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*J Immunol* 1998; 161:4268-4275; 
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Oleic Acid Increases Cell Surface Expression and Activity of CD11b on Human Neutrophils

Anthony M. Mastrangelo, Thomas M. Jeitner, and John W. Eaton

Traumatic bone injury frequently results in the release of marrow-derived fatty material into the circulation. This may lead to the syndrome of fat embolism, associated with the generation of free fatty acids, the sequestration of neutrophils in the lungs, and the subsequent development of acute respiratory distress. Neutrophil accumulation in tissues requires their adherence to vascular endothelial cells and involves the β2 integrin, CD11b/CD18 (Mac-1). We now report that the exposure of isolated human neutrophils to oleic acid causes a rapid increase in the cell surface expression and affinity state of CD11b, particularly under acidic conditions that are typical of inflammatory sites. Oleic acid also triggers neutrophil aggregation and neutrophil adherence to both fibrinogen-coated surfaces and confluent cultures of HUVEC. These processes are blocked by CD11b-specific inhibitors, including neutrophil-inhibitory factor and mAbs to CD11b. These observations may help explain the etiology of so-called fat embolism wherein trauma-induced release of fatty material causes pulmonary neutrophil accumulation and the development of acute respiratory distress. The Journal of Immunology, 1998, 161: 4268–4275.

Neutrophils are equipped with distinct granules and vesicles that are mobilized to the plasma membrane after activation (1). These intracellular stores each contain characteristic proteins that, collectively, execute the inflammatory functions of the neutrophil. Several of these proteins are involved in mediating the interaction of neutrophils with the endothelium, such as the β2 integrin CD11b/CD18, while others, including myeloperoxidase, are necessary for killing microorganisms. Because many of the microbialicidal factors released by neutrophils also are potentially harmful to host cells, it is vital that they not be evoked by circulating neutrophils. Thus, the earliest events of neutrophil activation involve positioning these cells at the site of inflammation before release of microcidal agents. Part of the process of targeting neutrophils to inflammatory sites involves CD11b/CD18-mediated adhesion to the vascular endothelium. This adhesion is accompanied by an increase in the affinity and cell surface expression of CD11b/CD18, both of which precede the release of myeloperoxidase and other primary granule products (2, 3).

Following traumatic bone injury, activated neutrophils often accumulate within lung tissue, causing pulmonary damage and the development of the adult respiratory distress syndrome (ARDS) (4, 5). Interestingly, long bone fractures also result in the deposition of marrow-derived fatty acids in the circulation (6). These fat emboli, which contain oleic acid as the major fatty acid component (6, 7), lead to the generation of free fatty acids upon hydrolysis (4). Previous observations from our laboratory indicate that free fatty acids amplify the mobilization of the myeloperoxidase-containing granules of neutrophils (8). Thus, elevations in plasma-free fatty acids might activate neutrophils, promoting the release of granular contents and enhancing damage to the pulmonary vascular endothelium and interstitium. This hypothesis is supported by the observation that the i.v. administration of oleic acid into experimental animals results in the accumulation of neutrophils in the lungs and the pathologic manifestations of ARDS (9). Moreover, the generation of fat emboli subsequent to trauma precedes the development of respiratory distress, suggesting that free fatty acids might promote the sequestration of neutrophils in the lungs (4).

Having previously demonstrated that oleic acid stimulates the mobilization of myeloperoxidase-containing granules, we hypothesized that oleic acid also might induce the fusion of CD11b/CD18-containing granules and vesicles with the plasma membrane. Interestingly, the cell surface expression of CD11b/CD18 is markedly increased on neutrophils from trauma patients both before and after the development of ARDS (10). In addition, specific inhibitors of CD11b/CD18-ligand binding attenuate the adherence of neutrophils to the pulmonary vascular endothelium in several in vivo models of respiratory distress (11, 12). In the present study, we have examined both the cell surface expression and affinity status of CD11b (and, by implication, CD18) in response to oleic acid and the role of these affinity states in oleic acid-mediated homotypic (aggregation) and heterotypic (endothelial attachment) neutrophil adhesion. Our results demonstrate that micromolar amounts of oleic acid increase the cell surface expression of CD11b and induce the high affinity state of this integrin. These responses are greatly enhanced when the extracellular pH is slightly acidic and are, at least in part, dependent on oleic acid-mediated cytosolic acidification. Furthermore, oleic acid, through a CD11b-mediated mechanism, induces neutrophil aggregation and neutrophil-endothelial cell attachment. These results implicate oleic acid as a possible agonist of neutrophil aggregation, sequestration, and activation. These observations may help explain the
etiology of ARDS arising from fat embolism in which trauma-induced release of fatty material is followed by the accumulation of neutrophils within the pulmonary circuit.

