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Modulation of ADP-Induced Platelet Activation by Aspirin and Pravastatin: Role of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1, Nitric Oxide, Oxidative Stress, and Inside-Out Integrin Signaling

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ABSTRACT

Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1), a receptor for oxidized-LDL, is up-regulated in activated endothelial cells, and it plays a role in atherothrombosis. However, its role in platelet aggregation is unclear. Both aspirin and HMG CoA reductase inhibitors (statins) reduce LOX-1 expression in endothelial cells. In this study, we investigated the effect of aspirin and pravastatin on LOX-1 expression on platelets. After ADP stimulation, mean fluorescence intensity of LOX-1 expression on platelets increased 1.5- to 2.0-fold. Blocking LOX-1 inhibited ADP-induced platelet aggregation in a concentration- and time-dependent manner. We also established that LOX-1 is important for ADP-stimulated inside-out activation of platelet $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrins (fibrinogen receptors). The specificity of this interaction was determined by arginine-glycine-aspartate-peptide inhibition. Furthermore, we found that LOX-1 inhibition of integrin activation is mediated by inhibition of protein kinase C activity. In other experiments, treatment with aspirin (1–10 mM) and pravastatin (1–5 μM) reduced platelet LOX-1 expression, with a synergistic effect of the combination of aspirin and pravastatin. Aspirin and pravastatin both reduced reactive oxygen species (ROS) released by activated platelets measured as malonyldialdehyde (MDA) release and nitrate/nitrite ratio. Aspirin and pravastatin also enhanced nitric oxide (NO) release measured as nitrite/nitrite + nitrate (NOx) ratio in platelet supernates. Small concentrations of aspirin and pravastatin had a synergistic effect on the inhibition of MDA release and enhancement of nitrite/NOx. Thus, LOX-1 is important for ADP-mediated platelet integrin activation, possibly through protein kinase C activation. Furthermore, aspirin and pravastatin inhibit LOX-1 expression on platelets in part by favorably affecting ROS and NO release from activated platelets.

Lectin-like oxidized LDL (ox-LDL) receptor-1 (LOX-1) is an ~50-kDa surface receptor initially found to be expressed on endothelial cells (Sawamura et al., 1997). It has been shown to bind to ox-LDL and to mediate endocytosis of ox-LDL by endothelial cells, causing endothelial activation and dysfunction (Moriwaki et al., 1998). The crystal structure of LOX-1 has been resolved, and it has a C-type lectin-like domain, neck domain, transmembrane domain, and a short 34-amino acid residue cytoplasmic domain (Ohki et al., 2005; Park et al., 2005). LOX-1 has been postulated to exert an important role in the pathogenesis of atherosclerosis. Numerous pathological effects of ox-LDL have been shown to be mediated by LOX-1, such as inflammation, oxidative stress, and apoptosis (Mehta et al., 2006). Mostly, the effects of LOX-1 in atherogenesis are mediated by LOX-1 expressed on endothelial cells. It is unclear whether LOX-1 expressed by other cell

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ABBREVIATIONS: LDL, low-density lipoprotein; ox-LDL, oxidized low-density lipoprotein; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; RGD, arginine-glycine-aspartate; PKC, protein kinase C; PRP, platelet-rich plasma; PPP, platelet-poor plasma; FACS, fluorescence-activated cell sorting; MDA, malonyldialdehyde; ROS, reactive oxygen species; NOx, nitrite + nitrate; MFI, mean fluorescence intensity; Ab, antibody.

types, such as immune cells or platelets, also contributes in disease progression, although inflammation and thrombosis are essential components of atherogenesis.

Platelets have also been shown to express small amounts of LOX-1 (Chen et al., 2001; Puccetti et al., 2005b). The HMG CoA reductase inhibitor atorvastatin was reported to decrease LOX-1 expression in hypercholesterolemic patients (Puccetti et al., 2005b). More recently, the role of oxidative stress in affecting platelet aggregation has been elucidated (Krötz et al., 2002, 2004). However, the role of LOX-1 in platelet aggregation is still largely unclear. It is tempting to speculate that LOX-1 plays a significant role in platelet aggregation, either indirectly through oxidative stress generated by ox-LDL or directly by influencing inside-out signaling of platelet integrin.

In this study, we report that the expression of LOX-1 on human platelets increases upon their exposure to ADP. We also report that blocking LOX-1 with a specific LOX-1 antibody inhibits ADP-induced platelet aggregation. Furthermore, we demonstrate that the inhibition of platelet aggregation occurs through interference with platelet binding to fibrinogen, which is mediated by platelet $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrins. We also show that treatment of platelets with aspirin or pravastatin reduces LOX-1 expression and that combination treatment of aspirin and pravastatin synergistically reduces LOX-1 expression.

Materials and Methods

Materials and Reagents. Monoclonal antibody against human LOX-1 (JTX92) was provided by Dr. T. Sawamura. This antibody is raised in mice with humanized Fc portion. Fluorescein isothiocyanate-conjugated secondary antibody was purchased from BD Biosciences (San Jose, CA). Monomeric fibrinogen conjugated with fluorescent Alexa Fluor 488 was purchased from Invitrogen (Carlsbad, CA). PepTag Assay for nonradioactive detection of protein kinase C (PKC) was obtained from Promega (Madison, WI). ADP and arginine-glycine-aspartate (RGD)-peptide were purchased from Sigma-Aldrich (St. Louis, MO).

Platelet Isolation. Platelet-rich plasma (PRP) was isolated from peripheral blood collected in 3.8% sodium citrate (nine parts of blood to one part of sodium citrate) from healthy donors. The blood was centrifuged at 200*g* for 15 min to obtain PRP. Platelet-poor plasma was isolated by centrifuging the plasma after PRP collection for another 10 min at 500*g* at room temperature. The protocol was approved by the Institutional Review Board, and informed consent was obtained from each subject.

