

The anti-inflammatory peptides, antinflammins, regulate the expression of adhesion molecules on human leukocytes and prevent neutrophil adhesion to endothelial cells

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ABSTRACT Antiflammin-1 and antiflammin-2 are nonapeptides corresponding to the region of highest similarity between glucocorticoid-inducible proteins lipocortin-1 and uteroglobin. We have studied whether antinflammins could affect expression of adhesion molecules on human leukocytes and coronary artery endothelial cells (HCAEC) and binding of neutrophils (PMNs) to HCAEC. Although neither antiflammin-1 nor antiflammin-2 affected expression of adhesion molecules on resting PMNs, monocytes, and lymphocytes in whole blood, they attenuated changes in L-selectin and CD11/CD18 expression evoked by platelet-activating factor or interleukin-8 with IC₅₀ values of 4–20 $\mu\text{mol/l}$. The maximum inhibition was similar to those seen with human recombinant lipocortin-1 (100 $\mu\text{g/ml}$). Unlike dexamethasone (100 nmol/l), the antinflammins had little effect on LPS-stimulated expression of E-selectin and ICAM-1 on HCAEC. Consistently, culture of HCAEC with dexamethasone, but not with antinflammins, decreased PMN binding to endothelial cells. Preincubation of PMNs with antinflammins markedly decreased their adhesion to LPS-activated HCAEC. Inhibition of adhesion was additive with function blocking anti-E-selectin and anti-L-selectin antibodies, but was not additive with anti-CD18 antibody. These results show that antinflammins inhibit PMN adhesion to HCAEC by attenuating activation-induced up-regulation of CD11/CD18 expression on leukocytes, and suggest that antinflammins may represent a novel therapeutic approach in blocking leukocyte trafficking in host defense and inflammation.—Zouki, C., Ouellet, S., Filep, J. G. The antiinflammatory peptides, antinflammins, regulate the expression of adhesion molecules on human leukocytes and prevent neutrophil adhesion to endothelial cells. *FASEB J.* 14, 572–580 (2000)

Key Words: lipocortin-1-derived peptides • L-selectin • integrin • neutrophil recruitment • inflammation

GLUCOCORTICOIDS ARE POWERFUL inhibitors of leukocyte trafficking in inflammation. Multiple mecha-

nisms have been proposed to account for this action, including inhibition of adhesion molecule expression on human endothelial cells (1) and neutrophil granulocytes (PMNs) (2). Glucocorticoid injection in humans induces synthesis and surface expression of lipocortin-1 on PMNs and monocytic cells (3, 4). Lipocortin-1 has been suggested to act as the 'second messenger' for the glucocorticoid inhibition of leukocyte migration (5). Recent evidence indicates that several regions of the lipocortin-1 molecule may be associated with biological activity.

Antinflammins, discovered as inhibitors of phospholipase A₂, are synthetic nonapeptides corresponding to a region of high amino acid sequence similarity between lipocortin-1 and uteroglobin (6). Antiflammin-1 (MQMKKVLDS) is equivalent to the carboxyl-terminal part of α -helix three in uteroglobin (7), whereas antiflammin-2 (HDMNKVLDL) corresponds to residues 246–254 of lipocortin-1 (8). Antinflammins show potent anti-inflammatory effects. Although an apparent controversy exists whether antinflammins can or cannot affect phospholipase A₂ activity (6, 9–11), *in vitro*, they inhibit synthesis of platelet-activating factor (PAF) (12), PMN and mononuclear cell chemotaxis and aggregation (12, 13), and platelet aggregation (14). Antinflammins also reduce the increase in vascular permeability and leukocyte infiltration induced in rats by an Arthus reaction or by intradermal injection of C5a (12), and inhibit murine ear edema (15) and endotoxin-induced uveitis in rats (16). These studies raised the possibility that antinflammins might be potent regulators of leukocyte trafficking, but provided little insight into the underlying mechanisms.

Leukocyte extravasation into inflamed areas is a multistep process that is regulated by several adhesion molecules (17, 18). The initial capture and

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tethering of circulating PMNs to endothelium is mediated by L-selectin (CD62L) constitutively expressed by most leukocytes (19, 20) and by P- and E-selectins expressed by activated endothelium (17, 18). L-selectin is rapidly shed after cell activation with a concomitant up-regulation of Mac-1 (CD11b/CD18) (21). The β_2 integrins Mac-1 and LFA-1 (CD11a/CD18) are largely responsible for subsequent tightening of the adhesion and transendothelial migration of PMNs via interactions with their endothelial counterreceptors, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 (17, 18). PAF and interleukin-8 (IL-8) are thought to serve as signals for PMNs to promote activation of β_2 integrins, leading to firm adhesion (22, 23).

In the present experiments, we studied the effect and the cellular mechanisms of action of antiinflammatories on expression of adhesion molecules on human leukocytes and human coronary artery endothelial cells (HCAEC) and on binding of PMNs to HCAEC.

