CRP-LOX-1 INTERACTION ASSAY BY ELISA

Recombinant human LOX-1 (0.1 μg) or BSA (0.1 μg, Sigma) was immobilized to each well of 384-well plates (Maxisorp, Nunc) by incubation overnight at 4 °C in PBS. After 2 washes with PBS, the plates were blocked with 80 μL of 20% ImmunoBolock (DS Pharma)/PBS at 4 °C for 8 h. After 2 washes with PBS, CRP in the reaction buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 1% BSA, pH 7.0) was added to each well, and incubated at 4 °C overnight. The plates were then washed 3 times with PBS and incubated for 2 h with horseradish peroxidase–conjugated antihuman CRP antibody (1:5000) (Bethyl) in PBS, 1% BSA. After 5 washes with PBS, peroxidase activity was determined with a TMB Peroxidase EIA Substrate kit (Bio-Rad). For the analyses of the binding of heat-denatured CRP, CRP solution in PBS was heated in boiling water for 5 min before use. The immunoreactivity of the denatured CRP to the anti-CRP antibody was examined by ELISA of immobilized CRP. The indicated amounts of CRP, denatured CRP, or BSA were immobilized, and blocking was performed as above. Then, the immobilized proteins were detected by the horseradish peroxidase–conjugated antihuman CRP antibody as above.

BIACORE ANALYSES

The K_d value of the CRP binding to LOX-1 was measured by surface plasmon resonance on a BIACORE 2000 (GE Healthcare). Recombinant LOX-1 was immobilized on a research-grade CM5 sensor chip (GE Healthcare) by use of an Amine coupling kit (GE Healthcare) according to the manufacturer's instructions. BSA was immobilized on a sensor chip by the same method described above, and used as reference. We then injected 30 μL of the analytes (CRP 0.45, 0.89, 1.78, and 3.56 μmol/L) in 10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 1% BSA (pH 7.0) at a flow rate of 20 μL/min for 120 s, and dissociation in the same buffer was monitored for 120 s. After each analysis, the sensor chip was regenerated with 5 μL of 50 mmol/L NaOH at a flow late of 60 μL/min. We calculated K_d values by using a 1:1 Langmuir binding model with BIAevaluation software version 3.0 (GE Healthcare).

RNA INTERFERENCE

We designed small interfering RNA (siRNA) duplex oligoribonucleotides targeting the bovine LOX-1 coding region (Genbank NM174132) by using the Block-iT RNAi (RNA interference) designer program from the Invitrogen website. The stealth RNAi negative control duplex (Invitrogen) was used as a negative control. The siRNA sequences are as follows: si1 for LOX-1 (204–228), 5′-UUCUUUAUGAGAUCAGAGACCUGGG-3′ and si2 for LOX-1 (396–420), 5′-ACUUCUUGGAGAUUCAGGUUCUGGC-3′.

Bovine aortic endothelial cells (BAEC) and bLOX-1-CHO (Chinese hamster ovary cells stably expressing bovine LOX-1) *(15)* were maintained with DMEM (Gibco)/10% fetal bovine serum/1% (vol/vol) Antibiotic-Antimycotic (Gibco) and Ham's F-12 medium/10% fetal bovine serum containing 10 mg/L blasticidin (Kaken Pharmaceutical), respectively. The cells were seeded 1 day before transfection. The following day, the cells at 50%–60% confluency were transfected with siRNA oligos or the control siRNA by use of Lipofectamin RNAiMAX tansfection reagent (Invitrogen) according to the manufacture's instructions. After incubation at 37 °C for 24 h, the effects of downregulation of expression of the LOX-1 gene [oxidized low density lipoprotein (lectin-like) receptor 1] were examined. Suppression of LOX-1 expression was confirmed by the immunostaining with anti–LOX-1 antibody (TS20) combined with Alexa488-antimouse IgG (1:2000) (Invitrogen) and quantitative analysis with the IN Cell analyzer. All transfections were performed in triplicate.

ANIMALS

All protocols were approved by the Institutional Animal Care and Use Committee of the National Cardiovascular Center. Male Wistar Kyoto (WKY) rats and stroke-prone spontaneously hypertensive (SHR-SP) rats (SLC) were used at 8 weeks of age for experiments.

IMMUNOHISTOCHEMICAL ANALYSIS

For the analysis by confocal laser microscope, rat dermal tissue was snap-frozen in liquid nitrogen and sectioned at 10 μm with a cryostat (Leica). The sections were fixed by 4% formaldehyde for 15 min, blocked with 20% Blockace (Snow brand) for 1 h at room temperature, and then stained with 2 mg/L Alexa633 anti–LOX-1 antibody (TS20) or 0.2 mg/L R-phycoeythrin–conjugated anti-rat CD31 antibody (TLD-3A12, BD). Labeling of the anti–LOX-1 antibody was performed with an Alexa Fluor 633 protein-labeling kit (Invitrogen) according to the manufacturer's instructions.

FLUORESCENT MILES PERMEABILITY ASSAY

The rats were anesthetized with Nembutal (50 mg/kg body weight, administered intraperitoneally), and warmed on the thermal control plate set at 37 °C (HI1220, Leica). Hairs in the dorsal skin region were shaved for fluorescence analyses. CRP, BSA, or vascular endothelial growth factor (Sigma) dissolved in 10 μL of PBS were intradermally injected to the shaved lumbar area of dorsal skin. In some experiments, anti–LOX-1 antibody (TS20, 3 μg) *(22)* or nonimmune mouse IgG (3 μg, Sigma) was coinjected with CRP. Thirty

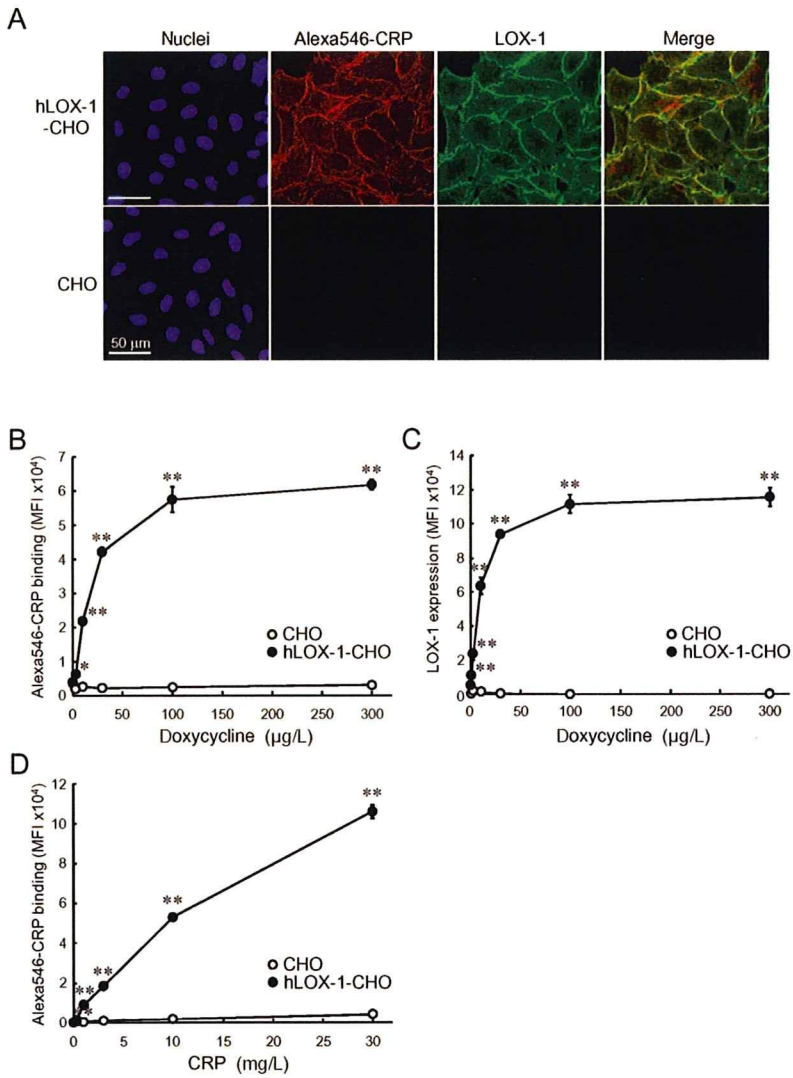

Fig. 1. Fluorescently labeled CRP binds to LOX-1.

(A) Analysis of hLOX-1-CHO (Chinese hamster ovary cell expressing human LOX-1 driven by tetracycline-inducible promoter) (*upper panels*) and control CHO cells (*lower panels*) stained with anti–LOX-1 antibody (*green*) or incubated with Alexa546-CRP (10 mg/L, *red*) by laser confocal microscopy. The nuclei of the cells were counterstained with DAPI (*blue*). The merged image of Alexa546-CRP and LOX-1 indicates colocalization of these molecules on the cell surface. (B, C) Alexa546-CRP (10 mg/L) binding (B) to hLOX-1-CHO in response to the induction of the expression of LOX-1 (C) by increasing dose of doxycycline (0–300 mg/L). (D) Dose-dependent binding of Alexa546-CRP (0–30 mg/L) to hLOX-1-CHO that were pretreated with 100 μg/L of doxycycline. hLOX-1-CHO or control CHO cells were pretreated with indicated concentration of doxycycline to induce the expression of human LOX-1. Then, cells were incubated with Alexa546-CRP for 1h at 4 °C. The binding of Alexa546-CRP and the expression of the introduced protein were analyzed by IN Cell analyzer. The asterisks indicate significant difference vs CHO ($*P < 0.05$, $**P < 0.01$). MFI, mean fluorescence intensity.

minutes later, 2% Evans blue (Wako) in saline (Otsuka Pharma) was injected via the tail vein at a dose of 20 mg/kg weight of the rats. Vascular permeability was assessed by the exudation of Evans blue into the animal dorsal skin, detected with the Maestro Imaging System (CRi). A bandpass filter from 575–605 nm was used for excitation, and the fluorescence intensity at 680 nm was measured for quantitative analysis. The fluores-

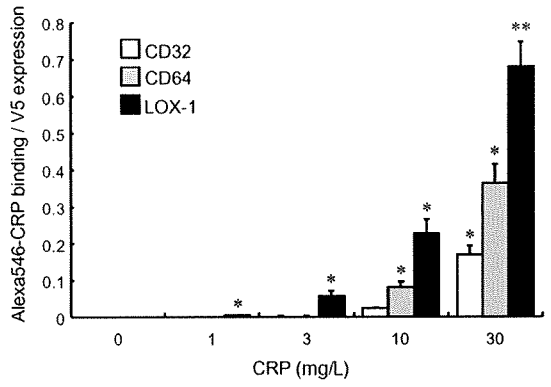

Fig. 2. Quantitative analyses of Alexa 546-CRP binding to COS7 cells expressing LOX-1 and Fcγ receptors CD32 and CD64.

cDNAs encoding human LOX-1 (Genbank NM002543), CD32 (Genbank NM021642), and CD64 (Genbank NM000566) subcloned into the pcDNA6.2/V5/GW/D-TOPO expression vector (V5) (Invitrogen) were transfected into COS7 cells. The binding of Alexa546-CRP and the expression of the introduced protein were analyzed by IN Cell analyzer. pcDNA3.1/V5-His/lacZ (Invitrogen) was used as a negative control. Signals observed in the cells transfected with pcDNA3.1/V5-His/lacZ were considered as nonspecific background. The asterisks indicate significant difference vs 0 mg/L of CRP (*$P < 0.05$, **$P < 0.01$).

cence of the uninjected area in the shaved dorsal skin was assumed as background, and the integral of the fluorescence intensity of the leaked dye minus the background value was calculated by use of Image J software (NIH). The fluorescence was measured at 3 h after the injection of CRP. To minimize individual variation among the animals, the effects of CRP on vascular permeability were expressed relative to that of 1 ng vascular endothelial growth factor.

STATISTICAL ANALYSIS

All data are presented as mean (SE). Statistical analyses were performed with the Student *t*-test. *P* values <0.05 were considered to be statistically significant.