Platelet Aggregation Study. Platelets were counted by flow cytometry (FACStar; BD Biosciences). Platelet count in PRP was adjusted at 300,000/mm^3 by adding PPP to PRP. PRP then was incubated at 37°C for 5 min before treatment. In the first set of experiments, before 10 μM ADP stimulation, platelets were incubated for 1, 5, and 10 min at room temperature with JTX92 at concentrations of 1, 5, and 10 μg/ml. For the second set of experiments, platelets were pretreated with aspirin at 1, 5, or 10 mM or with pravastatin at 1, 5, or 10 μM for 5 min at room temperature. Then, 10 μM ADP was added. Platelet aggregation was measured by using a four-channel platelet aggregometer (Bio/Data Corp., Horsham, PA).

LOX-1 Expression. In ADP-stimulated platelets, the expression of LOX-1 was studied with the use of JTX92 and fluorescein isothiocyanate-conjugated secondary antibody. First, platelets were washed and resuspended in FACS buffer containing phosphate-buffered saline, 2% fetal calf serum, and 0.1% sodium azide. Then, primary antibody against LOX-1 was added, and samples were briefly vortexed and incubated at room temperature for 10 min. Thereafter, primary antibody was washed with FACS buffer, secondary antibody was added, and samples were incubated for another 10 min at room temperature. Then, unbound secondary antibodies were washed and resuspended in FACS buffer before flow cytometric analysis. The expression levels were confirmed by FACS analysis using FACSCalibur (BD Biosciences).

Fibrinogen Binding to Platelets. Platelets were treated with anti-LOX-1 antibody (1, 5, and 10 μg/ml) and incubated for 10 min at room temperature. This was followed by the addition of 10 μM ADP together with 200 μg/ml Alexa Fluor 488-human fibrinogen, and the sample was incubated at 37°C for 15 min. Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry. For the RGD inhibition experiment, platelets were isolated from PRP, and then they were gel-filtrated on a Sepharose 4B column (GE Healthcare, Piscataway, NJ). Finally, they were eluted with elution buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.35 mM bovine serum albumin, 3.3 mM NaH_2PO_4, and 4 mM HEPES, pH 7.4. Then, 1 mM RGD-peptide was added together with 200 μg/ml Alexa Fluor 488-conjugated human fibrinogen supplemented with 1 mM $CaCl_2$ (Basani et al., 2001). Thereafter, 10 μM ADP was added followed by incubation at 37°C for 15 min. Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry.

In other experiments, platelets were initially treated with 10 mM aspirin or with 10 μM pravastatin for 5 min at room temperature. Then, 10 μM ADP was added together with 200 μg/ml Alexa Fluor 488-human fibrinogen, and the sample was incubated at 37°C for 15 min (Heilmann et al., 1994). Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry using FACSCalibur.

Protein Kinase C Activity Assay. For determination of protein kinase C activity, platelets were pelleted and homogenized. Then, protein was passed through a DEAE cellulose column, and it was eluted as per protocol. After treatment with protease inhibitors, brightly colored protein kinase C peptide substrate was added, and the reaction was allowed to proceed at 30°C for 30 min. After adding 80% glycerol, samples were run on 0.8% agarose gel in 50 mM Tris-HCl, pH 8.0, until separation. Phosphorylated substrate migrates toward the cathode, and unphosphorylated substrate migrates toward the anode.

MDA and Nitrite/Nitrate Measurement. Malonyldialdehyde (MDA) concentration in the platelet supernates was measured spectrophotometrically, as an index of ROS generation, using an MDA kit (Oxis International, Inc., Foster City, CA).

End products of nitric oxide (NO) interactions, nitrite and total nitrite + nitrate (NOx), were analyzed using a chemiluminescence analyzer (model NOA 280i; Seivers, Boulder, CO). Nitrate concentration was calculated by subtracting nitrite concentration from total NOx concentration. The aqueous samples of the exiting media collected during the experiments were drawn using a gas-tight syringe (Hamilton, Reno, NV). A 250-μl volume was injected into the respective reducing solution in a radical purge vessel (Seivers). The reducing agent converts nitrite or total NOx from injected samples to NO. The nitrite reducing solution was 0.2 M KI and glacial acetic acid mixed in a 1:3 volumetric ratio. The nitrate reducing solution was a saturated solution of VCl_3 in HCl (stock solution of 0.8 g VCl_3 and 8 ml of HCl diluted to 100 ml in deionized water) at 95°C. The reducing solution was continuously bubbled with N_2 to purge NO from the solution and to transport NO into the chemiluminescence detector wherein it reacts with ozone and emits light in the infrared region. The concentrations were obtained by comparing them with $NaNO_2$ or $NaNO_3$ standard calibration curves. The nitrate/nitrite and nitrite/NOx ratio is referred to as the ROS level and the NO level, respectively (Kavdia et al., 2000; Kavdia, 2006).

Statistical Analysis. Results are shown as mean ± S.D. All data were analyzed by Fisher's exact test. $p < 0.05$ was accepted as

indicating statistical significance. All calculations were performed using the SPSS library (SPSS Inc., Chicago, IL).

Results

Up-Regulation of LOX-1 Expression on Platelets by ADP Stimulation. We confirmed that LOX-1 is expressed on human platelets by using JTX92 by flow cytometry (Fig. 1A). ADP stimulation significantly increased LOX-1 expression on platelets. Mean fluorescence intensity (MFI) of LOX-1 expression was found to increase 1.5- to 2.0-fold ($p < 0.01$) versus basal LOX-1 expression (Fig. 1B).

