American Journal of Pathology, Vol. 146, No. 1, January 1995

In Vitro Effects of Oxidized Low Density Lipoprotein on CD11b/CD18 and L-Selectin Presentation on Neutrophils and Monocytes with Relevance for the *In Vivo* Situation

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Oxidized LDL (oxLDL) has been identified as a potent stimulus of leukocyte adhesion to endothelium, a hallmark of early atherogenesis. A cytofluorometric study was performed to further characterize the mechanisms by which oxLDL stimulates the rapid adhesion of leukocytes to endothelium in vitro and in vivo. Incubation (30 minutes at 37 C) of whole blood (diluted with buffered saline to 1 × 10^6 leukocytes/ml) with oxLDL (0.85 mg LDL cholesterol/ml; oxidized by 7.5 μmol/L Cu^{2+} for 18 hours) but not native LDL stimulated the upregulation of CD11b/CD18 adhesion receptors on neutrophils (anti-leu-15 binding: 178 ± 16% of baseline, P < 0.01, means ± SD of n = 10 experiments) and on monocytes (169 ± 34% of baseline, P < 0.01). This phenomenon was almost entirely inhibited by n-butanol or the vasoactive drug pentoxifylline (PTX), which also significantly reduced oxLDL-induced leukocyte adhesion to venular and arteriolar endothelium, as assessed by intravital microscopy on the dorsal skinfold chamber in hamsters (venules: 49 ± 19 versus 120 ± 34 cells/mm², P < 0.05; arterioles: 9 ± 4 versus 52 ± 7 cells/mm², P < 0.01) 30 minutes after intravenous injection of oxLDL (4 mg/kg body weight; means ± SD of n = 7 hamsters per group). Butanol and PTX also significantly reduced the upregulation of CD11b/CD18 by f-methionyl-leucyl-phenylalanine (fMLP) and platelet-activating factor (PAF) but not by phorbol myristate acetate (PMA). Whereas fMLP and PAF stimulate leukocytes via binding to specific cell surface receptors and triggering complex signal transduction pathways, PMA bypasses these pathways and directly activates intracellular protein kinase C. By analogy, we propose that oxLDL upregulates CD11b/CD18 through its previously documented ability to stimulate the generation of second messengers. The effect of n-butanol and PTX on receptor presentation cannot be explained by changes in plasma membrane fluidity, as both agents failed to reverse the decrease in plasma membrane fluidity of neutrophils after stimulation with oxLDL, as assessed by fluorescence anisotropy measurement of the membrane marker diphenylhexatriene. Incubation of isolated neutrophils but not of whole blood with oxLDL resulted in a significant loss of L-selectin from the neutrophil surface (anti-TQ-1 binding: 40 ± 13% of baseline, P < 0.01). A significant loss of this adhesion receptor on neutrophils and monocytes was also observed after stimulation of isolated neutrophils and whole blood with fMLP, PAF, and PMA. Analogous to the effects on upregulation of CD11b/CD18 by n-butanol and PTX, these agents almost entirely prevented shedding of L-selectin in response to oxLDL, fMLP, and PAF but not PMA, suggesting that complex signal transduction pathways within the plasma membrane of neutrophils and monocytes are required

Supported in part by the Deutsche Forschungsgemeinschaft through Hu408/1-4 and by a grant from Hoechst AG. U.H. von Andrian was supported by a stipend from the Deutsche Forschungsgemeinschaft.

Accepted for publication September 16, 1994.

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for L-selectin shedding induced by physiological activating agents. (Am J Pathol 1995, 146:218–227)

Oxidized low density lipoprotein (oxLDL) has been identified as a potent chemotactic agent involved in the infiltration of leukocytes into the subendothelial space during early atherogenesis.[1–3] This finding is based predominantly on experiments *in vitro* demonstrating chemotactic and adhesion-promoting effects of oxLDL on neutrophils and mononuclear leukocytes added to endothelial cell cultures.[4–6] Using a skinfold chamber model for intravital fluorescence microscopy in hamsters and mice, we have provided *in vivo* evidence that oxLDL but not native LDL elicits the rolling and adhesion of fluorescently stained leukocytes to the micro- and macrovascular endothelium within minutes after oxLDL injection.[7,8] Electron micrographs as well as differential leukocyte counts have revealed that the adhesion-promoting effect of oxLDL affects all leukocyte subpopulations, including monocytes, neutrophils, and lymphocytes.[7] The present *in vitro* study was undertaken to investigate the effect of oxLDL on adhesion molecules involved in oxLDL-induced leukocyte/endothelium interaction.