Results

We examined the binding of Alexa Fluor 546-labeled CRP (Alexa546-CRP) to a LOX-1–expressing cell line. To ensure the specificity of the binding, we used hLOX-1-CHO (Chinese hamster ovary cell expressing human LOX-1 driven by tetracycline-inducible promoter). After treatment with 100 μg/L of doxycycline, hLOX-1-CHO clearly bound to Alexa546-CRP, whereas control CHO cells did not (Fig. 1A). Analysis by confocal laser microscope showed LOX-1 protein and Alexa546-CRP colocalized on the cell surface (Fig. 1A). The binding of Alexa546-CRP increased in parallel with the expression of LOX-1 in hLOX-1-CHO depending on the dose of doxycycline (0–300 μg/L), whereas Alexa546-CRP did not significantly bind to control CHO cells regardless of doxycycline dose (Fig. 1B, C). After treatment with 100 μg/L of doxycycline, Alexa546-CRP bound to hLOX-1-CHO cells in a dose-dependent manner (0–30 mg/L), whereas control CHO cells did not show significant binding even at an Alexa546-CRP concentration of 30 mg/L of (Fig. 1D). Significant binding of Alexa546-CRP was clearly observed at concentrations as low as 0.3 mg/L.

We then used the transient expression system in COS7 cells to compare LOX-1 with the known receptors for CRP, CD32, and CD64. Expression level of the transfected cDNA was monitored by V5-tag fused to the C-terminus of each protein. Alexa546-CRP binding to CD32- and CD64-transfected cells was detected as reported *(13, 14)* at concentrations of 10 and 30 mg/L of Alexa546-CRP. Significant binding of Alexa546-CRP was again observed in COS7 cells transiently expressing LOX-1 from the concentration of 1 mg/L (Fig. 2).

We further characterized CRP–LOX-1 interaction in a cell-free system, to eliminate the possibility that the observed CRP binding might be due to indirect effects of LOX-1 expression. In this system, the recombinant extracellular domain of LOX-1 prepared with a mammalian cell line was coated to an ELISA plate, and CRP binding was detected by anti-CRP antibody. Therefore, with this sandwich ELISA, the possibility of artifactual detection of the binding of non-CRP proteins is lower. CRP bound to LOX-1 protein in a dose-dependent manner (0.3–30 mg/L) (Fig. 3A). Similar results were obtained by using another anti-CRP antibody for detection (data not shown). CRP preparations from 2 different commercial sources also showed significant binding to LOX-1 (data not shown), indicating that the CRP binding is independent of its type of preparation. The CRP that was heat denatured in boiling water lost its binding ability to LOX-1, although it was still recognized by anti-CRP antibody (Fig. 3A, B). All the preparations of CRP showed a single band in SDS-PAGE (data not shown). Supplementation of IgG up to 1 g/L did not affect the binding of CRP at the concentration of 3 mg/L (data not shown). Significant binding of CRP was clearly observed at 1 mg/L in the case of the Alexa546-CRP binding to LOX-1 expressed on the cells. To analyze the kinetics of CRP binding to LOX-1, we used a surface plasmon resonance detection system (Biacore). We observed specific binding of CRP to the

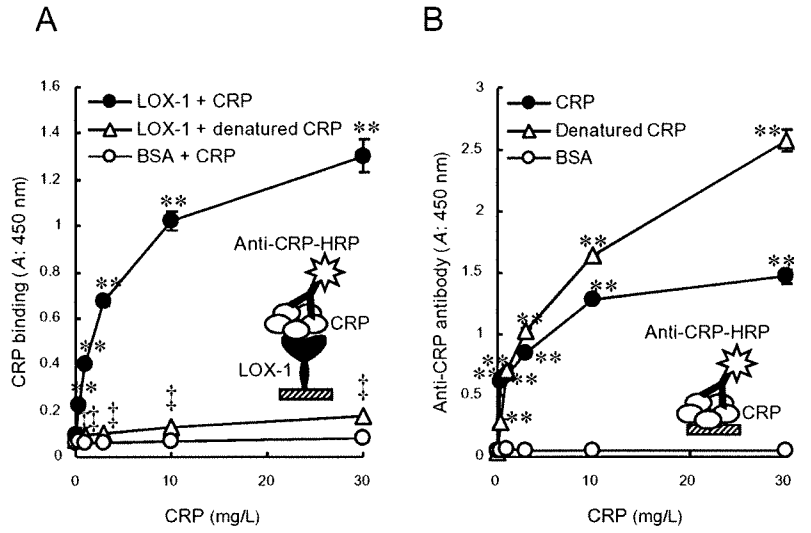

Fig. 3. (A) Interaction of CRP with recombinant human LOX-1 or BSA precoated on an ELISA plate.

CRP bound to recombinant human LOX-1 dose-dependently (closed circle) but not to BSA (open circle). Denatured CRP heated at 100 °C for 5 min showed minimal binding to LOX-1 (open triangle). Data are expressed as mean (SE) (n = 3). The asterisks and daggers indicate significant differences (*$P < 0.05$, **$P < 0.01$ vs BSA; ‡$P < 0.01$ vs CRP). (B) Recognition of native (closed circle) and heat-denatured (open triangle) CRP by anti-CRP antibody that is used for the analyses of LOX-1-CRP interaction. Anti-CRP antibody reacted with both native and heat-denatured CRP, but not BSA (open circle). Data are mean (SE) (n = 3). The asterisks indicate significant differences vs BSA (**$P < 0.01$). HRP, horseradish peroxidase.

LOX-1–coated flow cell. With this system, K_d was calculated to be 1.6×10^{-7} mol/L.

To examine whether LOX-1 works as the receptor for CRP in native cells, we then examined BAEC, in which LOX-1 is expressed at a high level *(15)*. We designed 2 different siRNAs for bovine LOX-1, which suppressed bovine LOX-1 expression in CHO cells and the binding of Alexa546-CRP (Fig. 4A). In BAEC, the siRNAs suppressed the binding of Alexa546-CRP to the cells more than 50% (Fig. 4B). The residual binding of Alexa546-CRP might be due to its binding to other receptors for CRP such as CD64 and CD32 or anionic phospholipids exposed on the surface of activated/dead cells.

Then, we investigated whether or not LOX-1 mediates the in vivo effects of exogenous CRP. It has been reported that administration of human CRP to rats yields an appropriate model for the analyses of the actions of human CRP, because human CRP activates both rat and human complement systems whereas endogenous rat CRP does not *(8)*. Therefore, we injected human CRP intradermally into rats and analyzed the changes in vascular permeability at the site of the injection by monitoring the exudation of Evans blue dye. Because the expression of LOX-1 in SHR-SP rats is much higher than in normotensive WKY rats in CD31-positive endothelial cells and interstitial cells (Fig. 5A), we compared the responses to CRP in these strains. We observed that 1 and 5 ng of vascular endothelial growth factor increased vascular permeability observed by Evans blue dye exudation. In good agreement with the higher expression of LOX-1 in SHR-SP rats, SHR-SP rats showed significantly larger responses of Evans blue dye exudation around the sites of CRP injection compared with WKY rats at doses of 1 and 3 μg (Fig. 5B). In SHR-SP rats intradermal injection of CRP dose-dependently induced the exudation of Evans blue dye, and coinjection of anti–LOX-1 antibody with CRP significantly suppressed the increase in vascular permeability (Fig. 5C). These results suggest that LOX-1 not only binds to CRP but also plays a key role in the vascular mechanisms of CRP-induced pathophysiology.

Discussion

In the present study we identified LOX-1 as a novel receptor for CRP. Fcγ receptors, CD64 and CD32, are also known receptors for CRP. In our experiments CRP bound to LOX-1, but also bound to those Fcγ receptors.

Expression patterns of LOX-1 and Fcγ receptors (*a*) are controlled in different manners, (*b*) exhibit different histological distributions, and (*c*) have different

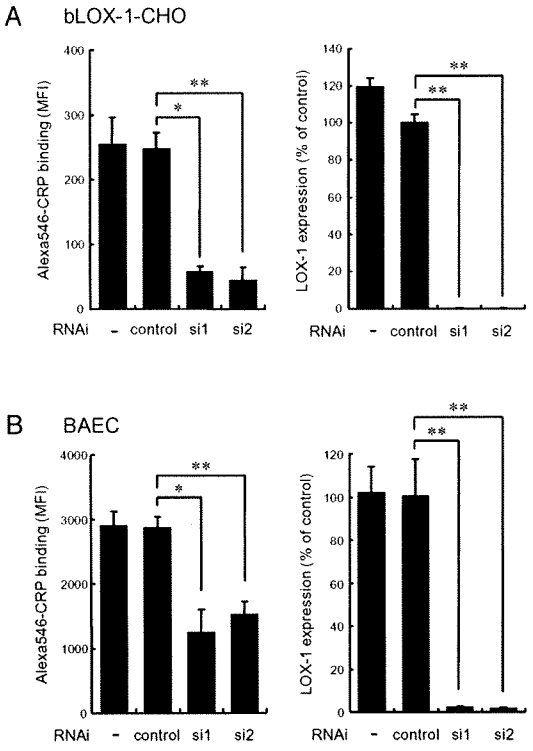

Fig. 4. Binding of Alexa546-CRP to bLOX-1-CHO (Chinese hamster ovary cells stably expressing bovine LOX-1) (A, *left*) and BAEC (B, *left*) were suppressed by siRNA against bovine LOX-1.

bLOX-1-CHO and BAEC were pretreated with 2 different siRNAs specific to bovine LOX-1 (si1 and si2), or with negative control siRNA (–Control) for 24 h. Then, cells were incubated with Alexa546-CRP (3 mg/L) for 1 h at 4 °C. The binding of Alexa546-CRP was quantified by IN Cell analyzer. Suppression of the expression of LOX-1 was confirmed in bLOX-1-CHO (A, *right*) and BAEC (B, *right*) by immunostaining of LOX-1 protein quantified by IN Cell analyzer. The asterisks indicate significant differences between the indicated groups ($*P < 0.05$, $**P < 0.01$). RNAi, RNA interference; MFI, mean fluorescence intensity.

original functions. There have been numerous reports that the biological activity of CRP in vitro, except for its complement activation, is mediated by Fcγ receptors *(12)*. One reported study, however, showed that CRP did not bind to cells that express Fcγ receptors unless IgG was added to medium *(31)*. It is also known that, in the presence of excess amounts of IgG or serum, CRP binding to Fcγ receptors is suppressed. These previous in vitro data indicate that the interaction of CRP and Fcγ receptors might be modulated by environmental IgG.

Because the gene for LOX-1 is an immediate early gene, LOX-1 expression is highly induced by various stimuli *(25)*. Within endothelial cells such stimuli include oxidized LDL and various inflammatory cytokines. In proatherogenic states such as hypertension, hyperlipidemia, and diabetes, LOX-1 expression soars *(25, 32)*. In cardiomyocytes, LOX-1 expression is enhanced by ischemia-reperfusion. On the other hand, Fcγ receptors are expressed constitutively with a distinct expression pattern, particularly in leukocytes, and there are reports that they also function in vascular endothelial cells and vascular smooth cells. Considering this difference in expression pattern, it can be assumed that LOX-1 plays a key role particularly at an acute phase of pathological progression. It can also be assumed that compared to Fcγ receptors LOX-1 functions strongly at the lumen of endothelial cells, where it is in direct contact with blood and therefore with abundant IgG.

We originally found and identified the LOX-1 molecule as the receptor for oxidized LDL *(15)*. FcγRII was been reported to bind to oxidized LDL *(33)*. Therefore a reasonable course of deduction suggests that both LOX-1 and Fcγ receptors bind to CRP, considering the possibility of structural similarity in their ligand-recognizing regions.

Furthermore, it is known that CRP also binds to oxidized LDL *(4)*. Both CRP and LOX-1 bind to such molecules as anionic phospholipids, apoptotic cells, and bacteria *(3, 21, 24, 34)*, although no common structural basis of LOX-1 ligands including CRP is known. LOX-1 has also been reported to be involved in the process of antigen cross-presentation in dendritic cells *(35)*. These observations suggest that LOX-1 and CRP may play significant roles together in innate immunity. The reason that oxidized LDL is well recognized by these molecules could be a result of their response as stimulated by the innate immune system reaction to oxidized LDL as a foreign body stemming from endogenous tissues.