MATERIALS AND METHODS

Antibodies and reagents

In these studies, the monoclonal antibodies (mAbs) used included FITC-conjugated mouse anti-human L-selectin mAb DREG-56 (PharMingen, San Diego, Calif.), R-phycoerythrin-conjugated mouse anti-human CD18 mAb MEM-48 (Monosan, Uden, The Netherlands), FITC-labeled mouse anti-human E-selectin mAb 1.2B6 (Serotec, Kidlington, England), and R-phycoerythrin-conjugated mouse anti-human ICAM-1 mAb HA58 (PharMingen). Appropriately labeled class-matched irrelevant mouse immunoglobulin G (IgG₁) was used as a negative control for each staining. The following murine mAbs were used in neutrophil-endothelial cell adhesion assays: anti-L-selectin mAb DREG-56 (IgG₁, PharMingen) at 20 μ g/ml (24); anti-E-selectin mAb ENA-2 (IgG₁, purified F(ab')₂ fragments, Monosan) at 10 μ g/ml (25); and anti-CD18 mAb L130 (IgG₁, Becton-Dickinson Immunocytometry Systems, Mountain View, Calif.) at 10 μ g/ml (26). The irrelevant mAb MOPC-21 (IgG₁, PharMingen) at 20 μ g/ml was used as a negative control.

Synthetic antiinflammin-1 and antiinflammin-2 were obtained from Bachem Bioscience (King of Prussia, Pa.). A scrambled sequence of antiinflammin-2 (MLNHKLDVD, synthesized by Biosynthesis Inc., Lewisville, Tex.) and the unrelated synthetic peptide VPVEAVNPM corresponding to residues 24–32 of the rat cholecystokinin prepro-sequence (Bachem) were used as controls. Purity of the peptides, as analyzed by the manufacturer, was >98%, amino acid composition, and molecular mass were confirmed by mass spectrometry. Lipopolysaccharide (LPS, *Escherichia coli* O111:B4) and dexamethasone 21-phosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.), PAF was from Calbiochem (La Jolla, Calif.); human recombinant IL-8 was purchased from R&D Systems (Minneapolis, Minn.). Recombinant human lipocortin-1 (8) was a gift from Dr. R. B. Pepinsky (Biogen, Cambridge, Mass.).

Whole blood incubation

Venous blood (anticoagulated with sodium heparin 50 U/ml) was obtained from nonsmoking healthy volunteers (male and female, 24–45 years of age) who had not taken any drugs for at least 10 days before the experiments. Informed consent was obtained from each volunteer, and the protocol was approved by the Clinical Research Committee. White blood cell counts were between 4500 and 9000 cells/ μ l. Whole blood aliquots were incubated with various concentrations of antiinflammin-1, antiinflammin-2, or lipocortin-1 for 30 min at 37°C, 95% air/5% CO₂ and then challenged with PAF (1 μ mol/l) or IL-8 (10 nmol/l) for 30 min.

Analysis of surface antigen expression

Direct immunofluorescence labeling of resting and treated leukocytes in whole blood was performed as described (2, 26). Leukocytes were stained with saturating concentration of fluorescence dye-conjugated anti-human L-selectin or anti-human CD18 mAb. Nonspecific binding was evaluated by using appropriately labeled mouse IgG₁. Double- or single-color immunofluorescence staining was analyzed by a cytofluorometer (FACScan, Becton Dickinson) with Lysis II software. Antibody binding was determined as mean fluorescence intensity after gating for PMNs, monocytes, and lymphocytes by their characteristic forward and side scatter properties.

Culture of endothelial cells

Normal HCAEC obtained from Clonetics Corp. (San Diego, Calif.) were cultured as described (26). HCAEC (passages 3 to 6) seeded into 24-well or 96-well microplates and grown to confluence were used in the experiments. Two days before the experiments, the cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum only.

Expression of E-selectin and ICAM-1

After incubation for 4 h at 37°C in a 5% CO₂ atmosphere with LPS (1 μ g/ml) in the absence or presence of antiinflammin-1, antiinflammin-2 (100 μ mol/l), or dexamethasone (100 nmol/l), HCAEC were removed from the 24-well microplates by exposure to EDTA (0.01%) in phosphate-buffered saline (PBS) for 10 min at 37°C, followed by gentle trituration. Cells were resuspended in ice-cold saline containing sodium azide (0.02%), incubated with saturating concentration of fluorescein dye-conjugated anti-E-selectin or anti-ICAM-1 mAb for 30 min at 4°C, washed, fixed in formaldehyde (3.7% in PBS), and immunofluorescence was analyzed with a cytofluorometer. Nonspecific binding was evaluated by using appropriately labeled class-matched, irrelevant mouse IgG₁.

Neutrophil-endothelial cell adhesion assay

The adhesion assay was performed as described previously (26). In brief, monolayers of HCAEC in 96-well microplates were stimulated with LPS (1 μ g/ml) with or without antiinflammin-1, antiinflammin-2 (100 μ mol/l), or dexamethasone (100 nmol/l) for 6 h at 37°C in a 5% CO₂ atmosphere. The cells were then washed three times and 2×10^5 ⁵¹Cr-labeled PMNs in 100 μ l were added. In some experiments, PMNs were preincubated with antiinflammin-1, antiinflammin-2 for 30 min or with dexamethasone (100 nmol/l) for 120 min before addition to HCAEC. In another set of experiments, LPS-activated HCAEC were incubated for 15 min with ENA-2 or MOPC-21 mAb before addition of PMNs. Radiolabeled PMNs

were incubated with DREG-56, L130, or MOPC-21 mAb for 15 min before addition to HCAEC. After incubation of HCAEC with PMNs for 30 min at 37°C on an orbital shaker at 90 rpm, loosely adherent or unattached leukocytes were washed three times, and the endothelial monolayer plus the adherent PMNs were lysed in 200 μ l of 0.1% Triton X-100. The number of adhered PMNs in each experiment was estimated from the radioactivity of a control sample. Treatment of HCAEC with antiinflammins did not affect the integrity of viable endothelial monolayers.

