β2 Integrin–Dependent Neutrophil Adhesion Induced by Minimally Modified Low-Density Lipoproteins Is Mainly Mediated by F2-Isoprostanes

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**Background**—Oxidation of LDL produces a series of biologically active, oxidized lipids. Among them, isoprostanes, and in particular iPF$_{2\alpha}$-III, seem to be crucial in mediating some of the key cellular events seen in myocardial ischemia-reperfusion injury.

**Methods and Results**—Minimally modified LDL (MM-LDL) triggers a dose-dependent, very rapid neutrophil adhesion to human fibrinogen. Rapid adhesion triggering correlates with degree of LDL oxidation and accumulation of isoprostanes. Isoprostanes accumulated in MM-LDL are major determinants of the proadhesive effect of oxidized LDL, as shown by experiments of receptor functional deletion. Moreover, evidence is provided of expression on human neutrophils of a biological active isoprostane receptor distinct from the classical thromboxane A$_2$ receptor.

**Conclusions**—These data suggest that isoprostanes are major contributors to the proadhesive effect induced by MM-LDL on neutrophils and provide additional evidence for the involvement of isoprostanes in the pathogenesis of myocardial ischemia/reperfusion injury. *(Circulation. 2002;106:2434-2441.)*

**Key Words:** leukocytes • lipoproteins • ischemia • reperfusion

Oxidation of low-density lipoproteins (LDLs) produces a series of biologically active lipids that mediate different cellular events seen in the developing atherosclerotic process. Among them, isoprostanes have been found to be increased in human atherosclerotic plaques and in oxidized LDL as long as endogenous antioxidants are consumed and subsequent breakdown of lipid hydroperoxides proceeds. F$_2$-isoprostanes (iPs), a series of prostaglandin (PG) F$_2$-like isomers formed primarily by a free-radical mechanism from arachidonic acid, have been shown to markedly increase in the coronary vessels during ischemia/reperfusion injury and acute percutaneous transluminal coronary angioplasty (PTCA) for myocardial infarction. They are also increased in association with several risk factors for the development of ischemic vascular disease. Moreover, iPF$_{2\alpha}$-III, one of the iPs formed during lipid peroxidation, has been found to be a potent vasoconstrictor and activator of neutrophil and platelet adhesion and a specific and quantitative marker of oxidant stress in vivo.

An inflammatory response involving neutrophils and leading to tissue injury has been demonstrated in the pathogenesis of ischemia-reperfusion syndrome and restenosis after PTCA, in which increased local levels of minimally modified LDL (MM-LDL) and isoprostanes are observed. Thus, we set out to explore the effects of MM-LDL on human neutrophil integrin-dependent rapid adhesion. We found that human MM-LDLs are able to trigger very rapid β$_2$ integrin–dependent adhesion of human polymorphonuclear neutrophils (PMNs) to human fibrinogen mainly through iPF$_{2\alpha}$-III or related isoprostanes. We also suggest that neutrophils express iPs receptors distinct from the classical thromboxane A$_2$ receptor (TP).

**Methods**

**Reagents and Antibodies**

fMLP and fibrinogen were from Sigma; Pertussis toxin (PTX) was from Alexis; iPF$_{2\alpha}$-III, U4419, and the TP antagonist SQ29,548 were from Cayman Chemical; blocking monoclonal Ab anti-human CD36 was from UBI (Hauppauge, NY). Blocking monoclonal Ab anti-human CD11a (Ts1/22) was from Dr T. Springer (Boston, Mass), anti-human CD11b was from Repligen Corp (Waltham, Mass), anti-human CD11c was from Santa Cruz Biotech (Santa Cruz, Calif), and anti-human CD18 (IB4) was from ATCC (Manassas, Va). Recombinant human ICAM-1 was purified as Ig-ICAM-1 fusion chimera (Ig H chain 2 to 3) from supernatants of stable transfected CHO cell lines. The monoclonal Ab anti-human lectin-like ox-LDL-receptor-1 (LOX-1) was kindly donated by Dr...
Sawamura (Department of Bioscience, Osaka, Japan). All lipids and peptides were dissolved immediately before use in PBS, pH 7.2.