Until recently, the biological function of CRP other than its role in complement activation has often been attributed to the influence of some impurity mixed in the solution *(11, 36)*. To avoid any such influence we made sure to use a CRP solution that gave a single band in SDS-PAGE and had a lipopolysaccharide level below the detectable limit. Human CRP was purchased from 3 different sources, and all 3 solutions regardless of the manufacturer demonstrated binding to LOX-1. The possibility of a minute amount of impure ingredient existing in the solution that affected the CRP binding cannot be totally excluded, but regardless of the influence of impurities, our results from using the cell-free system demonstrated that LOX-1 and CRP form a complex. It is important to assess the interaction between 2 very pure substances in a system that introduces no other impurities.

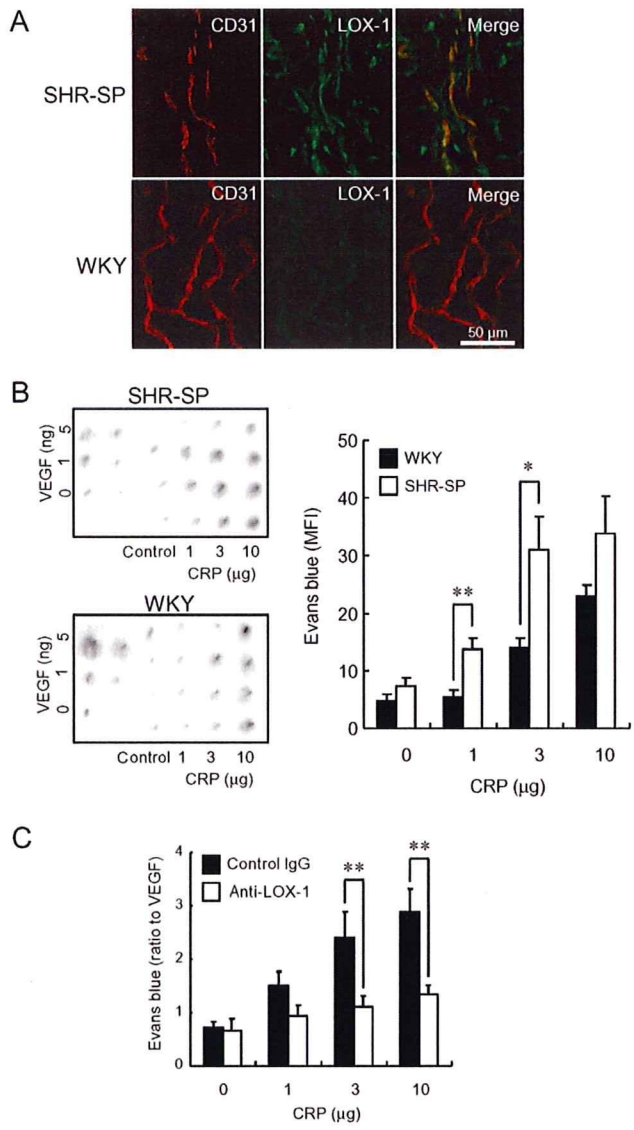

Fig. 5. CRP enhanced vascular permeability via LOX-1 in vivo.

(A) LOX-1 expression (*green*) in dermal blood vessels of SHR-SP (upper) and WKY rats (lower). Blood vessels were visualized by the staining of an endothelial marker CD31 (*red*). Higher expression of LOX-1 was observed in blood vessels and in nonvascular cells of SHR-SP compared with WKY rats. (B) Extravasation of Evans blue induced by intradermal injection of CRP (1, 3, and 10 μg) in SHR-SP (*upper left*) and WKY rats (*lower left*) 3 h after the injection. For negative control, 10 μg of BSA was injected. For positive control, 1 and 5 ng of vascular endothelial growth factor (VEGF) was injected. Evans blue was detected by its fluorescence by se of a Maestro Imaging System (CRi). Semiquantitative analysis of the exudation of Evans blue dye (*right*). Data are expressed as mean (SE) of 8 rats each injected at 4 points for each dose of CRP. The asterisks indicate significant differences between SHR-SP and WKY rats ($*P < 0.05$; $**P < 0.01$). (C) Effects of anti-LOX-1 antibody on CRP-induced enhancement of vascular permeability. CRP was coinjected with 3 μg of anti-LOX-1 antibody (IgG) or control mouse IgG intradermally to lumbar area in dorsal skin of SHR-SP rats. Fluorescence of the Evans blue was quantitatively analyzed by Maestro Imaging System (CRi). Evans blue exudation induced by the injection of 1 ng of vascular endothelial growth factor in the same rat was used to normalize the permeability. Data are expressed as mean (SE).m. ($n = 8$). The asterisks represent significant differences between IgG and anti-LOX-1 antibody groups ($**P < 0.01$).

Such a system helps clarify the physicochemical properties of the pure substances. To understand the biological properties of CRP and its receptors, however, monitoring them in their natural and biological context in the presence of numerous impurities is necessary. There are actually many potential molecules that can be ligands to CRP and CRP receptors in vivo. We found in this study that CRP induces changes in blood vessel permeability and that LOX-1 appears to mediate these changes. Pepys et al. reported that administration of CRP to normal animals does not yield any significant vascular effects *(36)*, whereas CRP administration to rat models of myocardial infarction increases infarct size *(8, 9)*. In the present study, we have made it possible to monitor the vascular response to CRP by using hypertensive rats in which LOX-1 expression is greatly enhanced. In a rat model of myocardial infarction, increases in LOX-1 expression have been reported *(27, 28)*. Therefore, it is entirely possible that LOX-1 is also involved in mediating the effects of CRP in the myocardial infarction model. In addition, the increase in vascular permeability observed in the present study might lead to retention of lipoproteins in the vascular wall, a process that is critical to promoting atherogenesis *(37)*.

To determine the K_d value for the binding of CRP to LOX-1 we used surface plasmon resonance analysis, which can more accurately calculate the binding constants. K_d was calculated to be 1.6×10^{-7} mol/L (18 mg/L). The hsCRP cutoff value for the risk for ischemic heart disease is reportedly $0.9–2.6 \times 10^{-8}$ mol/L (1–3 mg/L) *(7)*. In response to inflammatory stimuli, hsCRP concentrations can increase to more than 10^{-6} mol/L. Therefore, the K_d value of CRP is in the relevant range to explain the pathophysiological significance of CRP. Actually, in both cell-based and cell-free assay systems in the present study, CRP-binding was clearly observed at 1 mg/L.

It is commonly known that 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, statins, reduce the risk of cardiovascular diseases by reducing plasma LDL-cholesterol. Recent findings suggest that pleiotropic effects of the statins in addition to their cholesterol-reducing functions may also contribute to the beneficial cardiovascular effects *(38)*. Statins reportedly reduce the expression of oxidized LDL receptor LOX-1 *(39)*. In a recent epidemiological study, JUPITER (Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin), the observed reduction in cardiovascular risk even among those with low LDL-cholesterol demonstrated that patients with increased CRP benefit from statin treatment *(40)*. Thus, pharmacological approaches targeting cardiovascular risk factors are phenomenologically overlapping. In this study, we have demonstrated a direct molecular link connecting the major cardiovascular risk factors, which may be an initial step that leads to further novel studies and therapeutic approaches against cardiovascular diseases.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data or analysis, and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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LOX-1 dependent overexpression of immunoglobulins in cardiomyocytes in response to angiotensin II

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ABSTRACT

LOX-1, a cell surface lectin-like receptor, is upregulated by oxidized low-density lipoprotein (ox-LDL) and angiotensin II (Ang II), and plays an important role in host defense. The specific C-type lectin domain on LOX-1 is essential for ox-LDL binding and internalization, generation of oxidant species and eliciting immune response. Here, we show that LOX-1 deletion alters genes that relate to immune response. Microarray (and qPCR) analysis of cardiac tissues showed downregulated expression of several immunoglobulins (*Igk-V8, Igk-C, Igh-6, Igj, Ighg, Igh,* and *Igl-V1*) in the LOX-1 knockout (KO) mice [$p < 0.05$ vs. the wild-type (WT) mice]. The expression of these immunoglobulins was upregulated several-fold in the LOX-1 KO mice hearts when these mice were infused with Ang II ($p < 0.05$, vs. WT mice). Importantly, cultured mouse HL-1 cardiomyocytes expressed these immunoglobulins, and pretreatment of cardiomyocytes with a specific anti-LOX-1 antibody enhanced the generation of immunoglobulins upon subsequent exposure to Ang II. These observations mirrored the data obtained from WT and LOX-1 KO mice hearts in the resting state and following Ang II infusion. This study provides first set of data on immunoglobulin expression in cardiac tissues of WT and LOX-1 KO mice and in cultured HL-1 cardiomyocytes, and demonstrates that LOX-1 inactivation leads to upregulation of immunoglobulins in cardiomyocytes upon challenge with Ang II.

LOX-1, a cell surface lectin-like receptor for oxidized low density lipoproteins (ox-LDL), plays a pivotal role in host defense resulting from a broad ligand-binding spectrum [1,2]. LOX-1 molecule structurally belongs to the C-type lectin family molecules with high level of homology with NKR-P1 family of proteins expressed on the surface of natural killer (NK) cells, which are involved in target-cell recognition and NK cell activation [1,3]. The C-type lectin domain on LOX-1 is essential for ox-LDL binding, and mediates many of the biological effects of ox-LDL [1]. Although LOX-1 was initially thought to be an endothelial cell specific receptor, recent data shows its expression in cardiomyocytes, especially when these cells are exposed to cytokines, reactive oxygen species (ROS) or angiotensin II (Ang II) [4]. Ang II is a major stimulus for LOX-1 expression, and it is likely that LOX-1 plays a critical role in Ang II-mediated hypertension and subsequent cardiac remodeling [5,6].

Immunoglobulins are important members of the host immunity, and their primary function is to specifically bind one or few closely related antigenic determinants via their Fab fragment. Traditional view dictates that immunoglobulin expression is limited to B cells; however, recent data suggest that immunoglobulins are also expressed by several other cell lines, such as lung alveoli [7], cardiac cells [8–10], lung interstitium [11], multiple epithelial cell lines, and cancerous cells [12–14].

Recently, we observed upon microarray analysis of cardiac tissues that LOX-1 deletion alters genes relating to immune system. These genes appear to be downregulated in response to Ang II in the wild-type (WT) mice hearts. In order to examine gene expression of immunoglobulins during LOX-1 deletion, we investigated their differential gene expression in the hearts of WT and LOX-1 knockout (KO) mice given Ang II (or saline) infusion. The data observed *in vivo* were confirmed in cultured mouse cardiomyocytes. This study describes an important role of LOX-1 deletion in cardiomyocyte-specific immunoglobulins expression in response to Ang II.

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Materials and methods

Animals. C57BL/6 mice (WT) were obtained from Jackson Laboratories. The homozygous LOX-1 KO mice (LK) were developed on C57BL/6 background as described recently [15]. WT and LOX-1 KO mice, such as WT mice given saline (WTS), LOX-1 KO mice given saline (LKS), WT mice given Ang II (WTA), and LOX-1 KO mice given Ang II (LKA), weighing 22–26 g and 8–10 weeks of age were utilized in this study. The methodology for Ang II infusion has been recently published [6]. Hearts were removed and thoroughly washed with saline until free of blood. All animals received humane care in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals published by the National Institutes of Health. The present studies were approved by Institutional Animal Care and Usage Committee. In previously published studies [6], we showed attenuation of hypertensive response to Ang II infusion and a significant cardioprotection by LOX-1 deletion.

