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Sulfhydryl Regulation of L-Selectin Shedding: Phenylarsine Oxide Promotes Activation-Independent L-Selectin Shedding from Leukocytes[1]

Teresa A. Bennett,* Bruce S. Edwards,* Larry A. Sklar,*† and Snezna Rogelj[2]*

The L-selectin adhesion molecule mediates leukocyte recruitment to inflammatory sites and lymphocyte trafficking through the peripheral lymph nodes. In response to leukocyte activation, L-selectin is proteolytically released from the cell surface, disabling leukocytes from the subsequent L-selectin-dependent interactions. We have found that L-selectin shedding is sensitive to sulfhydryl chemistry; it is promoted by thiol-oxidizing or -blocking reagents and inhibited by reducing reagents. Phenylarsine oxide (PAO), a trivalent arsenical that interacts with vicinal dithiols, is most potent in inducing rapid shedding of L-selectin from isolated neutrophils, eosinophils, and lymphocytes as well as from neutrophils in whole blood. PAO does not cause cell activation, nor does it interfere with integrin function or alter the expression of several other cell surface molecules at the low concentrations that induce L-selectin shedding. PAO is not required to enter the cell to induce L-selectin shedding. TAPI-2 ((*N*-{D,L-[2-(hydroxyaminocarbonyl)-methyl]-4-methylpentanoyl}-L-3-(tert-butyl)-alanyl-L-alanine, 2-aminoethyl amide), which has previously been shown to inhibit the activation-dependent L-selectin shedding, is also capable of inhibiting PAO-induced L-selectin shedding. We hypothesize that PAO-induced L-selectin shedding involves a regulatory molecule, such as protein disulfide isomerase (PDI), an enzyme that plays a role in the formation and rearrangement of disulfide bonds, contains PAO-binding, vicinal dithiol-active sites, and is expressed on the neutrophil surface. Cell surface expression of PDI, L-selectin shedding induced by PDI-blocking Abs and by bacitracin, a known inhibitor of PDI activity, and direct binding of PDI to PAO, provide supporting evidence for this hypothesis. ***The Journal of Immunology*****, 2000, 164: 4120–4129.**

The recruitment of neutrophils to inflammatory sites and lymphocyte trafficking among blood, lymphoid, and nonlymphoid tissues involve a regulated sequence of adhesive interactions among adhesion molecules of the selectin, mucin, integrin, and ICAM families (1, 2). At the receptor level, expression, conformation, and proteolytic cleavage are among the mechanisms by which cellular adhesive interactions can be controlled. L-selectin is expressed on the majority of leukocytes, including peripheral blood T and B lymphocytes, neutrophils, eosinophils, basophils, monocytes, NK cells, and some subpopulations of thymocytes (3). Under flow-induced high shear forces, L-selectin plays a critical role in initiating the interactions of these cells with the activated endothelium at an inflammatory site (4, 5). In addition, L-selectin is thought to amplify the inflammatory process by permitting adherent neutrophils to recruit additional neutrophils (6–8). Lymphocytes require L-selectin for trafficking across the high endothelial venules into the peripheral lymph nodes (2) and to discriminate between the Th1- vs. Th2-type cytokine-producing T cells (9). L-selectin also plays a decisive role in the development of other neutrophil- and lymphocyte-mediated pathological processes, including ischemia-reperfusion injury, septic shock, graft rejection, autoimmune diseases, the metastasis of lymphoid tumors (10–13), and HIV-induced $CD4^+$ cell depletion (14).

L-selectin shedding is one important aspect of the normal physiologic regulation of L-selectin adhesive function. Cell surface expression of this adhesion molecule is characteristically down-modulated in response to cell activation (3, 15, 16). This proteolytic release from neutrophils inhibits subsequent L-selectin-dependent interactions with other neutrophils and endothelial cells at inflammatory sites (17, 18). Lymphocyte L-selectin is shed in response to activation by PMA (15), bacterial superantigens (19), or, like neutrophil L-selectin, by the treatment of cells with Abs to L-selectin (20, 21). L-selectin loss results in profound changes in T cell recirculation pathways (22), and studies with L-selectin-deficient mice have revealed a dramatic (70–90%) reduction in the number of lymphocytes in peripheral lymph nodes (22, 23). The released, soluble L-selectin retains binding capacity and may function as an adhesive buffer by preventing leukocyte adhesion at sites of subacute inflammation (24). Increased levels of plasma L-selectin are found in several disease states, including AIDS (25).

L-selectin shedding is the result of a proteolytic cleavage close to its transmembrane domain, conducted by a constitutively active membrane metalloprotease (26, 27), recently shown to be identical with TNF-α converting enzyme (28). Several groups, including ours, have shown that hydroxamic acid-based inhibitors of matrix metalloproteases, such as (*N*-{D,L-[2-(hydroxyaminocarbonyl)-methyl]-4-methylpentanoyl}-L-3-(tert-butyl)-alanyl-L-alanine,2-aminoethyl amide (TAPI-2),[3] inhibit the L-selectin sheddase and

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[3] Abbreviations used in this paper: TAPI-2, (*N*-{D,L-[2-(hydroxyaminocarbonyl)-methyl]-4-methylpentanoyl}-L-3-(tert-butyl)-alanyl-L-alanine, 2-aminoethyl amide; PAO, phenylarsine oxide; PDI, protein disulfide isomerase; DMP, 2,3-dimercapto-

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have used these compounds to study the physiological consequences of L-selectin retention (18, 29–31). While it is clear that the susceptibility to this protease is determined by the tertiary structure of L-selectin, the mechanism by which these conformational changes are modulated remains unclear (27, 32). Interestingly, calmodulin has recently been found to be associated with the cytoplasmic domain of L-selectin, and calmodulin inhibitors were shown to induce L-selectin shedding through a protease-dependent mechanism (33).

We report here on the regulation of L-selectin shedding by sulfhydryl reagents. In an effort to begin to understand the mechanisms of shedding, we have studied in detail the effect of phenylarsine oxide (PAO), which we found induces activation-independent L-selectin release from neutrophils, lymphocytes, and eosinophils. PAO is an organic trivalent arsenical that cross-links vicinal thiols in the Cys-x-y-Cys sequence by forming stable dithioarsine rings (34, 35). The dithiols 2,3-dimercaptopropanol (DMP), also known as British anti-lewisite, and its membrane-impermeable sulfonic acid analogue 2,3-dimercaptopropanesulfonic acid (DMPS), known to remove PAO from its protein target(s) (36), effectively block PAO-induced L-selectin shedding. PAO affects many cell functions, including receptor internalization (37), glucose uptake (38), neutrophil NADPH oxidase (39), platelet activation (40), protein tyrosine phosphatase activity (41), and IL-1 converting enzyme-related apoptosis (42). Although most of these effects are imparted at low concentrations of PAO ($\ll$10 μM), they may require PAO to enter the cell. Here we present evidence suggesting that PAO induces L-selectin shedding by interacting with a cell surface target and that the entrance of PAO into the cell is not required. Moreover, we propose that a likely target of PAO in this process is a membrane-resident protein disulfide isomerase (PDI) (43–45), a redox-sensitive enzyme that catalyzes oxidation-reduction reactions through an internal, vicinal dithiol-dependent, disulfide-sulfhydryl interchange.