Statistical analysis

Results are expressed as means \pm SE. Statistical comparisons were made by analysis of variance using ranks (Kruskal-Wallis test), followed by Dunn's multiple contrast hypothesis test to identify differences between various treatments or by the Mann-Whitney *U* test for unpaired observations. *P* values <0.05 were considered significant for all tests.

RESULTS

Antiinflammins attenuate activation-induced changes in L-selectin and CD11/CD18 expression on leukocytes in whole blood

Neither antiinflammin-1 nor antiinflammin-2 had a significant effect on expression of L-selectin and CD18 on resting PMNs, monocytes, and lymphocytes. For instance, L-selectin and CD18 expression by PMNs was 91 ± 4 and $109 \pm 4\%$ of control in the presence of 100 μ mol/l antiinflammin-1, and 97 ± 6 and $109 \pm 10\%$ of control in the presence of 100 μ mol/l antiinflammin-2, respectively. Addition of PAF (1 μ mol/l) to whole blood gave a significant decrease in L-selectin and a marked up-regulation of CD18 on leukocytes. **Fig. 1** reports representative results illustrating the effects of antiinflammin-2 on PAF-activated neutrophils. Addition of 'scrambled' antiinflammin-2 (MLNHKLDVD) (**Fig. 1**) or the nonapeptide corresponding to residues 24–32 of the rat prepro-cholecystokinin (data not shown) had no effect on PAF-induced changes. Preincubation of blood with antiinflammin-1 or antiinflammin-2 inhibited PAF-induced down-regulation of L-selectin and up-regulation of CD18 expression on PMNs, monocytes, and lymphocytes in a concentration-dependent fashion (**Fig. 2**). The apparent IC_{50} values for antiinflammin-1 and antiinflammin-2 were similar for PMNs (6.3 and 4.7 μ mol/l, respectively), monocytes (9.5 and 4.8 μ mol/l, respectively), and lymphocytes (5.2 and 7.8 μ mol/l, respectively). Addition of lipocortin-1 to whole blood gave a concentration-dependent attenuation of PAF-induced changes in leukocyte adhesion molecule expression (**Fig. 3**). The apparent maximum inhibition that can be achieved with antiinflammins was similar to that detected with lipocortin-1 (**Fig. 3**) and to that we had previously found with dexamethasone (2). Denaturation of lipocor-

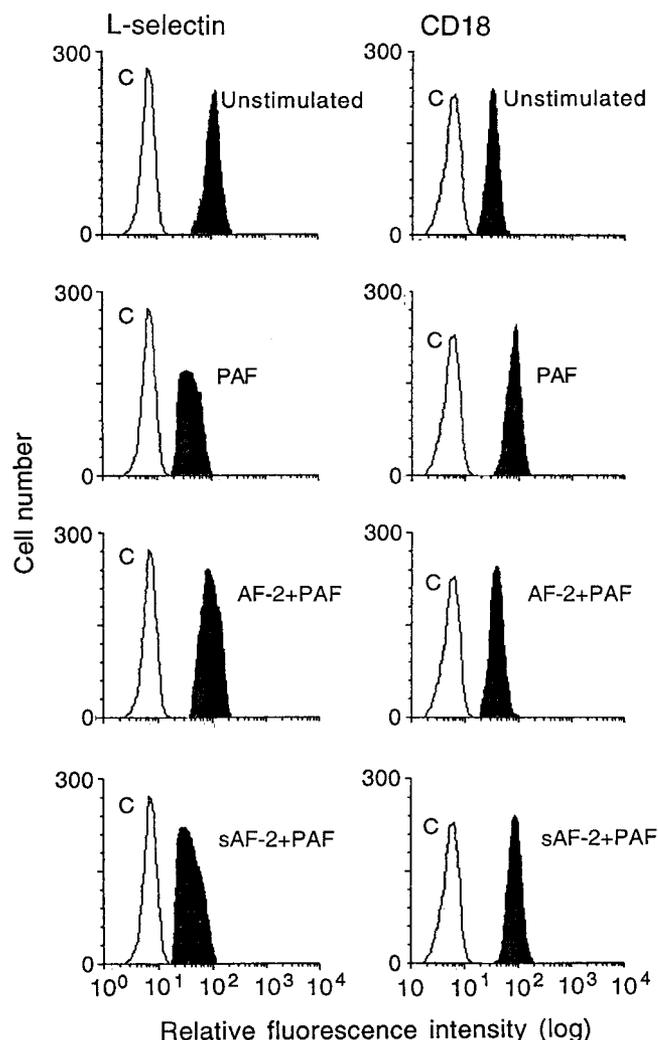


Figure 1. Whole blood actions of antiinflammin-2 on cell surface expression of L-selectin and CD18 by human neutrophils. Whole blood aliquots were incubated with antiinflammin-2 (AF-2, 100 μ mol/l) or the peptide MLNHKLDVD (scrambled antiinflammin-2, sAF-2, 100 μ mol/l) for 30 min and then challenged with PAF (1 μ mol/l) for 30 min at 37°C. In each histogram is also displayed the negative control of immunostaining with class-matched irrelevant antibodies (C). Shown are representative results of 5 experiments.

tin-1 resulted in a complete loss of its inhibitory actions (data not shown). As with PAF, both antiinflammin-1 and antiinflammin-2 partially blocked IL-8-induced changes in L-selectin and CD18 expression by PMNs with apparent IC_{50} values of 9 and 18 μ mol/l, respectively (**Fig. 4**). Immunostaining of leukocytes with an anti-CD11b mAb revealed changes similar to those observed with the anti-CD18 mAb (data not shown).