Under different pathophysiological conditions, leukocytes interact with the endothelium via specific cell adhesion molecules, some of which are constitutively expressed on the plasma membranes of leukocytes (L-selectin) or endothelial cells (ICAM-1 and -2), whereas others are upregulated by cytokines and inflammatory mediators (E-selectin, and VCAM-1) and by oxygen free radicals (P-selectin) on the surface of endothelial cells or leukocytes (CD11/CD18). The upregulation may either require a time period of several hours and involve the *de novo* synthesis of receptor molecules, as shown for E-selectin and VCAM-1 upregulation on endothelial cells, or occur within minutes due to receptor rearrangement or translocation of preformed receptors from intracellular stores, as shown for CD11b/CD18 adhesion receptors on leukocytes and P-selectin on endothelial cells (reviewed in References 9–11).

In light of the short time in which oxLDL stimulates leukocyte rolling and adhesion,[7,8] the most likely adhesion receptors on leukocytes that are involved in this event appear to be L-selectin and CD11b/CD18. L-selectin mediates the rolling of leukocytes along the endothelial lining, whereas CD11b/CD18 is essential for leukocyte adhesion and emigration, as demonstrated experimentally[12–14] and in studies on patients with genetic deficiencies of adhesion receptor expression and/or function.[15,16] Whereas L-selectin is constitutively expressed on the surface of leukocytes,[17,18] CD11b/CD18 is upregulated on neutrophils and monocytes by various inflammatory mediators[13,17,19–21] with a time course that perfectly matches oxLDL-induced leukocyte/endothelium interaction under *in vivo* conditions.[7,8] Because oxLDL has previously been shown to induce the upregulation of receptor molecules on the surface of monocytes[5] and endothelial cells,[6,22] a cytofluorometric approach was chosen in this study to investigate the effect of oxLDL on the presentation of L-selectin and CD11b/CD18 on human neutrophils and monocytes and to test whether these changes are susceptible to pharmacological manipulation with relevance for the *in vivo* situation.

Materials and Methods

Lipoproteins

Lipoprotein isolation and modification was performed as previously described in detail.[7] Briefly, whole blood was collected from nonsmoking, drug-free human subjects (20 to 30 years of age) into tubes containing 1.5 mg of dipotassium EDTA per ml of blood. LDL was isolated by density gradient centrifugation, the density cut being d = 1.045 to 1.065 g/ml. Before oxidative modification of LDL, EDTA was removed by chromatography on Sephadex columns (PD-10, Sephadex G-25M, Pharmacia Fine Chemicals, Uppsala, Sweden). Cholesterol content was determined enzymatically and the LDL suspension was diluted with phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}, pH 7.3, at 21 C) to reach a final concentration of LDL total cholesterol of 0.85 mg/ml. Oxidative modification was achieved by incubation of the LDL suspension (1 to 1.5 ml) with 7.5 μmol/L $CuSO_4$ (18 hours at 37 C). The efficacy of LDL oxidation has been verified by the demonstration of tocopherol utilization, degradation of polyunsaturated fatty acids, and the formation of lipid peroxides[7]: α-tocopherol and γ-tocopherol, the major antioxidants in LDL, which have been observed to disappear within 6 hours after similar incubation conditions,[23] dropped from 3.6 ± 0.8 μg/ml and 0.4 ± 0.2 μg/ml, respectively, to values below detection limits. The antioxidant capacity being no longer sufficient to protect the polyunsaturated fatty acids from oxidation, the weight percentage of total polyunsaturated fatty acids dropped from 43.2 ± 3.0 to 13.4 ± 7.8 ($P < 0.02$, Mann-Whitney test) and the ratio of saturated to *cis*-unsaturated fatty acids increased from 0.4 ± 0.2 to 1.0 ± 0.2 ($P < 0.02$, Mann-Whitney test). The electrophoretic mobility of the LDL on agarose gel increased (native LDL R_f =

0.17 ± 0.08 *versus* oxLDL R_f = 0.70 ± 0.16, $P < 0.02$, Mann-Whitney test), reflecting increased negative surface charge.[23] Finally, lipid peroxidation was confirmed by the demonstration of increased lipoperoxide formation (native LDL: 0.06 ± 0.02 μmol/ml *versus* oxLDL: 0.31 ± 0.04 μmol/ml, $P < 0.02$, Mann-Whitney test).

Whole Blood Incubation

Whole blood (10 ml) was obtained by venipuncture from 10 healthy nonsmoking, drug-free human subjects (male and female, 20 to 35 years of age) and anticoagulated with sodium heparin (50 U/ml blood). The blood samples were diluted with PBS to a concentration of 1×10^6 leukocytes/ml, aliquotted (200 μl in 5-ml polypropylene centrifuge tubes), and incubated for 30 minutes in a water bath at 37 C in the presence of various concentrations of *n*-butanol (0.1, 0.25, and 0.5% in PBS) from Sigma Chemical Co. (Deisenhofen, Germany) or pentoxifylline (PTX; 30, 300, 3000 μg/ml in PBS), kindly provided by Hoechst AG. Even in the highest concentrations tested, both agents did not reduce cell viability (>95% in all samples), as assessed by trypan blue exclusion and propidium iodide staining of isolated cells. Native LDL or oxLDL were then added to the preincubated whole blood aliquots to reach a final concentration of 0.85 mg of LDL cholesterol per ml of blood and incubated for another 30 minutes in a water bath at 37 C. As negative control we used normal saline and as positive controls formyl-methionyl-leucyl-phenylalanine (fMLP, 10^{-7} mol/L; Sigma), platelet-activating factor (PAF; 1-*o*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, 10^{-7} mol/L; Sigma) and phorbol 12-myristate 13-acetate (PMA, 10^{-7} mol/L; Sigma).