Materials and Methods

Neutrophil, lymphocyte, and eosinophil isolation

Human venous blood was collected from healthy volunteers into sterile syringes containing heparin (10 U/ml of blood; Elkins-Simms, Cherry Hill, NJ). The blood was separated on Mono-Poly resolving medium (ICN Biochemicals, Aurora, OH) by centrifugation at 500 $\times$ *g* for 22 min at 12°C. The granulocyte and mononuclear (for lymphocytes) layers were collected separately and washed in HHB buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, and 30 mM HEPES, pH 7.40), then pelleted at 400 $\times$ *g* for 10 min. The cells were resuspended in HHB buffer containing 0.1% human serum albumin (Armour, Kankakee, IL) and 1.5 mM $CaCl_2$, at 10^7 cells/ml. The buffer was depleted of endotoxin by affinity chromatography over columns containing polymyxin B-Sepharose (Detoxi-gel, Pierce, Rockford, IL) and autoclaving for 1 h. All plastic ware was autoclaved for at least 45 min. Eosinophils were identified by labeling the granulocyte population with VLA-4 mAb (IgG1, PE-anti human CD49d, PharMingen, San Diego, CA) at 0.50 μg/ml, then gating on the FL2-positive population with a FACScan cytometer (Becton Dickinson, Lincoln Park, NJ). This method of identifying eosinophils was verified by flow cytometry cell sorting (Elite, Coulter, Miami, FL) of very late Ag-4 (CD49d) and L-selectin-positive granulocytes and subsequent immunohistochemical analysis of the sorted population (Ref. 46 and E. B. Lynam and L. A. Sklar, unpublished observations).

Reagents

Neutrophils were activated with fMLF (Sigma, St. Louis, MO) for 10 min at 37°C at a final concentration of 100 nM. PAO, DMP, DMPS, *N*-ethylmaleimide, *N*-acetyl-L-cysteine, glutathione, iodoacetate, nitro blue tetrazolium, iodoacetamide, mersalyl acid (*o*-((3-hydroxomercurio-2-methoxypropyl)carbomyl)phenoxyacetic acid), thimerosal (mercury-((*O*-carboxyphenyl)thio)ethyl sodium salt), 5,5′-dithio-bis-(2-nitrobenozoic acid) (DTNB), PMSF, and *p*-aminophenylmercuric acetate were all obtained from Sigma. Bromobimanes (monobromobimane, dibromobimane, and monobromotrimethylammoniobimane) were purchased from Molecular Probes (Eugene, OR), and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt were purchased from Fluka (Buchs, Switzerland). Diamide (azodicarboxylic acid bis-dimethylamide), azodicarbonamide, As_2O_3, $CdCl_2$, and Sb_2O_3 were obtained from Aldrich (Milwaukee, WI). Stock solutions of DMPS were prepared in sterile water. PAO solutions were prepared in DMSO (Sigma) and gently heated until PAO went into solution. PAO-induced shedding was accomplished by incubating cells with PAO (at 100 nM unless otherwise specified) for 10 min at 37°C. DMP and DMPS were used at a final concentration of 50 μM unless otherwise stated. Neutrophils were incubated with these reagents for 10 min at 37°C. TAPI-2 (provided by Dr. Roy A. Black, Immunex, Seattle, WA) was prepared in DMSO and used at a final concentration of 100 μM; it was administered to the cells for 10 min at 4°C before stimulation with fMLF or addition of PAO.

PAO reversal assays

PAO reversal assays were performed by first preincubating cells with 100 nM PAO for 10 min at 4°C, then either DMP or DMPS was added. Cells were incubated for another 10 min at 4°C, followed by an additional 10-min incubation at 37°C.

Analysis of surface Ag expression

Direct immunofluorescence labeling of control and treated cells was performed in a final volume of 200 μl at 10^6 cells/ml by incubating cells with mAb for 1 h at 4°C. Leu 8-FITC (IgG2a; Becton Dickinson Monoclonal Antibodies, Lincoln Park, NJ), a fluorescent mAb that recognizes L-selectin, was used at a final concentration of 0.625 μg/ml. Likewise, Leu 15-PE (IgG2a; Becton Dickinson Monoclonal Antibodies), a fluorescent mAb that recognizes the α-subunit (CD11b) of Mac-1, was used at 1.25 μg/ml. The relative expression of the receptors was quantitated using a FACScan Flow Cytometer (Becton Dickinson).

Immunophenotyping assay

Control cells and PAO-treated cells (100 nM for 10 min at 37°C) were labeled for surface expression of several epitopes. Direct immunofluorescence labeling of cells was performed for detection of L-selectin and β_2 integrin with Abs Leu 8-FITC and Leu 15-PE. Indirect immunofluorescence was used to detect the remaining epitopes, including PDI. Cells (1×10^6) in 200 ml of HHB were incubated for 40 min at 4°C with appropriate Abs. The Abs were against CD14, CD16 (both at 10 μg/ml; Dako, Carpinteria, CA), CD43 (8 μg/ml; IgG2a; Camfolio (Becton Dickinson), San Jose, CA), CD54 (8 μg/ml; BioSource, Camarillo, CA), PSGL-1 (PL1; IgG1; 10 μg/ml; a gift from Dr. Rodger McEver, University of Oklahoma, Oklahoma City, OK). After incubation the cells were washed by centrifugation for 10 min at 400 $\times$ *g* at 4°C. The second Ab, goat anti-mouse IgG-FITC polyclonal Ab (Becton Dickinson Antibodies) at a concentration of 6.25 μg/ml, was added, and cells were incubated for an additional 20 min at 4°C. After a final wash, the specific labeling for each Ab was analyzed by flow cytometry. Expression of PDI on the cell surface was determined similarly. Anti-PDI mAbs (clone RL90 (IgG2a) and clone RL77 (IgG2b), both 1.5 mg/ml) were obtained from Affinity BioReagents (Golden, CO). Both were used at the final dilution of 5 μl/100 μl (10^5) cells. Matched isotype control Abs (Coulter, Hialeah, FL) were used to measure any nonspecific staining. The results are reported as the relative mean channel fluorescence.

PAO time-course experiments

For these experiments, isolated neutrophils, eosinophils, or lymphocytes were warmed to 37°C, and a zero point sample was withdrawn and placed on ice. PAO was then added (1 μM for neutrophils and eosinophils, and 5 μM for lymphocytes). Cell samples were withdrawn at 1-min intervals and placed on ice. Thereafter, the cells were labeled for 40 min with Leu 8-FITC for the lymphocyte preparation or with Leu 8-FITC plus anti-VLA-4-PE for the granulocyte population. This permits the simultaneous identification and quantitation of L-selectin on neutrophils and eosinophils as described above.

propranol; DMPS, 2,3-dimercaptopropanesulfonic acid; DTNB, 5,5′-dithio-bis-(2-nitrobenozoic acid); PSGL-l, P-selectin glycoprotein ligand-1; SL-selectin; soluble L-selectin.