HL-1 cardiomyocyte cell culture. HL-1 adult mouse cardiomyocytes were a gift from Dr. W.C. Claycomb (Louisiana State University, New Orleans, LA) and were cultured [16]. In brief, HL-1 cells were grown at 37 °C under 5% CO_2 in 0.02% gelatin coated flasks containing Claycomb medium (JRH Biosciences) supplemented with heat-inactivated 10% fetal bovine serum (JRH Biosciences), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen) and 0.1 mM norepinephrine (Sigma). Twenty-four hours after seeding, the cells were pretreated with LOX-1 antibody (10 μg/ml) for 3 h and then exposed to Ang II (10^{-8}nM) for 24 h.

RNA isolation and real-time quantitative PCR (qPCR). Total RNA of heart (left ventricle) was isolated from mouse hearts and HL-1 cardiomyocytes using RNeasy Mini-Kit (Invitrogen, Carlsbad, CA). To remove genomic DNA contamination, RNA samples were treated with 1 U DNase/μg total RNA (Invitrogen) for 15 min at 25 °C. To validate immunoglobulin expression due to LOX-1 deletion, qPCR was performed using the I-cycler IQ detection system (Bio-Rad, Hercules, CA). We designed qPCR specific primer using ProbeFinder (http://www.roche-applied-science.com) web-based software from Mouse Universal ProbeLibrary from Roche Applied Science. All qPCR reactions were carried out in a final volume of 25 μl containing 1X of SYBR Green PCR Master (Applied Biosystems, Foster City, CA), 300 nM of each gene specific primers, 50 ng cDNA, and then added sterile deionized water. The standard cycling condition was 50 °C for 2 min, 90 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 1 min. The comparative threshold cycles (C_t) values for *in vivo* (LKS/WTS, WTA/WTS, and LKA/WTA groups) and *in vitro* (anti-LOX-1 Ab/Control, Ang II/Control, and anti-LOX-1 Ab/Ang II) study were normalized for GAPDH reference genes and compared with a calibrator using the $2^{-\Delta\Delta Ct}$ method [17]. qPCR was performed in triplicate to ensure quantitative accuracy. Final results were expressed in relation to the reference gene (GAPDH). Immunoglobulin primer sequences, forward and reverse, used for the validation of selected candidate genes were: *Igk-V8* (5'-TTCTGCTCTGGGTATCTGGTG-3', 3'-CCTTCTGTCC TACTGACATAGCC-5'), *Igk-C* (5'-CAGCTTCATGCTAATCAGTTTCA-3', 3'-CTGACACTGCAGGTGATGGT-5'), *Igh-6* (5'-GCTTGGTGTGGTG GAAGAAC-3', 3'-GAAACCCAGGCTGT GACACT-5'), *Igj* (5'-GAACTTT GTATACCATTTGTCAGACG-3', 3'-CTGGGTGGCAGTAACAA CCT-5'), *Ighg* (5'-GATCTTTCTCTTCCTGTTGTCAGTAAC-3', 3'-ACTGAGGCC CCAGGTTTT-5'), *Igh* (5'-CCTCTTCTTGGTAGCAACAGC-3', 3'-TGAAG CTCCAGGCCTCAC-5'), *Igl-V1* (5'-TCTCTCCTGGCTCTCAGCTC-3', 3'-C ACCAGGTGATGTGGTGAGT-5'), and *GAPDH* (5'-TT T GATGTTAGTG GGGTCTCG-3', 3'-AGCTTGTCATCAACGGGAAG-5').

Microarray analysis. To identify differential gene expression patterns associated with different groups of mice (LKS vs. WTS; WTA vs. WTS; LKA vs. WTA), we performed Affymetrix Mouse Genome GenChip 430 2.0 gene expression arrays according to standard Affymetrix protocol. Primary data obtained from scanning microarray signals were analyzed using the Affymetrix MicroArraySuite 5.0. For further analysis, microarray data in the form of CEL files were imported into BRB ArrayTools developed by Drs. Simon and Peng Lam (http://linus.nci.nih.gov/BRB-ArrayTools.html). MG-430 2.0 microarray raw expression intensities of each experimental group were scaled to a target intensity of 100 U, normalized independently, using the robust multichip average algorithm for the quantification of the expression level of target genes, and passed by the filtering and subletting criteria with any one absent (A) or marginal call (M). Genes with $\geqslant$50% missing data across all observations were excluded from the analysis. Fold change was transformed based on $\log_2$ (LKS/WTS), $\log_2$ (WTA/WTS) and $\log_2$ (LKA/WTA), respectively. Fold change above 2.0 was defined as differentially expressed genes between two groups.

In order to categorize differential gene expression between LOX-1 KO mice and WT mice, we performed hierarchical clustering analysis on the filtered microarray data using the Database for Annotation, Visualization and Integrated Discovery (DAVID 2008, http://david.abcc.ncifcrf.gov). It was classified into biological process, cellular component, and molecular functions using the terminology proposed by the Gene Ontology (GO) Consortium [18] using EASE software. We focused on genes of known function that were classified into immune response categories and subcategories.

Statistical analysis. Data are presented as means ± SE, and were analyzed with unpaired *t*-test. A value of $p < 0.05$ was considered significant.

Results

From over 34,000 well-characterized mouse genes of Affymetrix Mouse Genome GenChip 430 2.0 gene expression arrays, we filtered for transcripts that were present (expressed) in at least 50% of the left ventricle samples per group, and were increased or decreased $\geqslant$2-fold (vs. WTS). The 399 (LKS/WTS), 446 (WTA/WTS), and 240 (LKA/WTA) transcripts passed the filtering criteria to be differentially regulated between the groups with 95% confidence. Of those transcripts, we filtered 19 (LKS/WTS), 18 (WTA/WTS), and 11 (LKA/WTA) transcripts related to immune response using functional annotation analysis in DAVID (Table 1). The relevant biological functions of these genes are summarized in Table 2.

The Immune response genes that were differentially expressed between LKS vs. WTS, WTA vs. WTS, and LKA vs. WTA groups are shown in Fig. 1. Several of these genes belonging to the immunoglobulin family that were altered are (1) light chain; immunoglobulin kappa chain variable 8 (*Igk-V8*) immunoglobulin kappa chain, constant region (*Igk-C*), and immunoglobulin lambda chain, variable 1 (*Igl-V1*), (2) heavy chains; immunoglobulin heavy chain 6 (heavy chain of IgM) (*Igh-6*), immunoglobulin heavy chain (gamma polypeptide) (*Ighg*), and immunoglobulin heavy chain complex (*Igh*), and (3) joining chain; immunoglobulin joining chain (*Igj*).

A cluster of immunoglobulin genes related to immune function, such as, *Igk-V8* (−6.7-fold), *Igk-C* (−4.0-fold), *Igh-6* (−3.5-fold), *Igj* (−2.5-fold), *Ighg* (−3.4-fold), *Igh* (−2.2-fold), and *Igl-V1* (−2.0-fold) were downregulated in hearts of LOX-1 KO mice (LKS vs. WTS). These genes were also downregulated in the hearts of WT mice given Ang II (WTA vs. WTS), including *Igk-V8* (−3.1-fold), *Igk-C* (−2.6-fold), *Igh-6* (−3.2-fold), *Igj* (−2.3-fold), *Ighg* (−2.4-fold), *Igh* (−1.2-fold), and *Igl-V1* (−1.9-fold). Interestingly, the expression of these immunoglobulins was strongly upregulated in the hearts of LOX-1 KO mice given Ang II (LKA vs. WTA, 9.9-fold, 4.4-fold, 9.6-fold, 5.3-fold, 3.1-fold, 2.5-fold, and 2.5-fold, respectively, all $p < 0.05$) (Fig. 1). Other specific genes of interest included *Igk-V8* and *Igj* which were the most up-regulated genes in LKA compared to WTA hearts.

Table 1
Immune response genes that differentially expressed between LKS vs. WTS, WTA vs. WTS, and LKA vs. WTA groups.

Gene title	Gene symbol	LKS/WTS	WTA/WTS	LKA/WTA
Tnf receptor-associated factor 6	*Traf6*	−1.1	1.3	−2.0
Sp110 nuclear body protein	*Sp110*	2.1	1.0	−1.2
Mannan-binding lectin serine peptidase 1	*Masp1*	−2.1	1.3	−1.2
Chemokine (C–C motif) ligand 5	*Ccl5*	−1.2	−2.0	−1.2
Complement factor B	*Cfb*	−2.1	−1.1	−1.1
2′–5′ oligoadenylate synthetase 3	*Oas3*	−1.1	−2.1	−1.1
Pro-platelet basic protein	*Ppbp*	−1.8	−2.0	−1.1
Tumor necrosis factor (ligand) superfamily, member 4	*Tnfsf4*	2.2	−1.3	1.0
Eukaryotic translation initiation factor 2-alpha kinase 2	*Eif2ak2*	2.0	−1.1	1.1
Interleukin 7	*Il7*	−1.6	−2.0	1.2
T-cell immunoglobulin and mucin domain containing 4	*Timd4*	−1.1	−2.0	1.2
V-set and immunoglobulin domain containing 1	*Vsig1*	1.0	−2.2	1.3
Histocompatibility 28	*H28*	2.2	1.0	1.3
Histocompatibility 2, Q region locus 10	*H2-Q10*	−2.1	−2.3	1.5
Histocompatibility 2, class II, locus Mb2	*H2-DMb2*	−1.2	−2.2	1.5
Similar to gamma-2a immunoglobulin heavy chain	*LOC100047788*	−2.3	−2.4	1.5
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F	*Sema3f*	2.3	2.6	1.6
CD79B antigen	*Cd79b*	−1.9	−2.0	1.9
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	*Sema3b*	1.4	−1.1	2.2
Immunoglobulin lambda chain, variable 1	*Igl-V1*	−2.0	−1.9	2.5
Immunoglobulin heavy chain complex	*Igh*	−2.2	−1.2	2.5
Chemokine (C–X–C motif) ligand 13	*Cxcl13*	−3.7	−2.9	2.9
Immunoglobulin heavy chain (gamma polypeptide)	*Ighg*	−2.5	−2.4	3.1
Immunoglobulin kappa chain, constant region	*Igk-C*	−4.0	−2.6	4.4
Complement factor D (adipsin)	*Cfd*	−4.6	−4.4	5.1
Immunoglobulin joining chain	*Igj*	−3.4	−2.3	5.3
Immunoglobulin heavy chain 6 (heavy chain of IgM)	*Igh-6*	−3.5	−3.1	9.6
Immunoglobulin kappa chain, variable 8	*Igk-V8*	−6.7	−3.1	9.9

Table 2
Biological function of immunoglobulins (LKA vs. WTA).

Gene symbol	*p* Value	Gene ontology (GO)			Chromosomal location
		Biological process	Cellular component	Molecular function	
Igk-V8	0.029	Unknown	Unknown	Unknown	Chr6 30.0 cM
Igk-C	0.035	Humoral immune response	Unknown	Antigen binding	Chr6 30.0 cM
Igh-6	0.023	Activation of MAPK activity Antibody-dependent cellular cytotoxicity Positive regulation of types I & IIa hypersensitivity Humoral immune response mediated by circulating Ig Positive regulation of B cell proliferation Defense response to bacterium Positive regulation of immune response	Multivesicular body, external side of plasma membrane	Antigen binding, Transmembrane receptor activity, protein binding	Chr12 58.0 cM
Igj	0.023	Humoral immune response	Extracellular space	Antigen binding	Chr5 E1 (Syntenic gene)
Ighg	0.005	Positive regulation of types I & IIa hypersensitivity, Humoral immune response mediated by circulating Ig, Ig mediated immune response	Membrane, Immunoglobulin complex, circulating	Antigen binding	Chr12 F2 (Syntenic gene)
Igh	0.006	Mucosal immune response, Humoral immune response mediated by circulating Ig, Ig mediated immune response	Immunoglobulin complex, circulating	Antigen binding	Chr12 58.0 cM
Igl-V1	0.008	Humoral immune response	Extracellular region	Antigen binding	Chr16 13.0 cM

In order to determine if the immunoglobulins are generated in the mouse cardiomyocytes, we carried out *in vitro* experiments using HL-1 cardiomyocytes. We observed that HL-1 cardiomyocytes expressed mRNA for these immunoglobulins, and that treatment with Ang II exerted inhibitory effect on their expression (all $p < 0.05$ vs. control: *Igk-V8* (−5.7-fold), *Igk-C* (−7.5-fold), *Igj* (−17.2-fold), *Ighg* (−13.0-fold), and *Igl-V1* (−19.7-fold). Similar to our microarray findings in the mouse hearts, pretreatment of HL-1 cardiomyocytes with a specific anti-LOX-1 antibody followed by their exposure to Ang II resulted in the upregulation of immunoglobulins; ($p < 0$ 05 vs. Ang II treatment without LOX-1 antibody: *Igk-V8* (4.9-fold), *Igk-C* (2.0-fold), *Igj* (1.5-fold), *Ighg* (1.4-fold), and *Igl-V1* (1.2-fold).