Engaging LOX-1 Receptor with Anti-LOX-1 Antibody Inhibits Platelet Aggregation. To determine whether LOX-1 has any role in platelet aggregation function, we used JTX92. This monoclonal antibody has been reported previously to block the LOX-1 effect (Li et al., 2003a,b). We found that pretreatment with anti-LOX-1 antibody blocked ADP-stimulated platelet aggregation in a concentration-dependent manner (Fig. 2A). Because JTX92 is an antibody against LOX-1 raised in mice but its Fc portion has been switched to human Fc (humanized), we used PPP as a control because it contains a considerable amount of human γ-globulin. Time course experiments were performed by preincubating PRP with 10 μg/ml anti-LOX-1 antibody for 1, 5, and 10 min. Incubation was not done longer than 10 min because antibody binding to receptors may induce internalization and may confound the data. The experiment revealed that inhibition of platelet aggregation occurs as early as 1 min into incubation of platelets with the antibody (Fig. 2B). These data suggest that LOX-1 plays an important role in ADP-mediated platelet aggregation.

Anti-LOX-1 Antibody Inhibits Integrin-Mediated Platelet Binding to Fibrinogen. ADP, through its ADP receptor, is known to activate platelet integrin by the inside-out integrin signaling pathway (Li et al., 2003c; Abrams, 2005). It is possible that LOX-1 receptor engagement by antibody activates intracellular signaling events that block the "inside-out" signaling pathway of integrin activation.

To identify more precisely the step(s) in platelet aggregation that is(are) inhibited by anti-LOX-1 antibody, we tested the binding of platelets to fibrinogen monomer conjugated with Alexa Fluor 488. Platelets were then analyzed by flow cytometry to determine their binding capacity to fibrinogen monomers. This assay enabled us to confirm that ADP stimulation increased binding of platelets to fibrinogen. Figure 3A shows the increase in fluorescently labeled fibrinogen binding to platelets after ADP stimulation. The level of binding to fibrinogen after ADP stimulation (open histogram) is 3-fold higher compared with the unstimulated level (solid histogram). This confirms that integrin adhesion to ligand containing a repetitive pattern of binding sites, such as fibrinogen, is stronger compared with adhesion to ligand with a single binding site, because FACS-based assay using soluble monomeric ligand to integrin, such as intercellular adhesion molecule-1 binding to leukocyte-integrin lymphocyte function-associated antigen 1, is known to be undetected by flow cytometry. These data, therefore, supported the importance of an increase in clustering as opposed to an increase in

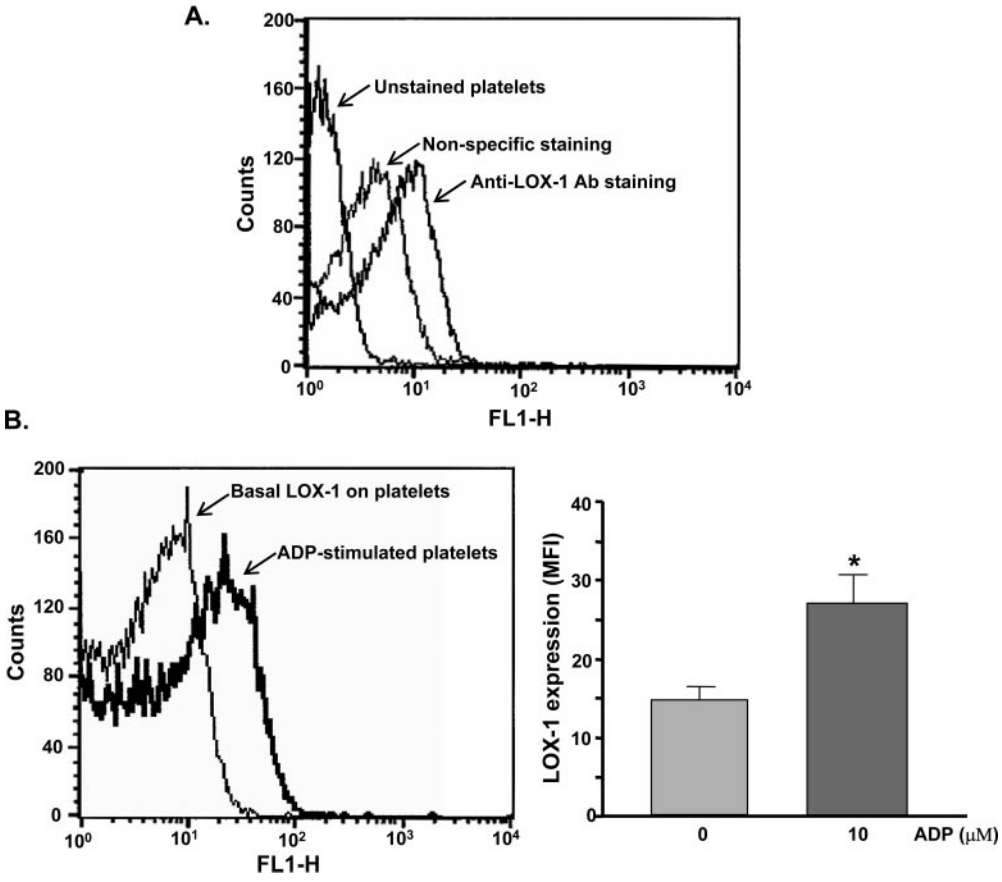


Fig. 1. FACS analysis of platelets showing the expression of LOX-1. A, representative histograms of unstained platelets, nonspecific binding, and platelets after treatment with anti-LOX-1 antibody (Ab) are shown. B, left, representative LOX-1 expression level without and with 10 μM ADP stimulation. Right, summary of data derived from FACS analysis. LOX-1 level is expressed as the MFI with data from three different healthy donors. *, $p < 0.01$ versus control (0 μM ADP).

