, Western blotting of MCP-1, ICAM-1, TF, and PAI-1 was performed and the same filters were re-probed with an anti-GAPDH antibody for normalization of cell lysates. Data are expressed as means ± SD of ratios of each expression to GAPDH of densitometric analyses from five separate experiments. Representative immunoblots from five similar experiments are shown at the top. *$P < 0.01$ and †$P < 0.05$. B: Effect of knockdown of LOX-1 by siRNA on LOX-1 protein expression in ECs. LOX-1 protein expression was determined by fluorescent immunohistochemistry and the data were quantitatively analyzed by the ImageJ. *$P < 0.0001$. C: Endothelial LOX-1 was inhibited by siRNA and monocytes were then added. Data are expressed as means ± SD of three independent experiments. Representative immunoblots from three similar experiments are shown. *$P < 0.01$ and †$P < 0.05$.

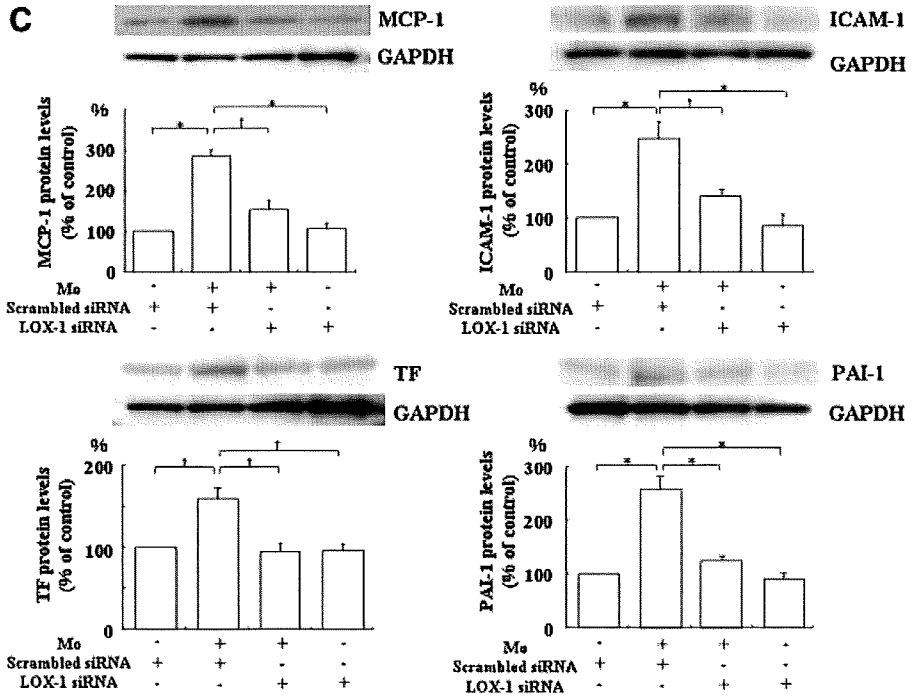

Fig. 3. (*Continued*)

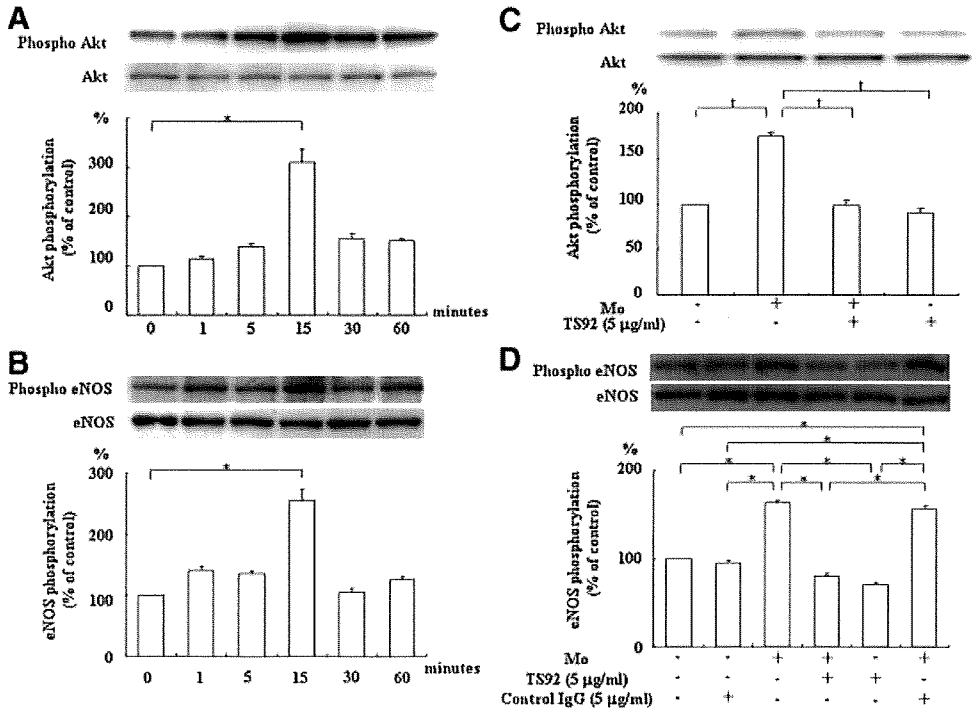

Fig. 4. Role of LOX-1 in monocyte adhesion-triggered Akt/eNOS activation in ECs. Time course of Akt (A) and eNOS (B) phosphorylation induced in ECs by monocyte adhesion up to 60 min after adding monocytes. Nonphosphorylated expression of Akt and eNOS was not significantly altered by monocyte adhesion up to 60 min. Data are expressed as means ± SD of ratios of each phosphorylated expression to nonphosphorylated expression of densitometric analyses from three separate experiments. Representative immunoblots from three similar experiments are shown. Effect of inhibition of LOX-1 by TS92 on increased phosphorylation of Akt (C) and eNOS (D) 15 min after adding monocytes. Data are expressed as means ± SD of ratios of each phosphorylated expression to nonphosphorylated expression of three separate experiments. The same concentration of control with nonimmune IgG had a negligible effect on eNOS phosphorylation and expression in the presence and absence of monocyte adhesion. Representative immunoblots of three similar experiments are shown. *$P < 0.01$ and †$P < 0.05$.