Effect of antiinflammins on expression of E-selectin and ICAM-1 on LPS-stimulated endothelial cells

After stimulation by LPS, HCAEC increased on average 25.8-fold and 2.2-fold the expression of E-selectin

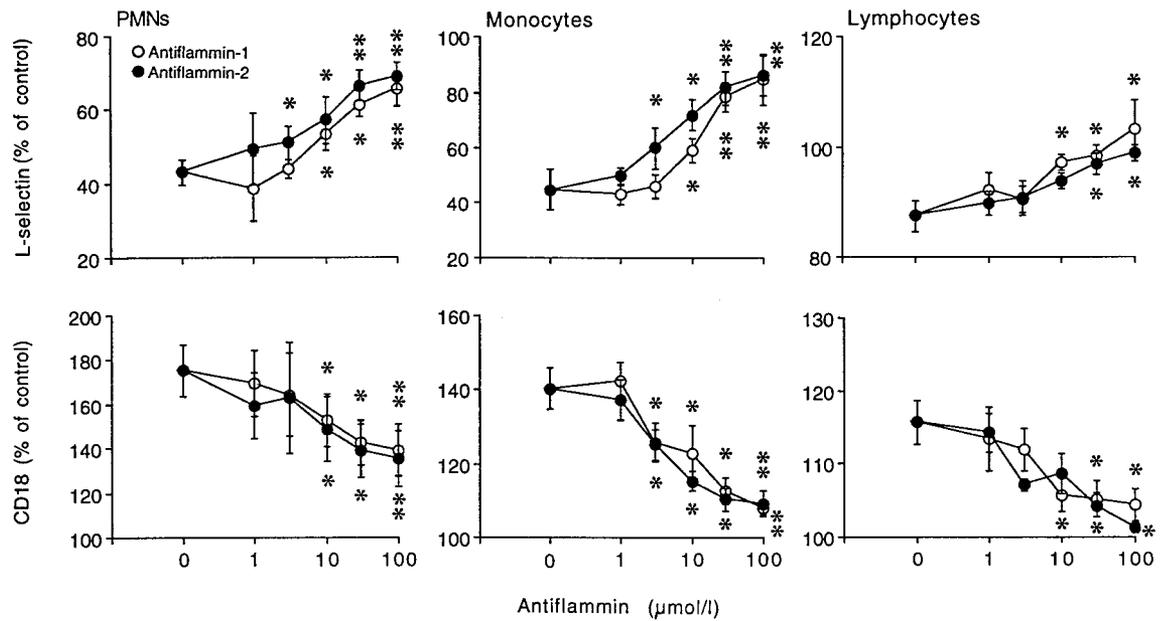


Figure 2. Antiflammins inhibit PAF-induced changes in expression of L-selectin and CD18 on human leukocytes. Whole blood aliquots were incubated with antiflammins for 30 min and then challenged with PAF (1 $\mu\text{mol/l}$) for 30 min at 37°C. Adhesion molecule expression is presented as percentage of control (unchallenged cells). Mean fluorescence intensity for L-selectin: PMNs, control, 91 ± 14 , PAF, 41 ± 6 ; monocytes, control, 43 ± 6 , PAF, 21 ± 5 ; lymphocytes, control, 50 ± 9 , PAF, 45 ± 9 , $n = 6$, all $P < 0.05$. Mean fluorescence intensity for CD18: PMNs, control, 52 ± 5 , PAF, 83 ± 6 ; monocytes, control, 74 ± 9 , PAF, 105 ± 10 ; lymphocytes, control, 16 ± 2 , PAF, 18 ± 2 , $n = 6$, all $P < 0.05$. The results are the mean \pm SE of three to six experiments with different donor cell preparation. * $P < 0.05$, ** $P < 0.01$ vs. PAF-stimulated cells.

tin and ICAM-1, respectively ($n=3$, both $P < 0.05$) (Fig. 5). Neither antiflammin-1 nor antiflammin-2 affected expression of these adhesion molecules on unstimulated HCAEC (data not shown). Furthermore, antiflammin-2 produced only a slight inhibi-

tion of LPS-induced changes (Fig. 5). The maximum inhibition did not exceed 7%. Similar results were obtained with antiflammin-1 (data not shown). Treatment of HCAEC with dexamethasone (100 nmol/l) inhibited $\sim 60\%$ of the LPS-stimulated ex-