Discussion

The present study enabled us to make three novel hitherto-unknown observations. First, the mouse hearts produce a variety of immunoglobulins. Second, the LOX-1 KO mice have low levels of these immunoglobulins in the heart (vs. the WT mice). Third, the expression of these immunoglobulins in the LOX-1 KO mice is upregulated several-fold when the mice are infused with Ang II (vs. WT mice given Ang II). Most importantly, cultured mouse HL-1 cardiomyocytes expressed these immunoglobulins suggesting that these immunoglobulins were of cardiomyocyte origin. Further, the expression level of these immunoglobulins in HL-1 cardiomyocytes decreased upon treatment with Ang II; in contrast,

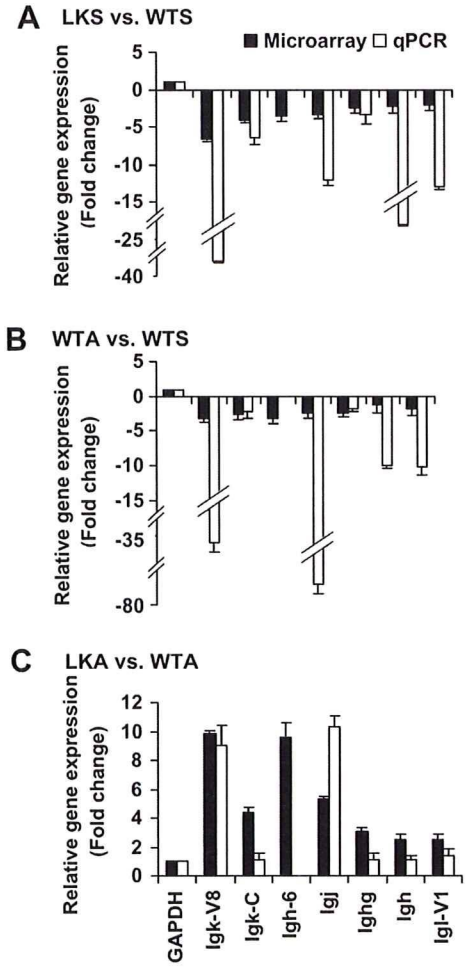


Fig. 1. Relative expression level for immunoglobulin genes in hearts from different groups of mice using microarray and qPCR analysis. Data are shown as following comparisons: LKS vs. WTS, WTA vs. WTS, and LKA vs. WTA groups. See text for description of groups. The expression of genes determined by microarray was validated by qPCR. For qPCR, the quantity of RNA for each gene was normalized to GAPDH in the same sample. For microarray data, the expression values are given in a linear scale after background correction, normalization, and scaling down. Data shown are means ± SE.

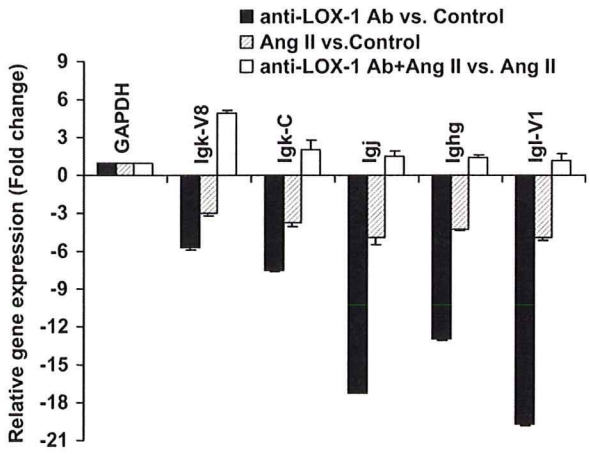
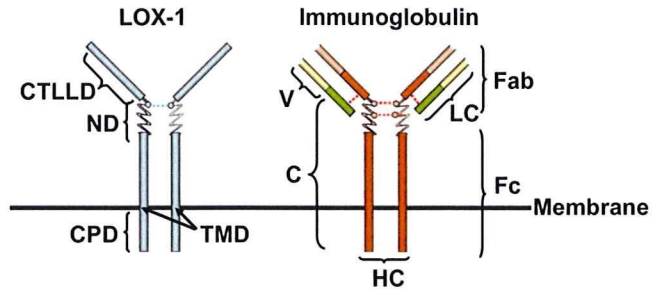


Fig. 2. Relative gene expression of immunoglobulin genes by real-time quantitative PCR in HL-1 cardiomyocytes. The expression of genes determined by microarray was validated by qPCR. For qPCR, the quantity of RNA for each gene was normalized to GAPDH in the same sample. Data shown are means ± SE.



Mouse	LOX-1	Igs	Function
Wild-type (basal)	++	++	Normal
Wild-type (stress)	++++	+	Heart injured
LOX-1$^{-/-}$ (basal)	-	++	No discernible change
LOX-1$^{-/-}$ (stress)	-	++++	Heart protected

Abbreviations: CTLLD- C-type lectin-like domain; ND-Neck domain; CPD- Cytoplasmic domain; TMD- Transmembrane domain; V-Variable region ; C-Constant region; HC- Heavy chain ; LC-Light chain

Fig. 3. Hypothesized model of the interaction between LOX-1 and immunoglobulins.

pretreatment of these cardiomyocytes with a specific anti-LOX-1 antibody sharply enhanced the expression of these immunoglobulins in response to Ang II (Fig. 2). These observations parallel the data obtained from WT and LOX-1 KO mice hearts in the resting state and following Ang II infusion.

Expression of immunoglobulins has been previously reported in non-lymphoid tissues. Ashton *et al.* [9] observed expression of *Ighg* in hearts and its pronounced upregulation with aging. Yun et al. [10] showed significant changes in immunoglobulin gene transcription associated with the hypertrophy phenotype. Recently, Norkina et al. [19] showed a significant increase in the expression of the Igk-V5, Igk-V8, and Igk-V28 in the intestine of the cystic fibrosis mouse. Guilbbault et al. [11] also reported an increase in the mRNA expression in the lungs of cystic fibrosis transmembrane conductance regulator (*Cftr*) KO mice compared with their WT controls for several immunoglobulin genes, including *Igh-4*, *Igh-VJ558*, *Igk-V8*, and *Igj*. Vasu et al. [8] have previously reported observations similar to ours in genomic profiles in the hearts of alpha-tocopherol transfer protein null mice.

The mechanisms and biological significance of immunoglobulin production in non-lymphoid tissues is unclear. Qiu et al. [14] reported that immunoglobulin G secreted by human epithelial cancers of unidentified specificity promoted growth and survival of tumor cells. Zheng et al. [12,20] showed that blockade of the cancerous tissue-derived immunoglobulin alpha heavy chain suppressed the growth and viability of cancer cells. They also found that *IgA* increased the access percentage of S phase from early mitosis of cancer cells [12,20]. These findings suggest a growth promoting action of immunoglobulins at least in cancer cells.

Immunoglobulins are generally thought to play a role in cardioprotection [21]. Our model of the interaction between LOX-1 and immunoglobulins is shown in Fig. 3. This model, based on similarity of the C-type lectin-like domain of LOX-1 and immunoglobulins, suggests equilibrium between the expression of LOX-1 and immunoglobulins in the resting state. During stress of Ang II infusion, there is a relative increase in the expression (and activity) of LOX-1 in the WT mice and, hence, the tissue injury response. However, in the absence of LOX-1 (KO state or use of blocking antibody) there is a shift in this equilibrium with a marked increase in the expression and activity of immunoglobulins particularly during the stress of Ang II infusion. Shift in this equilibrium is at east one mechanism by which LOX-1 KO mice hearts are protected from ischemia [22] and hyper-

tensive remodeling [6]. At this time, this remains a speculation and this concept needs to be tested, particularly in response to other stressful stimuli.

We recognize that the expression of immunoglobulins needs to be quantified at protein level. The subcellular localization (cytosolic vs. membrane) as well as presence or absence of secretory component of immunoglobulins also needs to be studied. Further, putative mediators for immunoglobulin expression need to be examined. Finally, the precise mechanism by which Ang II modulates immunoglobulin expression in cardiomyocytes need to be understood.

Our study is the first to demonstrate that a host of immunoglobulin genes (both heavy and light chains, kappa variable) are expressed in cardiomyocytes in basal state both *in vivo* and in HL-1 cardiomyocytes *in vitro*. When stimulated by Ang II, expression of these immunoglobulins is decreased in the WT mice; however, deletion of LOX-1 paradoxically causes them to be upregulated.

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Increased Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Expression in the Maternal Vasculature of Women With Preeclampsia

Role for Peroxynitrite

Sowndramalingam Sankaralingam, Yi Xu,* Tatsuya Sawamura, Sandra T. Davidge

Abstract—Preeclampsia is a hypertensive disorder unique to pregnancy, in which the placenta may release factors into the maternal circulation resulting in systemic effects. Small dense low-density lipoprotein (LDL; which is susceptible for oxidation) is increased in preeclampsia. Lectin-like oxidized LDL receptor-1 (LOX-1) is a receptor for oxidized LDL. However, the expression levels and the regulation of LOX-1 in the maternal vasculature of women with preeclampsia are unknown. We hypothesized that there is an increased LOX-1 expression in arteries from women with preeclampsia. We further hypothesized that circulating factors in the plasma of women with preeclampsia would upregulate the LOX-1 expression in vascular endothelial cells and contribute to vascular endothelial oxidative stress. We observed abundant LOX-1 expression and the presence of oxidized LDL in arteries from women with preeclampsia, which was negligible in arteries from normotensive pregnant women. Human umbilical vein endothelial cells treated for 24 hours with 2% plasma from preeclamptic women increased LOX-1 expression and oxidized LDL uptake, as well as induced oxidative stress, as evidenced by increased NADPH oxidase activity and superoxide and peroxynitrite levels. These effects were significantly reduced by pretreatment with blocking antibody or small interfering RNA to LOX-1, as well as 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride (FeTPPS), a peroxynitrite scavenger. Exogenous peroxynitrite and 3-morpholino sydnonimine (SIN-1) increased LOX-1 protein and mRNA expression. In conclusion, increased LOX-1 expression in the systemic vasculature of preeclampsia women provides a fundamental insight into the pathology of preeclampsia and likely contributes to the induction and maintenance of vascular oxidative stress. (*Hypertension*. 2009;53:270-277.)