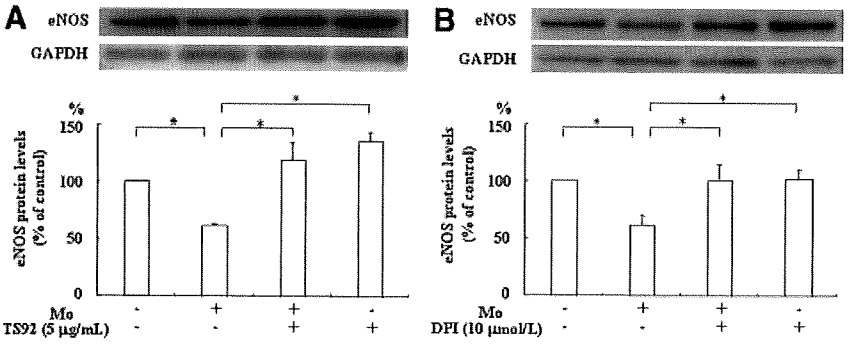

Fig. 5. Effect of inhibition of LOX-1 by TS92 (A) and DPI (B) on monocyte adhesion-triggered downregulation of eNOS protein expression 18 h after adding monocytes. Data of Western blotting are expressed as means ± SD of four separate experiments. *$P < 0.01$.

Interaction between Ca^{2+} and NADPH oxidase/ROS

The finding (Hu et al., 2002) that the inhibition of ROS attenuates Ca^{2+} signaling in ECs prompted us to further examine the cross-talk between Ca^{2+} signaling and ROS generation in monocyte–EC interaction. Pretreatment of ECs with 10 μmol/L BAPTA-AM, a Ca^{2+} chelator, for 30 min indicated that Ca^{2+} chelation attenuated the membrane translocation of Rac1 and $p47^{phox}$ induced in ECs by monocyte adhesion 30 min after adding monocytes (Fig. 8A). Monocyte adhesion-induced ROS generation in ECs was markedly inhibited by pretreatment with BAPTA-AM similar to inhibition of NADPH oxidase by DPI (Fig. 8B). We finally examined the effect of inhibition of NADPH oxidase on monocyte attachment-triggered $[Ca^{2+}]_i$ increase in ECs. Figure 8C shows that inhibition of NADPH oxidase by DPI induced a suppression of monocyte attachment-induced $[Ca^{2+}]_i$ similar to Ca^{2+} chelation by BAPTA-AM in the presence of external Ca^{2+}.

Discussion

The present study shows that monocyte adhesion-triggered NADPH oxidase-dependent, Akt/eNOS and Ca^{2+} signaling pathways are mediated via LOX-1 independent of the oxidized LDL–LOX-1 axis in ECs.

The binding of oxidized LDL to LOX-1 reportedly generates superoxide in ECs, which is canceled by the antibody to LOX-1 (Cominacini et al., 2000). It was also reported that the antibody to LOX-1 suppresses NADPH oxidase-dependent ROS generation induced by remnant lipoprotein particles (RLPs), suggesting that some molecule(s) of RLPs may bind to LOX-1 (Shin et al., 2004). In fact, LOX-1 has been shown to recognize phosphatidylserine and negatively charged phospholipids (Kakutani et al., 2000). In the present study, we found that monocyte adhesion increased Rac1 and $p47^{phox}$ membrane translocation, NADPH oxidase activity, ROS generation, NF-κB phosphorylation, and redox-sensitive molecular expression in the absence of oxidized LDL in ECs, which was blocked by pretreatment of ECs with DPI and TS92. This is, to our knowledge, the first evidence to clearly show that NADPH oxidase-dependent redox signaling pathway through LOX-1 is independent of an oxidized LDL–LOX-1 axis in monocyte–EC interaction. It would be of interest to identify the molecule(s) in monocytes, which activate endothelial LOX-1, and this issue should be extensively investigated.

Li et al. (2001) suggested LOX-1-mediated Akt activation by oxidized LDL in cultured EC. However, little was known about the Akt/eNOS signaling in monocyte–EC interaction. We found that monocyte adhesion-triggered redox signaling pathway in ECs was accompanied by the transient activation of Akt/eNOS via LOX-1 15 min after adding monocytes, followed by normalization with further time intervals. However, NO production of culture medium did not significantly alter when the endothelial phosphorylation of Akt/eNOS was increased 15 min after adding monocytes because generated ROS probably increased NO breakdown (Martindale and Holbrook,

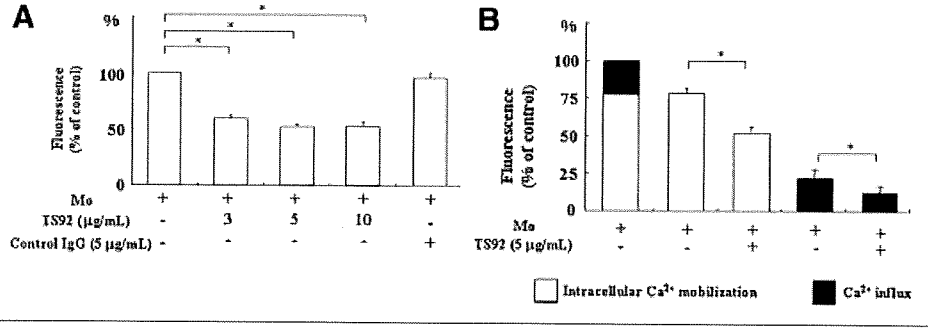

Fig. 6. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in ECs in response to monocyte attachment. A: Effects of various concentrations of TS92 on the $[Ca^{2+}]_i$ increase in ECs caused by monocyte attachment. ECs were pretreated with 3, 5, and 10 μg/ml TS92 for 1 h and then monocytes were added. Antibody isotope control (5 μg/ml) had a negligible effect on $[Ca^{2+}]_i$ induced in ECs by monocyte attachment. B: Effect of inhibition of LOX-1 by TS92 on monocyte attachment-triggered intracellular Ca^{2+} mobilization and Ca^{2+} influx, which are defined in Materials and Methods Section. Data are expressed as means ± SD of % fluorescent values of 100 ECs compared with control in an experiment representative of four similar experiments. *$P < 0.0001$.