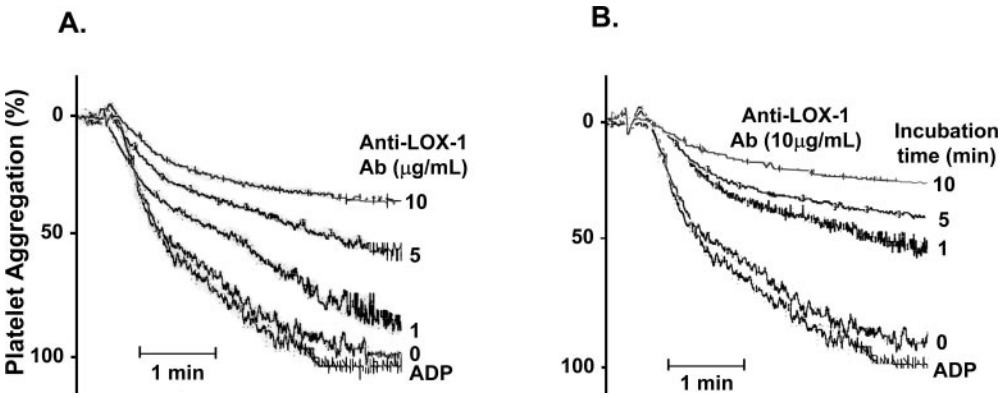


Fig. 2. Anti-LOX-1 Ab inhibits platelet aggregation. PRP was isolated from normal human volunteers. Platelet aggregation was measured by light transmission after stimulation with 10 μM ADP. Inhibition of platelet aggregation occurs after incubation of PRP with anti-LOX-1 antibody. The inhibition of platelet aggregation by LOX-1 antibody occurs in a dose- (A) and time-dependent manner (B).

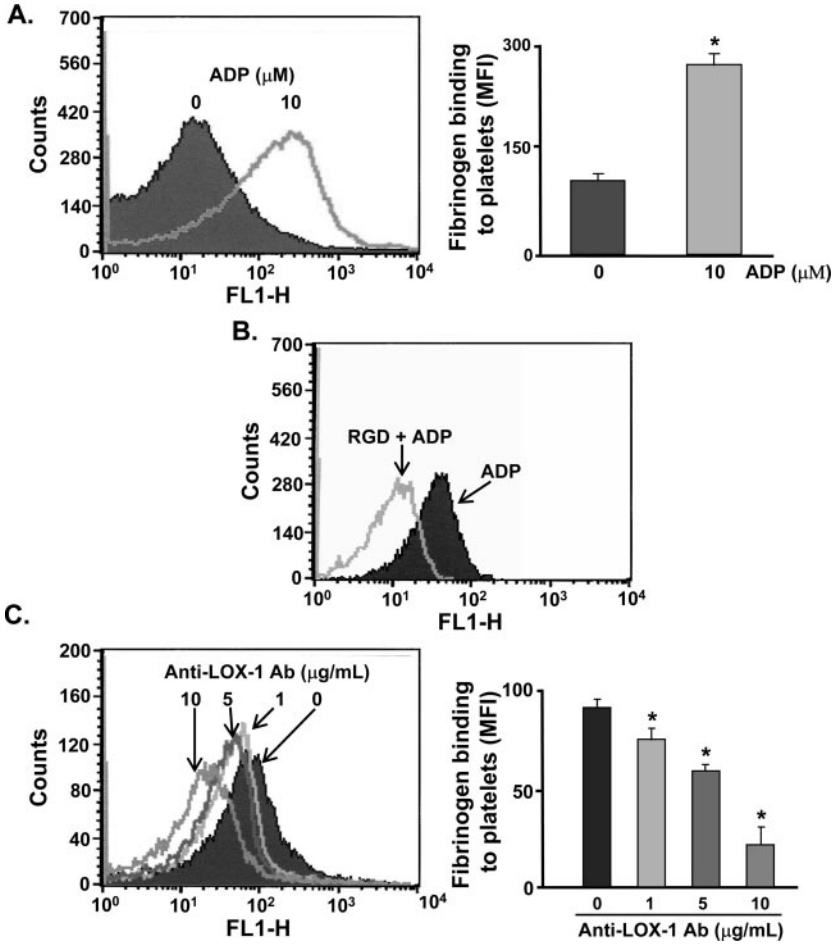


Fig. 3. Alexa Fluor 488-conjugated fibrinogen binding to platelets was tested by flow cytometry. A, increase in binding of Alexa Fluor 488-conjugated fibrinogen after 10 μM ADP stimulation compared with unstimulated platelets (*, $p < 0.01$). B, specificity of fibrinogen binding to platelet integrin is confirmed by the inhibition of 1 mM RGD peptide. C, left, inhibition of platelet integrin-mediated binding of platelets to fibrinogen by anti-LOX-1 antibody. Platelets were initially treated with anti-LOX-1 antibody and incubated for 10 min at room temperature with 10 μM ADP together with Alexa Fluor 488-conjugated fibrinogen and incubated for an additional 15 min. C, right, summary data from three separate experiments (*, $p < 0.01$ versus 0 μM anti-LOX-1 Ab).

affinity (conformational change) on integrin activity (Carman and Springer, 2003). To confirm that this interaction is specifically mediated by integrin, we used RGD-peptide as the inhibitor. RGD-peptide is known to act as a competitive inhibitor to integrin binding, because it is the 3-amino acid sequence of fibrinogen that binds to integrin. (Basani et al., 2001). Figure 3B shows RGD inhibition of fibrinogen-platelet interaction after ADP stimulation. Thus, we confirmed that platelet interaction to fibrinogen is specifically integrin-mediated.

In keeping with this hypothesis, treatment with anti-LOX-1 antibody decreased binding of fibrinogen monomer to platelets in a concentration-dependent manner (Fig. 3C). In this experiment, we first treated PRP with anti-LOX-1 antibody for 10 min at room temperature and then with ADP together with Alexa Fluor 488-conjugated fibrinogen at 37°C for 15 min. Even with a short incubation time, we could identify inhibition of fibrinogen binding to platelets. This confirms that LOX-1 is important in ADP-mediated inside-out signaling of platelet integrins.