Key Words: preeclampsia ■ LOX-1 ■ NADPH oxidase ■ endothelium ■ peroxynitrite

Preeclampsia is a pregnancy-specific disorder in humans, characterized by hypertension and proteinuria occurring after the 20th week of gestation. These symptoms resolve after delivery, suggesting that the placenta plays a central role in the pathogenesis of this disorder. It is generally agreed that poor invasion of the uterine spiral arteries by the trophoblast leads to an ischemic placenta that subsequently releases a number of circulating factors into the maternal circulation.[1] The factors released into the maternal circulation include a number of vasoactive molecules and proinflammatory cytokines, which can potentially cause dysfunction of the maternal endothelium. Such factors can induce the endothelial cells to generate excess of oxygen-derived free radicals, resulting in the development of oxidative stress.[2]

One of the early changes that may occur as a result of endothelial injury in the uterine spiral arteries is the accumulation of neutral lipids, a phenomenon called "acute atherosis" of pregnancy.[3] Whether lipid accumulation occurs in the maternal systemic vasculature and, if so, the possible mechanisms involved remain unknown. Several studies have shown increased serum levels of triglycerides, low-density lipoproteins (LDLs), and lipid peroxides in women with preeclampsia when compared with normotensive pregnant women.[4,5] In addition, small dense LDL is also increased in the plasma of women with preeclampsia.[6] Small dense LDLs are more susceptible to oxidation, resulting in the generation of oxidized LDL (oxLDL).[7,8] OxLDL can bind to the lectin-like oxidized LDL receptor-1 (LOX-1) on endothelial cells.[9] LOX-1 is a type II membrane protein cell surface receptor identified on endothelial cells, vascular smooth muscle cells, and monocyte macrophages. LOX-1 is expressed in atheroscle-

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rotic lesions in humans[10] and has also been shown to be elevated in hypertensive rats.[11] LOX-1 is responsible for the binding, uptake, and degradation of oxLDL. During this process, the binding of oxLDL activates the NADPH oxidase enzyme system, resulting in the excessive generation of superoxide.[12] Scavenging of NO by superoxide may not only reduce NO bioavailability but also generate a more potent oxidant, peroxynitrite. Peroxynitrite formation has been observed in both the placenta[13] and the maternal vasculature of women with preeclampsia.[14] One recent study has observed elevated LOX-1 expression in the placenta of women with preeclampsia.[15] However, the expression, regulation, and significance of LOX-1 in the maternal systemic vasculature of preeclampsia remain unknown.

We hypothesized that LOX-1 is upregulated in small resistance-sized arteries obtained from women with preeclampsia. We further hypothesized that the circulating factors in the plasma of women with preeclampsia via the formation of peroxynitrite provide a feed-forward loop to upregulate LOX-1.

Methods

Subjects

Pregnant subjects were recruited at the time of delivery, and nonpregnant subjects were recruited at the time of abdominal surgeries at the Royal Alexandra Hospital (Edmonton, Canada). The protocols were approved by the University of Alberta Ethics Committee, and the studies were conducted according to the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All of the subjects provided informed consent before inclusion in the study. Twelve subjects had preeclampsia characterized by the de novo onset of hypertension and proteinuria after the 20th week of gestation. Hypertension was defined as a blood pressure of >140/90 mm Hg on 2 occasions 6 hours apart and proteinuria of >500 mg in a 24-hour urine collection or more than +2 on a dip stick.[14] Normal pregnant subjects (n=12) were normotensive throughout pregnancy. Nonpregnant subjects (n=12) were also normotensive. None of the subjects had a past history of chronic hypertension, renal, liver, or other metabolic diseases and were not on any medications. Either blood samples at the time of admission or fat biopsies during surgeries were collected from these women as detailed below. Blood was collected by routine forearm venipuncture at the time of admission (before delivery) in tubes containing EDTA. Blood samples were immediately centrifuged at 2000*g* for 20 minutes and then aliquoted under sterile conditions and stored at −80°C. Omental fat biopsies were obtained at the time of cesarean section for normotensive pregnant and preeclamptic women or during abdominal surgeries for nonpregnant women who were admitted for indications such as dysfunctional uterine bleeding, ovarian cyst, menorrhagia, and dysmenorrhea and then snap frozen in liquid nitrogen and stored at −80°C.[14] The patient characteristics are shown on Tables S1 and S2 (see online data supplement at http://hyper.ahajournals.org). In the nonpregnant group, all of the subjects were white, nonsmokers, not previously pregnant, and not on any medications.

Immunohistochemistry

Our first aim was to compare the expression of LOX-1 and to detect the presence of oxLDL in small arteries from nonpregnant, pregnant, and preeclamptic women. Omental fat biopsies were flash frozen in liquid nitrogen and stored at −80°C. Later they were cut into ≈0.5-cm diameter in size and frozen in optimal cutting temperature compound, cut into 8-μm sections, mounted on glass slides at −25°C, and stored at −80°C until use. All of the arteries in the section were counted. The slides were immunostained using rabbit polyclonal antibodies for LOX-1 (1:100; Santa Cruz Biotechnologies) and oxLDL (1:100; Calbiochem). Antirabbit secondary antibody (1: 200; Alexa fluor 488, Invitrogen) was used to detect the primary antibody and was visualized using a fluorescein isothiocyanate filter.

To address the mechanisms of LOX-1 upregulation, we performed a bioassay by observing the effects of plasma from the 3 groups of women on endothelial cells in culture. Human umbilical vein endothelial cells were treated with 2% plasma from nonpregnant, pregnant, and preeclamptic women for 24 hours. Individual plasma samples but not pooled plasma from each group were used in this study. In response to treatment with plasma, DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)- labeled oxLDL uptake, intracellular superoxide levels using dihydroethidine, NADPH oxidase activity by lucigenin chemiluminescence, and nitrotyrosine staining as a marker of peroxynitrite formation were measured. In some experiments, cells treated with plasma were pretreated with 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride (FeTPPS; 5 μmol/L), a peroxynitrite scavenger; monoclonal antibody to LOX-1 (mAbLOX-1; 10 μg/mL); or small interfering RNA (siRNA; 30 nmol/L) to LOX-1 before exposure to plasma. In a separate set of experiments, human umbilical vein endothelial cells were treated with either 3-morpholino sydnonimine (SIN-1; 0.25 mmol/L) or peroxynitrite (25 μmol/L) for 6 hours. LOX-1 protein and mRNA expressions were assessed by Western blot and PCR, respectively. Please see the online data supplement for detailed methods used in this study.

Statistical Analysis

Values are expressed as means±SEMs. Comparison of ≥3 groups was done using a 1-way ANOVA followed by a Tukey's posthoc test. Comparison of 2 groups was conducted using a Student *t* test. A *P* value of <0.05 was deemed significant.

Results

Arterial Expression of LOX-1 and OxLDL

We detected abundant LOX-1 expression ($P<0.001$) in arteries from women with preeclampsia (15.00±6.50 arbitrary units [AU]), whereas there was negligible expression in arteries from nonpregnant (0.18±0.09 AU) and pregnant women (0.25±0.22 AU; Figure 1). In arteries from preeclamptic women, LOX-1 expression was localized to both the endothelium and the vascular smooth muscle cells. However, LOX-1 expression is greater in the endothelial layer.

Because LOX-1 is involved in the binding and uptake of oxLDL, we performed immunohistochemistry to identify the presence of oxLDL in these arteries. OxLDL was present only in arteries from women with preeclampsia (6.00±0.50 AU; $P<0.001$) but not in arteries from nonpregnant and pregnant women (0.07±0.02 and 0.07±0.06 AU; Figure 1). Also, OxLDL appears to accumulate immediately beneath the endothelial layer.

Endothelial Response to Plasma

LOX-1 Expression in Response to Plasma

LOX-1 expression was significantly increased in endothelial cells exposed to plasma from women with preeclampsia (0.419±0.018 AU; $P<0.01$) in comparison with cells treated with plasma from nonpregnant and pregnant women (0.1450±0.0039 and 0.1930±0.0053 AU), respectively (Figure 2).

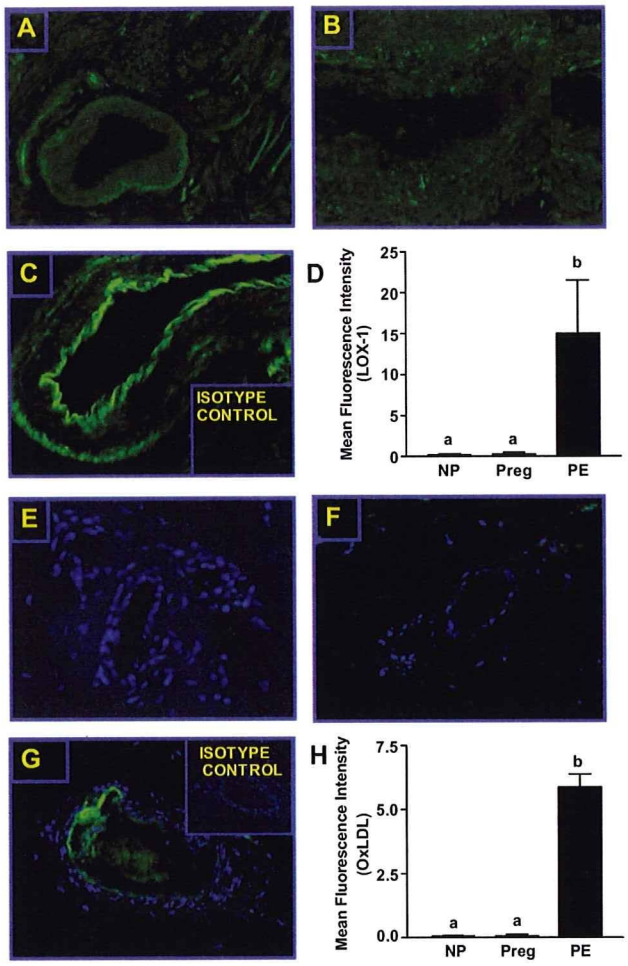

Figure 1. LOX-1 expression and detection of oxLDL in maternal arteries. Immunohistochemical staining for LOX-1 expression in representative sections of small arteries from omental fat biopsies of (A) nonpregnant, (B) pregnant, and (C) preeclamptic women shown at magnification ×200. Immunohistochemical staining for oxLDL in representative sections of small arteries from omental fat biopsies of (E) nonpregnant, (F) pregnant, and (G) preeclamptic women shown at magnification ×200. Summary graph for (D) LOX-1 expression and (H) presence of oxLDL in small arteries from 6 subjects in each group. Isotype staining with rabbit IgG as a negative control are shown as insets in C and G. Bars represent means±SEs. Different letters denote significant difference ($P<0.05$) from each other.

DiI-Labeled OxLDL Uptake

We observed significantly increased oxLDL uptake by endothelial cells in response to treatment with preeclamptic plasma when compared with treatment with nonpregnant and pregnant plasma. This uptake of oxLDL was significantly reduced by competition with unlabeled oxLDL and mAbLOX-1 (Figure 3).

NADPH Oxidase Activity

NADPH oxidase activity was significantly increased in endothelial cells treated for 24 hours with plasma from women with preeclampsia (53.92±1.40 AU/mg of protein; $P<0.01$) when compared with treatment with plasma from nonpregnant and pregnant women (31.37±1.10 and 29.60±1.20; AU/mg of protein), respectively (Figure 4A). LOX-1 blockade with mAbLOX-1 caused a marked reduction in NADPH oxidase

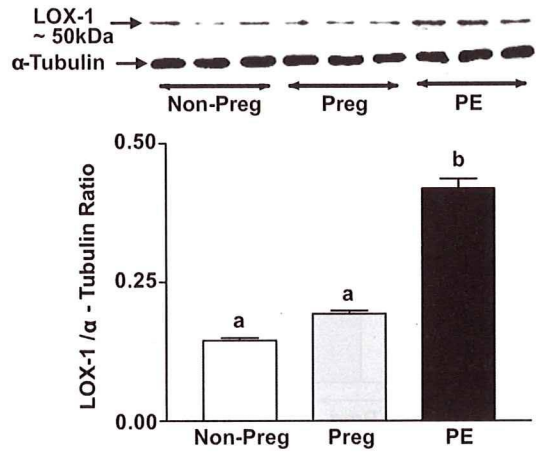

Figure 2. LOX-1 protein expression. A representative Western blot for LOX-1 expression from endothelial cells treated for 24 hours with 2% plasma from nonpregnant, pregnant, and preeclamptic women. Summary graph showing densitometric analysis of LOX-1 expression normalized to tubulin from 6 samples in each group. Bars represent means±SEs. Different letters denote significant difference ($P<0.05$) from each other.

activity only in the preeclamptic group (24.70±1.05 AU/mg of protein; $P<0.01$) but did not significantly affect NADPH oxidase activity in the nonpregnant and pregnant groups (26.58±1.50 and 26.10±1.30 AU/mg of protein, respectively). An isoimmune IgG control did not affect the NADPH oxidase activity in endothelial cells in response to preeclamptic plasma. These results were also confirmed by using siRNA to LOX-1 preeclamptic, which reduced preeclamptic plasma-induced NADPH oxidase activity to 25.90±0.50 AU/mg of protein (Figure 4B).