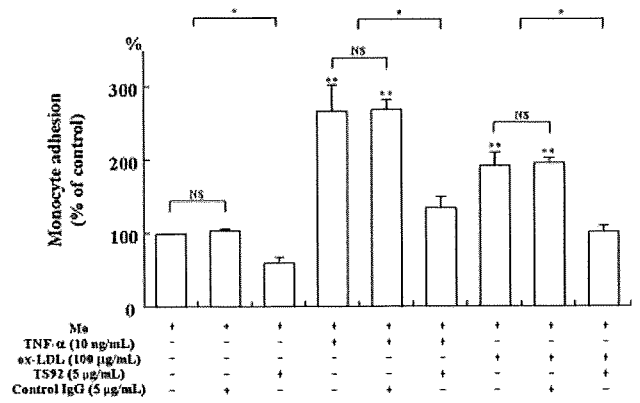

Fig. 7. Effect of inhibition of LOX-1 by TS92 on monocyte adhesion to ECs pretreated with or without 10 ng/ml TNF-α or 100 μg/ml oxidized LDL overnight. ECs were pretreated with TS92 or control antibody at concentrations of 5 μg/ml for 1 h and washed three times before adding monocytes. After 30 min of coculture, adhered monocytes were measured as described in Materials and Methods Section. Data are expressed as means ± SD of an experiment representative of three independent experiments (n = 6, each experiment). *P < 0.01 versus each stimulation and **P < 0.001 versus without stimulation.

2002), suggesting that monocyte adhesion-triggered Akt/eNOS activation via LOX-1 may be preventing action to ROS-mediated reducing NO bioavailability.

The gene expression of ICAM-1 and VCAM-1 has been shown to be regulated by ROS (Cominacini et al., 1997; Min et al., 2005). For our part, we observed that blockade of LOX-1, ICAM-1, and VCAM-1, but not PECAM-1, inhibited NADPH oxidase-dependent ROS generation induced in ECs by monocyte adhesion, as well as monocyte adhesion, suggesting that monocyte adhesion induces redox-sensitive and Ca^{2+} signaling pathways which are equally through LOX-1, ICAM-1, and VCAM-1. The results of our in vitro experiments suggest that any of LOX-1, ICAM-1, and VCAM-1, which are involved in monocyte adhesion, appears to induce identical signaling and that LOX-1 is not the sole mediator signaling upon monocyte adhesion.

Since LOX-1 is constitutively expressed in ECs, one would expect that circulating nonactivated monocytes would be able to bind to endothelium through LOX-1 in physiologic conditions. The present study showed that proinflammatory molecules and compounds such as TNF-α and oxidized LDL markedly enhanced monocyte adhesion to cultured ECs via LOX-1. It was reported that a major part of enhanced adhesiveness in ECs induced by CRP and glucose stimuli is attributable to LOX-1 (Li et al., 2003, 2004), suggesting that LOX-1 plays a role in monocyte adhesion in inflammation and diabetes mellitus. Taken together, the present study suggests