**LDL Isolation, Oxidation, and Characterization**

Whole blood from healthy volunteers was collected into Vacutainer tubes (Becton Dickinson) containing EDTA (1 mg/mL), immediately centrifuged at 2000 rpm for 20 minutes at 4°C, and processed for LDL separation. LDL was isolated by sequential flotation in NaBr solution containing 1 mg/mL EDTA (density 1.019 to 1.063 g/mL). LDL was stored sterile in the dark at 4°C and used within 3 days. Immediately before oxidation or use, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 Sephadex G-25 mol/L gel (Amersham Pharmacia Biotech) in 10 mmol/L PBS. MM-LDL was prepared by exposure of LDL at room temperature to UV light for 24 hours to several days under sterile conditions. The extent of LDL oxidation was determined by evaluating the level of thiobarbituric acid–reactive substances, as reported. Protein was measured by the Pierce BCA protein assay reagent.

**Quantification of iPF<sub>2α</sub>-III Isoprostane Content in Native and Oxidized LDL**

iPF<sub>2α</sub>-III was purified using a double extraction protocol followed by quantification using a commercial enzyme-immunoassay (Cayman Chemical), according to Wang et al., with modification. Briefly, samples were quickly loaded on a solid-phase extraction cartridge (Supelchem LC18, Supelco), washed, and eluted with ethyl acetate. Elute was diluted and loaded onto a second solid-phase extraction cartridge (Supelchem LC-Si, Supelco), washed, and eluted. Elute was taken to dryness under vacuum using a SpeedVac Rotary Evaporator (Savant), reconstituted in 0.5 mL EIA buffer, and analyzed at serial dilutions. Results were corrected for the recovery and expressed as picogram per milligram of protein. Only free (not esterified in phospholipids) isoprostanes can be detected using the double extraction protocol described.

**Isolation of Human Polymorphonuclear Cells**

Blood was collected from healthy donors and citrate-anticoagulated. Human blood PMNs were isolated by dextran sedimentation and...
centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech), as described previously. Contaminating erythrocytes were lysed by hypotonic saline, and then neutrophils were washed with PBS and finally resuspended in PBS containing 10% heat-inactivated FCS, CaCl$_2$ 1 mmol/L, MgCl$_2$ 1 mmol/L, pH 7.2. All of the above procedures were done under sterile conditions using reagents prepared in endotoxin-free water for clinical use.

Isolation of Human Platelets
Platelets were isolated from whole blood. A final concentration of 11.8 mmol/L citric acid, 18 mmol/L dextrose, and 14 mmol/L sodium citrate was added to blood, and platelet-rich plasma was obtained by centrifugation at 300 g for 10 minutes. The platelet-rich plasma was recentrifuged at 700 g for 15 minutes, and platelets were gently suspended at 10$^8$/mL in PBS containing 1 mg/mL glucose, pH 7.4. The platelet suspensions were kept at room temperature and used within 1 hour.

Rapid Adhesion Assay
Eighteen-well glass slides were coated for 16 hours at 4°C with human fibrinogen (20 μg/well in endotoxin-free PBS) or with purified human ICAM-1 (3000 sites/μm$^2$). Neutrophils (10$^6$/well; 5$^5$/5 mL in PBS containing 10% heat-inactivated FCS, CaCl$_2$ 1 mmol/L, MgCl$_2$ 1 mmol/L, pH 7.2; adhesion buffer) were added, incubated for 10 minutes at 37°C, and then stimulated by the addition of the agonists for 3 minutes before washing, fixation on ice in 1.5% glutaraldehyde for 60 minutes, and computer-assisted enumeration of cells bound in 0.2 mm$^2$, as described. For the desensitization experiments, neutrophils (10$^6$/mL in adhesion buffer) were pretreated with 1 μmol/L iPF$_2$-III or 1 μmol/L U4419 at 37°C for 30 minutes, rapidly washed 3 times in adhesion buffer, and immediately used.

Chemotaxis Assays
PMN migration was assessed using 1-μm-pore-size transwells (Biorad, Becton Dickinson). Neutrophils were at 2×10$^6$/mL in PBS containing 10% heat-inactivated FCS, CaCl$_2$ 1 mmol/L, MgCl$_2$ 1 mmol/L, pH 7.2. Added to the top well was 100 μL of cell suspension, and 600 μL of medium, containing agonists, was added to the bottom well. Chemotaxis was for 2 hours. After fixation with 1.5% glutaraldehyde, migrated cells were counted by fluorescence-activated cell sorting using polystyrene beads (Polyscience) as an internal standard.