Incubation of Isolated Neutrophils

Neutrophils were isolated by density gradient centrifugation (400 × *g* for 30 minutes at 21 C, Ficoll separation medium, d = 1.077; Seromed, Berlin, Germany) from heparinized blood that had been mixed with dextran to allow sedimentation of red blood cells (10 ml of blood plus 5 ml of dextran 3%, M_r, 300,000) for 30 minutes at 21 C. The cell suspension was washed twice in PBS. The preparation consisted of greater than 80% neutrophils and approximately 10% eosinophils. The leukocyte suspensions (1×10^6 cells/ml) were preincubated (30 minutes at 37 C) in the absence or presence of various concentrations of *n*-butanol or PTX and subsequently incubated for another 30 minutes with native or oxLDL (final concentration, 0.85 mg of LDL cholesterol per ml of culture medium; 37 C). Even in the highest concentrations, both agents did not reduce cell viability (>95% in all samples) as assessed by trypan blue exclusion and propidium iodide staining of isolated cells. Control samples were incubated with PBS.

Flow Cytometry

Anti-leu-15 (Becton Dickinson, Heidelberg, Germany), an antibody directed against the α-chain (CD11b) of the CD11b/CD18 adhesion receptor complex, and anti-TQ-1 (Coulter Electronics, Krefeld, Germany), an antibody directed against L-selectin, were used in the fluorocytometric studies. Anti-leu-15 and anti-TQ-1 were conjugated with phycoerythrin. A total of 5 μl of the conjugated antibodies were added to the pretreated whole blood aliquots (200 μl). After incubation for 30 minutes at 4 C, 2 ml of a lysing medium (FACS lysing solution, Becton Dickinson) were added for 10 minutes to fix leukocytes and to lyse erythrocytes. The samples were centrifuged, washed, resuspended in PBS, and stored at 4 C in the dark until fluorocytometric analysis. Negative controls were obtained by omitting the monoclonal antibody. Also, class-matched irrelevant phycoerythrin-conjugated monoclonal antibodies were added to evaluate nonspecific antibody binding. Single color immunofluorescence analysis was performed on a FACS analyzer (Becton Dickinson) equipped with an argon laser and a standard fluorescein isothiocyanate-phycoerythrin filter pack providing an excitation wavelength of λ_{ex} = 488 nm and the collection of fluorescence signals at λ_{em} = 530 ± 15 nm (green fluorescence) and λ_{em} = 595 ± 30 nm (red fluorescence). The data on volume (Coulter principle), side scatter, and fluorescence intensity properties were recorded for 1×10^4 events and collected in list mode on a Consort 30 data handling system (Becton Dickinson) for subsequent analysis. Antibody binding was determined as mean channel fluorescence after gating for neutrophils or monocytes by their characteristic volume and side scatter properties. Likewise, suspensions of isolated neutrophils were incubated under identical conditions with the conjugated antibodies and binding was assessed as described above.

Animal Model

As a functional test of leukocyte/endothelium interaction, we used the dorsal skinfold chamber model on Syrian golden hamsters, which we had previously

used to demonstrate the adhesion-promoting effects of oxLDL.[7,8] Observation chambers and indwelling catheters were implanted in pentobarbital-anesthetized hamsters (50 to 70 g body weight, age 6 to 8 weeks) as previously described.[7] The study was performed according to a protocol approved by the local ethics committee. Quantification of leukocyte/endothelium interaction was performed in four to six postcapillary venules (diameter, 20 to 60 μ) per observation chamber, as well as in four to six arterioles (diameter, 20 to 60 μ) before and at various times until 60 minutes after intravenous injection of oxLDL (4 mg/kg body weight). For contrast enhancement, leukocytes were stained *in vivo* with acridine orange injected intravenously (0.5 mg/kg/min; Sigma) and classified by fluorescence microscopy according to their interaction with the endothelial lining as adherent, rolling, or free-flowing cells.[7] At all time points before and after injection of oxLDL, microvessel diameters were assessed by means of a computer-assisted microcirculation analysis system and centerline red cell velocities were assessed by dual slit cross correlation. To test the effect of PTX on leukocyte/endothelium interaction, hamsters were pretreated by intravenous injection of PTX (10 mg/kg body weight, in 200 μl normal saline, 10 minutes before injection of oxLDL). The toxicity of *n*-butanol *in vivo* precluded testing of this agent in the intravital model.