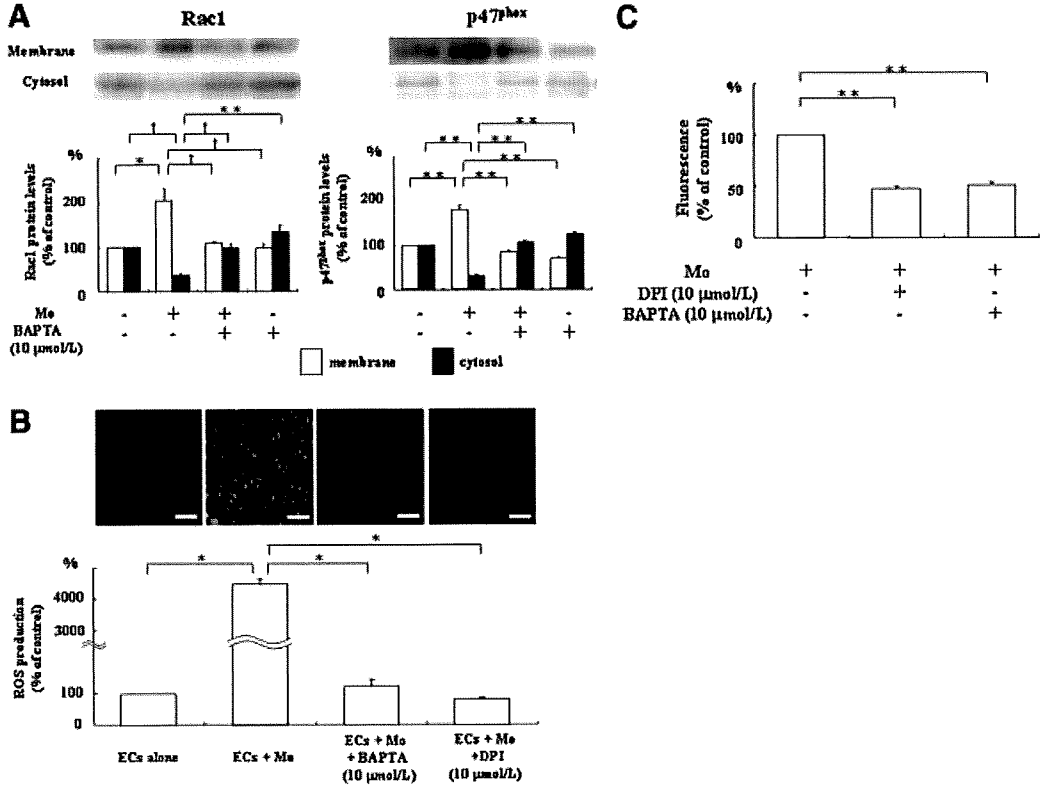

Fig. 8. Cross-talk between Ca^{2+} and NADPH oxidase/ROS in monocyte adhesion to ECs. Effect of Ca^{2+} chelation by 10 μmol/L BAPTA-AM on Rac1 and $p47^{phox}$ activation (A) and ROS generation (B). ECs were pretreated with or without 10 μmol/L BAPTA-AM for 30 min and the activation of Rac1 and $p47^{phox}$ was determined by membrane translocation 30 min after adding monocytes, whereas ROS generation levels were measured 60 min after adding monocytes. A: Rac1 and $p47^{phox}$ levels in membrane and cytosol fractions of ECs. Data are the means ± SD of densitometric analyses of Western blotting from three separate experiments. B: Representative photomicrographs of ROS generation from three similar experiments are shown. Bars are the means ± SD of quantitative analyses from three separate experiments. Scale bars, 100 μm. C: Effect of inhibition of NADPH oxidase by 10 μmol/L DPI on monocyte attachment-induced $[Ca^{2+}]_i$ increase in ECs in the presence of external Ca^{2+}. Data are expressed as means ± SD of % fluorescent values of 100 ECs compared with control in an experiment representative of three similar experiments. *P < 0.001, †P < 0.005, and **P < 0.0001.

that in some proatherogenic situations, nonactivated or activated circulating monocytes can bind to activated ECs via LOX-1.

We have recently shown that BAPTA-AM prevents the activation of NF-κB and the enhancement of TF expression induced in ECs by monocyte adhesion, indicating Ca^{2+} signaling is upstream of NF-κB in monocyte adhesion to ECs (Sakamoto et al., 2005). However, the potential cross-talk between ROS and Ca^{2+} signaling is still poorly understood. NADPH oxidase reportedly affects Ca^{2+} signaling in ECs (Hu et al., 2002). Zimmerman et al. (2005) also reported that NADPH oxidase-derived superoxide modulates angiotensin II-stimulated influx of extracellular Ca^{2+} in neural cells. In contrast, Price et al. (2003) showed that Ca^{2+} signaling regulates the membrane translocation and activation of Rac1 and $p47^{phox}$ in response to certain agonists. These findings suggest the cross-talk between that NADPH oxidase-dependent ROS and Ca^{2+}. In the present study, we found Ca^{2+}-dependent Rac1 and $p47^{phox}$ activation in ECs caused by monocyte adhesion, and also showed that inhibition of ROS generation suppressed monocyte attachment-triggered Ca^{2+} signaling in the same culture system, indicating that Ca^{2+} signaling is both upstream and downstream of NADPH oxidase and ROS. Our study demonstrates the potential cross-talk between Ca^{2+} signaling and NADPH oxidase-dependent ROS in monocyte–EC interaction. This might open new avenues in understanding the pathogenesis of endothelial dysfunction.

Although we showed that monocytes adhesion activated Rac1 and $p47^{phox}$ and generated ROS via LOX-1 in ECs, the issue raised by the present study is that ROS generated in adhered monocytes other than ECs might be responsible for the observed molecular changes in ECs as Cathcart (2004) reported, even if monocytes were extensively washed out. Further study is needed to clarify the role of monocyte-derived ROS in monocyte adhesion-induced endothelial signaling and molecular changes.

Endothelial dysfunction plays a pivotal role in cardiovascular diseases including coronary artery disease, hypertension, stroke, and peripheral artery disease. In the presence or absence of LOX-1 activation by oxidized LDL, angiotensin II, etc., monocyte adhesion induces endothelial responses including ROS generation, reducing NO synthesis, increased permeability, and impaired coagulation, and fibrinolysis, resulting in the acceleration of endothelial dysfunction. Our results demonstrate that monocyte adhesion-triggered redox and Ca^{2+} signaling were induced in ECs independent of oxidized-LDL, and these NADPH oxidase- and Ca^{2+} signaling-dependent NF-κB signaling pathways were inhibited by blocking LOX-1. Our study also showed that, with or without oxidized-LDL, LOX-1 can participate in monocyte adhesion in certain situations, although LOX-1 activation is known to induce ICAM-1 and VCAM-1 on ECs. Therefore, our study has broad implications for its insight into the signaling pathways of endothelial dysfunction caused by monocyte adhesion and highlights LOX-1-mediated signaling pathways as an attractive target of cardiovascular disease.

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Evidence for Increased Methylglyoxal in the Vasculature of Women With Preeclampsia

Role in Upregulation of LOX-1 and Arginase

Sowndramalingam Sankaralingam, Han Xu, Yanyan Jiang, Tatsuya Sawamura, Sandra T. Davidge

Abstract—Preeclampsia is characterized by vascular endothelial dysfunction partly attributed to oxidative stress. In the vasculature of preeclamptic women, we have shown increased lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) and arginase expression, which can contribute to vascular oxidative stress. However, the mechanisms of such upregulation are unknown. Methylglyoxal (MG) that plays a role in the vascular complications of diabetes mellitus and the development of hypertension can be one potential factor that can affect LOX-1 and arginase through its ability to induce oxidative stress in vascular cells. MG also reacts with lysine residues in proteins to generate advanced glycation end product, N^{ε}-carboxy ethyl lysine, which also serves as a marker of MG. We hypothesized that markers of MG formation will be increased in the vasculature of preeclamptic women and that exogenous MG will induce oxidative stress by the upregulation of LOX-1 via arginase. We observed increased N^{ε}-carboxy ethyl lysine expression in the vasculature of women with preeclampsia in comparison with normotensive pregnant women. Moreover, glyoxalase I and II, enzymes that detoxify MG, and glutathione reductase, which generates reduced glutathione, a cofactor for glyoxalase, are also reduced in preeclampsia. In cultured endothelial cells, MG increased arginase expression by 6 hours and LOX-1 expression by 24 hours. Inhibition of arginase or NO synthase significantly reduced MG-induced LOX-1 expression, superoxide levels, and nitrotyrosine staining. In conclusion, MG-induced LOX-1 expression is mediated via arginase upregulation likely because of uncoupling of NO synthase, which may have implications in preeclampsia. **(*Hypertension*. 2009;54:897-904.)**