Anti-LOX-1 Antibody Treatment Inhibits Protein Kinase C Activation. The finding that anti-LOX-1 antibody can block integrin-mediated platelet binding to fibrinogen suggests that LOX-1 engagement with antibody may inter-

fere with intracellular signaling of inside-out activation of integrin. Protein kinase C has been reported to mediate this pathway (Abrams, 2005). One of the responses of platelets to most agonists is the activation of phospholipase C. Phospholipase Cβ is primarily responsible for the rapid burst of phosphoinositide hydrolysis that occurs during platelet activation by agonists. Activated phospholipase C hydrolyzes phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol-1,4,5-trisphosphate. Diacylglycerol activates protein kinase C, and it contributes to protein phosphorylation. Inositol-1,4,5-trisphosphate binds to receptors in the dense tubular system and the cell membrane, and it allows an influx of Ca^{2+} into the cytoplasm (Abrams, 2005; Han et al., 2006; Yacoub et al., 2006)

We studied the activity of protein kinase C to phosphorylate its substrate. Treatment with LOX-1 antibody indeed inhibited protein kinase C activity (Fig. 4). Phosphorylated protein kinase C, which represents the activated form, travels toward the cathode (up), whereas the unphosphorylated protein migrates toward the anode (down). The inhibition seems to readily take place with the lowest concentration of antibody used, i.e., 0.1 μg/ml. In essence, we established that LOX-1 antibody inhibits activation of protein kinase C intracellular signaling, which is important for platelet integrin activation. As a control, we included the well-known PKC inhibitor staurosporine to show that protein kinase C activation by ADP is specific.

Inhibition of Platelet Aggregation by Aspirin and Pravastatin. In other experiments, we studied platelet inhibitory effects of aspirin and pravastatin on platelet aggregation and the role of LOX-1 in this process.

As shown in Fig. 5A, aspirin inhibited ADP-induced platelet aggregation in a concentration-dependent manner. Pravastatin, in contrast, did not induce significant inhibition of platelet aggregation. The combination of aspirin and pravastatin showed a modest synergistic effect. With 1 mM aspirin and 1 μM pravastatin, inhibition of platelet aggregation was 17 $\pm$ 4% (versus 22 $\pm$ 6% with aspirin alone; $p < 0.05$).

LOX-1 Down-Regulation by Aspirin and Pravastatin. As mentioned earlier, LOX-1 is expressed on human platelets, and ADP stimulation significantly increases LOX-1 expression (Fig. 1B). Upon treatment with 1 to 10 mM aspirin, LOX-1 expression was found to be reduced in a concentration-dependent manner ($p < 0.05$) (Fig. 5B). Likewise, 1 to 10 μM pravastatin treatment reduced LOX-1 expression in a concentration-dependent manner ($p < 0.05$). Combination of 1 mM aspirin and 1 μM pravastatin significantly reduced LOX-1 expression level ($p < 0.05$ versus aspirin or pravastatin alone).

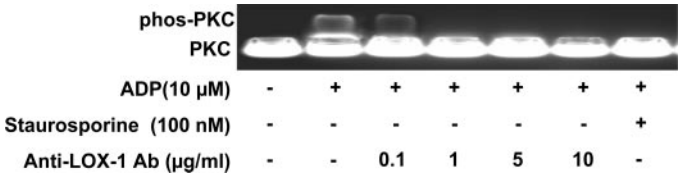


Fig. 4. Protein kinase C activity is inhibited by anti-LOX-1 antibody treatment. Phosphorylated protein kinase C substrate migrates up, and unphosphorylated protein kinase C substrate migrates down. The first lane (left-most) represents untreated platelets, the second lane represents ADP stimulation, the third through sixth lanes represent 10 μM ADP stimulation in the presence of anti-LOX-1 antibody (0.1, 1, 5, and 10 μg/ml, respectively). The seventh lane represents ADP stimulation in the presence of 100 nM staurosporine, a protein kinase C inhibitor, as a positive control. This figure is representative of three separate experiments.

Treatment with Aspirin and Pravastatin Reduces Oxidative Stress. Because LOX-1 has been widely implicated as a receptor for ox-LDL, which is up-regulated in the presence of oxidative stress (Mehta et al., 2006), we measured the level of oxidative stress in ADP-treated platelets. Using MDA as a measurement of oxidative stress, we found that ADP increased MDA concentration, and aspirin and/or pravastatin treatment reduced MDA levels in the PRP (Fig. 6A). Furthermore, the combination of small amounts of aspirin (1 mM) and pravastatin (1 μM) synergistically reduced MDA levels ($p < 0.01$ versus aspirin alone or pravastatin alone).

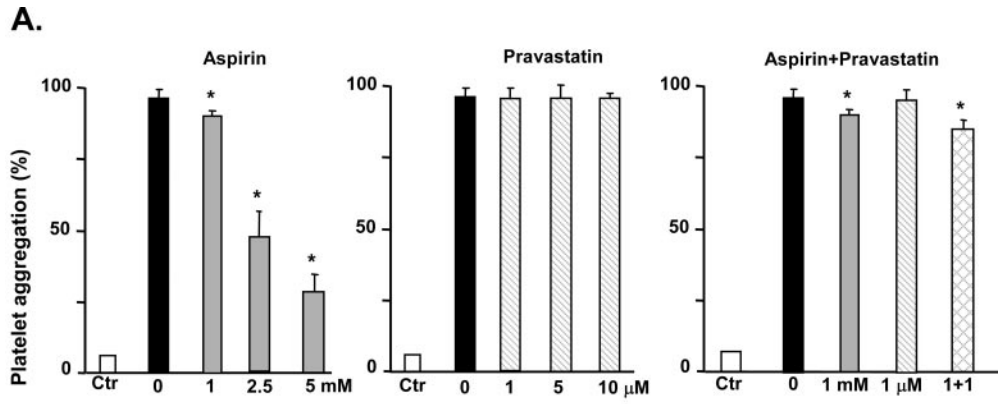
NO reacts readily with molecular oxygen and O_2^--derived free radicals. The oxidation of NO at physiological pH by O_2 leads to nitrite formation via intermediates nitrogen dioxide and nitrous anhydride. With superoxide, NO forms peroxynitrite that leads to formation of nitrate as the main end product. Thus, in two different treatments, if total NOx concentration is constant, increase in nitrate-to-nitrite ratio can be interpreted by increased ROS, whereas decrease in nitrate-to-nitrite ratio can be interpreted by increased NO levels and decreased ROS (Kavdia et al., 2000; Kavdia, 2006). Treatment with aspirin and pravastatin, the nitrate-to-nitrite ratio decreased with increase in respective dose compared with control (Fig. 6B). This indicated that both these agents inhibited ROS and increased NO levels. This trend is also supported by the increase in nitrite-to-total NOx ratio, as seen in Fig. 6C, with increase in drug dose, because a reduction in ROS level increases the nitrite formation rates through metabolism with molecular oxygen.