Fluorescence Anisotropy Measurements

Steady state fluorescence anisotropy of diphenylhexatriene in isolated human neutrophils was performed as previously described in detail for mononuclear cells.[24] Measurements were performed five times on each sample. Neutrophil isolation and incubation were performed as described above.

Statistical Analysis

All data were expressed as arithmetic means ± SD. Statistical comparisons were made by the Mann-Whitney test or the Wilcoxon's signed rank test with Bonferroni correction. *P* values <0.05 and <0.01 were considered statistically significant.

Results

Effect of oxLDL, fMLP, PAF, and PMA on Receptor Presentation

In whole blood samples that had been incubated in the absence of *n*-butanol or PTX, oxLDL challenge resulted in a significant increase in the binding of anti-leu-15 on the surface of neutrophils and monocytes (Figure 1), suggesting an upregulation of CD11b/CD18 on the surface of these cells. No upregulation was observed in response to incubation with native LDL (Figure 1). Neither oxLDL nor native LDL affected the binding of anti-TQ-1 to neutrophils and monocytes when LDL incubation was performed in whole blood, suggesting that L-selectin presentation was not altered (Figure 1). In contrast, oxLDL and, to a lesser degree, native LDL induced a loss of anti-TQ-1 binding to neutrophils that had been isolated by density gradient centrifugation (Figure 2). However, it should

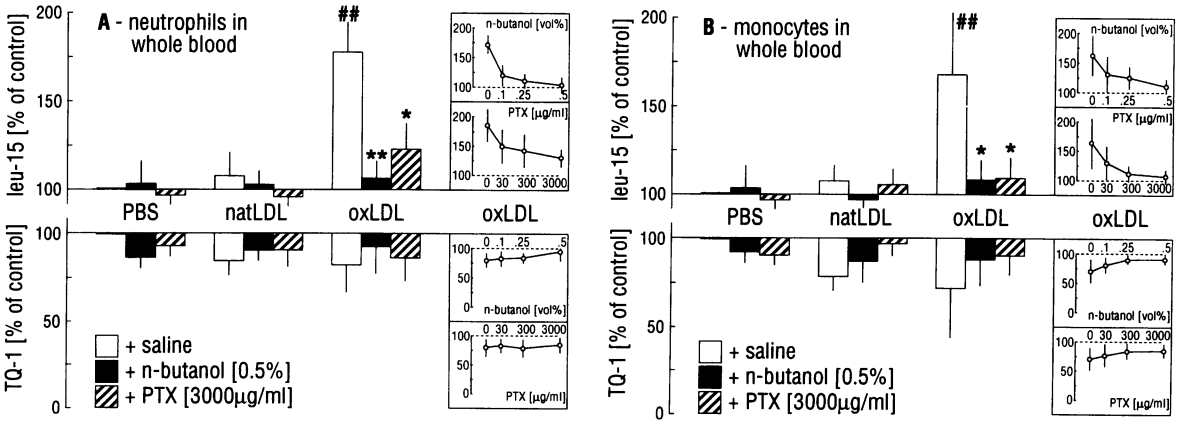

Figure 1. *CD11b/CD18 and L-selectin presentation on neutrophils* (A) *and monocytes* (B) *after incubation of whole blood with native LDL or oxLDL. Cytofluorometric quantification of anti-leu-15 binding to the CD11b subunit of CD11b/CD18 and of anti-TQ-1 binding to an epitope on L-selectin on human neutrophils* (A) *and monocytes* (B) *after preincubation of whole blood (diluted with PBS to 1 × 10⁶ leukocytes/ml) in various concentrations of* n-*butanol and PTX (30 minutes at 37 C) and subsequent incubation in PBS, native LDL (nat LDL), or oxLDL (final concentration, 0.85 mg of LDL cholesterol per ml). Relative fluorescence is expressed as percent of baseline values (mean channel fluorescence after 60 minutes of incubation in PBS at 37 C, 100%). Data are means ± SD of n = 10 experiments (controls and highest drug concentrations) and n = 4 experiments (dose-response experiments,* inset). *##P < 0.01, oxLDL* versus *PBS or native LDL, *P < 0.05 and **P < 0.01, oxLDL (+ preincubation in n-butanol or PTX)* versus *corresponding values in the absence of these agents; Wilcoxon test.*