Key Words: preeclampsia ■ methylglyoxal ■ LOX-1 ■ arginase ■ peroxynitrite ■ endothelium

Preeclampsia is a disorder of pregnancy characterized by hypertension and proteinuria occurring after the 20th week of gestation.[1] A history of hypertension, diabetes mellitus, hyperlipidemia, obesity, renal disease, and extremes of age are some of the risk factors for the development of preeclampsia. Preeclampsia is also characterized by inflammation, oxidative stress, and endothelial dysfunction.[2,3] The maternal vascular endothelial dysfunction is thought to occur because of the actions of factors and molecules released by the placenta into the maternal circulation.[4] Because diabetes mellitus is one of the risk factors for the development of preeclampsia, factors involved in vascular complications of diabetes mellitus may also influence the development and maintenance of vascular abnormalities and possibly hypertension in preeclampsia. One factor involved in the vascular complications of diabetes mellitus is methylglyoxal (MG).[5,6] MG also plays a role in the development of hypertension in spontaneously hypertensive rats.[7,8] Moreover, MG treatment resulted in increased blood pressure in Wistar Kyoto rats.[9] Other than increasing blood pressure, MG induces oxidative stress, resulting in the generation of reactive oxygen species and peroxynitrite in cultured vascular smooth muscle cells.[10,11]

MG is a highly reactive dicarbonyl compound formed by both enzymatic and nonenzymatic processes from glucose, proteins, and amino acids.[12] The enzymatic sources of MG includes not only glucose but also fatty acids and proteins by the action of the enzymes, MG synthase, acetol-mono-oxygenase,[13] and semicarbazide-sensitive amine oxidase (SSAO),[14] respectively. MG is a highly reactive molecule that reacts with various amino acid residues in proteins to form advanced glycation end products (AGEs). Although AGEs can be formed from glyoxal as well, MG is the most reactive[15] and most important precursor for AGEs.[6,16] MG is degraded by the glyoxalase system.[17] Glyoxalase I catalyzes the conversion of hemithioacetyl (an adduct of reduced glutathione [GSH] and MG) into S-D-lactoylglutathione, which is acted on by glyoxalase II to regenerate GSH. Glutathione peroxidase and glutathione reductase (GR) play roles in the formation and regeneration of GSH that serve to detoxify MG.

Received April 25, 2009; first decision May 16, 2009; revision accepted July 22, 2009.

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Hypertension **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.109.135228**

Whether MG is increased in the vasculature of women with preeclampsia and, if so, the mechanisms by which it affects endothelial function are unknown. We have shown previously in the vasculature of women with preeclampsia an enhanced expression of lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1).[18] LOX-1 is responsible for the binding, uptake, and degradation of oxidized low-density lipoprotein (oxLDL).[19] On activation via binding of oxLDL, LOX-1 generates superoxide via NADPH oxidase.[20] Other than LOX-1, increased arginase expression can also result in the generation of reactive oxygen species.[21] Increased arginase expression can deplete L-arginine[22,23] as a substrate, which can result in the decreased synthesis of NO[24] and the increased generation of superoxide by NO synthase (NOS).[25,26] This phenomenon by which NOS generates superoxide because of deficiency of the substrate, L-arginine, or the cofactor tetrahydrobiopterin is known as uncoupling of NOS.[25,26] Thus, overexpression of arginase in endothelial cells can lead to the uncoupling of NOS. Studies have shown increased arginase expression in both the placenta[21] and the maternal vasculature of women preeclampsia[27] and could contribute to decreased NO and enhanced superoxide formation. It is well known that MG can generate reactive oxygen species and peroxynitrite when added to vascular smooth muscle cells.[10] However, it is not known whether MG can generate reactive oxygen species via the arginase/LOX-1 pathway and contribute to endothelial dysfunction and oxidative stress in preeclampsia.

We hypothesize that markers of MG formation will be elevated in the maternal vasculature of women with preeclampsia. Also, MG will upregulate LOX-1 expression and oxidative stress markers via upregulation of arginase in endothelial cells in culture.

Methods

Subjects

Nonpregnant, pregnant, and preeclamptic subjects (6 per group) were recruited for this study at the Royal Alexandra Hospital (Edmonton, Alberta, Canada). Omental fat biopsies were obtained from these subjects during abdominal surgeries or cesarean section and stored at −80°C. For details of the inclusion and exclusion criteria and the subject characteristics (Table S1), please see the online Data Supplement, available at http://hyper.ahajournals.org.

Experimental Protocol

Immunohistochemistry

Our first aim was to assess the expression of AGEs, N^{ε}-carboxy methyl lysine (CML) and N^{ε}-carboxy ethyl lysine (CEL), that also serve as markers of MG in small arteries from nonpregnant, pregnant, and preeclamptic women. Although CML can be formed via a number of reactions, such as oxidative cleavage of Amadori products, Schiff base, auto-oxidation of glucose, and from MG, CEL is formed primarily from MG as its precursor.[28] CEL levels also serve as an index of MG in tissues.[29] We have, therefore, assessed both CML and CEL, which are considered markers of MG formation. The circulating concentrations of MG have been reported to vary from nanomoles per liter[30] to micromoles per liter,[7,31] the variations being attributed to analytic techniques.[17] However, the tissue levels of MG may be higher and also more important, because MG tends to react rapidly and readily with proteins in tissues.[32] We have, therefore, assessed markers of MG in tissues. The arteries in omental fat biopsies were immunostained using rabbit monoclonal antibodies for CML (1:100) and CEL (1:100). Antirabbit secondary antibody (1:200 Alexa Fluor 488, Invitrogen Canada, Inc) was used to detect the primary antibody and was visualized using a fluorescein isothiocyanate filter.