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Soluble L-selectin ELISA

Fifty-microliter aliquots of neutrophils suspended in HEPES buffer at 10^6 cells/ml were treated with 100 nM PAO, 100 nM PAO followed by 50 μM DMPS, 100 μM TAPI-2 followed by 100 nM PAO, 100 nM fMLF, or 100 μM TAPI-2 followed by 100 nM fMLF, with necessary incubations as outlined above. Also included was an untreated control kept at 4°C and a DMSO-treated sample subjected to a 10-min 37°C incubation. Aliquots were then centrifuged, and the supernatants were removed and prepared according to the test protocol of the Bender MedSystems (Boehringer Ingelheim Group, Vienna, Austria) sL-selectin ELISA kit. Neutrophils were resuspended in buffer and labeled for L-selectin expression with Leu 8-FITC (as above), then analyzed by FACScan.

Neutrophil aggregation measurements

Methods for aggregation measurements have been described previously (6). Briefly, control and treated cells, in a volume of 500 μl at 4×10^6 cells/ml, were labeled with the nucleic acid stain LDS-751 (Exciton, Dayton, OH) at 0.04 μg/ml for 7 min at 37°C. Samples were equilibrated for 2 min at 37°C under conditions of shear mixing using a small bar magnet (7×2 mm; VWR Scientific, Media, PA) above a heated stir device at 500 rpm. Samples were then activated with 0.1 μM fMLF, and data were acquired at specific intervals after stimulation. We report the percentage of cells that formed aggregates.

Dual population aggregation of neutrophils with ICAM-1 transfectants

This method has been previously described (6). Briefly, a transfected murine melanoma cell line expressing ICAM-1, Uill/E3, was aggregated with neutrophils to test neutrophil integrin activity. For dual population aggregation experiments, Ui11/E3 cells were labeled using a membrane-linked stain, PKH2-GL (Sigma). Labeled Ui11/E3 were suspended in HHB buffer containing 1.5 mM $CaCl_2$ and 0.1% human serum albumin, and 250 μl of labeled Uill/E3 cells at 3×10^6 cells/ml were combined with 250 μl of LDS-75 (40 ng/ml; Exciton)-labeled neutrophils at 3×10^6 cells/ml. The singlet and aggregate events were quantitated using FACScan research software. An analysis gate was placed around each specific cluster of events. We report here the percentage of neutrophils that were involved in two-color heterotypic aggregates.

Induction of L-selectin shedding by anti-PDI Abs

Neutrophils were preincubated in the absence or the presence of anti-PDI mAbs (clone RL90 (IgG2a) and clone RL77 (IgG2b), Affinity BioReagents) or matched isotype control mAbs (Coulter). Five microliters of Ab was added to 4×10^5 cells in a final volume of 200 μl, then the sample was incubated for 30 min on ice. Following a 10-min incubation at 37°C, the cells were washed with ice-cold HEPES buffer and assayed for L-selectin expression with Leu 8-FITC as described above.

Induction of L-selectin shedding with bacitracin

Bacitracin (100 mM; Sigma) or purified bacitracin A (a gift from Leo Kesner, Biology Department, State University of New York Health Sciences Center, Brooklyn, NY) stock was prepared in HHB and used to treat neutrophils at a final concentration of 3 mM. To rule out LPS contamination, some neutrophil samples were pretreated for 30 min at 4°C with 20 μg/ml of MY4 (Coulter), a mAb that blocks the LPS receptor CD14 (8). After the 30-min incubation at 37°C, the cells were placed on ice and assayed for L-selectin expression with Leu 8-FITC.

Interaction of PDI with PAO affinity resin

ThioBond (Invitrogen, San Diego, CA), an agarose-based support covalently modified with PAO, was washed twice with PBS (pH 7.2; Life Technologies, Grand Island, NY). A 500-μl aliquot placed in a 1.5-ml microfuge tube was activated with 1 ml of 20 mM 2-ME (Sigma). The tube was rocked at room temperature for 60 min. The resin was allowed to settle by gravity, and the supernatant was decanted. The resin was washed three times with PBS. PDI (20 μg; Calbiochem, San Diego, CA) was solubilized in 500 μl of PBS and added to the activated ThioBond. The sample was rocked for 90 min at room temperature. The resin was gravity settled, and the PDI solution was decanted and saved. Five subsequent washes were performed. To elute the bound protein, 500 μl of 0.5 M β-ME was added, and the sample was rocked at room temperature for 30 min. The eluate was then collected. Twenty-microliter fractions from the flow-through volume, each wash, elution, and a PDI control were assayed by SDS-PAGE. A control was generated by incubating 500 μl of ThioBond in 1 ml of a 10 mM DTT (Sigma) solution. DTT irreversibly inactivates the resin. PDI (20 μg) was then added to the resin, followed by the washing and elution procedure described above.

Table I. *Thiol oxidizing and blocking reagents induce L-selectin shedding from neutrophils*[a]

Blocking Reagents	Concentration
PAO	<1 μm
Aminophenylmercuric acetate	10 μM
Nitroblue tetrazolium	100 μM
Monobromobimane	100 μM
Dibromobimane	100 μM
Hydrogen peroxide	150 μM
DIDS*	200 μM
N-ethylmaleimide	250 μM
Quaternary bromobimane*	300 μM
Iodoacetate	300 μM
Mersalyl acid*	500 μM
Thimerosal*	500 μM
DTNB*	1 mM
PMSF	1 mM
Diamide	1 mM
Azodicarbonamide	1 mM
Iodoacetamide	10 mM

[a] The approximate concentrations of the reagent, resulting in complete shedding (as compared to the fMLF-positive control sample) within 10 min is indicated. The reported membrane-impermable reagents are marked with an *.

Results

Sulfhyrdryl reagents regulate L-selectin shedding

We have previously reported on the activation of integrin function by sulfhydryl reactive agents (47, 48). During the course of these investigations we observed that thiol-reactive agents also regulated L-selectin shedding. In general, the oxidizing and thiol-blocking reagents promote shedding (Table I). These include membrane-permeable thiol-reactive iodoacetate, monobromobimane, dibromobimane, 4-aminophenylmercuric acetate, and *N*-ethylmaleimide. The membrane-impermeable reagents include DTNB, mersalyl acid, thimerosal (sodium ethylmercurithiosalacylate), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, and quaternary BBr. Their monothiol reactivity, rather than membrane impermeability, is likely to account for their uniformly low effectiveness. Nitro blue tetrazolium is a superoxide scavenger and, like hydrogen peroxide, a potent oxidant. Azodicarbonamide is a structural analogue of diamide that is well known for its ability to cross-link thiols (49), while PMSF is reactive with thiol nucleophils. In addition, diagnostic inhibitors of enzymes with active site dithiol groups, such as arsenite and Cd^{2+} (50, 51), also induce L-selectin shedding (not shown). Trivalent arsenite (As_2O_3) and antimony (Sb_2O_3) are the most potent (inducing full shedding in 10 min at about 50 μM), while the divalent cadmium ($CdCl_2$) requires higher concentrations (1 mM). In contrast, high concentrations (5 mM) of the dithiol reducing agents were shown not to induce L-selectin shedding, but, rather, to block shedding when neutrophils were activated with formyl peptide (Fig. 1). Monothiol reducing reagents do not affect the rate of fMLF-induced shedding. We have shown previously that activation of cell adhesion occurs normally (47).