Results
Minimally Modified LDL Triggers Selective Integrin-Dependent Rapid Neutrophil Adhesion
Figure 1A shows that MM-LDL triggered, in a dose-dependent manner, rapid neutrophil adhesion to human fibrinogen, already at the lowest concentration used (10 μg of LDL protein/mL). At 150 μg of LDL protein/mL concentration, triggered adhesion was 86% of the maximal binding induced by fMLP. Native LDL (TBARS 0.083 nmol/mg LDL protein) was ineffective in adhesion triggering (Figure 1A), whereas the capability of MM-LDL to trigger neutrophil adhesion correlated with the degree of oxidation (TBARS 0.437 to 1.068 nmol/mg LDL protein) (Figure 1B). Interestingly, MM-LDL did not trigger binding to purified human ICAM-1, even at the highest concentration (Figure 1C). To evaluate the specificity of induced adhesion, we used blocking monoclonal antibodies. The efficacy of the antibodies was confirmed by their capability to prevent fMLP-triggered rapid adhesion to fibrinogen as well as to ICAM-1 (Figures 2A and 2B). Monoclonal antibodies anti-CD11b/CD18 and anti-CD11c/CD18 blocked MM-LDL–induced triggered adhesion to fibrinogen, with an average inhibition of 72% and 50%, respectively. Thus, CD11b/CD18 and CD11c/CD18 play a cooperative role in adhesion triggering to fibrinogen by MM-LDL. This was additionally confirmed by the capability of IB4, an anti-CD18 common chain blocking monoclonal antibody, to almost completely prevent induced adhesion...
Notably, the incapability of MM-LDL to trigger adhesion to ICAM-1 not only excludes activation of CD11a/CD18 by MM-LDL but also implies that MM-LDLs do not upregulate the capability of CD11b/CD18 to selectively recognize ICAM-1. Indeed, these two integrins are both involved in ICAM-1 recognition, as shown by the capability of the blocking antibodies to prevent adhesion to ICAM-1 induced by fMLP (Figure 2B). MM-LDL was also unable to direct neutrophil chemotaxis (data not shown). Together, these data suggest that MM-LDL, by triggering adhesion to fibrinogen but not to ICAM-1, are oxidation-dependent selective β2-integrin activators.

Integrin Triggering by MM-LDL Is Dependent on a Gβi-Linked Signaling Pathway

Figure 3A shows that PTX pretreatment completely abolished MM-LDL–induced binding of neutrophils to fibrinogen. The inhibitory effect of PTX was not attributable to a generic toxic effect of PTX pretreatment, because neutrophils still retained the capability to fully adhere on PMA triggering (data not shown). We next tested the role of classical ox-LDL receptors as mediators of the MM-LDL–stimulated adhesion. As shown in Figure 3B, monoclonal antibodies anti-CD36 and anti-LOX-1 were completely ineffective in blocking MM-LDL–triggered neutrophil adhesion. Together, these data show that MM-LDL induces neutrophil integrin activation through a heterotrimeric G protein–linked receptor and generated signaling pathway.

Isoprostanes Mediate Minimally Modified LDL–Induced Rapid Integrin Activation

To evaluate the role of iPF2α-III as mediator of MM-LDL proadhesive activity, we took advantage of the capability of G-protein–linked serpentine receptors to undergo homologous desensitization on ligand engagement. As shown in Figure 4A, iPF2α-III pretreatment completely abolished a subsequent iPF2α-III–stimulated adhesion of neutrophils to fibrinogen. In contrast, neutrophil pretreatment with iPF2α-III did not prevent adhesion triggering by the classical chemoattractants fMLP, C5a, LTb4, and PAF or by the chemokines IL-8 and GROα, thus showing that iPF2α-III is unlikely to trigger heterologous receptor desensitization (Figure 4A). Figure 4B shows that in neutrophils functionally deleted by 30 minutes of pretreatment with iPF2α-III, the capability of MM-LDL to trigger rapid adhesion to fibrinogen was severely impaired, with an average inhibition of 70%. Notably, quantification of isoprostane in oxidized MM-LDL used in this experimental context revealed a content of iPF2α-III 3 times higher than in native LDL (486 pg/mg protein in native LDL, TBARS = 0.11; 1409 pg/mg protein in oxidized LDL, TBARS = 0.52). Taken together, these data show that the proadhesive activity of MM-LDL is mainly mediated by iPF2α-III, and this correlates with isoprostane content in MM-LDL.