Aspirin, but Not Pravastatin, Inhibits Integrin-Mediated Binding of Platelets to Fibrinogen. As shown in Fig. 7, aspirin treatment reduced the binding capacity of fibrinogen to platelets, whereas pravastatin had a small effect. These data demonstrate that aspirin-mediated reduction in LOX-1 expression is associated with reduction in ROS generation and platelet aggregation. However, pravastatin-mediated decrease in LOX-1 is only associated with reduction in ROS, but only minimal inhibition of platelet aggregation, suggesting separate mechanisms linking platelet aggregation and ROS reduction.

Discussion

We have demonstrated that blocking LOX-1 inhibits platelet aggregation and that this phenomenon is mediated by inhibition of protein kinase C activity.

LOX-1, a receptor for oxidized-LDL, was initially reported to be mainly expressed in endothelial cells (Sawamura et al., 1997). It is present in human atherosclerotic lesions (Kataoka et al., 1999). Its role in the pathogenesis of atherosclerosis has been mainly attributed to endothelial dysfunction (Mehta et al., 2006). Some studies have shown the presence of LOX-1 on human platelets. The expression of LOX-1 in endothelial cells has been shown to mediate its interaction with platelets (Kakutani et al., 2000). Because platelets are also important players in atherogenesis (Gawaz et al., 2005), we postulated that LOX-1 may influence platelet function (Chen et al., 2001; Puccetti et al., 2005b). In the current



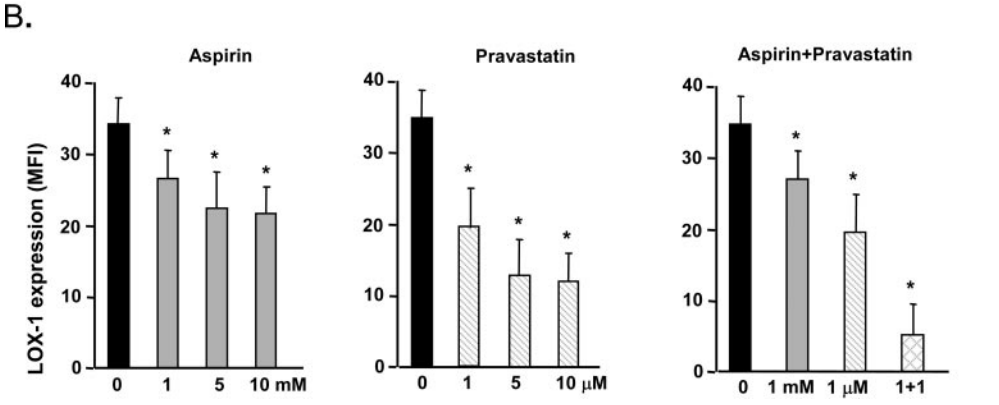
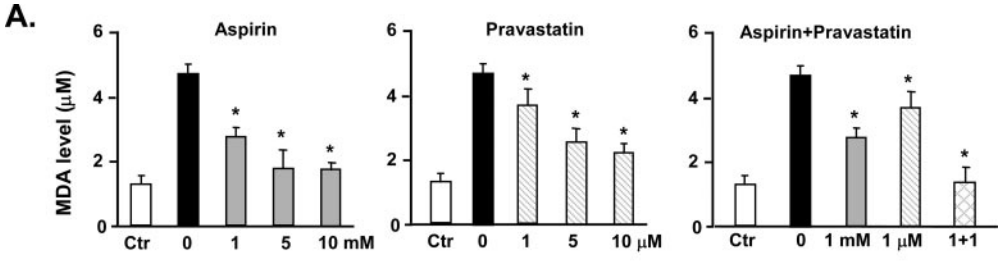
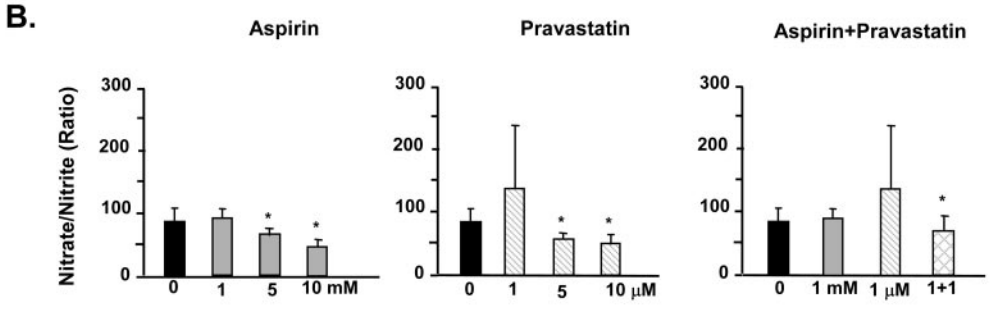


Fig. 5. Inhibition of platelet aggregation and LOX-1 expression by aspirin and pravastatin. A, aspirin inhibits platelet aggregation in a dose-dependent manner (left), but pravastatin does not have a significant inhibitory effect (middle). The combination of both aspirin and pravastatin (each in lowest concentration) inhibits platelet aggregation (right). B, LOX-1 expression from FACS analysis demonstrated as graph bars. Platelets collected from PRP are stimulated with ADP in the presence of aspirin, pravastatin, and their combination. LOX-1 level is expressed as the MFI with data from three different healthy donors. Aspirin (left), pravastatin (middle), and combination of both (right) reduce the expression level of LOX-1. *, $p < 0.05$ versus control.