We further assessed the expression of enzymes that are involved in the synthesis and degradation/detoxification of MG. We chose to measure the expression of SSAO, because it is widely studied in relation to MG formation.[14] We also assessed the expressions of glyoxalase I and II, enzymes that detoxify MG. In addition, we compared the expressions of glutathione peroxidase and GR, enzymes that generate GSH, a cofactor necessary for the detoxification of MG. Rabbit antibodies against human SSAO, glyoxalase I and II, as well as glutathione peroxidase and GR, were used at 1:100 dilution. Antirabbit secondary antibody (1:200 Alexa Fluor 488, Invitrogen Canada, Inc) was used to detect the primary antibody and was visualized using a fluorescein isothiocyanate filter.

Cell Culture Studies

The first aim of our cell culture study was to examine the effect of MG on LOX-1 and arginase expression in human umbilical vein endothelial cells. For details on human umbilical vein endothelial cell isolation please see the online Data Supplement.

Human umbilical vein endothelial cells were incubated with MG from 0 to 1000 μmol/L for 24 hours on the basis of previous studies and our own preliminary studies.[10,31] On the basis of our preliminary experiments, a dose of 30 μmol/L was chosen, and further studies were carried out at 6, 12, 18, and 24 hours. Interestingly, the plasma levels of MG in spontaneously hypertensive rats at 13 and 20 weeks are 30 and 33 μmol/L, respectively, thus justifying the dose used.[7]

LOX-1 and arginase expressions were assessed by Western blot, as described previously.[18,27] Arginase activity was determined by measuring urea formation, also as described previously.[33] In some experiments, the arginase inhibitor (*S*)-(2-Boronoethyl)-L-cysteine (BEC) (100 μmol/L), NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L), or peroxynitrite scavenger 5,10,15,20-tetrakis (4-sulfonatophenyl)prophyrinato iron (III), chloride (FeTPPS; 10 μmol/L) were used 30 minutes before the addition of MG to cells in culture.

In response to MG (30 μmol/L for 24 hours), nitrotyrosine, a marker of peroxynitrite, was measured by immunocytochemistry using rabbit polyclonal antinitrotyrosine antibodies. Superoxide generation in live endothelial cells in response to MG (30 μmol/L) was measured at 24 hours using dihydroethidine.[18] To determine a role for arginase as a source of superoxide, cells were exposed to MG in the presence or absence of BEC, an arginase inhibitor, or L-NAME, an NOS inhibitor. Inhibition of superoxide generation by L-NAME, an NOS inhibitor, provides evidence for NOS as a source of superoxide and, therefore, suggests uncoupling of NOS.[34] In addition, 3,3′-dioctadecylindocarbocyanine–labeled oxLDL uptake in response to MG in the presence or absence of BEC or L-NAME was performed as described previously.[18]

Statistical Analysis

Values are expressed as mean±SEM. Comparison of ≥3 groups was done using a 1-way ANOVA followed by a Tukey post hoc test. A $P<0.05$ was deemed significant.

Results

Arterial Expression of CEL, CML, and SSAO

We assessed the arterial expression of CEL and CML. Arteries from women with preeclampsia show significantly increased fluorescent staining for CEL and CML (26.7±3.5 and 22.6±3.1 arbitrary units; $P<0.01$), respectively, primarily in the endothelial layer when compared with that of nonpregnant (3.9±1.4 and 5.7±1.1 arbitrary units) and pregnant women (4.3±1.5 and 5.1±1.2 arbitrary units; Figure 1).

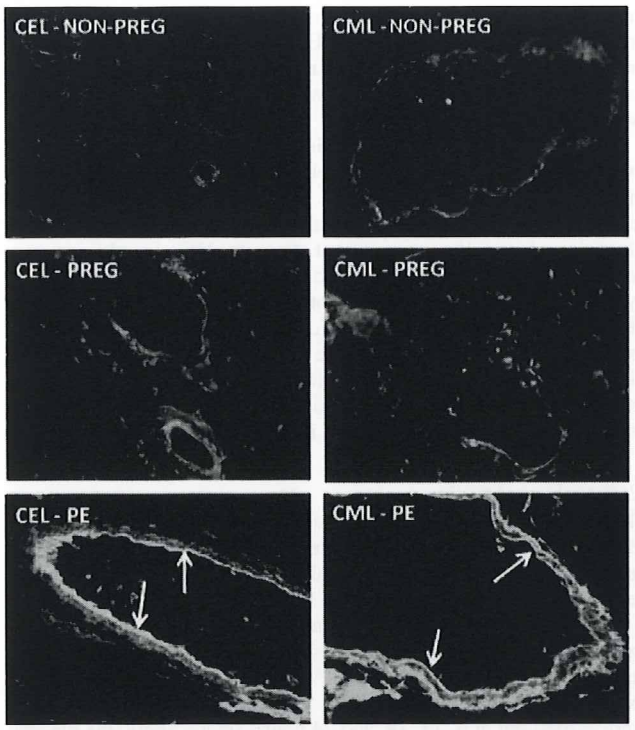

Figure 1. Evidence for MG formation in preeclampsia. Representative images for CEL and CML staining (arrows) in the vasculature of nonpregnant (NON-PREG), pregnant (PREG), and preeclamptic (PE) women. Nuclei are shown in blue, whereas CEL and CML are shown in green. The images shown are magnified ×100.

We also compared the expression of SSAO, an enzyme that generates MG. Interestingly, we observed an abundance of SSAO in arteries from women with preeclampsia when compared with arteries from nonpregnant and pregnant women (Figure 2).