A Gβi Protein–Linked Receptor Distinct From the Classical TP Receptor Mediates Isoprostane-Triggered Rapid Integrin Activation in Neutrophils

We then wished to investigate the nature of the receptor involved in rapid adhesion triggering by MM-LDL. As shown
in Figure 5A, the TP receptor antagonist SQ29,548 only partially prevented rapid adhesion triggering to fibrinogen by MM-LDL, with a maximal inhibition of 56%. Higher doses did not additionally inhibit the adhesion (not shown). Thus, as previously shown for iPF2α-III,13 the TP receptor partially mediates the proadhesive activity of MM-LDL. To corroborate this data, we carried out adhesion assays in neutrophils desensitized with the TP agonist U46619 or with iPF2α-III. As shown in Figure 5B, in neutrophils functionally deleted for the TP receptors, rapid adhesion induced by U4419 was almost completely blocked, whereas no more than 30% of the rapid adhesion triggered by iPF2α-III was prevented. In contrast, in neutrophils functionally deleted by iPF2α-III pretreatment, the capability of U4419 to induce rapid adhesion was almost unaffected, whereas iPF2α-III adhesion triggering was severely impaired. None of the pretreatments altered fMLP-induced adhesion triggering. Together, these data suggest that human PMN express biologically active heterotrimeric G_{i}-linked isoprostane receptors distinct from the classical TP receptor, which cooperate with the TP receptor to mediate the proadhesive effects of isoprostanes and of MM-LDL. To exclude the possibility that few contaminating activated platelets could be partially responsible for MM-LDL-mediated neutrophil adhesion, we also performed the adhesion assay in the presence of known number of human platelets. As shown in Figure 6, MM-LDL-mediated and fMLP-mediated neutrophil adhesion was dose-dependently inhibited by adding increasing numbers of platelets, thus ruling out a potential proadhesive effect of platelets in our experimental setting.

**Discussion**

Oxidative stress is able to trigger the generation of biological active substances within LDL. Among them, the best characterized are lysophosphatidylcholine, oxidized phospholipids, 9-hydroxyeicosatetraenoic acid, oxysterols, and iPs. However, the real significance of oxidized lipids, and especially iPs, in MM-LDL as biological mediators of neutrophil function remains to be established.

This aim of this study was to determine the proadhesive effects of MM-LDL on human neutrophils. From our results, the following conclusions can be drawn: (1) MM-LDL triggers in human neutrophils a PTX-sensitive signaling pathway, which suggests the involvement of a serpentine receptor linked to heterotrimeric G_{i} family.13 Moreover, neither MM-LDL nor iPF2α-III triggered adhesion to ICAM-1 or induced chemotaxis. Therefore, we wished to evaluate whether MM-LDL–triggered neutrophil adhesion could be mediated, at least in part, by iPF2α-III. The role of iPF2α-III as a mediator of...
MM-LDL-induced rapid neutrophil adhesion is supported by the following evidence: (1) MM-LDL-induced neutrophil adhesion was oxidation-dependent; (2) homologous desensitization by iPF2α-III-inhibited MM-LDL induced rapid neutrophil adhesion by 70%; and (3) adhesion triggering is PTX sensitive and partially inhibited by TP receptor antagonist. However, it seems unlikely that iPF2α-III could be solely responsible for the MM-LDL-triggered rapid neutrophil adhesion. Indeed, it is likely that other iPF2α-III analogues share the same receptor; therefore, the receptor functional deletion induced by pretreatment with iPF2α-III could prevent other isoeicosanoids, concurrently generated by oxidation of LDL, to signal their message into the cell. Thus, our data highlight the general role of isoprostanes as mediators of MM-LDL proadhesive activities in human neutrophils.