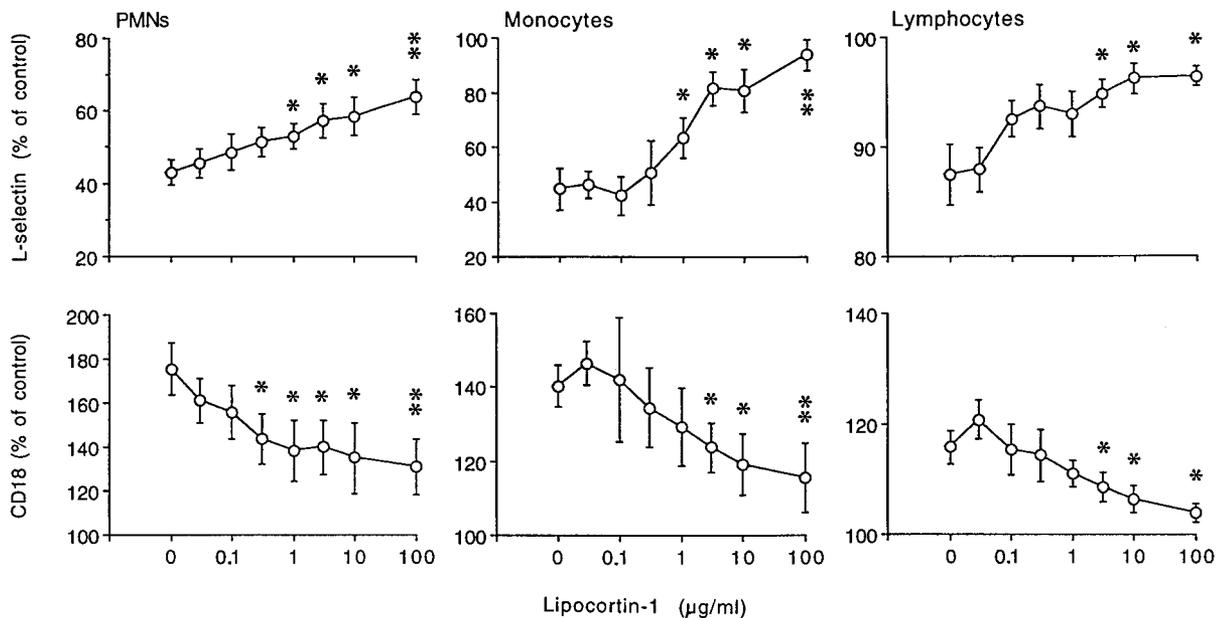


Figure 3. Lipocortin-1 blocks PAF-induced changes in L-selectin and CD18 expression on human leukocytes. Whole blood aliquots were incubated with lipocortin-1 for 30 min and then challenged with PAF (1 $\mu\text{mol/l}$) for 30 min at 37°C. Adhesion molecule expression is presented as percentage of control (unchallenged cells). Mean fluorescence intensity for L-selectin and CD18 as in legend to Fig. 1. Values are means \pm SE of three experiments with different donor cell preparations. * $P < 0.05$, ** $P < 0.01$ vs. PAF-stimulated cells.

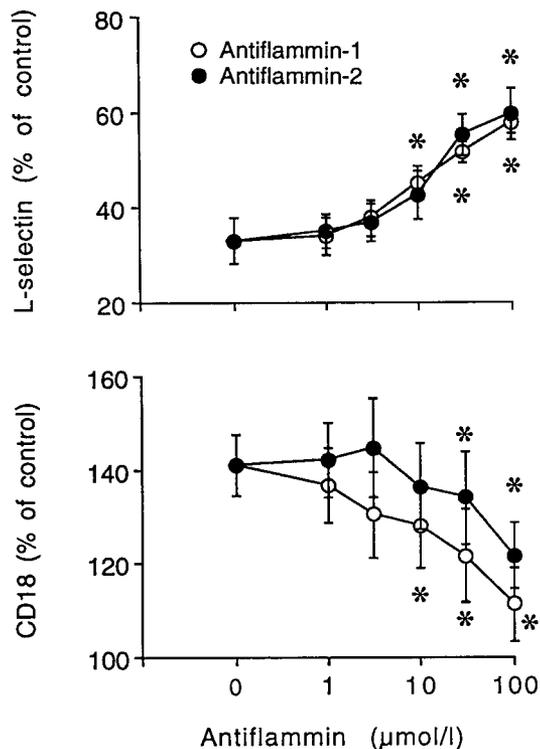


Figure 4. Antiflammins inhibit IL-8-induced changes in L-selectin and CD18 expression on human PMNs. Whole blood was incubated with antiflammins for 30 min at 37°C and then challenged with 10 nmol/l IL-8 for 30 min. Adhesion molecule expression is presented as percentage of control (unchallenged cells). Mean fluorescence intensity for L-selectin: control, 89 ± 10 , IL-8, 43 ± 5 ; CD18: control, 42 ± 4 , IL-8, 58 ± 5 , $n = 4$, both $P < 0.05$. Values are means \pm SE of four experiments with different donor cell preparations. * $P < 0.05$ vs. IL-8-stimulated cells.

pression of E-selectin and ICAM-1 (Fig. 5) without altering basal expression of these molecules (data not shown).