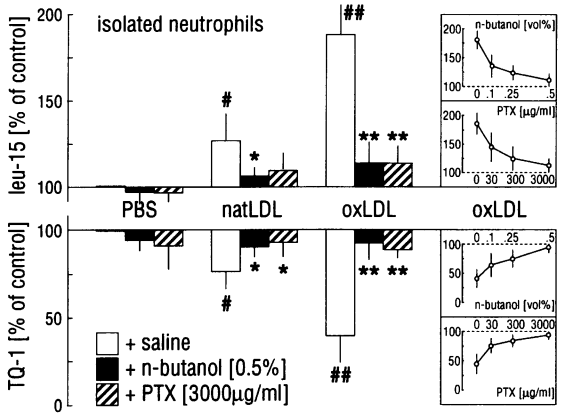

Figure 2. *CD11b/CD18 and L-selectin presentation on neutrophils after incubation of isolated neutrophils with native LDL or oxLDL. Cytofluorometric quantification of anti-leu-15 binding to the CD11b subunit of CD11b/CD18 and of anti-TQ-1 binding to an epitope on L-selectin on human neutrophils after preincubation of isolated neutrophils (1 × 10⁶ cells/ml) in various concentrations of* n*-butanol and PTX (30 minutes at 37 C) and subsequent incubation in PBS, native LDL, or oxLDL (final concentration, 0.85 mg of LDL cholesterol per ml). Relative fluorescence is expressed as percent of baseline values (mean channel fluorescence after 60 minutes of incubation in PBS at 37 C, 100%). Data are means ± SD of n = 6 experiments (controls and highest drug concentrations) and n = 3 experiments (dose-response experiments,* inset*). #P < 0.05 and ##P < 0.01, native LDL and oxLDL* versus *PBS, *P < 0.05 and **P < 0.01, native LDL and oxLDL (+ preincubation in n-butanol or PTX)* versus *corresponding values in the absence of the agents; Wilcoxon test.*

be noted that in agreement with previous reports in the literature,[19,21,25,26] the process of isolation and purification resulted in a significant activation of neutrophils, as evident in a 212 ± 37% increased binding of anti-leu-15 ($P < 0.01$ *versus* values of neutrophils in whole blood) and a significant decrease of anti-TQ-1 binding (to 77 ± 12%, $P < 0.05$). Incubation of whole blood with fMLP, PAF, and PMA stimulated both an upregulation of CD11b/CD18 adhesion receptors as well as a significant decrease of L-selectin on neutrophils and monocytes, suggesting the shedding of this adhesion receptor from the surface of these cells (Figure 3). Qualitatively and quantitatively similar effects on CD11b/CD18 and L-selectin presentation were seen after stimulation of isolated neutrophils with fMLP, PAF, and PMA (data not shown). The effects of fMLP, PAF, and PMA are in agreement with previously published data.[13,14,17,19–21] These stimuli were included in our study as positive controls to induce well characterized, differential effects on adhesion receptor presentation.

Effect of n-Butanol and PTX on Receptor Presentation

Pretreatment of whole blood (Figure 1) or isolated neutrophils (Figure 2) with *n*-butanol and PTX did not affect the baseline presentation of CD11b/CD18 or of L-selectin but resulted in a dose-dependent inhibition of CD11b/CD18 upregulation on neutrophils and monocytes in response to oxLDL (Figures 1 and 2)