Materials and Methods

Materials

Oleic acid, oleic acid methyl ester, and PMA were purchased from Sigma (St. Louis, MO). Stock solutions of fatty acids and PMA were prepared in ethanol and dimethylsulfoxide, respectively, and stored at −70°C. The following mAb were used: LM2/1 (anti-CD11b, IgG1) was purified from hybridomas supernatants (American Type Culture Collection, Rockville, MD) by the method described by Harlow and Lane (13); CBM15/1 (mAb specific for a high affinity form of CD11b, IgG1) was a gift of Dr. T. Springer (Springer, Boston, MA); CL15.2 (anti-CD54, IgG1) was purchased from Leinco Technologies (St. Louis, MO); and 2′,7′-bis(2-carboxyethyl)-5′-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR). Nigericin and carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) were from Sigma.

Cell preparation

Human neutrophils were isolated from venous blood of healthy volunteers (following informed consent) by the method of Redl et al. (14). Typically, 40 ml of blood was drawn into a 60-ml syringe containing 1000 U/ml of heparin and 20 ml of 6% Hetastarch. After gentle mixing, the blood was allowed to settle for 45 min. The leukocyte-rich plasma was removed and centrifuged at 500 × g for 7 min. Erythrocytes were then lysed with H2O, and leukocyte purity was restored with hypertonnic NaCl. After low speed centrifugation, the leukocytes were resuspended in HBSS without Ca2+ and Mg2+ (HBSS-) and applied to the top of a solution of isosmotic NaCl and Percoll at a specific density of 1.075. The cells were centrifuged at 10,000 × g for 20 min at 4°C, and the neutrophils were collected from the bottom layer. The cells were washed twice and resuspended in HBSS- and held briefly at 4°C. This procedure yielded >95% neutrophils according to light scatter analysis using a FACScan (Becton Dickinson, San Jose, CA).

Whole blood was drawn from human donors approximately 12 h postprandially. Neutrophils were enriched by fluorescence-activated cell sorting according to the method described by Jaffe et al. (15). Briefly, an umbilical cord (<2 h old) was rinsed externally with distilled H2O and then with 70% alcohol. A blunt-tipped cannula was inserted into each end of the cord and secured with sterile umbilical tape. Blood was removed from the cord by connecting a 50-cc sterile syringe to one cannula and flushing HBSS through the vein. This procedure yielded >95% neutrophils according to light scatter analysis using a FACScan (Becton Dickinson, CA). Isolated neutrophils were analyzed for their surface expression of specific Ags using indirect flow cytometry, as described by Sengelov et al. (2). The cells were fixed in 2% paraformaldehyde, 0.5% gluteraldehyde in PBS for 15 min at 4°C and then washed twice in PBS containing 0.5% (w/v) BSA. To block FcR, the cells were resuspended at 5 × 10^6 cells/ml in PBS containing 100 μg/ml of human IgG. After 30 min at 4°C, an Ag-specific mAb or mouse IgG1 was added at a final concentration of 10 μg/ml. The cells were placed at 4°C for 1 h, washed twice with PBS, and resuspended at 5 × 10^6 cells/ml in PBS containing 100 μg/ml of human IgG. Fluorescence-activated cell sorting (FACS) gated anti-mouse IgG1 was then added at 1:200 dilution. After a 30-min incubation at 4°C in the dark, the cells were washed twice with PBS, resuspended in 300 μl of 1% formaldehyde, and analyzed by flow cytometry (FACScan and Cell Quest; Becton Dickinson). The mean fluorescence intensity for specific Abs was corrected for nonspecific fluorescence by subtracting the mean fluorescence obtained with mouse IgG1. Approximately 15,000 to 20,000 events were analyzed per sample. For flow-cytometric analysis of neutrophils in whole blood, the granulocyte population was identified and analyzed on the basis of their forward and side scatter characteristics.

Flow cytometry

Isolated neutrophils were analyzed for their surface expression of specific Ags using indirect flow cytometry, as described by Sengelov et al. (2). The cells were fixed in 2% paraformaldehyde, 0.5% gluteraldehyde in PBS for 15 min at 4°C and then washed twice in PBS containing 0.5% (w/v) BSA. To block FcR, the cells were resuspended at 5 × 10^6 cells/ml in PBS containing 100 μg/ml of human IgG. After 30 min at 4°C, an Ag-specific mAb or mouse IgG1 was added at a final concentration of 10 μg/ml. The cells were placed at 4°C for 1 h, washed twice with PBS, and resuspended at 5 × 10^6 cells/ml in PBS containing 100 μg/ml of human IgG. Fluorescence-activated cell sorting (FACS) gated anti-mouse IgG1 was then added at 1:200 dilution. After a 30-min incubation at 4°C in the dark, the cells were washed twice with PBS, resuspended in 300 μl of 1% formaldehyde, and analyzed by flow cytometry (FACScan and Cell Quest; Becton Dickinson). The mean fluorescence intensity for specific Abs was corrected for nonspecific fluorescence by subtracting the mean fluorescence obtained with mouse IgG1. Approximately 15,000 to 20,000 events were analyzed per sample. For flow-cytometric analysis of neutrophils in whole blood, the granulocyte population was identified and analyzed on the basis of their forward and side scatter characteristics.