The most potent shedding reagent, PAO (reactive with vicinal dithiols), was examined more closely. Neutrophils shed their L-selectin in response to PAO in a dose-dependent fashion (Fig. 2*a*). A 10-min incubation with 100 nM PAO reduces L-selectin levels to that comparable with a 5-min treatment with 100 nM fMLF.

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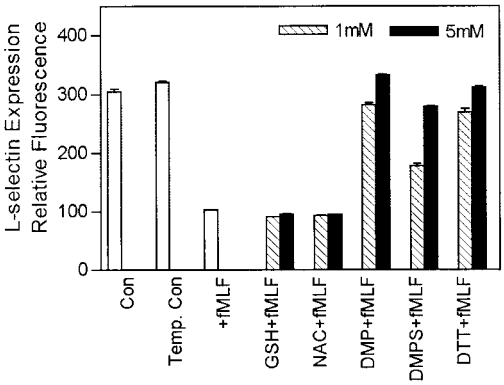

FIGURE 1. Inhibition of fMLF-induced shedding of L-selectin by dithiol reducing agents. Neutrophils were pretreated with the reducing reagents for 5 min on ice before the addition of fMLF (0.1 μM) and immediately thereafter were incubated for 5 min at 37°C. The reaction was terminated by placing the samples on ice, and the levels of the remaining cell surface L-selectin were determined by labeling with anti-L-selectin-FITC Ab and FACScan analysis. The monothiol reducing agents, glutathione and *N*-acetyl-L-cysteine, did not inhibit L-selectin shedding. High concentrations of dithiol reducing agents were able to inhibit shedding of L-selectin induced by fMLF. Experiments were performed at least three times and were conducted in triplicate. L-selectin levels are reported here as the mean ± SEM of a representative experiment.

Activation-dependent down-regulation of L-selectin is tightly coordinated with an increase in the surface expression of the Mac-1 (CD11b/CD18) β_2 integrin (6). Marginal up-regulation of Mac-1 in response to PAO (Fig. 2*b*), much less than that induced by activation with fMLF, indicates that PAO induces L-selectin shedding without activating the neutrophils. Similarly, PSGL-1 (a ligand for P- and L-selectin), CD14 (LPS receptor), CD16 (Fcγ receptor), CD43 (sialophorin; a major sialoglycoprotein shown to interact with ICAM-1), and CD54 (ICAM-1) were not affected by 100 nM PAO (Fig. 2*b*).

Neutrophils also shed their L-selectin when whole blood was treated with PAO (Fig. 2*c*), with 50% shedding at 20 min with 1 μM PAO. The requirement for an increased concentration of PAO in whole blood compared with the purified neutrophil populations probably reflects an increased number of PAO-reactive sites present in whole blood.

Reversibility of PAO binding

The dithiol, heavy metal chelating compound DMP was developed as an antidote for the arsenical war gas and has been extensively used for treatment of arsenical or mercury poisoning (52, 53). DMP (Fig. 3) is able to reverse the binding of PAO to its target (36). It is thought that DMP competes for PAO on the PAO-protein complex by reducing the vicinal sulfhydryls, stripping PAO from its target protein(s), and forming a stable, soluble chelate (52) (Fig. 3).

To determine whether PAO binding was DMP reversible, neutrophils were treated with 100 nM PAO for 10 min at 4°C. Unbound PAO was removed by centrifuging the cells, aspirating the supernatant, and resuspending the cells in fresh buffer containing 50 μM DMP. The suspension was left at 4°C for an additional 10 min, incubated at 37°C for 10 min, then labeled with mAb for L-selectin. Under these conditions L-selectin expression remained near control levels (Fig. 4*a*). DMP at this concentration was not able to inhibit fMLF-induced shedding of L-selectin, indicating a selective reversal of PAO effects by DMP rather than an inhibition of fMLF signaling or the proteolytic cleavage of L-selectin.

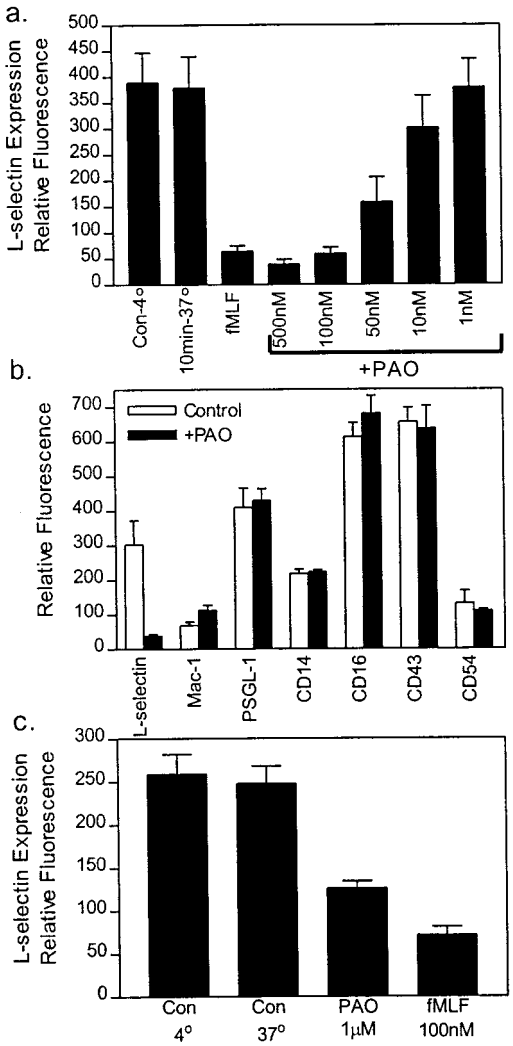

FIGURE 2. Effect of PAO on neutrophil adhesion molecules. a, PAO induces L-selectin shedding from neutrophils in a dose-dependent manner. Control-4°C, a neutrophil population that has remained at 4°C since isolation; Control-10 min @37°C, an untreated population that underwent a mock 10-min incubation along with the PAO- and fMLF-treated cells. b, Phenotypic analysis of PAO-treated neutrophils. PAO (100 nM) does not appreciably up-regulate Mac-1 or induce the shedding of other cell surface molecules known to undergo proteolytic cleavage. c, Activity in whole blood. Peripheral venous blood was diluted 1/10 with HHB buffer and treated with 1 μM PAO for 20 min at 37°C. L-selectin expression was measured by subsequent FACScan analysis of the LDS-751 and Leu 8-FITC labeled cells. The mean channel number of fluorescence is reported ±SEM. Experiments were performed two or three times with duplicate or triplicate samples in each.