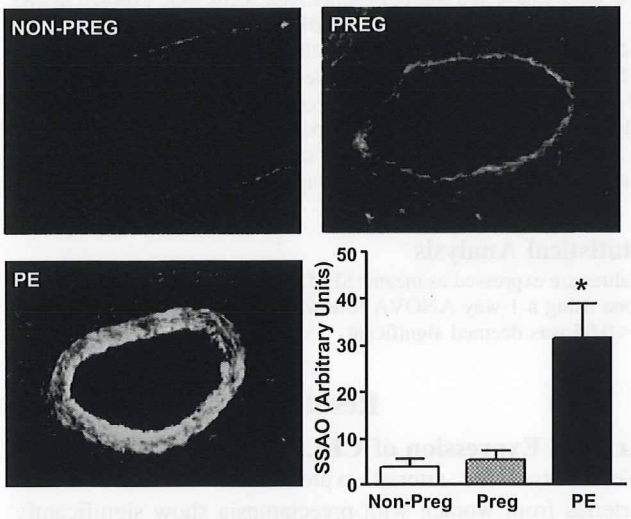

Figure 2. SSAO expression in maternal vasculature. Representative images (×100) show SSAO expression in the vasculature of nonpregnant (NON-PREG), pregnant (PREG), and preeclamptic (PE) women. Nuclei are shown in blue and staining for SSAO is shown in green. The summary graph shows mean SSAO expression from 6 subjects per group. *$P<0.05$.

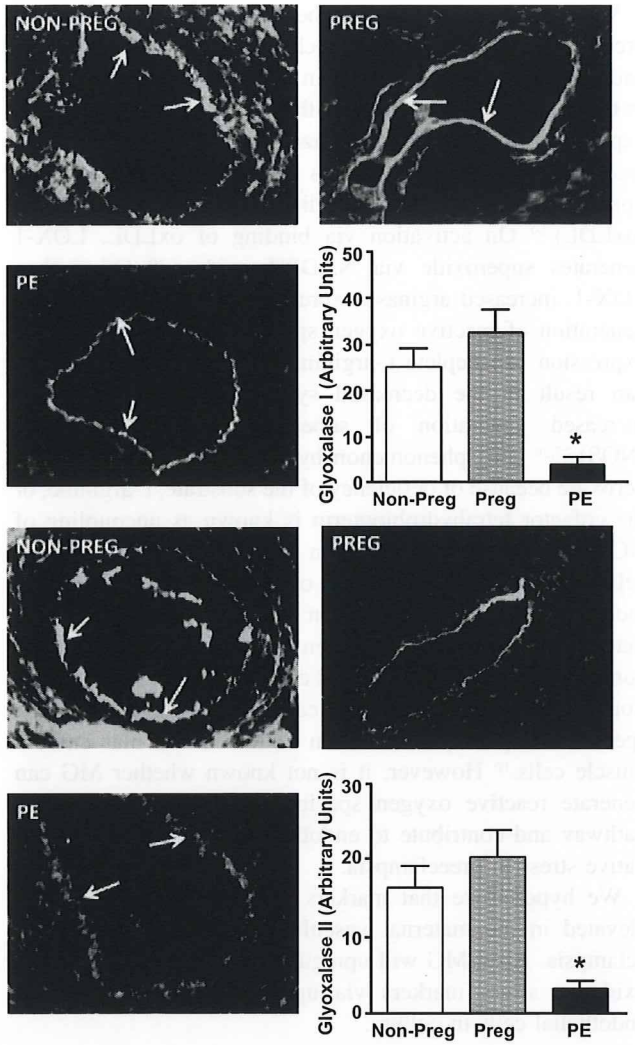

Figure 3. Glyoxalase I and II expression in maternal vasculature. Representative figures (×100) for glyoxalase I (top) and II (bottom) expression in arteries from nonpregnant (NON-PREG), pregnant (PREG), and preeclamptic (PE) women. Nuclei are shown in blue and staining for glyoxalase I and II are shown in green. A summary graph from 6 subjects per group is shown. *$P<0.05$.

Arterial Expression of Glyoxalase I and II, GR, and Glutathione Peroxidase

We observed a significant decrease in the expression of glyoxalase I and II, the detoxifying enzymes in the vasculature of women with preeclampsia, when compared with arteries from normotensive pregnant women (Figure 3). Interestingly, GR that regenerates GSH, a cofactor necessary for the detoxification of MG, is reduced, whereas the expression of glutathione peroxidase, the enzyme that depletes GSH, is increased in the preeclamptic vasculature (Figure 4). These findings further strengthen the evidence for increased MG levels in the vasculature of women with preeclampsia.

Endothelial Response to MG

Effect of MG on LOX-1 Expression

Endothelial cells treated with MG (0 to 1000 μmol/L) for 24 hours induced LOX-1 expression (≈100%, $P<0.05$) that was