Neutrophil adhesion assay

HUVEC were cultured on 48-well tissue culture plates and washed twice with PBS containing 1 mM Ca2+ and Mg2+ (PBSB) before use. Neutrophils (1 × 10^6 cells/ml) were exposed to oleic acid in Ringer’s buffer at pH 6.65 or pH 7.40 for 5 min at 37°C under 5% CO2. Each sample was aliquoted (3 × 10^5 neutrophils in 300 μl) into four wells containing HUVEC monolayers. Neutrophil contact with HUVEC was promoted by centrifugation of the plate for 3 min at 500 × g. The plate was then incubated for 5 min under tissue culture conditions. Unattached cells were gently washed away four times with PBSB. The adherent cells were then fixed for 15 min with 0.1% paraformaldehyde in PBSB. Neutrophil attachment was quantitated by averaging the number of cells counted in three fields in each well under ×100 magnification. This average was used to calculate the number of cells per well according to the following formula: Cells per well = average cell number per field × 76.23, where 76.23 = (area of well)/(area of ocular grid) = (100.81 mm^2)/(1.3225 mm^2).

Neutrophil aggregation assay

Neutrophil homotypic aggregation in response to oleic acid was investigated using a modification of the method described by Philips et al. (16). Isolated neutrophils (5 × 10^6 cells/ml) were suspended in Ringer’s buffer containing 10 mM HEPES and then equilibrated for 5 min at 37°C. Before addition of oleic acid, light transmission through 500 μl of sample was reinforced by 1 ml to establish a stable baseline of no aggregation (Chrono-Log Aggregometer, model 430 VS). Oleic acid was then added, and light transmission through the sample was measured continuously for 5 min. The aggregometer records the transmission of light through a cell suspension as a function of time (minutes). Because light transmission is inversely proportional to the numbers of individual (unaggregated) cells in the suspension, an increase in light transmission indicates a reduction in individual cells due to aggregation. The aggregometer is standardized to 0 and 100% aggregation before each experiment. Therefore, the slope of the line generated by the aggregometer also indicates the percentage of the total cells aggregating per minute. Thus, aggregation was reported as percentage of total cells aggregated per minute.

Intracellular pH measurements

The effects of oleic acid on intracellular pH were investigated using the pH-sensitive dye BCECF-AM. Neutrophils were suspended at 5 × 10^6 cells/ml in PBS and incubated for 20 min with 4 μM of the acetoxymethyl ester form of BCECF-AM. Cells were washed twice with PBS and suspended at 1 × 10^6 cells/ml in a modified Ringer’s buffer. Fluorescence determination of intracellular pH was performed at 37°C. The ratio of excitation intensity at 505 nm to that at 439 nm is pH sensitive and was utilized to cancel signal errors due to variations in BCECF-AM concentration, path length, esterase activity, and subcellular localization (Molecular Probes). The emission was observed at 535 nm. Baseline intracellular pH was determined for 1 min before the addition of oleic acid. The calibration of fluorescence versus intracellular pH (pH) was performed by the K’/nigericin method described by Thomas et al. (17).
Exposure of neutrophils to oleic acid increases cell surface expression of CD11b

The effects of oleic acid on the cell surface expression of CD11b (and, by implication, CD18) on neutrophils were assessed using flow cytometry. These experiments were performed at pH 7.4, 6.9, and 6.6 to elucidate whether the effects of oleic acid on CD11b expression could be influenced by the acidic environment prevailing in inflammatory sites, which ranges between pH 5.7 and 7.2 (18). Cell surface CD11b increased approximately twofold above control cells at pH 7.4 and fivefold at pH 6.6 after a 5-min exposure to oleic acid (Fig. 1). The oleic acid-mediated increase in CD11b was both time and dose dependent. For example, exposure of neutrophils to oleic acid at pH 7.4 caused an increase in CD11b expression after 5 min to a level 2.5-fold above that of the control cells (Fig. 2A). The concentrations of oleic acid used in these experiments were well below the concentration of circulating oleic acid measured in patients at risk for ARDS, such as those undergoing surgical implantation of hip prostheses (152.2 mM) (7).