DMPS (Fig. 3) is a membrane-impermeable analogue of DMP that has previously been used to define a site of PAO action with respect to the surface of the plasma membrane (54). Lower concentrations (50 μM) of DMPS, like the membrane-permeable DMP, did not interfere with the fMLF-induced shedding. DMPS was, however, still able to inhibit the PAO-induced shedding of L-selectin from neutrophils (Fig. 4*a*). This suggests that the mechanism by which PAO causes L-selectin to be shed is an extracellular process and is not due to intracellular signaling. Several other thiol reagents noted in Table I, reported to be membrane impermeable, induced L-selectin shedding. In contrast, most other previously reported PAO-induced events in neutrophils appear to be related to intracellular actions (39, 55, 56). Neither DMP nor

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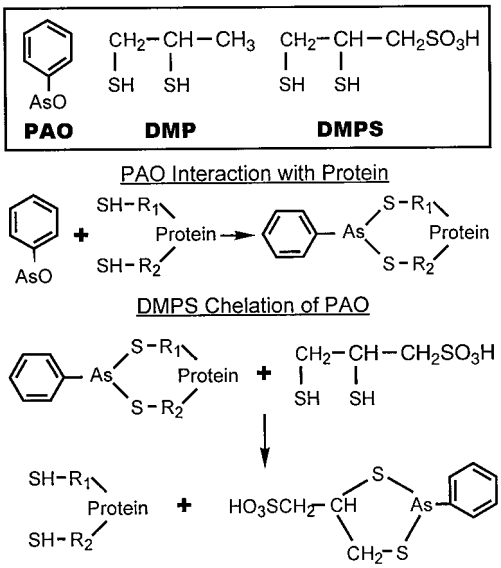

FIGURE 3. DMP, and DMPS structures. PAO interacts covalently with vicinal dithiol-containing proteins. The addition of DMP or DMPS effectively chelates PAO and restores the protein to its original reduced dithiol state.

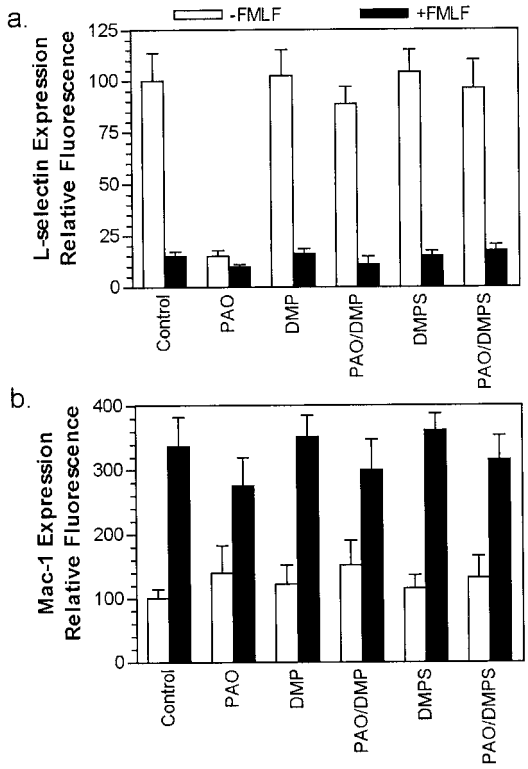

FIGURE 4. Inhibition of PAO-induced shedding of L-selectin. a, DMP and DMPS (at a final concentration of 50 μM) do not modulate L-selectin expression on their own; however, both reagents interfere with PAO (100 nM) induced shedding of L-selectin without inhibiting fMLF (100 nM) induced shedding of L-selectin. Data are normalized, with the control (without fMLF) representing 100%. Error bars indicate the SEM. b, Neither PAO, DMP, nor DMPS up-regulated Mac-1 or inhibited fMLF induced up-regulation. The experiment was repeated three times with duplicate determinations for each.

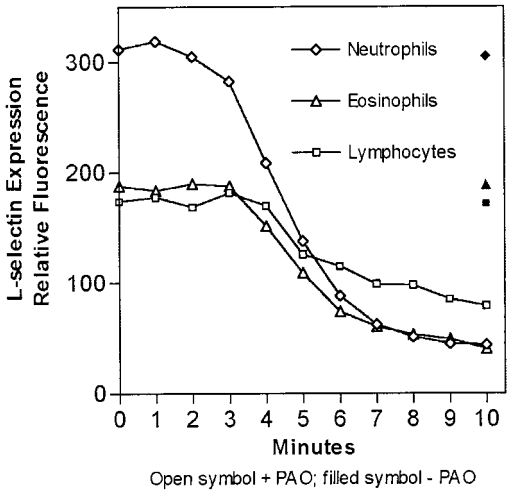

FIGURE 5. Time course of PAO-induced shedding of L-selectin from neutrophils, eosinophils, and lymphocytes. Isolated leukocyte populations were warmed to 37°C, and PAO was added at 1 μM for the neutrophil and eosinophil populations and 5 μM for the lymphocytes. Duplicate 100-μl samples were removed at 1-min intervals and placed on ice. Controls (filled symbols) at 10 min represent untreated populations that underwent the incubation at 37°C along with the PAO-treated cells. Anti-L-selectin Ab Leu 8-FITC was subsequently added to the cells on ice, and cell-associated L-selectin levels were measured using the FACScan. Anti-CD49-PE, also added to the granulocyte preparation, was used to simultaneously identify eosinophils in the granulocyte population. Mean channel number is reported. The experiments were performed three or more times, each in duplicate.

DMPS had adverse effects on Mac-1 (Fig. 4*b*). These compounds did not up-regulate the integrin on their own and did not interfere with fMLF-induced up-regulation. As a result, we conclude that it is unlikely that either DMP or DMPS interacts with the pathway that regulates L-selectin shedding. We envision that at low concentrations DMP and DMPS prevent the PAO-induced shedding of L-selectin by abstracting PAO from its distinct binding site(s) on the cell surface.

Rate of L-selectin cleavage in neutrophils, eosinophils, and lymphocytes

To determine whether PAO had a similar effect on L-selectin levels in other leukocytes, we examined L-selectin expression over time in lymphocytes and eosinophils along with neutrophils (Fig. 5). Eosinophils initially expressed lower levels of L-selectin and shed this molecule in response to PAO more slowly than neutrophils within the same granulocyte population. Similarly, lymphocytes also showed a lower basal level of L-selectin expression and, even at increased concentrations of PAO, a considerably slower rate of PAO-induced L-selectin shedding compared with neutrophils. PAO (5 μM), however, induced complete L-selectin release from lymphocytes in 30 min. When the incubation was conducted in the absence of PAO, none of these three leukocyte types experienced significant spontaneous L-selectin shedding (Fig. 5). These results show that although expressing different initial levels of L-selectin and responding with different rates of shedding, PAO induces shedding in neutrophils, eosinophils, and lymphocytes.