Antiflammins inhibit PMN adhesion to endothelial cells

Activation of HCAEC with LPS resulted in a 3.7-fold increase in the number of adherent PMNs (Fig. 6A). Culture of HCAEC with LPS in the presence of antiflammin-1 or antiflammin-2 produced only slight decreases in adhesion, whereas culture of HCAEC with LPS in the presence of dexamethasone (100 nmol/l) resulted in (on average) a 40% decrease in the number of adherent PMNs (Fig. 6A). Pretreatment of PMNs with either antiflammin-1 or antiflammin-2 before addition to LPS-activated HCAEC attenuated their attachment to endothelial cells in a concentration-dependent fashion, with apparent IC_{50} values of 1.2 and 3.0 μ mol/l, respectively (Fig. 6B). At 100 μ mol/l, antiflammin-1 and antiflammin-2 inhibited PMN adhesion by 54 ± 4 and $43 \pm 4\%$, respectively ($n=3$, both $P < 0.05$) (Fig. 6B). No

adhesion experiments were done when PAF or IL-8-activated neutrophils were added to HCAEC stimulated in the presence of antiflammins, since both PAF and IL-8 provoke neutrophil aggregation, making interpretation of the results difficult. 'Scrambled' antiflammin-2 or the rat prepro-cholecystokinin nonapeptide (1–100 μ mol/l) had no detectable effect on the number of adherent neutrophils (data not shown).

Since PMN adhesion to LPS-stimulated HCAEC is mediated by multiple adhesion receptors (26), we assayed the contribution of L-selectin, E-selectin, and CD18 to the binding interaction. A significant proportion of PMN-HCAEC attachment was blocked by mAbs binding to L-selectin ($24 \pm 4\%$, $n=3$), CD18 ($27 \pm 4\%$), or E-selectin ($35 \pm 5\%$) (Fig. 7). The combination of these mAbs inhibited PMN adhesion by $\sim 90\%$. Treatment of PMNs with antiflammin-1 and anti-CD18 mAb resulted in only a slightly greater inhibition of adhesion than observed with PMNs treated with either antiflammin-1 or anti-CD18 mAb (Fig. 7). The combination of antiflammin-1 with either anti-L-selectin mAb or anti-E-selectin mAb resulted in additive inhibition, and the degree of inhibition was similar to that observed when anti-L-selectin mAb or anti-E-selectin mAb was combined with anti-CD18 mAb, respectively (Fig. 7). Combining antiflammin-1, anti-L-selectin mAb, and anti-E-

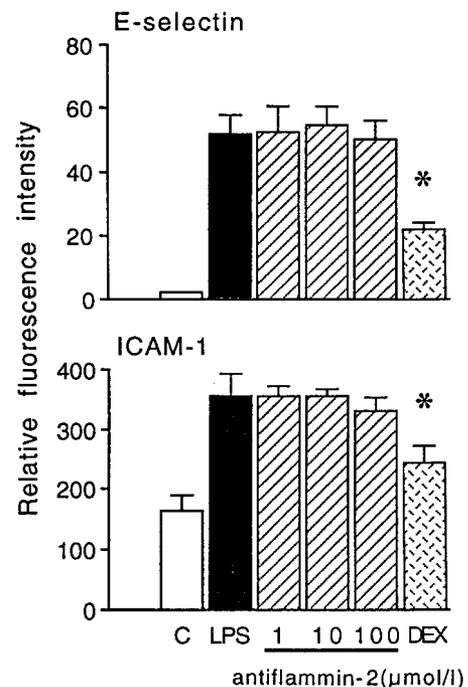


Figure 5. Effects of antiflammin-2 and dexamethasone on E-selectin and ICAM-1 expression on human coronary artery endothelial cells (HCAEC). Confluent monolayers of HCAEC were left unstimulated (control, C) or were challenged with 1 μ g/ml LPS in the presence of antiflammin-2 (100 μ mol/l) or dexamethasone (100 nmol/l) for 4 h at 37°C. Values represent the mean \pm SE of three independent experiments. * $P < 0.05$ vs. LPS-treated.