The increase in CD11b expression due to oleic acid was compared with that induced by PMA, because PMA is known to induce an increase in cell surface CD11b/CD18 via granule mobilization (19). In agreement with previously published observations, PMA stimulated a time-dependent increase in CD11b expression on neutrophils (Fig. 2, A and B). At pH 7.4, the increase in CD11b due to PMA was of greater magnitude than that induced by oleic acid (Fig. 2A). In contrast, the oleic acid-mediated increase in CD11b was greater than that induced by PMA when the extracellular pH was lowered to 6.9 (compare Fig. 2A to 2B). The effect of PMA on CD11b expression is essentially independent of the extracellular pH (compare Fig. 2A and 2B). Thus, oleic acid induces a time- and dose-dependent increase in CD11b expression, and the extent of this effect is modulated by extracellular pH.

The effects of oleic acid on the expression of CD11b on neutrophils in whole blood also were investigated to determine whether this fatty acid could induce CD11b expression under more physiologic conditions. Whole blood contains approximately 0.5 mM albumin, which has three high affinity fatty acid binding sites per molecule (20). The results shown in Figure 3 indicate that the...
Regulation of CD11b/CD18 on neutrophils involves modulation of both the cell surface expression of this integrin and the affinity of this integrin for its ligands. As shown in Figure 4, both PMA and oleic acid induced the expression of a high affinity epitope of CD11b, as indicated by the binding of CBRM1/5, a mAb specific for the high affinity state of this integrin (3). In the case of oleic acid, this response was regulated by the extracellular pH in a manner similar to the oleic acid-mediated increase in CD11b expression described earlier. For example, exposure of neutrophils to oleic acid at pH 6.6 caused a 10-fold increase in CBRM1/5 binding above control cells versus a 0.01-fold increase at pH 7.4 (Fig. 4). Furthermore, at pH 6.6, oleic acid increased the expression of the high affinity epitope of CD11b to a greater extent than PMA (Fig. 4). Thus, in addition to promoting cell surface expression of CD11b on neutrophils, oleic acid also increases the affinity of CD11b/CD18 in a pH-dependent manner.

**Oleic acid promotes both heterotypic and homotypic adherence of neutrophils**

Expression of the high affinity epitope of CD11b/CD18 would be expected to promote the attachment of neutrophils to CD11b/CD18 ligands such as fibrinogen and ICAM-1. Indeed, when neutrophils were exposed to oleic acid at pH 7.4, their adherence to HUVEC monolayers and to fibrinogen increased 3.4-fold and 4.1-fold above control cells, respectively (Table I). This increased adherence was mediated by CD11b because the enhanced attachment of oleic acid-treated neutrophils to both fibrinogen and HUVEC was blocked by neutrophil-inhibitory factor (NIF), a specific inhibitor of CD11b/CD18-mediated adherence (21) (Table II). The attachment of oleic acid-treated neutrophils to HUVEC also was completely prevented by the CD11b-blocking mAb, CBRM1/5 (Table II). In this case, we should note that the likely counterligand on HUVEC, ICAM-1, is constitutively expressed under our culture conditions (22). Inexplicably, oleic acid reduced the attachment of neutrophils to fibrinogen or to HUVEC when the extracellular pH was lowered to 6.6 (Table I). Reducing the extracellular pH to 6.6 did not affect the general adherence capabilities of the neutrophils, since acidic pH did not affect the baseline level of neutrophil attachment and did not alter neutrophil attachment induced by PMA (Table I).

When the effects of oleic acid on the adhesive properties of neutrophils were investigated further, we observed that neutrophils also aggregated after exposure to the fatty acid. This response was enhanced approximately threefold when the pH was lowered to 6.6 (Fig. 5). Neutrophil aggregation induced by oleic acid was completely inhibited by CBRM1/5 and partially blocked by NIF, once again indicating the involvement of CD11b (Table III). In contrast, the mAb LM2/1, which recognizes CD11b/CD18, but is not specific for an activated epitope, was relatively ineffective (Table III). Interestingly, an Ab against ICAM-1 also inhibited neutrophil aggregation, suggesting the possible involvement of ICAM-1 as a counterligand for CD11b/CD18 in this homotypic aggregation.

![Figure 3](image)

**FIGURE 3.** Oleic acid increases the expression of CD11b on neutrophils in whole blood. Venous blood was drawn (after overnight fast) and aliquots were directly exposed to the indicated concentrations of added oleic acid. The surface expression of CD11b was then determined by flow cytometry using the mAb LM2/1. The fluorescence for LM2/1 was corrected for background binding by subtracting the fluorescence obtained with a matched isotype control Ab. Each point represents the mean ± SEM from three separate experiments.