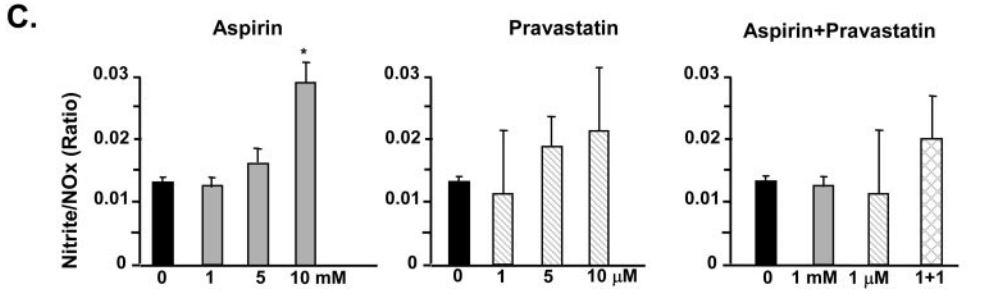


Fig. 6. A, dose-dependent decrease in nitrate-to-nitrite ratio and MDA levels following platelet treatment with aspirin, pravastatin, and their combination compared with control, indicating the inhibition of ROS and increase in NO levels by both aspirin and pravastatin. B, dose-dependent decrease in the nitrite/nitrate ratio. C, dose-dependent increase in nitrite/NOx ratio, which supports the results of the inhibition of ROS and increase in NO levels. *, $p < 0.05$ versus control.

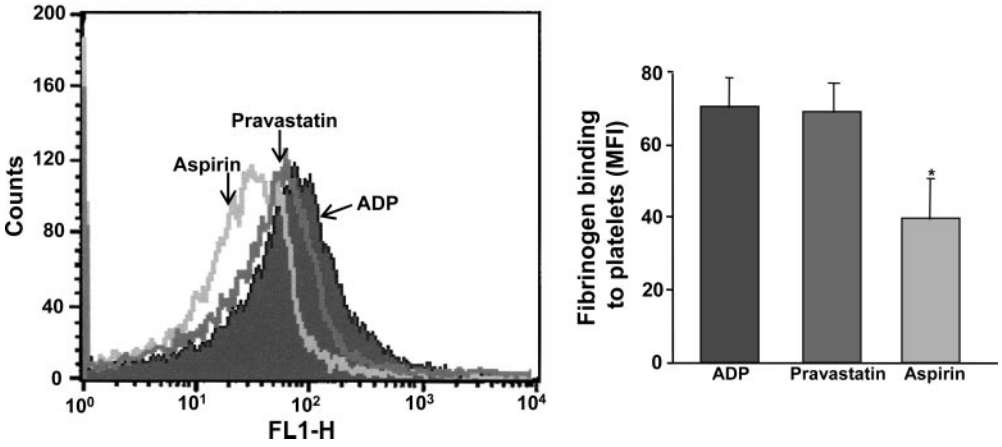


Fig. 7. Alexa Fluor 488-conjugated fibrinogen binding to platelets was examined by flow cytometry. Platelets were initially treated with 10 mM aspirin or 10 μM pravastatin, and then 10 μM ADP was added together with 200 μM Alexa Fluor 488-fibrinogen, and the mixture was incubated at 37°C for 15 min. Left, inhibition with aspirin of Alexa Fluor 488-conjugated fibrinogen to 10 μM ADP-stimulated platelets; pravastatin has a minimal effect in a representative experiment. Right, bar graphs of MFI from three independent experiments. *, $p < 0.05$ versus ADP alone.

study, we addressed the question of whether LOX-1 on platelets plays a role in platelet aggregation.

LOX-1 is known to mediate binding to ox-LDL, which itself is a product of oxidative stress. The role of oxidative stress in thrombosis has become appreciated recently (Krötz et al., 2004). ROS released by endothelial cells and immune cells (neutrophils, monocytes, and macrophages) and by platelets may play an important role in thrombus formation. Here, we provide another possible contribution of LOX-1 in modifying platelet aggregation. LOX-1 is an important component of integrin-mediated platelet binding to fibrinogen, which is the ultimate step in thrombosis.

ADP stimulation activates platelet integrins, mainly $\alpha_{IIb}\beta_3$ and to a lesser extent $\alpha_2\beta_1$, through a process called inside-out signaling (Li et al., 2003c; Abrams, 2005). This signaling pathway, common in all other integrins, induces conformational changes of these important adhesion molecules (Takagi et al., 2002; Vinogradova et al., 2002). In the present study, we show that LOX-1 affects the inside-out signaling of platelet integrins by inhibiting protein kinase C activation. To confirm the specificity of this reaction, we used RGD-peptide to inhibit integrin binding to fibrinogen (Basani et al., 2001; Xiong et al., 2002), and we were able to show similar response. Protein kinase C has been reported to be involved in inside-out signaling (Abrams, 2005; Han et al., 2006; Yacoub et al., 2006). It is likely that antibody engagement of LOX-1 on platelet triggers other signaling events that interfere with this process. The downstream signaling pathway of LOX-1 is still largely unknown. However, it is clear that antibody engagement may induce clustering of LOX-1 receptors and activate its downstream signaling cascades, or that it may block them (Li et al., 2003a,b). Further studies are warranted to clarify this issue.