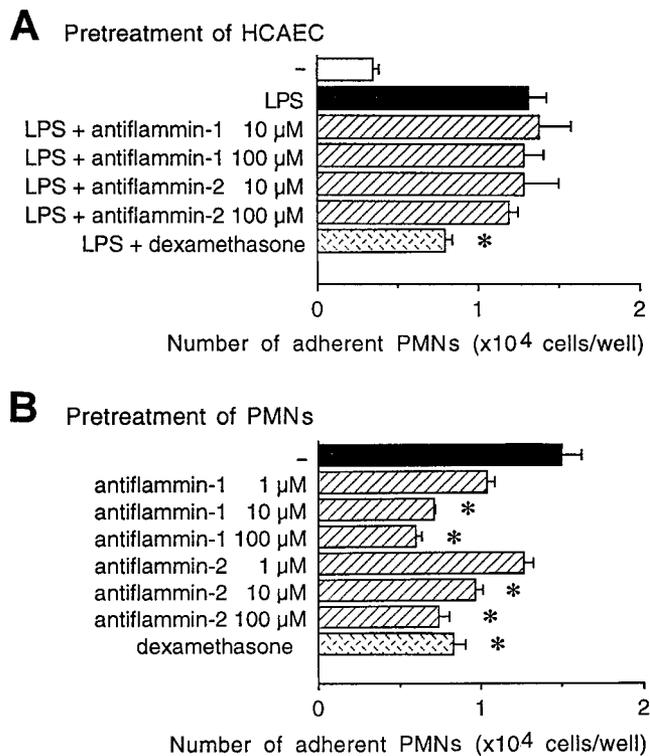


Figure 6. Inhibition of PMN binding to endothelial cells by anti-inflammamines and dexamethasone. *A*) Confluent HCAEC monolayers were cultured with LPS (1 μg/ml) in the presence of anti-inflammamin-1 (100 μmol/l), anti-inflammamin-2 (100 μmol/l), or dexamethasone (100 nmol/l) for 6 h at 37°C before addition of radiolabeled PMNs. *B*) PMNs were preincubated for 30 min with medium or anti-inflammamines; or with dexamethasone (100 nmol/l) for 120 min as indicated prior to addition to LPS-activated HCAEC. PMN adhesion to unstimulated HCAEC was $0.35 \pm 0.04 \times 10^4$ cells per well. Values are means \pm SE of three experiments using PMNs from different donors. **P* < 0.05 vs. LPS-treated HCAEC.

selectin mAb blocked ~82% of adhesion. Similar results were obtained with anti-inflammamin-2 (data not shown).

DISCUSSION

Here we propose a novel mechanism by which anti-inflammamines can affect the inflammatory response, namely, through modulation of surface expression of adhesion molecules on activated leukocytes and inhibition of neutrophil-endothelial cell adhesion.

The glucocorticoid-inducible protein lipocortin-1 appears to be an important negative effector of leukocyte emigration in various experimental models (27–30). Three regions of the lipocortin-1 molecule—a 188 amino acid NH₂-terminal fragment (5), peptide N-acetyl-2–26 (5), and peptide 246–254 (termed anti-inflammamin-2) (6)—have been reported to inhibit leukocyte accumulation. In our experiments, anti-inflammamin-2 and anti-inflammamin-1 (the carboxyl-terminal part of α-helix three in uteroglobin) appeared

to be equally potent inhibitors. The first two residues (which differ in anti-inflammamin-1 and anti-inflammamin-2) can be replaced, but not deleted, without loss of activity, indicating that the length of anti-inflammamines is critical for the biological activity (6). Oxidation of the Met residue in position 1 in anti-inflammamin-1 has been suggested to account for the rapid (within 5 min) decline in the inhibitory action of anti-inflammamin-1 incubated with human isolated PMNs (12). However, this Met residue is not a prerequisite for activity, since it is substituted with His in anti-inflammamin-2 (6). We could not detect any significant loss of inhibitory action of anti-inflammamin-1 in our experiments. Besides differences in the experimental conditions (e.g., presence of serum in our experiments), the reasons for this apparent discrepancy are not known at present. The inhibitory action of anti-inflammamines observed in this study are specific for these peptide sequences, because no inhibitory effects were detected with the peptide MLNHKLDVD (a scrambled sequence of anti-inflammamin-2) or the unrelated nonapeptide VPVEAVNPH in the assays used.

Our study documents that although anti-inflammamines had no effect on expression of adhesion molecules on human resting leukocytes in whole blood *in vitro*, they markedly attenuated changes in L-selectin and CD11/CD18 expression evoked by PAF or IL-8. Thus, anti-inflammamines can fully duplicate the actions of lipocortin-1 on leukocyte adhesion molecule expression. Assuming that no lipocortin-1 molecule has been denatured during the experiments (8), on a molar concentration basis, 30-fold more anti-inflammamines than native lipocortin-1 was needed to achieve a similar degree of inhibition. It is likely that higher concentrations of anti-inflammamines may be needed to occupy the putative lipocortin-1 receptor. Nevertheless, it is impressive that these nonapeptides are

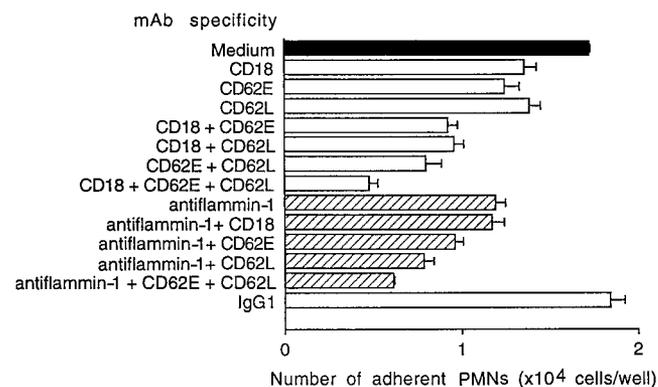


Figure 7. Inhibition of PMN binding to endothelial cells by anti-E-selectin, anti-L-selectin and anti-CD18 mAbs and anti-inflammamin-1. PMNs were treated with anti-inflammamin-1 (100 μmol/l) or the indicated mAbs before and during the assay. PMN adhesion to unstimulated HCAEC was $0.37 \pm 0.02 \times 10^4$ cells per well. The irrelevant mAb MOPC-21 (IgG₁) was used as a negative control. The results are means \pm SE of three experiments using PMNs from different donors.

active in the whole blood and overcome interactions with blood components to specifically regulate leukocytes. Human PMNs and monocytic cells possess specific and saturable binding sites for lipocortin-1 (30) that mediate the action of lipocortin-1 on these cells. Our results demonstrate that in addition to consolidating binding to the target cells (31), the antiinflammin-2 sequence (residues 246–254 in lipocortin-1) exerts actions similar to those of the parent protein. These findings are also consistent with our previous study, which showed that dexamethasone attenuates by ~60% the PAF and formyl-Met-Leu-Phe-induced changes in L-selectin and CD11b/CD18 expression by human neutrophils through induction of protein synthesis (2), most likely lipocortin-1 (5).