![Figure 4](image)

**FIGURE 4.** Cell surface expression of a high affinity epitope of CD11b is increased after exposure of neutrophils to either PMA or oleic acid. Isolated peripheral blood neutrophils were exposed to 200 nM PMA or 80 μM oleic acid for 5 min at pH 6.6 (dark bars) or pH 7.4 (hatched bars). Control cells received vehicle (5 μl of EtOH). The cell surface expression of CD11b and the expression of a high affinity epitope of CD11b were analyzed by flow cytometry using mAb LM2/1 or CBRM1/5, respectively. The fluorescence for LM2/1 and CBRM1/5 were corrected for background binding by subtracting the fluorescence obtained with a matched isotype control Ab. Data are expressed on a log scale and represent the mean ± SEM from three separate experiments.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Substrate</th>
<th>pH&lt;sub&gt;7.4&lt;/sub&gt;</th>
<th>pH&lt;sub&gt;6.6&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Fibrinogen</td>
<td>7,410 ± 3,916&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3,268 ± 183</td>
</tr>
<tr>
<td>Control</td>
<td>HUVEC</td>
<td>3,149 ± 553</td>
<td>3,083 ± 348</td>
</tr>
<tr>
<td>PMA</td>
<td>Fibrinogen</td>
<td>92,891 ± 9,550</td>
<td>83,139 ± 5,760</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>Fibrinogen</td>
<td>30,378 ± 7,263</td>
<td>1,529 ± 148</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>HUVEC</td>
<td>10,653 ± 2,416&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,547 ± 252</td>
</tr>
</tbody>
</table>

<sup>a</sup>Neutrophils were exposed to 200 nM PMA or 80 μM oleic acid for 5 min at either pH 7.4 or 6.6. The attachment of these cells to fibrinogen-coated plates and to monolayers of HUVEC was then determined as described in Materials and Methods.

<sup>1</sup>Mean ± SEM from three separate experiments.

<sup>b</sup>Not significantly different from fibrinogen control at pH 6.6.

<sup>c</sup>p < 0.05 vs. oleic acid sample at pH 6.6.
Intracellular acidification by oleic acid and its role in the up-regulation of CD11b

Several investigators have demonstrated an intracellular acidification caused by fatty acid-mediated transport of protons into cells, but the possible importance of this acidification in the regulation of cellular processes has not been elucidated (23). The influence of extracellular pH on oleic acid-mediated responses described above suggested that oleic acid might exert its effects by intracellular acidification. To determine whether oleic acid might lower cytosolic pH, neutrophils were loaded with the pH-sensitive fluorescence indicator, BCECF-AM, and then exposed to oleic acid. Fluorescence ratio analysis indicated that oleic acid reduced pH, in a manner dependent on extracellular pH (Fig. 6A). For example, when the extracellular pH was 7.4, 6.9, and 6.6, oleic acid acutely reduced pH, by 0.16, 0.21, and 0.32 pH units, respectively (Fig. 6A).

Fatty acid-mediated transport of protons across phospholipid bilayers may occur through initial protonation of the carboxyl group (24). Indeed, when the carboxyl group of oleic acid was replaced by a methyl ester (oleic acid methyl ester), this derivative did not reduce the pH of neutrophils. Interestingly, oleic acid methyl ester only slightly enhanced expression of CD11b (Fig. 6C), suggesting that mobilization of CD11b-containing granules might involve, or absolutely require, fatty acid-mediated reduction in intracellular pH. This presumption was experimentally examined by treating neutrophils with the ionophore, FCCP. Under these conditions, the extracellular and cytosolic concentrations of $\text{H}^+$ are equalized, and oleic acid will no longer cause changes in pH, (data not shown). When the intracellular pH is clamped in this manner, oleic acid-mediated increase in the cell surface expression and high affinity epitope of CD11b was reduced (Fig. 7A and B). Thus, oleic acid may promote the increased cell surface expression and affinity of CD11b/CD18, at least in part, via cytosolic acidification.

Discussion

The goal of this study was to elucidate the effects of oleic acid on neutrophil activation with respect to the cell surface expression and affinity state of the $\beta_2$ integrin CD11b/CD18. Neutrophils constitutively express CD11b/CD18 at the cell surface and also store CD11b/CD18 within intracellular secretory vesicles, specific granules, and gelatinase granules (2). These granules and vesicles are mobilized to the plasma membrane during neutrophil activation (2). In the present study, we observed an increase in the cell surface expression of CD11b on isolated neutrophils after exposure to oleic acid. The increase in CD11b expression by oleic acid is undoubtedly the result of a degranulation response, since oleic acid also increases cell surface expression of the TNF-$\alpha$ receptor R1 (data not shown) and stimulates the release of myeloperoxidase (8; Jeitner and Eaton, in preparation), which are contained within the specific granules and azurophil granules, respectively (25). In addition, oleic acid does not affect the cell surface expression of $\beta_2$ integrins that are not stored within granules or vesicles, such as CD11a/CD18 (data not shown). Therefore, it appears that oleic acid causes a generalized degranulation response, probably involving each type of granule produced by neutrophils.