Inhibition of neutrophil aggregation by PAO

To verify the functional integrity of neutrophils treated with PAO, we examined the ability of neutrophils to aggregate with one another. We have previously shown that homotypic aggregation,

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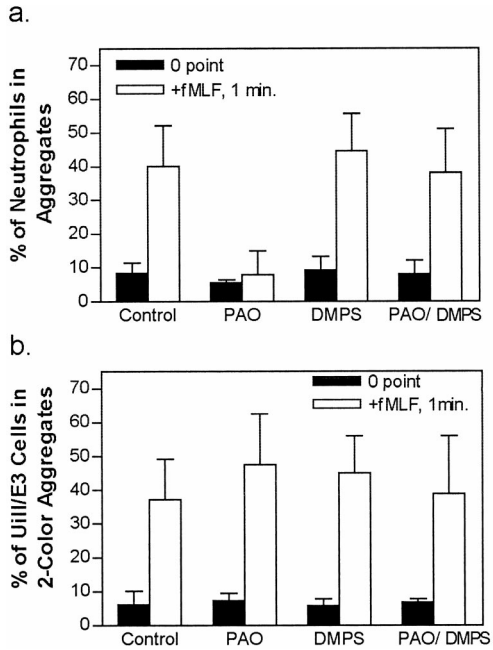

FIGURE 6. Measurement of neutrophil homotypic and heterotypic aggregation. a, Neutrophil homotypic aggregation was inhibited in 100 nM PAO-treated cells. DMPS (30 μM) alone had no affect on aggregation and was able to interfere with PAO-induced inhibition of aggregation presumably by removing bound PAO from the cell surface and preventing PAO-induced shedding of L-selectin. Aggregates were measured before stimulation (0 point) and at 1 min after stimulation with 100 nM fMLF. The number of neutrophils in aggregates and SEM is reported. b, ICAM-1-transfected cells were used to test the effect of PAO on integrin function. Data are expressed as the percentage of Ui11/E3 cells that aggregated with activated neutrophils at 1 min after stimulation. Isolated neutrophils and Ui11/E3 mouse melanoma cells transfected with human ICAM-1 were prepared and labeled as described in *Materials and Methods*. Neutrophils were pretreated with PAO, DMPS, or both. The cells were washed once and combined at 3×10^6cells/ml each, then stimulated with fMLF. The data represent two experiments, with duplicate determinations in each.

which occurs when neutrophils are exposed to fMLF or leukotriene B4 under shear stress (6, 7), involves two sequential steps that are analogous to leukocyte-endothelial cell adhesion. The first step is a low affinity interaction between neutrophil L-selectin and its mucin counterstructure PSGL-1 on the opposing neutrophil (7). The second step is a high affinity adhesion between a β_2 integrin (CD18) and its neutrophil ligand, most likely ICAM-3 (18, 57). Accordingly, the aggregation of PAO-treated neutrophils was inhibited (Fig. 6*a*) due to the loss of L-selectin. Although DMPS alone at low micromolar concentrations did not have an adverse effect on aggregation, DMPS was able to rescue the ability of the cells to aggregate, presumably by removing the PAO from the critical target protein. This allowed L-selectin to remain on the surface and to initiate the aggregation process upon stimulation with fMLF.

Effects of PAO on β_2 integrin function

To verify that the absence of aggregation in PAO-treated neutrophils was due to the loss of L-selectin and was not the result of PAO interfering with intracellular signaling or integrin activation, a murine melanocyte cell line transfected with ICAM-1 was used to assess β_2 integrin function (Fig. 6*b*). The adherence of neutrophils with the ICAM-1-transfected cells is dependent solely on the integrin step (58). Although treatment with 100 nM PAO inhibited homotypic neutrophil aggregation (Fig. 6*a*), it did not inhibit the adhesion of neutrophils to target cells (Fig. 6*b*). This demonstrated that at this concentration PAO treatment does not interfere with the signaling pathways that lead to an increase in the adhesive competence of neutrophil integrins.

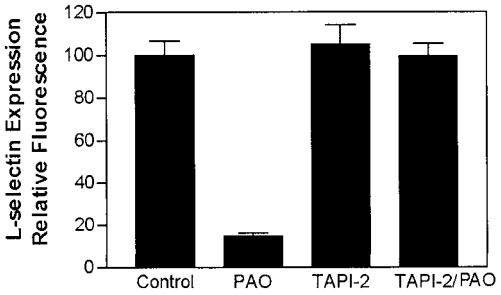

FIGURE 7. TAPI-2 inhibits PAO-induced shedding of L-selectin. Cells were pretreated with 100 μM TAPI-2 for 10 min at 4°C. PAO (100 nM) was then added, and cells were incubated for 10 min at 37°C. L-selectin expression was normalized to control levels, set to represent 100%. The experiment was performed twice, with triplicate samples in each. The mean and SEM are given.

Inhibition of PAO-induced shedding of L-selectin by TAPI-2

TAPI-2, a hydroxamate-based inhibitor of matrix metalloproteases, has previously been shown to inhibit the activation-induced shedding of L-selectin from neutrophils, eosinophils, and lymphocytes (18, 29–31). Here we show that TAPI-2 also inhibits PAO-induced shedding in neutrophils (Fig. 7). Thus, TAPI-2 appears to be able to inhibit the activation-independent release of L-selectin as well as the activation-dependent release.

ELISA for soluble L-selectin

To confirm that the L-selectin analysis by flow cytometry represented shedding from the neutrophil surface, an sL-selectin ELISA was performed on cell supernatants. Neutrophils were treated with 100 nM PAO, 100 nM PAO followed by 50 μM DMPS, 100 μM TAPI-2 followed by 100 nM PAO, 100 nM fMLF, or 100 μM TAPI-2 followed by 100 nM fMLF. Control samples and samples containing TAPI-2 displayed negligible levels of sL-selectin ($\ll$0.20 ng/ml). The PAO/DMPS samples registered slightly higher reading at ~0.4 ng/ml, while the PAO and fMLF samples displayed sL-selectin levels in the 1.6–2.0 ng/ml range. This indicates that PAO, like fMLF treatment, results in the release of the L-selectin molecule into the medium. As further conformation, the cells from which the supernatants were taken were subsequently labeled for L-selectin surface expression. The results mimicked those shown in Figs. 2*a*, 4*a*, and 7, in which control, DMPS, and TAPI-2 samples maintained near normal levels of cell surface L-selectin expression, while PAO- and fMLF-treated cells displayed minimal levels of L-selectin.