We also show that aspirin and pravastatin reduce LOX-1 expression. This finding correlates with reduction of ROS generation and enhanced NO release. It is noteworthy that aspirin and pravastatin had synergistic effects on these measurements. Because oxidative stress stimulates LOX-1 expression (Mehta et al., 2006) and LOX-1 expression itself induces oxidative stress, it is conceivable that the reduction in LOX-1 expression by aspirin and pravastatin is either the basis and/or a consequence of reduction in oxidative stress.

In a previous study in endothelial cells (Mehta et al., 2004), aspirin in a dose- and time-dependent manner was shown to reduce ox-LDL-mediated LOX-1 expression. The effect of aspirin was thought to be the effect of salicylate moiety, because treatment of endothelial cells with salicylate, but not indomethacin, resulted in the suppression of LOX-1 expression, an effect similar to that of aspirin. It is noteworthy that both aspirin and its component salicylate decreased superoxide anion generation. Other studies in endothelial cells have shown that the HMG CoA reductase inhibitors simvastatin and atorvastatin reduce LOX-1 expression, up-regulate protein kinase B activity, and reduce ^{125}I-ox-LDL uptake in endothelial cells (Mehta et al., 2001). This was thought to result in an increase in endothelial nitric-oxide synthase expression and activity.

It is possible that inhibition of platelet aggregation by aspirin reflects oxidative stress reduction. Oxidative stress has clearly been shown to enhance platelet aggregation and thrombosis (Krötz et al., 2004). Platelets produce ROS because they have NAD(P)H-oxidase activity (Seno et al., 2001; Krötz et al., 2002). ROS affect thrombosis by increasing platelet recruitment into growing thrombus (Krötz et al., 2002). It is noteworthy that we did not see inhibition of platelet aggregation by pravastatin, even though ROS was significantly reduced. However, this does not necessarily mean that pravastatin does not have antithrombotic inhibition. It is possible that by reducing ROS level, pravastatin reduces platelet recruitment into the growing thrombus. In support of this hypothesis are the observations from our laboratory that another HMG CoA reductase inhibitor, atorvastatin, reduced the weight of thrombus in the rat aorta exposed to oxidative stress (Gaddam et al., 2002). Furthermore, there is evidence that regulation of integrins may be affected by redox condition owing to the modifications of its disulfide bonds that may affect its affinity through conformational changes (Lahav et al., 2002; Walsh et al., 2004). Again, we observed that aspirin, but not pravastatin, by reducing ROS, inhibited integrin-mediated binding of platelet to fibrinogen. It is still unclear whether the redox state that can affect integrin disulfide bonds can be replicated in our fibrinogen binding assay, or for that matter, whether the redox state that alters disulfide bonds occurs in vivo. It is conceivable that ROS produced during oxidative stress may increase platelet LOX-1 expression. Clues in favor of this assumption

are emerging from preliminary observations on the effects of direct ROS generation by the Fe(III)-ascorbate reaction (Ciuffi et al., 1999) on thrombus formation in carotid arteries of wild-type and LOX-1-deficient mice.

In hypercholesterolemic patients, atorvastatin has been reported to reduce LOX-1 expression on platelets (Puccetti et al., 2005b). The level of expression of LOX-1 was measured by flow cytometry with a different clone of monoclonal antibody (JM90) than the antibody we used in this study. Both monoclonal antibodies are humanized, but most probably they recognize different epitopes of LOX-1 (Puccetti et al., 2005b). Even though the data of Puccetti et al. (2005b) were presented as percentage of LOX-1-positive platelets rather than MFI, this supports our findings of LOX-1 expression on platelets. This study and other studies show that platelet activation with thrombin, as opposed to ADP, also up-regulates expression levels of LOX-1 on the surface of platelets (Bruni et al., 2005; Puccetti et al., 2005a,b). Moreover, statins reduce the expression levels of P-selectin (Bruni et al., 2005; Puccetti et al., 2005a). This finding has been suggested to explain the antiplatelet effect of statins. Interestingly, in agreement with our study is the report that the effect of statins on down-regulation of LOX-1 on platelets occurs much earlier than the reduction in serum LDL-cholesterol level. This suggests that the antiplatelet effects of statins are independent of serum cholesterol effects. It is not clear whether HMG-CoA reductase is fully functional in platelets. In our system, this confounding factor was excluded. It is unlikely that the effect of pravastatin seen in the present study could have been mediated by the HMG-CoA inhibitory effect, because the LOX-1 down-regulation effect was observed almost immediately.

In conclusion, we propose that platelet LOX-1 plays an important role in ADP-induced platelet integrin binding to fibrinogen via interaction with protein kinase C and that LOX-1 thereby participates in inside-out signaling of platelet integrins (Fig. 8). Our study also shows that aspirin and pravastatin can reduce LOX-1 expression level associated with reduction in ROS production by platelets. These data suggest the importance of LOX-1 in platelet aggregation. Furthermore, they elucidate its role in the mechanism of antiplatelet effect of combination of aspirin and pravastatin. More importantly, we also demonstrate the synergistic effect of aspirin and pravastatin as antiplatelet agents.

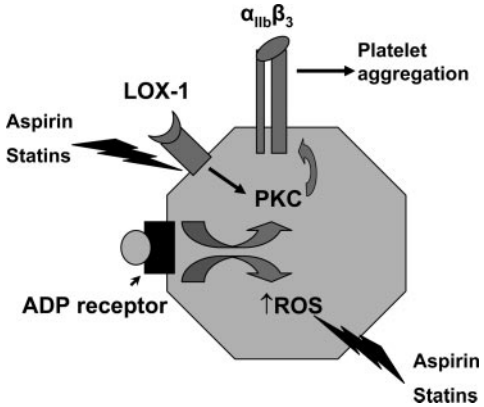


Fig. 8. ADP stimulation through ADP receptor activates inside-out signaling pathway of platelet integrins, mainly $\alpha_{IIb}\beta_3$, which involves PKC. LOX-1 blocking with anti-LOX-1 monoclonal antibody inhibits integrin activation by inhibiting PKC activation. Aspirin and pravastatin reduce both expression of LOX-1 and ROS.

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