In a separate set of experiments, LOX-1 expression and NADPH oxidase activity in endothelial cells in response to plasma were assessed at a time point (6 hours) before LOX-1 expression was increased. Although LOX-1 expression did not change at 6 hours (data not shown), NADPH oxidase activity was increased significantly ($P<0.05$) in the pre-

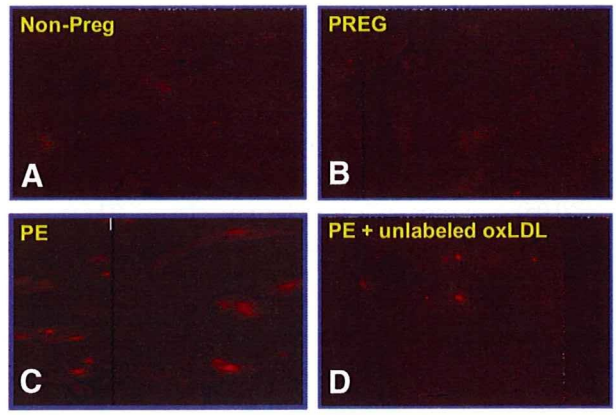

Figure 3. DiI-labeled oxLDL uptake. A representative image showing endothelial cells incubated with DiI-labeled oxLDL (10 μg/mL) for the last 3 hours of a 24-hour treatment period with plasma from (A) nonpregnant, (B) pregnant, and (C) preeclamptic women and (D) competition with excess unlabeled oxLDL (50 μg/mL) in the presence of preeclamptic plasma. Six samples of plasma from each group were used.

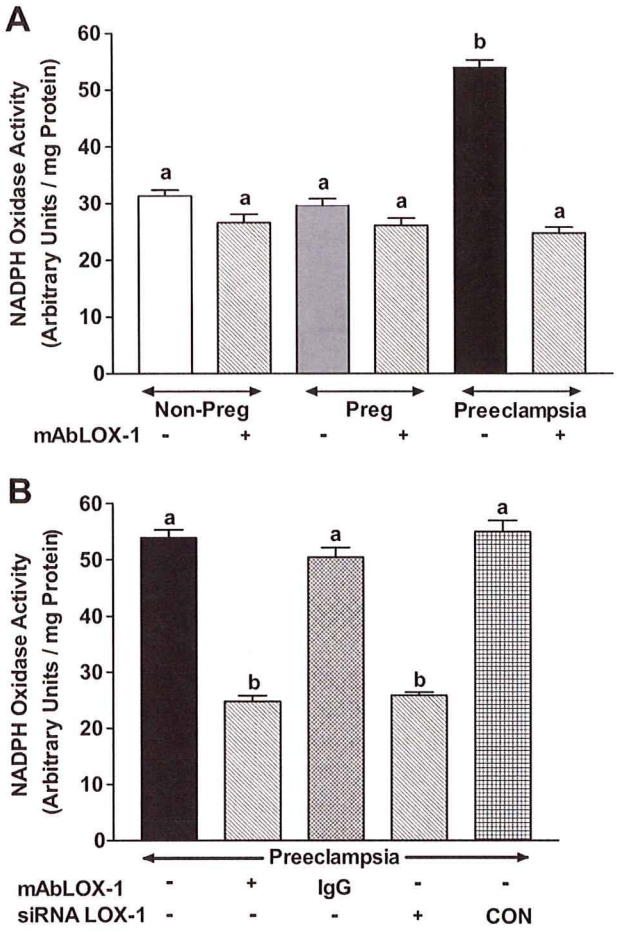

Figure 4. NADPH oxidase activity assay. A, Summary graph showing NADPH oxidase activity from endothelial cells treated for 24 hours in the presence or absence of mAbLOX-1 (10 μg/mL) with 2% plasma from nonpregnant, pregnant, and preeclamptic women. B, Summary graph showing NADPH oxidase activity from cells treated with 2% plasma from women with preeclampsia for 24 hours in the presence or absence of mAbLOX-1 (10 μg/mL) or siRNA LOX-1 (30 nmol/L) and their respective controls, nonimmune IgG (10 μg/mL) and control siRNA (30 nmol/L). Different letters denote significant difference ($P<0.05$) from each other.

eclamptic group as early as 6 hours (11.21±1.00 AU/mg of protein) in comparison with nonpregnant (8.30±1.00 AU/mg of protein) and pregnant (8.10±1.00 AU/mg of protein) groups. Furthermore, mAbLOX-1 also reduced this increase in NADPH oxidase activity (8.40±1.00 AU/mg of protein; $P<0.05$) in response to preeclamptic plasma. These results suggest that increased plasma levels of ligands to LOX-1 are responsible for this early increase in NADPH oxidase activity, although at a later time point (24 hours), the increased NADPH oxidase was likely attributable to increases in both LOX-1 expression and the higher levels of ligands in the preeclamptic plasma.

Superoxide Detection in Live Cells

Endothelial cells treated with plasma from women with preeclampsia generated significantly high levels of superoxide (1.309±0.032 AU; $P<0.01$) when compared with treatment with plasma from nonpregnant (0.574±0.027 AU) and pregnant (0.265±0.019 AU) women (Figure 5). This increase in superoxide generation in response to preeclamptic plasma was reduced by pretreatment with κ-carrageenan (0.445±0.018 AU), a nonspecific LOX-1 blocker; mAbLOX-1 (0.649±0.023 AU), a specific monoclonal blocking antibody to LOX-1; and NADPH oxidase inhibitors apocynin (0.629±0.017 AU) and diphenylene iodonium chloride (0.0676±0.027 AU). Endothelial cells treated with superoxide dismutase were used as a negative control (data not shown).

Detection of Nitrotyrosine as a Marker of Peroxynitrite

Endothelial cells treated with plasma from women with preeclampsia (18.769±4.022 AU; $P<0.01$) showed enhanced nitrotyrosine formation when compared with endothelial cells treated with plasma from nonpregnant and pregnant women (1.016±0.132 and 0.722±0.106 AU), respectively (Figure 6). Also, mAbLOX-1 and siRNA to LOX-1 reduced preeclamptic plasma–induced nitrotyrosine levels significantly (10.347±0.580 and 09.633±0.639 AU, respectively; $P<0.05$). Also, FeTPPS almost completely abolished (0.762±0.051 AU) the generation of superoxide by endothelial cells in response to plasma from women with preeclampsia.

Effect of Peroxynitrite on LOX-1 Expression

Having observed increased peroxynitrite generation in endothelial cells treated with plasma from women with preeclampsia, we sought to determine whether peroxynitrite plays a role in the upregulation of LOX-1 in response to plasma. LOX-1 expression was assessed by Western blot, in response to preeclamptic plasma in the presence of FeTPPS, a peroxynitrite scavenger. Interestingly, FeTPPS, significantly reduced LOX-1 expression by ≈30% ($P<0.05$), suggesting that peroxynitrite may play a role in inducing LOX-1 in preeclampsia (Figure 7).

In a separate series of experiments, we observed that exogenous peroxynitrite induced a modest but significant increase in LOX-1 protein expression by ≈40% (Figure 8A). This was also confirmed by using SIN-1, an agent that generates endogenous peroxynitrite by increasing both NO and superoxide production. SIN-1 also increased LOX-1 protein expression (Figure 8B; $P<0.05$). We also determined whether peroxynitrite can upregulate LOX-1 mRNA. We assessed LOX-1 mRNA expression in response to peroxynitrite or SIN-1. Both peroxynitrite and SIN-1 induced a 1.5-fold ($P<0.05$) increase in LOX-1 mRNA expression in 6 hours (Figure 8C and 8D).

Discussion

In women with preeclampsia, there is evidence for focal accumulation of lipid-laden macrophages in decidual vessels[16] and accumulation of neutral lipids in uterine spiral arteries.[3] These phenomena have been termed "acute atherosis," which is analogous to atherosclerosis. However, reports of such vascular abnormalities in the maternal systemic vasculature have not been described. In the present study, we report for the first time the accumulation of oxLDL and increased LOX-1 expression in the maternal vasculature of women with preeclampsia. Our data also indicate that per-

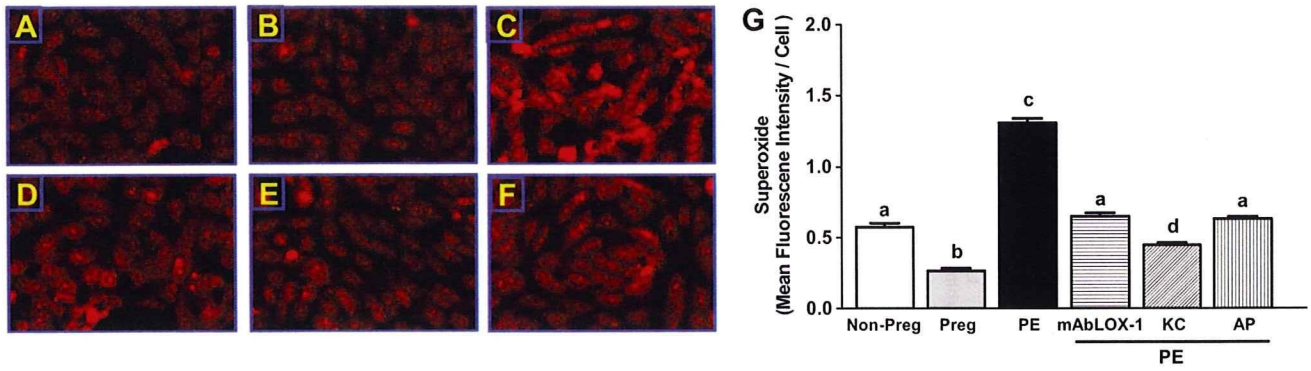

Figure 5. Live cell dihydroethidine staining for superoxide. Representative image showing superoxide production by live endothelial cells in response to treatment with 2% plasma for 24 hours from (A) nonpregnant, (B) pregnant, or (C) preeclamptic women. Additionally, (D) mAbLOX-1 (10 μg/mL), (E) κ-carrageenan (KC; 250 μmol/L), or (F) apocynin (AP; 30 μmol/L) was added in the presence of preeclamptic plasma. G, Summary graph showing live cell superoxide production in response to plasma from 6 subjects in each group. Bars represent means±SEs. Different letters denote significant difference ($P<0.05$) from each other.

oxynitrite generated secondary to LOX-1 upregulation, in turn, provides a feed-forward loop to further increase LOX-1 in preeclampsia. In women destined to develop preeclampsia, the symptoms continue to progress until delivery, suggesting a feed-forward mechanism of vascular endothelial dysfunction that could be possibly mediated by peroxynitrite through LOX-1 pathway.