A hypothesis was developed in which extracellular PAO regulates the susceptibility of the L-selectin molecule to a constitutively active, TAPI-2-inhibitable, protease. We postulated that PDI (43–45), known for its ability to rearrange disulfide bonds within a variety of substrate proteins, could promote an interchange between its thiols and the disulfide bonds of the 24 cysteine residues of L-selectin. To explore this, we first determined that PDI is indeed expressed on the neutrophil cell surface (Fig. 8*a*). Additionally, two anti-PDI monoclonals, both previously reported to inhibit PDI activity (59, 60), were found to induce L-selectin shedding

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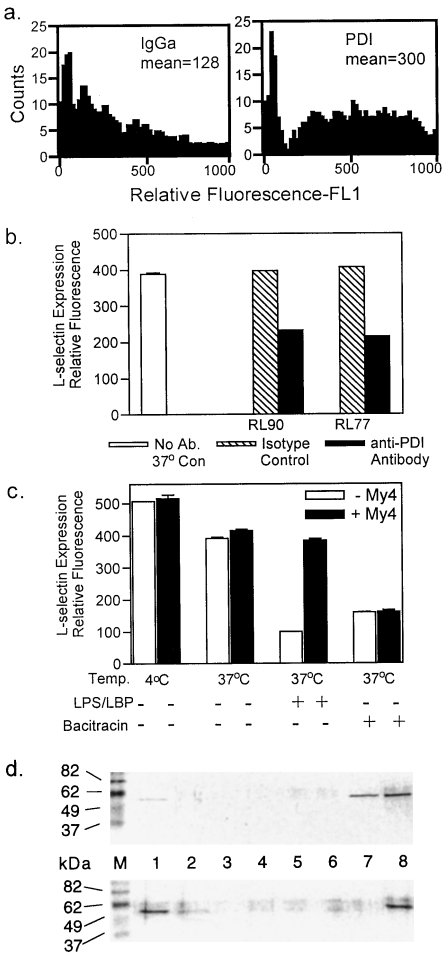

FIGURE 8. A role for PDI in L-selectin shedding. a, Neutrophils express cell surface PDI. Neutrophils were labeled with the monoclonal anti-PDI Ab (clone RL90 or the isotype control (IgG2a)) and washed, and the bound anti-PDI was detected by the secondary, goat anti-mouse FITC Ab. A representative FACScan histogram is shown, demonstrating a fluorescence increase in the anti-PDI-labeled cells of about 170 channels over that of the IgGa isotype control. The experiment with this clone and with another anti-PDI clone, RL77 (an IgG2b isotype), was repeated in duplicate at least five times, and comparable results were obtained. b, Inhibition of PDI activity with anti-PDI mAbs induces L-selectin shedding. Neutrophils were incubated in the presence of anti-PDI Abs RL90, RL77 (filled bars), or their isotype control Abs, IgG2a or IgG2b, respectively (hatched bars), for 30 min on ice (2 μl/10^5 cells) to allow Abs to bind, and then were incubated at 37°C for 15 min. Cells were assayed for L-selectin expression with Leu 8-FITC, and means are shown. Analogous experiments, performed four or more times, were conducted in duplicate. c, Bacitracin induces L-selectin shedding in an LPS/LPS binding protein-independent manner. Cells were preincubated for 30 min on ice with or without the LPS receptor CD14 blocking Ab My4 (20 μg/ml). Bacitracin (3 mM), the standard concentration used to inhibit cell surface PDI activity, or LPS/LPS binding protein (10 ng/ml) were added where indicated and incubated, along with duplicate control samples, at 37°C for 30 min. Cells were assayed for L-selectin expression with Leu 8-FITC, and the means are shown. Experiments were conducted three times in duplicate. d, PDI specifically binds to PAO affinity resin (ThioBond). SDS-PAGE analysis of flow-through (*lane 1*), washes (*lanes 2–6*), elution (*lane 7*), and PDI control (*lane 8*). The upper gel represents fractions collected from ThioBond resin activated with 20 mM β-ME, then incubated with 20 μg of PDI. The majority of PDI was bound to the resin, only coming off when eluted with 0.5 M β-ME (*lane 7*). The lower gel represents fractions from ThioBond inactivated with 10 mM DTT. Resin treated this way was not able to bind PDI. The initial flow-through contained most of the PDI (*lane 1*).

(Fig. 8*b*). We further verified the involvement of PDI using another known inhibitor of PDI activity, bacitracin (Fig. 8*c*). This antibiotic inhibits PDI, but not thioredoxin, the other enzyme also present at the cell surface and known to catalyze oxido-reduction reactions (60). LPS receptor CD14-blocking Abs (My4) were used to verify that LPS contamination of bacitracin was not involved in the induction of L-selectin shedding from these bacitracin-treated neutrophils. Moreover, bacitracin-induced shedding was not due to neutrophil activation, which would result in the characteristic quantitative up-regulation of the cell surface β_2 integrins (data not shown). These results were confirmed using bacitracin further purified by Dr. Kesner (State University of New York). Lymphocytes also express PDI on the cell surface (61) (as confirmed by us) and respond to anti-PDI Abs by shedding L-selectin, albeit much more slowly than neutrophils. Lymphocytes only respond to prolonged (>30-min) bacitracin treatment (data not shown). Finally, we have obtained the first direct evidence for the interaction of PAO and PDI. ThioBond resin (Invitrogen), an agarose-based support covalently attached to PAO, specifically binds purified PDI (Fig. 8*d*). ThioBond did not bind BSA, and resin inactivated by treatment with DTT was not able to bind PDI.

Discussion

L-selectin shedding and the sheddase

The predominant mechanism for regulating L-selectin-mediated adhesion is its proteolytic shedding from cell surfaces during an immunological or inflammatory response (15). Several laboratories have concluded that the L-selectin sheddase is a constitutively active protease, most likely identical with TNF-α converting enzyme (28), and that it is the conformational status of L-selectin molecule that determines the susceptibility of the L-selectin molecule to the proteolytic cleavage (26, 27, 32). It has been postulated that ligand binding or cellular activation induces the protease-susceptible conformation in the membrane-proximal region of L-selectin. Cellular activation has also been reported to induce a transient conformational change in L-selectin molecules that correlates with an increase in L-selectin avidity for its ligand PPME (polyphosphomannan monoester core) (62). Thus, ligand and cell activation-induced changes in L-selectin conformation may be a mechanism that ensures that the rapid increase in L-selectin receptor avidity is efficiently down-modulated through its subsequent proteolytic release (62).

PAO and sulfhydryl regulation

We have followed a lead that suggests regulation of shedding through extracellular sulfhydryl chemistry (Table I and Fig. 1). Moreover, purified neutrophils and eosinophils respond rapidly to low micromolar doses of PAO without compromising the signaling and adhesive functions of other adhesion molecules such as Mac-1 (Figs. 2, 4, and 6). Lymphocytes also respond to PAO by shedding their L-selectin, although they require somewhat higher concentrations and longer incubation times (Fig. 5). Homotypic aggregation, here used as a model for the L-selectin-dependent adhesive processes and known to play a physiologic role in inflammatory amplification, is inhibited by PAO (Fig. 6). Other L-selectin-dependent interactions, such as the recruitment of leukocytes to the inflammatory sites and lymphocyte recirculation through the lymph nodes, are expected to be profoundly affected by the PAO-induced L-selectin loss.