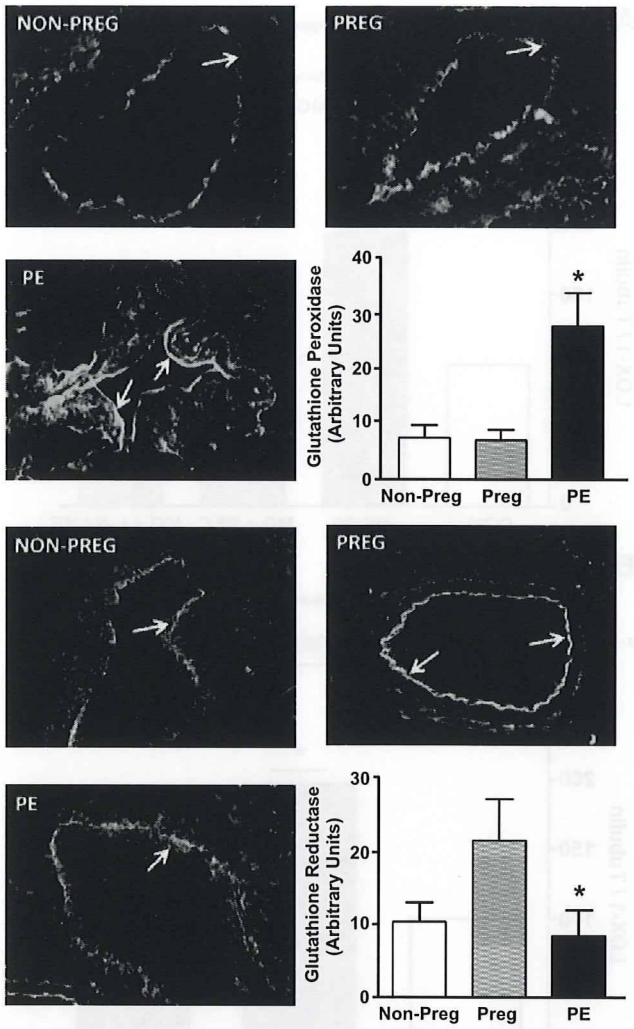

Figure 4. Glutathione peroxidase and GR expression in maternal vasculature. Representative figures (×100) for glutathione peroxidase (top) and GR (bottom) expression in arteries from nonpregnant (NON-PREG), pregnant (PREG), and preeclamptic (PE) women. Nuclei are shown in blue and staining for glutathione peroxidase and GR are shown in green. A summary graph from 6 subjects per group is shown. *$P<0.05$.

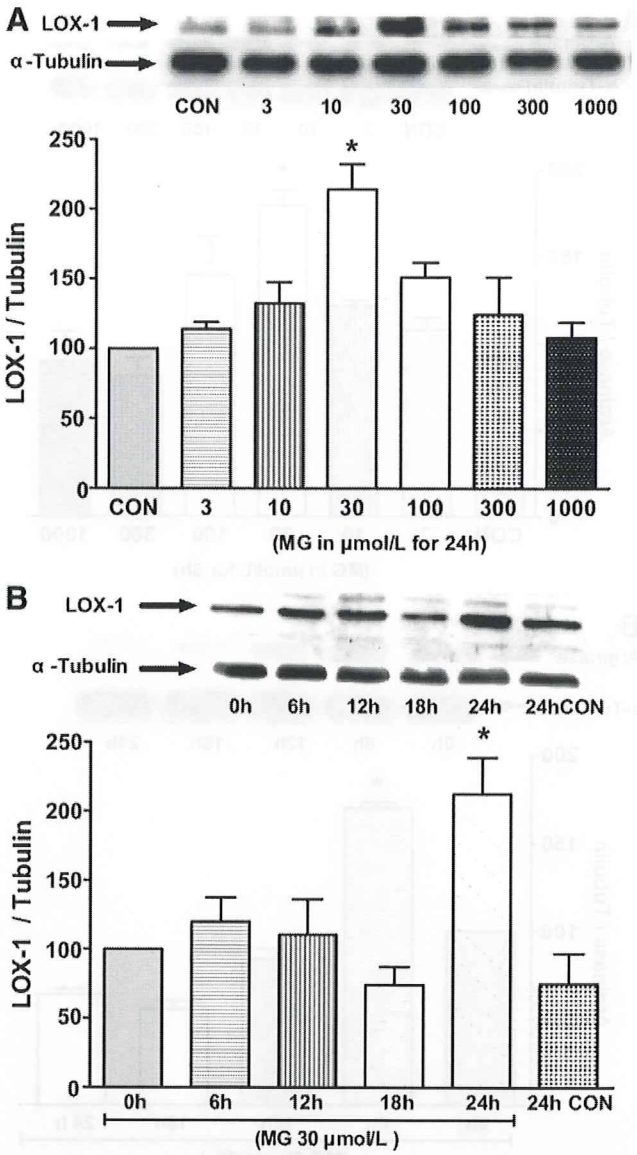

Figure 5. Effect of MG on endothelial LOX-1 expression. Representative Western blot shows increased LOX-1 expression in endothelial cells (A) treated with MG (0 to 1000 μmol/L) for 24 hours and (B) treated with 30 μmol/L of MG for 24 hours. LOX-1 expression was normalized to α-tubulin. Graphs shown are a summary of 5 independent experiments. *$P<0.05$.

maximal at a dose of 30 μmol/L (Figure 5A). In the next set of experiments, MG (30 μmol/L) induced ≈100% of the LOX-1 expression that was maximal at 24 hours (Figure 5B). Thus, MG increased LOX-1 expression in a dose- and time-dependent manner.

Effect of MG on Arginase Expression and Activity

Endothelial cells treated with MG (0 to 1000 μmol/L) for 6 hours showed significantly increased arginase II expression (≈70%; $P<0.05$) that was maximal at a dose of 30 μmol/L (Figure 6A). Interestingly, endothelial cells treated with 30 μmol/L of MG showed maximal expression as early as 6 hours (Figure 6B). Also, endothelial cells treated with MG (0 to 30 μmol/L) for 6 hours showed significantly increased arginase activity (0.053±0.020 to 0.133±0.020 U/mg of protein; $P<0.05$) in a dose-dependent manner that was maximal at 30 μmol/L.

Effect of Arginase and NOS Inhibition on MG-Induced Oxidative Stress

MG (30 μmol/L) treatment for 24 hours resulted in an increased generation of superoxide compared with untreated cells (62.3±5.7 versus 25.7±3.5; $P<0.01$) that was significantly reduced to similar levels after arginase (32.3±3.5) or NOS inhibition (29.4±2.9). In parallel to increased superoxide levels, nitrotyrosine staining was also increased by MG (30 μmol/L) treatment for 24 hours (78.28±4.90 versus 17.34±2.00; $P<0.01$) that was also reduced after arginase (38.13±3.70) and NOS inhibition (3.20±0.60).

Effect of Arginase and NOS Inhibition on MG-Induced LOX-1 Expression and 3,3'-Dioctadecylindocarbocyanine–Labeled oxLDL Uptake

We wanted to determine whether arginase upregulation and, therefore, the subsequent generation of oxidative stress by