Preeclampsia is characterized by hyperlipidemia,[4,5] including alterations in LDL. For instance, small dense LDLs are significantly elevated in the circulation of women with preeclampsia.[5,6] These small dense LDL particles are more atherogenic[7] and are more susceptible for oxidative modification,[4,7,17] resulting in the formation of oxLDL. OxLDL is immunogenic and subsequently results in the formation of autoantibodies to oxLDL in the circulation.[18] Thus, circulating autoantibodies to oxLDL have also provided evidence for the presence of oxLDL. Data from a previous study has shown increased levels of autoantibodies to oxLDL in the circulation of women with preeclampsia.[19] In contrast, however, other studies have reported no change in levels of autoantibodies to oxLDL in the plasma of women with preeclampsia.[20–22] In addition, another recent study has shown decreased plasma levels of oxLDL in preeclamptic women, which the authors suggested could be attributed to

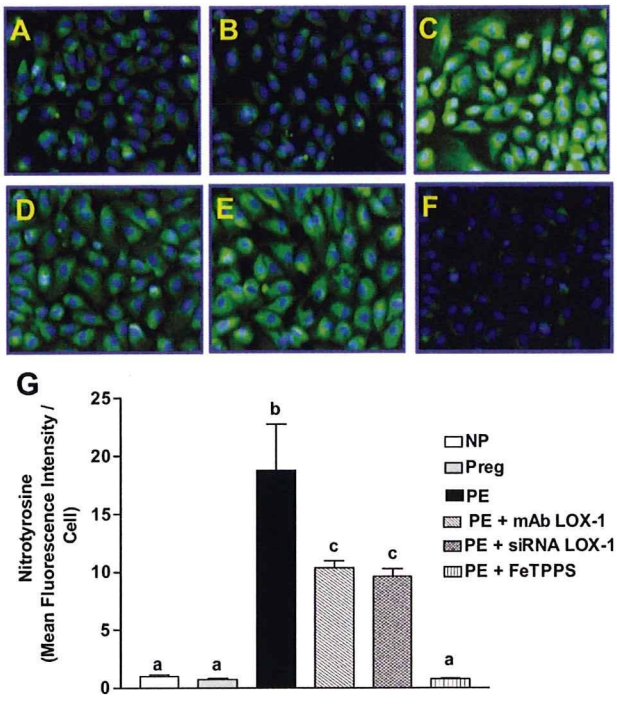

Figure 6. Nitrotyrosine staining as a marker of peroxynitrite. Representative figure showing nitrotyrosine staining (green) in endothelial cells treated with 2% plasma for 24 hours from (A) nonpregnant, (B) pregnant, and (C) preeclamptic women. The effect of (D) mAbLOX-1 (10 μg/mL), (E) siRNA LOX-1 (30 nmol/L), and (F) FeTPPS (5 μmol/L) in the presence of plasma from women with preeclampsia is shown. Nuclei stained with Hoescht 33342 are shown in blue. G, Graph showing mean fluorescence intensity of nitrotyrosine staining from 6 subjects per group. Bars represent means±SEs. Different letters denote significant difference ($P<0.05$) from each other.

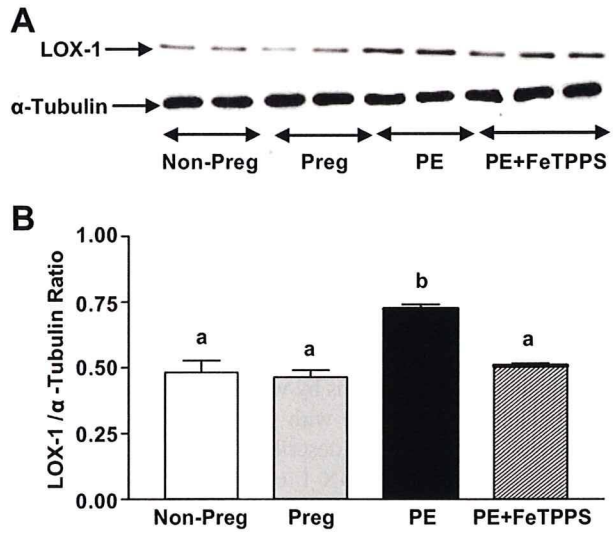

Figure 7. Effect of FeTPPS on LOX-1 expression. A, A representative Western blot for LOX-1 expression from endothelial cells treated with 2% plasma from nonpregnant, pregnant, and preclamptic women for 24 hours. Some groups of cells were pretreated with FeTPPS before incubation with plasma from women with preeclampsia. B, Summary graph showing densitometric analysis of LOX-1 expression normalized to tubulin from 6 samples in each group. Different letters denote significant difference ($P<0.05$) from each other.

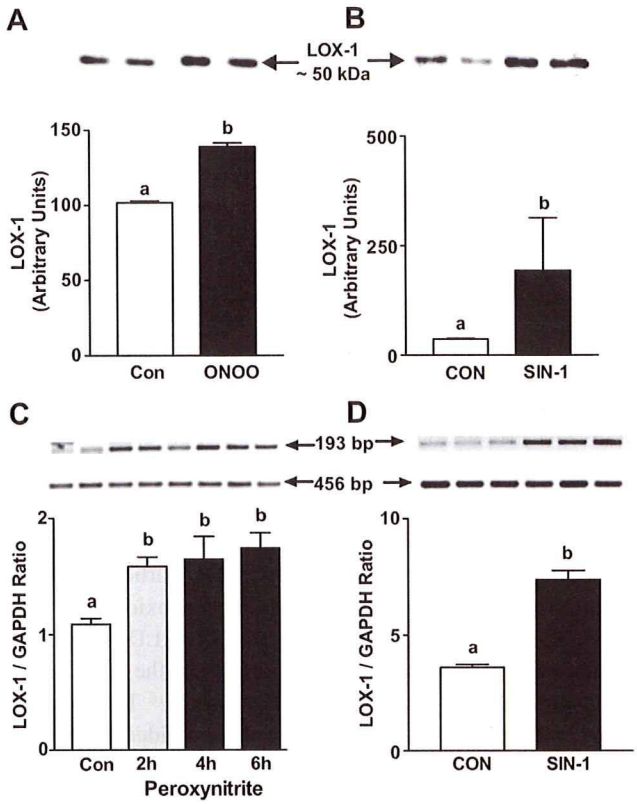

Figure 8. Peroxynitrite- and SIN-1–induced LOX-1 expression. A and B, Representative Western blots showing LOX-1 protein expression in endothelial cells treated with (A) vehicle (Con; 0.3 mol/L NaOH) or peroxynitrite ($ONOO^-$; 25 μmol/L) for 6 hours and in endothelial cells treated with (B) vehicle (water) or SIN-1 (0.25 mmol/L) for 6 hours. C and D, Also shown are representative DNA gels in response to treatment with either (C) peroxynitrite for 2, 4, and 6 hours or (D) SIN-1 for 6 hours. The band corresponding with 193 bp is LOX-1, and 456 bp is GAPDH. Bars represent means±SEs. Different letters denote significant difference ($P<0.05$) from each other.

increased levels of autoantibody to oxLDL.[23] It has also been shown that women with increased circulating levels of oxLDL have a significantly higher risk for developing preeclampsia.[24,25] Thus, the data regarding circulating oxLDL or its autoantibodies have shown evidence to suggest increased circulating levels of oxLDL in preeclamptic women; however, some of the data are conflicting. Our data provide the first direct evidence for the presence of increased oxLDL that has accumulated within the vasculature of women with preeclampsia.

The possible mechanisms by which oxLDL accumulates in the vasculature of women with preeclampsia and their consequences have not been described previously. It is known that oxLDL binds to LOX-1 on vascular cells, including endothelial cells and smooth muscle cells. LOX-1 is a major endothelial receptor for the uptake of 50% to 70% of oxLDL.[26] In the present study, we found enhanced expression of LOX-1 primarily in the endothelial cell layer of the small arteries in women with preeclampsia. This could lead to the enhanced uptake and accumulation of oxLDL in the arterial walls, which could have deleterious effects by inducing and maintaining oxidative stress that may subsequently lead to endothelial cell dysfunction.

Binding of oxLDL to LOX-1 could activate the NADPH oxidase enzyme system to generate superoxide.[12] We demonstrated increased NADPH oxidase activity in cultured endothelial cells in response only to plasma from women with preeclampsia, which was significantly reduced by blocking with mAbLOX-1 or siRNA to LOX-1. This suggests that ligands to LOX-1, possibly oxLDL, play a role in upregulating the NADPH oxidase enzyme system specifically in preeclampsia. To further address whether the increased NADPH oxidase activity and the observed increase in superoxide levels were attributable not only to increased LOX-1 expression but also to increased levels of ligands circulating in the plasma, we examined LOX-1 protein expression and NADPH oxidase activity at an earlier time point (6 hours) before LOX-1 expression was induced. We found that, despite normal LOX-1 expression in response to plasma from 3 groups of women at 6 hours, NADPH oxidase activity was increased significantly only in the preeclamptic group, suggesting that initial increases in superoxide levels might stem from increased circulating levels of ligands to LOX-1. Long-term increases in NADPH oxidase and subsequent superoxide levels could be a combined effect of both increases in oxLDL levels and increased LOX-1 expression, as seen in our 24-hour experimental protocol.

In this study, we have also shown a novel pathway for the regulation of LOX-1 by peroxynitrite. Peroxynitrite increased both LOX-1 mRNA and protein expression. Furthermore, we have demonstrated a feed-forward loop by which peroxynitrite further upregulates and maintains a higher LOX-1 expression. Thus, it appears that generation of peroxynitrite through LOX-1 further upregulates LOX-1 and may be a key player in perpetuating oxidative stress in preeclampsia. Indeed, blocking LOX-1 in endothelial cells in response to preeclamptic plasma significantly reduced superoxide and peroxynitrite levels.

Other than peroxynitrite, LOX-1 can be upregulated by a number of factors in the plasma, such as tumor-necrosis factor-α, transforming growth factor-β, oxLDL, angiotensin II, endothelin I, C-reactive protein, and 8-isoprostane, to mention a few.[27] Many of these factors have been shown to be elevated in the plasma of women with preeclampsia[2]; thus, the upregulation of LOX-1 and activation of NADPH oxidase could be through the action of multiple factors. In our experiments, mAbLOX-1 reduced NADPH oxidase activity by >50%, suggesting that this receptor could be a major factor for inducing oxidative stress in preeclampsia. Moreover, apocynin, an NADPH oxidase inhibitor, reduced superoxide generation in response to preeclamptic plasma to the same extent as mAbLOX-1, suggesting that most of the NADPH oxidase activity in preeclampsia could be through LOX-1, as demonstrated in this bioassay. This does not exclude the role of other factors in activating the NADPH oxidase enzyme system, because mAbLOX-1 did not completely suppress NADPH oxidase activity. Importantly, apart from oxLDL, there is an array of structurally different, negatively charged molecules, such as polyanionic chemicals (polyinosinic acid and carrageenan), anionic phospholipids (phosphatidylserine and phosphatidylinositol), and cellular

ligands, such as apoptotic/aged cells, activated platelets, and bacteria, that can act as ligands for LOX-1.[27,28] Although this study did not focus on the ligands, but on the receptor itself, it is possible that multiple factors could be involved in the activation of LOX-1, thus having broad implications for a common mechanism for vascular dysfunction in women with preeclampsia.

Because of the heterogeneity of preeclampsia, different circulating factors could play a role in different women or during different stages of the disease process. Nonetheless, LOX-1 pathway appears to be a predominant pathway in inducing cellular oxidative stress in response to circulating factors in the plasma of women with preeclampsia. Moreover, because the effect of a number of circulating factors converges on LOX-1 pathway, LOX-1 could be a potential target for therapeutic intervention.

Finally, preeclampsia is often considered the extreme of a pregnancy continuum, with evidence of inflammation and oxidative stress increased in pregnancy when compared with the nonpregnant state.[29] However, in our study, the responses in the nonpregnant and the pregnant groups were similar in most of the outcome measures in the vasculature and in isolated endothelial cells. However, the levels of superoxide were in fact reduced in endothelial cells treated with pregnant plasma relative to nonpregnant plasma. Thus preeclampsia, in part, could be a lack of adaptation to pregnancy, in addition to circulating factors that activate the endothelium.

Perspectives

Preeclampsia is likely a multifactorial disorder, with inflammation, oxidative stress, immune mechanisms, and other pathways playing a role. Although a number of studies have clearly shown evidence for vascular oxidative stress in preeclampsia,[14,30–32] the antioxidant trials with vitamins C and E have failed to reduce the incidence of preeclampsia[33] and in some cases have even been detrimental by increasing the rate of low birth weight babies.[34] These antioxidants are designed to scavenge oxidants and not to inhibit generation of such molecules. Furthermore, they would not provide the first line of defense in scavenging intracellular superoxide. In light of such evidence, identification of pathways, such as LOX-1, that could be blocked may prove to be more effective in reducing intracellular oxidative stress. Our study clearly suggests that LOX-1 pathway could be a major pathway involved in promoting and maintaining a vicious cycle of events resulting in oxidative stress and ultimately leading to endothelial cell dysfunction in preeclampsia.

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Disclosures

None.

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