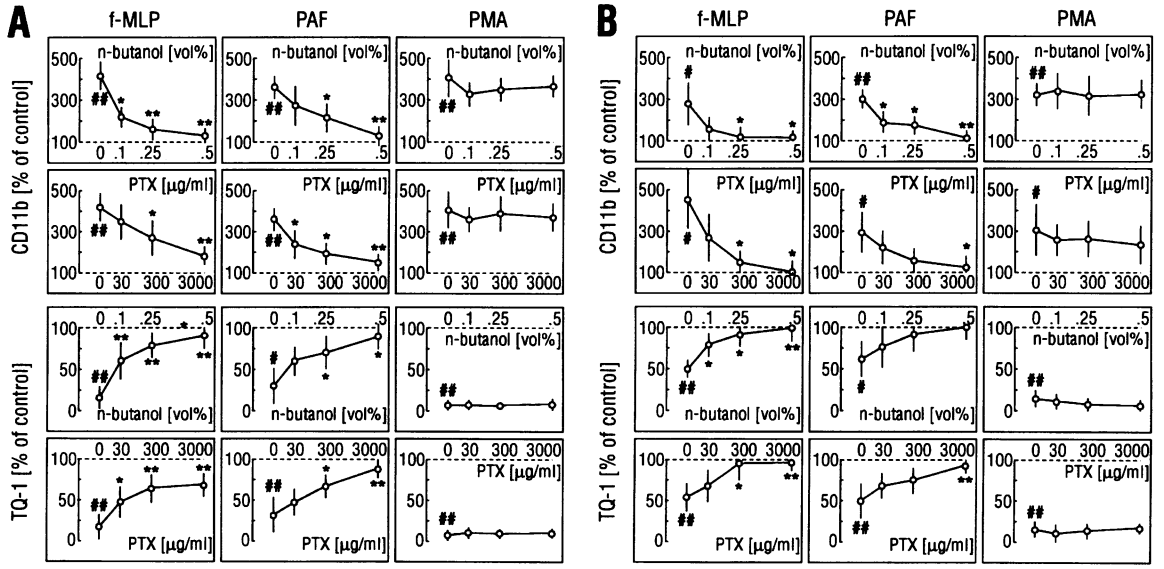

Figure 3. *Effect of fMLP, PAF, and PMA on CD11b/CD18 and L-selectin presentation on neutrophils* (A) *and monocytes* (B). *Cytofluorometric quantification of anti-leu-15 binding to the CD11b subunit of CD11b/CD18 and of anti-TQ-1 binding to an epitope on L-selectin on human neutrophils* (A) *and monocytes* (B) *after preincubation of whole blood (diluted with PBS to 1 × 10⁶ leukocytes/ml) in various concentrations of* n*-butanol and PTX (30 minutes at 37 C) and subsequent incubation in fMLP (1 × 10⁻⁷ µmol/L), PAF (1 × 10⁻⁷ µmol/L), or PMA (1 × 10⁻⁷ µmol/L). Relative fluorescence is expressed as percent of baseline values (mean channel fluorescence after 60 minutes of incubation in PBS at 37 C). Dotted line = 100%. Data are means ± SD of n = 10 experiments. #P < 0.05 and ##P < 0.01* versus *baseline values (of cells incubated in the absence of fMLP, PAF, or PMA; 100%); *P < 0.05 and **P < 0.01* versus *corresponding values in cells incubated in the absence of* n*-butanol or PTX; Wilcoxon test.*

and in response to fMLP and PAF (Figure 3). In contrast, *n*-butanol and PTX, even at the highest concentrations, failed to inhibit the upregulation of CD11b/CD18 and the shedding of L-selectin in response to PMA (Figure 3).

Effect of PTX on Leukocyte/Endothelium Interaction In Vivo

In untreated control hamsters, injection of oxLDL resulted in a significant increase in leukocyte rolling and adhesion to the microvascular endothelium of venules and arterioles (Figure 4). In contrast, pretreatment of the animals with PTX significantly reduced the adhesion of circulating leukocytes in both microvascular segments (Figure 4). Yet, PTX did not affect oxLDL-induced leukocyte rolling along the venular endothelium (Figure 4). The effect of PTX on oxLDL-induced leukocyte/endothelium interaction was not due to changes in local shear force conditions, as the microhemodynamic parameters red blood cell velocity and microvessel diameter were not significantly different between the experimental groups (not shown).

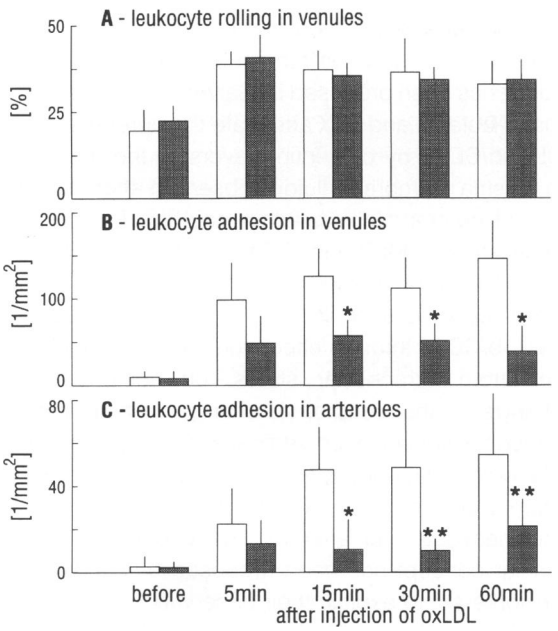

Figure 4. *OxLDL-induced leukocyte/endothelium interaction* in vivo. *Leukocyte/endothelium interaction was assessed in 4 to 6 postcapillary venules* (A *and* B) *and 4 to 6 arterioles* (C) *before and 5, 15, 30, and 60 minutes after intravenous injection of oxLDL. For contrast enhancement, leukocytes were stained* in vivo *with acridine orange and classified by fluorescence microscopy according to their interaction with the endothelial lining as adherent, rolling, or free-flowing cells.*[7] *Adherent leukocytes were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 seconds and are given in the Figure as the number of cells per square millimeter of vessel surface, as calculated from the diameter (in microns) and length (200 μ) of the vessel segment studied. Rolling leukocytes are given as a percentage of all nonadherent leukocytes passing through the microvascular segment within 30 seconds. Measurements were performed in control hamsters (white bars) and in hamsters that had been pretreated with PTX (10 mg/kg body weight i.v. in 200 μl of normal saline 10 minutes before injection of oxLDL; gray bars). Data are means ± SD of n = 7 animals per group.* *P < 0.05 *and* **P < 0.01 versus *corresponding values in control animals.*

Effect of n-Butanol and PTX on Plasma Membrane Fluidity of Neutrophils

Incubation of isolated human neutrophils with oxLDL, but not with native LDL, resulted in a significant increase of fluorescence anisotropy of the membrane marker DPH, indicating a marked reduction of plasma membrane fluidity (saline control, $r = 0.223 \pm 0.009$; native LDL, $r = 0.234 \pm 0.009$; oxLDL, $r = 0.286 \pm 0.008$; means ± SD of n = 7 experiments; $P < 0.01$ oxLDL *versus* saline control, Mann-Whitney test). In contrast to previous reports in the literature,[27–29] no membrane-fluidizing effect was observed for *n*-butanol and PTX even in the highest concentrations used, despite multiple variations in experimental conditions (incubation times, temperatures, and cell densities). Likewise, both agents failed to prevent or attenuate the decrease in leukocyte plasma membrane fluidity in response to oxLDL (data not shown).