The mechanism(s) responsible for oleic acid-mediated mobilization of neutrophil granules is not fully understood. The well-known profusogenic actions of free fatty acids might be important in promoting fusion of intracellular granules with the plasma membrane, which is a phenomenon well described in other circumstances (26, 27). A second, but not mutually exclusive, mechanism

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**Table II.** CD11b mediates oleic acid-induced attachment of neutrophils to endothelial cells and fibrinogen at pH 7.4$^a$

<table>
<thead>
<tr>
<th>Condition</th>
<th>Substrate</th>
<th>Cells Attached$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Fibrinogen</td>
<td>2,383 ± 204</td>
</tr>
<tr>
<td>PMA</td>
<td>Fibrinogen</td>
<td>78,221 ± 6,632</td>
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<tr>
<td>Oleic acid</td>
<td>Fibrinogen</td>
<td>3,548 ± 1,096</td>
</tr>
<tr>
<td>Oleic acid + NIF</td>
<td>Fibrinogen</td>
<td>10,748 ± 2,059</td>
</tr>
<tr>
<td>Oleic acid + NIF</td>
<td>Fibrinogen</td>
<td>1,117 ± 134</td>
</tr>
<tr>
<td>Control</td>
<td>HUVEC</td>
<td>4,250 ± 199</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>HUVEC</td>
<td>18,069 ± 2,672</td>
</tr>
<tr>
<td>Oleic acid + NIF</td>
<td>HUVEC</td>
<td>4,131 ± 978</td>
</tr>
<tr>
<td>Oleic acid + CBRM</td>
<td>HUVEC</td>
<td>5,667 ± 580</td>
</tr>
<tr>
<td>Oleic acid + MgG1</td>
<td>HUVEC</td>
<td>16,334 ± 1,113$^c$</td>
</tr>
</tbody>
</table>

$^a$ Neutrophils were treated with either 100 nM NIF, 5 $\mu$g/ml CBRM1/5, or 5 $\mu$g/ml MgG1 for 5 min. The cells were then exposed to 200 nM PMA or 80 $\mu$M oleic acid for 5 min at pH 7.4. The attachment of these cells to fibrinogen-coated plates and to monolayers of HUVEC was then determined as described in Materials and Methods.

$^b$ Mean ± SEM from three separate experiments.

$^c$ Not significantly different when compared to oleic acid/HUVEC.

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**Table III.** CD11b mediates oleic acid-induced neutrophil aggregation at pH 6.6$^a$

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells Aggregated (% per minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>8.5</td>
</tr>
<tr>
<td>Oleic acid + NIF</td>
<td>2.72</td>
</tr>
<tr>
<td>Oleic acid + CBRM</td>
<td>0.0</td>
</tr>
<tr>
<td>Oleic acid + LM21</td>
<td>6.38</td>
</tr>
<tr>
<td>Oleic acid + α-ICAM-1</td>
<td>0.0</td>
</tr>
<tr>
<td>Oleic acid + MgG1</td>
<td>6.38</td>
</tr>
</tbody>
</table>

$^a$ Neutrophils were treated with either 600 nM NIF or 5 $\mu$g/ml of CBRM1/5, LM21, α-ICAM-1, or MgG1 for 5 min. The cells were then exposed to 200 $\mu$M oleic acid and analyzed for aggregation over a 10-min period as described in Materials and Methods. These results are representative of a single experiment that was performed twice with high reproducibility.

**FIGURE 5.** Neutrophil aggregation after exposure to oleic acid. Isolated peripheral blood neutrophils were exposed to the indicated concentrations of oleic acid (added as indicated by the small arrow) with constant stirring at either pH 6.6 (A) or pH 7.4 (B). Changes in light transmission (ΔT) over time were then recorded in a standard platelet aggregometer. Data shown are representative of three independent experiments.
might involve oleic acid-mediated cytosolic acidification. This is supported by the observation that the oleic acid-mediated increase in CD11b expression was enhanced when the extracellular pH was reduced from 7.4 to 6.6. Furthermore, addition of oleic acid caused a substantial drop in intracellular pH, which also was enhanced when the extracellular milieu was acidic. Indeed, free fatty acids, including oleic acid, are known to cause intracellular acidification by transporting protons across cell membranes via their carboxyl group (24). Hence, when we exposed neutrophils to oleic acid methyl ester, which is incapable of carrying a proton on its carboxylate moiety and does not cause intracellular acidification, we did not observe an increase in CD11b expression. In addition, prevention of cytosolic acidification by a proton ionophore partially inhibited the oleic acid-mediated increase in CD11b expression, which further supports a role for intracellular acidification in the degranulation response induced by oleic acid.