Because the membrane-impermeable PAO-reversing reagent DMPS blocks PAO-induced L-selectin release, the critical PAO

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target protein is likely to reside on the outside of the plasma membrane (Fig. 4). This extracellular location of the L-selectin shedding regulatory protein is substantiated by the analogous, although far less potent, effect of the membrane-impermeable monothiol-reactive reagents. The higher specificity of the dithiol-reactive PAO combined with its extracellular site of action provide an opportunity to cause L-selectin shedding with membrane-impermeable analogues of PAO. Restricting PAO access to the cell surface promises to minimize toxicities associated with intracellular PAO.

Mechanism of PAO action

Although we have not yet formally excluded the idea that PAO interacts directly with L-selectin, we have obtained evidence for a regulatory molecule such as PDI (Fig. 8). Inhibition of PDI activity by DTNB, anti-PDI Abs, and bacitracin lead to L-selectin shedding. If cell surface PDI could act as a regulatory protein that retains L-selectin in a noncleavable conformation, then the inhibition of its oxido-reductive capacity by PAO, through interaction with a thioredoxin-like active site Cys-Gly-His-Cys, is expected. Similarly, mono-thiol-reactive *N*-ethylmaleimide and DTNB, both inducers of L-selectin shedding, are routinely used to block PDI activity, albeit at high concentrations (63). Although PAO has not yet been reported to block PDI active sites, it is known to cross-link the homologous active site in thioredoxin (64). PDI is a subunit of the tri-iodothyronine receptor (65), and the recombinant rat tri-iodothyronine receptor has been shown to bind specifically to a PAO affinity column (66). Additionally, we have shown that purified PDI specifically binds to a PAO affinity column, providing further evidence for an interaction between PDI and PAO.

One remaining speculation is that L-selectin is a substrate for PDI. PDI is known to catalyze disulfide bond interchange in a spectrum of substrate proteins, and this isomerase function depends on the integrity of the vicinal-dithiol active sites. PDI is also a chaperone whose function does not depend on its isomerase activity (43–45). The chaperone activity is thought to be due to PDI binding to proteins that have a tendency to aggregate in the denatured state by promoting the correct folding of the protein. PDI is a critical component of protein complexes such as the α-subunit of prolyl-hydroxylase, *N*-glycosyl transferase, and the triglyceride transfer protein complex, where it is required to maintain triglyceride transfer protein in catalytically active form and to prevent its aggregation (67).

Despite its Lys-Asp-Glu-Leu endoplasmic reticulum retention signal, PDI has been detected on the surface of many cell types, including hepatocytes, platelets, and lymphocytes (60, 61, 68, 69), and is implicated in many cell surface processes. A PDI homologue, cognin, plays a role the adhesion-dependent aggregation of retinal cells (70). PDI modulates the conformation of thrombospondin where the isomerization of disulfide bonds is likely to have a profound effect on its ligand binding and adhesive capacity (71). Couet et al. have demonstrated that cell surface PDI is involved in the shedding of human thyrotropin receptor ectodomain (60). By analogy, our results implicate PDI in the release of L-selectin. One crucial difference is that it is the inhibition of neutrophil PDI that permits L-selectin shedding. Moreover, L-selectin shedding can be induced by treatment with the same PDI-blocking reagents (DTNB, bacitracin, and anti-PDI Abs) that impede the release of the thyrotropin receptor. It remains to be determined whether this PDI-mediated mechanism operates independently or is a part of the calmodulin-controlled, L-selectin shedding pathway (33).

Neutrophils contain appreciable immunoreactive PDI in their specific granules, and degranulation with PMA treatment releases PDI into the medium (72). We now show that PDI is present on the resting neutrophil cell surface. Neither the oxidative status of the PDI Cyc-x-y-Cys active sites, the impact of cell activation, nor the role of the released PDI in shedding of L-selectin is known.

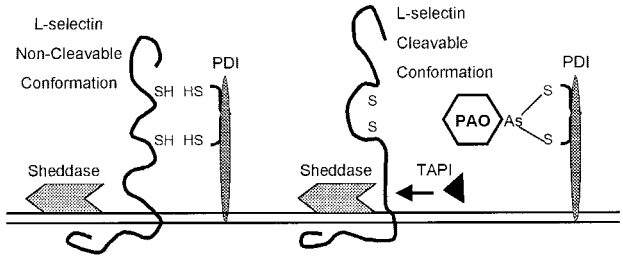

FIGURE 9. Regulation of L-selectin shedding. Reduced cell surface PDI constitutively maintains L-selectin in the reduced, noncleavable conformation. Chemical blockade or direct oxidation of the PDI vicinal dithiol active sites led to the formation of a critical disulfide bridge within the L-selectin molecule. The resulting conformation change in L-selectin permited cleavage by the sheddase. In the presence of TAPI, L-selectin shedding was blocked.

Because L-selectin initiates the interaction of leukocytes with activated endothelium, L-selectin appears to play a pivotal role in inflammatory disease. These include acute respiratory distress syndrome, ischemia-reperfusion injury that follows myocardial infarction and stroke, the pathogenesis of multiorgan failure that follows sepsis, and the diseases of eosinophilic inflammation, such as asthma and dermatitis. Cell surface PDI-blocking agents, such as impermeable PAO analogues that interfere with both leukocyte adhesion to the endothelium and leukocyte-leukocyte interactions, could play an important role in limiting or perhaps preventing damage in acute as well as chronic inflammatory diseases. Furthermore, HIV-induced $CD4^+$ lymphocyte depletion may be due to L-selectin signaling (14). Abrogation of this signaling by shedding L-selectin might mitigate the decrease in $CD4^+$ cell count that is typical of late stages of AIDS pathogenesis.

In summary, we present our model, illustrated in Fig. 9. This model proposes a novel mechanism by which the cellular chaperone and oxido reductase, PDI, regulates the susceptibility of leukocyte L-selectin to shedding. Cell surface PDI constitutively acts upon L-selectin to maintain disulfide bonds in the reduced, noncleavable state. Blockade of PDI function permits reversion of L-selectin to the oxidized, cleavable conformation. In the presence of the sheddase inhibitor, TAPI-2, L-selectin is retained on the cell surface. We speculate that following physiological activation, L-selectin conformation is modulated by oxidation of its critical sulfhydryls, rendering it sensitive to proteolysis. Our preliminary data suggest that reactive oxygen and/or nitrogen species, released by activated cells, might modulate PDI activity under physiologic conditions. This PDI-mediated mechanism, analogous to that which mediates chloroplast translational activation (73), offers a simple, extracellular switch for the regulation of L-selectin shedding.

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