Within minutes of activation with PAF or IL-8, leukocytes release L-selectin from their surface by a proteolytic enzyme. Inasmuch as this enzyme appears to be constitutively active, formation of an appropriate 3-dimensional structure of L-selectin near the membrane is thought to regulate this proteolytic process (32, 33). Although the nature of conformational changes required for the cleavage is not understood at present, our study suggests that this can be partially prevented by antiinflammins. Phosphorylation of serine in the intracellular tail of L-selectin (34) and calmodulin inhibitors (35) were reported to induce proteolytic shedding of L-selectin. Whether the action of antiinflammins involves activation of calmodulin or interference with a phosphorylation step remains to be investigated. Leukocyte integrins change their conformation during cell activation with characteristics of the active molecule (i.e., increase in the binding avidity or affinity), depending on the method of stimulation (36, 37). Activation of leukocytes with stimuli that increase intracellular Ca^{2+} concentration, such as PAF or IL-8, induce clustering of β_2 integrins, thereby increasing the overall strength of binding without affecting affinity (37). However, it seems unlikely that antiinflammins might interfere with Ca^{2+} signaling, because recent results suggest that antiinflammin-2 does not inhibit calcium-dependent mobilization of arachidonic acid (13). Activation of leukocytes from the outside of the cell with Mg^{2+} in the presence of EGTA results in the formation of a higher affinity form of the integrins by either unmasking the ligand binding site or by tertiary changes within the ligand binding domain (36, 38). Although both mechanisms may operate in integrins, it is not known how these mechanisms are activated.

Despite inhibition of L-selectin shedding from PMNs, which would be expected to promote their adhesion to HCAEC, antiinflammins markedly reduced the number of adherent neutrophils. This

inhibition can be attributed primarily to their effect on PMNs rather than on HCAEC, because antiinflammins had little effect on LPS-stimulated expression of E-selectin and ICAM-1 on HCAEC. The antiinflammins or a function-blocking anti-CD18 mAb resulted in similar decreases in PMN adhesion to HCAEC. Furthermore, the actions of antiinflammin-1 or antiinflammin-2 and anti-CD18 mAb were not additive, indicating that inhibition of PMN-HCAEC adherence by antiinflammins is predominantly attributable to attenuation of up-regulation of CD11/CD18 expression on PMNs. Since the inhibition with antiinflammins was additive with anti-E-selectin and anti-L-selectin mAbs, it is unlikely that antiinflammins interfered with E-selectin or L-selectin function or ligands. Inhibition of neutrophil adhesion by antiinflammins resembles that of peptide N-acetyl-2–26 of lipocortin-1 (5). This peptide can also inhibit PMN adhesion when using a leukocyte stimulus, but not when using endothelial cell stimulation, although the underlying mechanisms of action are not known at present.

This and earlier studies (1, 2) point to similarities and striking differences between the actions of antiinflammins and glucocorticoids. Antiinflammins exert similar inhibitory actions as glucocorticoids on activation-induced changes in adhesion molecule expression by PMNs, resulting in attenuation of PMN adhesion to HCAEC. By contrast, dexamethasone, but not antiinflammins, inhibits LPS-induced expression of ICAM-1 and E-selectin on human umbilical vein endothelial cells (1) as well as on HCAEC (present study), leading to decreases in PMN adherence. While most of the actions of glucocorticoids are mediated by translocation of the glucocorticoid–glucocorticoid receptor complex to the nucleus and its binding to genes containing glucocorticoid-responsive elements (1, 39), the absence of a glucocorticoid-responsive element in the gene for E-selectin (40) suggests that glucocorticoids either interfere directly with a transcriptional regulator or induce the synthesis of a secondary regulatory element (1). The lack of effect of antiinflammins on E-selectin and ICAM-1 expression on HCAEC would suggest that lipocortin-1 may not be this regulatory element.

Our results may have relevance to inhibition of excessive trafficking of leukocytes both in acute and chronic inflammation. By inhibiting up-regulation of CD11/CD18 expression, antiinflammins attenuate firm adhesion of PMNs to the endothelium and consequently their transendothelial migration, key events for leukocyte accumulation in tissues. Therefore, antiinflammins may be useful therapeutic agents to prevent and/or attenuate the neutrophil-mediated tissue injury that accompanies myocardial reperfusion injury (41, 42), as well as other chronic disease states such as rheumatoid arthritis (43). The

mechanisms of action of antinflammins differ from those of nonsteroid anti-inflammatory drugs or the acute-phase reactant C-reactive protein, which inhibit PMN binding to endothelial cells by inducing shedding of L-selectin from the leukocyte surface without affecting CD11/CD18 expression (26, 44).

In conclusion, this study demonstrates that antinflammins mimic the actions of glucocorticoids on adhesion molecule expression on human leukocytes, but not on endothelial cells, and attenuate PMN adhesion to HCAEC via inhibition of cell activation-induced changes in CD11/CD18 expression. Therefore, antinflammins may represent a novel therapeutic approach in blocking leukocyte trafficking in host defense and inflammation. **[F]**

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