Discussion

The principal observation of this study is that incubation of whole blood with oxLDL, but not native LDL, stimulates the upregulation of the CD11b/CD18 adhesion receptor complex on the surface of human neutrophils and monocytes (Figure 1). The key role of CD11b/CD18 in oxLDL-induced leukocyte/endothelium interaction *in vivo* has been documented previously by intravital microscopy on the skinfold chamber model in mice.[30] The extent of upregulation of CD11b/CD18 by oxLDL corresponds well with data recently published for CD11b/CD18 upregulation by tumor necrosis factor under similar experimental conditions.[29] OxLDL-induced upregulation of CD11b/CD18 adhesion receptors occurs within 30 minutes, corresponding to the time point when leukocyte adhesion was observed to reach a plateau after intravenous injection in hamsters (Figure 4)[7,8] and mice.[30] The short time period also concurs with reports demonstrating a rapid upregulation of CD11b/CD18 by other stimuli such as fMLP, PAF, PMA, or LTB_4[13,14,17,19,20] and suggests that oxLDL increases CD11b/CD18 presentation not through *de novo* synthesis of receptor molecules. Rather, oxLDL-induced upregulation could involve the rearrangement or

translocation of preformed receptors from intracellular storage vesicles, which have been described to exist in neutrophils and monocytes and to fuse with the plasma membrane within several minutes after cell activation.[19,20] Similar effects of oxLDL were also observed on isolated neutrophils (Figure 2). However, in agreement with the literature,[21,25,26] isolation and purification procedures had induced a significant activation of neutrophils, resulting in a significant upregulation of CD11b/CD18 and a loss of L-selectin from the neutrophil surface. When incubated with isolated and thus primed neutrophils, not only oxLDL but also native LDL induced a slight but statistically significant upregulation of CD11b/CD18 and a shedding of L-selectin (Figure 2). This finding may help to explain the previously reported hyperadhesiveness of isolated leukocytes in response to stimulation not only with oxLDL but also, albeit less pronounced, with native LDL.[5,31] Our data once again emphasize the caveat that isolation procedures may induce an activation of neutrophils and monocytes,[19,21,25,26] resulting in a priming of these cells that bears the potential to significantly alter biological responses. For this reason, all experiments in our study were performed on both isolated neutrophils and in whole blood (with the exception of membrane fluidity studies, in which red blood cells in whole blood preparations would have rendered the fluorescence anisotropy measurements uninterpretable).

OxLDL-induced upregulation of CD11b/CD18 adhesion receptors was significantly reduced by preincubation of the whole blood and of isolated neutrophil samples with *n*-butanol or the vasoactive drug PTX (Figures 1 and 2). This *in vitro* suppression of oxLDL-induced CD11b/CD18 upregulation by PTX was also reflected in a functional *in vivo* assay, in which pretreatment of hamsters with PTX significantly reduced oxLDL-induced leukocyte adhesion to venular and arteriolar endothelium (Figure 4). Our combined *in vitro* and *in vivo* data are thus consistent with, and extend, previous observations that both *n*-butanol and PTX inhibit the upregulation of CD11b/CD18 in response to other stimuli and affect leukocyte activation and adhesion *in vitro*.[29,32,33]