The precise role of cytosolic acidification in the mobilization of neutrophil granules by oleic acid remains elusive. However, one possibility is that oleic acid-mediated intracellular acidification reorganizes the cytoskeleton in a manner that favors degranulation. In resting neutrophils, the actin cortex might prohibit degranulation by forming a barrier between intracellular granules and the plasma membrane. The actin-binding protein, gelsolin, regulates the length of actin filaments by severing the noncovalent bonds.
between actin subunits within the filament, leading to isolation of actin networks and fluidization of the cell cortex (28). Gelsolin activity is induced by elevated intracellular Ca\textsuperscript{2+} levels (28). However, at pH 6.75, the Ca\textsuperscript{2+} requirement for the severing activity of gelsolin is reduced significantly and, at pH <6, gelsolin severs actin in the absence of Ca\textsuperscript{2+} (29). Thus, oleic acid might increase the activity of gelsolin by reducing the cytosolic pH, which in turn would act to sever the actin cortex, thereby allowing granules passage to the plasma membrane.

Most agonists that increase the cell surface expression of CD11b also increase the ligand-binding activity of this integrin (3). CBRM1/5 is a mAb that recognizes an activation epitope on a subset of CD11b molecules on neutrophils after stimulation with chemotacticants or phorbol esters, but does not recognize CD11b on resting cells (3). Our results demonstrate that oleic acid induces the expression of a high affinity epitope of CD11b on neutrophils, as indicated by CBRM1/5 binding.

The increase in the high affinity epitope of CD11b by oleic acid involves oleic acid-mediated intracellular acidification. This is supported by the observation that enhanced expression of the CBRM1/5 epitope occurred preferentially under conditions of low extracellular pH, which corresponded to exaggerated reductions in cytosolic pH mediated by oleic acid. In addition, induction of the high affinity epitope of CD11b by oleic acid was partially inhibited with a proton ionophore, thus further supporting a role for cytosolic acidification in mediating this response. It is well known that integrin-ligand binding can cause integrins to signal the activation of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter and increase intracellular pH (30). However, to the best of our knowledge, this is the first report that demonstrates modulation of integrin affinity by intracellular pH itself. This suggests that cytosolic acidification is involved in "inside-out" signaling and may be a novel mechanism for regulation of integrin activation. In this regard, we note that charge reversal mutations within the cytoplasmic domains of conserved amino acid sequences of integrin \(\alpha\) and \(\beta\) subunits have been shown to constitutively activate the \(\alpha_{\text{IIb}}\beta_{3}\) integrin (31). This may occur through disruption of salt bridges between the \(\alpha\) and \(\beta\) subunits that are thought to be important in regulating integrin activity (31). Therefore, oleic acid-mediated cytoplasmic acidification might induce the high affinity epitope of CD11b by altering the ionic charge of the membrane-proximal amino acids that participate in forming the salt bridge. This could modify the association between the \(\alpha\) and \(\beta\) subunits, resulting in conformational changes that expose the high affinity epitope of CD11b/CD18.

In addition to direct effects on the \(\alpha\) and \(\beta\) subunits of CD11b/CD18, cytosolic acidification mediated by oleic acid can potentially alter the affinity state of CD11b/CD18 by modifying its association with the cytoskeleton. The function of integrins is thought to be at least partially regulated by their interactions with the actin network via actin-binding proteins (32). Interestingly, talin, which associates with \(\beta\) integrins in vitro (33), has been demonstrated to interact with actin more frequently under acidic conditions (34). Perhaps by lowering the cytosolic pH, oleic acid can affect integrin-actin interactions by modifying the association of actin-binding proteins with actin. Recently, it has been shown that disruption of the actin cytoskeleton is necessary for the expression of a high affinity epitope of CD11a/CD18 on resting lymphocytes (35). As previously discussed, reductions in cytoplasmic pH by oleic acid can potentially reduce actin filament formation by activating proteins such as gelsolin. The disruption of actin filaments in this manner may alter the interaction between CD11b/CD18 and the cytoskeleton, which could lead to conformational changes of CD11b/CD18 that increase the affinity state of this integrin.