It is of interest that both MM-LDL and iPF2α-III selectively trigger binding only to fibrinogen through CD11b/CD18 and CD11c/CD18. Fibrinogen is recognized by the β1 integrins CD11b/CD18 and CD11c/CD18 but not CD11a/CD18. To our knowledge, this is the first known example of a subtype-selective integrin agonist. Moreover, CD11b/CD18 can also mediate binding (through a distinct extracellular domain) to ICAM-1. Thus, the inability of MM-LDL and iPF2α-III to trigger adhesion to ICAM-1 suggest that these agonists trigger signaling pathways leading to subtype-restricted and domain-specific integrin activation.

SQ29,548 blocked only 56% of MM-LDL-induced adhesion. The residual activity cannot be attributed to the LDL scavenger receptor CD36 or to LOX-1, because blockade of these receptors did not prevent the MM-LDL-triggered adhesion. This led us to investigate the possibility that human PMNs express specific isoprostane receptors. This hypothesis seems to be supported by the experimental evidence. Indeed, TP receptor functional deletion only minimally prevented iPF2α-III–induced biological effects (30% inhibition), whereas receptor functional deletion by iPF2α-III did not
Figure 6. Platelets do not mediate MM-LDL-induced polymorphonuclear neutrophil rapid adhesion. Eighteen glass wells were coated with human fibrinogen. Human polymorphonuclear neutrophils were mixed with platelets immediately before the assay. Adhesion was done in presence of the indicated number of platelets and was stimulated for 3 minutes with buffer (no agonist), 100 nmol/L fMLP, or 100 μg/mL of MM-LDL (average TBARS=1)

Effects of antioxidants in the prevention of restenosis after PTCA may be partly related to the prevention of isoprostane generation within LDL. Importantly, our data could be potentially significant in the context of the recently described role of subendothelial retention of atherogenic lipoproteins in early atherosclerosis and in the context of the recently described role of widespread activation of neutrophils across the coronary vascular bed in patients with unstable angina.

In conclusion, our data characterize the dose- and oxidation-dependent effects of MM-LDL on rapid neutrophil adhesion and demonstrate that this phenomenon is mainly mediated by IPF_{2α}-III and, likely, other isoeicosanoids that cooperatively engage either TP or a novel, G_{i}-linked receptor expressed on human polymorphonuclear neutrophils. These data additionally strengthen the clinical relevance of the development of new drugs targeting the inhibition of F_{2α}-isoprostane generation and activity in the clinical conditions where oxidative stress, neutrophil activation, and platelet activation coexist.

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References
11. Audoly LP, Rocca B, Fabre JE, et al. Cardiovascular responses to the inflammatory response that may additionally exacerbate vascular and tissue damage. This vascular reperfusion injury is largely mediated by free radicals generated by neutrophils on adhesive interactions with platelets and the fissurated arterial wall. Thus, it is conceivable that iPS-rich LDL might directly trigger firm adhesion of neutrophils at sites of fibrin and platelet deposition, thus mediating the earliest phases of ischemia-reperfusion injury. Notably, compelling evidence from a variety of animal models indicates that blockade of neutrophil adhesion to endothelium attenuates ischemia-reperfusion injury. Furthermore, it has been demonstrated that aspirin does not prevent PTCA-induced vasospasm or restenosis after PTCA and that despite standard aspirin therapy, leukocyte activation and platelet adherence still occur after coronary angioplasty. In contrast, experimental data and preliminary clinical studies have suggested that antioxidants, such as vitamin E, may suppress isoprostane generation in vivo, reduce atherosclerosis in apolipoprotein E-deficient mice, and prevent restenosis after angioplasty. Furthermore, in a large double-blind randomized trial, probucol, a well-known antioxidant, improved vascular remodeling after PTCA. We hypothesize that the beneficial effect of antioxidants in the prevention of restenosis after PTCA may be partly related to the prevention of isoprostane generation within LDL. Importantly, our data could be potentially significant in the context of the recently described role of subendothelial retention of atherogenic lipoproteins in early atherosclerosis and in the context of the recently described role of widespread activation of neutrophils across the coronary vascular bed in patients with unstable angina.

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