The finding that oxLDL-induced upregulation of CD11b/CD18 can be inhibited by *n*-butanol and PTX (Figures 1 and 2) suggests that oxLDL acts via a mechanism similar to fMLP or PAF and not like PMA, which stimulates CD11b/CD18 upregulation by a mechanism that is not affected by either of these agents (Figure 3). In contrast to PMA, which stimulates CD11b/CD18 upregulation through direct activation of protein kinase C (PKC), fMLP and PAF, as well as other stimuli such as tumor necrosis factor and LTB_4, have been shown to act indirectly on PKC through binding to specific cell surface receptors and through complex postreceptor signal transduction pathways, involving G proteins and the generation of second messengers, in particular of diacylglycerol.[34–38] The relevance of this mode of CD11b/CD18 upregulation has been emphasized in studies on rabbit neutrophils, in which G protein inhibition by pertussis toxin completely prevented CD11b/CD18 upregulation in response to fMLP (but not to PMA, which bypasses the second messenger pathway),[36] and on human neutrophils, in which *n*-butanol[39] and PTX[33,40] suppressed another PKC-dependent phenomenon (the generation of superoxide anion) in response to fMLP but not to PMA. Inasmuch as oxLDL has been shown to stimulate the formation of second messengers in human leukocytes,[41] the data of the present study strongly suggest that oxLDL stimulates CD11b/CD18 adhesion receptor upregulation via a second messenger-dependent mechanism. The mechanism of action by which *n*-butanol and PTX affect cell surface receptors and/or postreceptor signal transduction pathways remain a matter of speculation. It has been proposed by Salyer and coworkers[29] that *n*-butanol and PTX attenuate the upregulation of CD11b/CD18 by preventing/reversing the decrease in plasma membrane fluidity observed after stimulation of neutrophils with tumor necrosis factor[9] and other stimuli (fMLP and PAF).[42–44] It has been proposed that a decrease in membrane fluidity of stimulated leukocytes could induce an upregulation of CD11b/CD18 through effects on receptor translocation from intracellular stores, on conformational changes of the receptor via changes in heterodimer subunit association, on vertical and/or rotational displacement of receptors within the plane of the plasma membrane, and/or on the formation of receptor clusters necessary for effective ligand binding.[45–49] A similar concept has been proposed by Calder and coworkers[50] to explain their observation of a significantly increased adhesion of leukocytes to plastic after the reduction of plasma membrane fluidity by the introduction of saturated fatty acids and by Galdiero and coworkers[51] to explain the inverse effect, namely of decreased adhesion of human neutrophils after the increase of plasma membrane fluidity by the incubation with *cis*-unsaturated fatty acids. Although we observed that oxLDL and not native LDL significantly decreased plasma membrane fluidity of neutrophils (presumably through changes in the weight ratio of saturated to unsaturated fatty acids, as suggested from previous studies[52]) we (and others[53]) could not document a membrane fluidizing effect of *n*-butanol or PTX on either unstimulated or oxLDL-stimulated

neutrophils. Although this finding does not preclude that the decrease in membrane fluidity after stimulation with oxLDL may have accounted for, or contributed to, the upregulation of CD11b/CD18, it suggests that the inhibition of this phenomenon by *n*-butanol and PTX in our study occurred through a different mechanism. In consistence with the observation that both *n*-butanol[39] and PTX[33,40] affect leukocyte responses to stimuli that elicit the formation of second messengers (ie, fMLP) but not to stimuli that directly activate PKC, *n*-butanol was found to inhibit fMLP-induced superoxide production via the blockade of second messenger generation.[39]

In contrast to CD11b/CD18, binding of the TQ-1 antibody (which recognizes an epitope on the L-selectin receptor) to the surface of neutrophils and monocytes was not affected by incubation of whole blood with oxLDL (Figure 1), but was significantly reduced by fMLP, PAF, and PMA (Figure 3). The latter finding is in agreement with previous reports in the literature[13,18] and suggests that leukocyte activation by a variety of stimuli induces a rapid downregulation of L-selectin, probably through receptor shedding as a result of proteolytic cleavage of the extracellular part of L-selectin.[18] Although the data of the present study do not provide an adequate explanation for the inability of oxLDL to stimulate L-selectin shedding, it is conceivable that this relatively weak stimulus (as compared with fMLP, PAF, or PMA) was not sufficient to induce receptor shedding from unprimed leukocytes (Figure 1) but that priming of neutrophils by isolation and purification procedures facilitated the loss of L-selectin from the cell surface (Figure 2). However, an interesting observation has been made by Molad and coworkers,[54] who reported that in contrast to classical chemotactic stimuli, which induce both the upregulation of CD11b/CD18 and the shedding of L-selectin from the neutrophil surface, occupancy of Fc receptors by immunocomplexes stimulated the upregulation of CD11b/CD18 at concentrations that did not affect L-selectin expression. Since oxLDL has been shown to bind to Fc receptors,[55] it is intriguing to assume that under the conditions of this experiment, oxLDL may have induced the upregulation of CD11b/CD18 via binding to Fc receptors. The observation that oxLDL-induced upregulation of CD11b/CD18 could be blocked by *n*-butanol and PTX could then be explained by the fact that Fc receptor occupancy induces postreceptor signal transduction involving G proteins and second messengers in both neutrophils[56] and monocytes.[57] Alternatively, lysophosphatidylcholine, the major active component of oxLDL, could serve as a substrate for phospholipase C, resulting in the generation of mono- and diacylglycerol[58] and thus in the activation of PKC.[59] This notion is also supported by the recent demonstration that oxLDL stimulates the upregulation of ICAM-1 adhesion receptors on the endothelial cell surface via a PKC-dependent (and thus staurosporine-inhibitable) mechanism.[6]

Irrespective of the specific effect of oxLDL, the observation that L-selectin shedding by fMLP and PAF but not by PMA is effectively prevented by *n*-butanol and PTX suggests that L-selectin shedding may involve similar second messenger pathways as found to be operative in the upregulation of CD11b/CD18 (Figure 3).

Acknowledgments

We gratefully acknowledge the excellent technical assistance of B. Sehringer-Mansour and I. Wernicke.

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