Since oleic acid induces the expression of a high affinity epitope of CD11b/CD18, we investigated the consequences of this response on neutrophil heterotypic and homotypic adherence. We found that oleic acid stimulated CD11b/CD18-dependent neutrophil aggregation, and neutrophil attachment to fibrinogen-coated plates and to endothelial cell monolayers. Thus, expression of the high affinity epitope of CD11b by oleic acid corresponds to an increase in the function of this integrin. Interestingly, neutrophil aggregation was preferentially induced by oleic acid under conditions of low extracellular pH, which also promoted enhanced expression of the high affinity epitope of CD11b. However, under neutral conditions, the weak induction of the CBRM1/5 epitope by oleic acid preferentially stimulated heterotypic adherence. The reason for this differential response on neutrophil adherence by oleic acid is not fully understood. Perhaps neutrophil heterotypic attachment involves a low to moderate affinity state of CD11b, while neutrophil homotypic aggregation occurs, and is favored, when a large number of CD11b molecules are in a high affinity state. It has recently been demonstrated that cytosolic acidification prevents neutrophil spreading and retains neutrophils in a round shape (36). Hence, maybe under acidic conditions, the combination of high affinity CD11b/CD18 integrins and a persistent round morphology may favor neutrophil aggregation over heterotypic attachment.

Induction of CD11b/CD18-dependent neutrophil aggregation and heterotypic attachment by oleic acid may help explain neutrophil accumulation in the lungs of patients with ARDS secondary to fat embolus. In this disease state, large numbers of neutrophils are retained as aggregates within the pulmonary microcirculation and also emigrate into the alveolar interstitium (37, 38). Clinical disorders that result in ARDS are accompanied by increases in circulating phospholipase A\(\text{2}\) activity and elevations in plasma-free fatty acids, particularly oleic acid (5, 39). Interestingly, one experimental animal model of ARDS involves i.v. infusion of oleic acid, which causes the pathologic manifestations of ARDS, including the accumulation of neutrophils within the pulmonary microcirculation (6). However, it is still not known why mobilization of fatty acids into the bloodstream leads to neutrophil-mediated lung injury and the subsequent development of ARDS.

Our observations suggest that protein-free oleic acid might play a role in sequestering neutrophils in the lungs by increasing the cell surface expression and activity of CD11b/CD18 on neutrophils. The concentrations of oleic acid used in our experiments involving purified neutrophils were within the pathophysiologic range measured in patients at risk for ARDS, such as those undergoing hip prosthesis implantation surgery (7). Given that whole blood contains approximately 0.5 mM albumin, which has three to six fatty acid binding sites per molecule, the concentrations of protein-free oleic acid in our whole blood experiments were probably also within pathologic concentrations seen in vivo. Thus, it is likely that oleic acid can induce the cell surface expression and activity of CD11b on circulating neutrophils when concentrations of oleic acid exceed the fatty acid binding capacity of albumin, since we observed an oleic acid-mediated increase in the cell surface expression of CD11b on neutrophils in whole blood. Indeed, the expression of CD11b/CD18 is up-regulated on circulating neutrophils in trauma patients with ARDS when compared with healthy individuals (10). In addition, adherence of neutrophils to the pulmonary vascular endothelium in several in vivo models of respiratory distress is attenuated by anti-CD11b/CD18 and NIF (11, 12). Therefore, the induction of CD11b activity by oleic acid implicates this fatty acid as an agonist of neutrophil recruitment during ARDS.

The acidic environment in which we observed an enhanced effect of oleic acid on CD11b activity, neutrophil degranulation, and
neutrophil aggregation can potentially occur in the pulmonary microcirculation during ARDS (as well as in areas of focal inflammation). In general, inflammatory sites are usually acidic (pH 5.7–7.2) (18), due in part to the release of fatty acids from both microbial metabolism and the actions of phospholipases from host cells. Similarly, fatty acids released into the circulation during ARDS can potentially reduce the pH in the microcirculation. In addition, ischemia and hypoxia, which often occur during ARDS, can cause localized areas of acidosis within pulmonary tissue. In this regard, it is also noteworthy that the dissociation of oleic acid from albumin is favored in acidic environments (40). Taken together, our results suggest that, in the pulmonary microcirculation during ARDS and in intermittent foci of inflammation, neutrophil adhesion and degranulation and CD11b/CD18-dependent neutrophil aggregation may be greatly enhanced by the combination of low pH and free fatty acids, such as oleic acid.

Acknowledgments

We thank Dr. Timothy Springer for providing CB1RM1/5; Dr. Mathew Moyle and Corvas for providing neutrophil-inhibitory factor; Dr. Stanley Mudzinski, Todd Christian, Jim Mittler, David Devernoe, and Scott Menzie for skilled technical assistance with the flow-cytometric studies; Dr. Paul Gudewicz for critical suggestions of the manuscript; Drs. John Mahey, Mark Scott, and Susan LaFlame for valuable suggestions and advice; Diane Konzen for preparation of the manuscript; and Lisa Mas-trangelo for technical review of the manuscript.

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