Elevated Concentrations of Nonesterified Fatty Acids Increase Monocyte Expression of CD11b and Adhesion to Endothelial Cells

Wei-Yang Zhang, Eric Schwartz, Yingjie Wang, Jeanne Attrep, Zhi Li, Peter Reaven

Objective—Monocyte proinflammatory activity has been demonstrated in obesity, insulin resistance, and type 2 diabetes, metabolic conditions that are frequently associated with elevated levels of nonesterified fatty acids (NEFA). We therefore tested the hypothesis that NEFA may induce monocyte inflammation.

Methods and Results—Monocytes exposed to NEFA for 2 days demonstrated a dose-related increase in intracellular reactive oxygen species (ROS) formation and adhesion to endothelial cells. All of these effects were inhibited by the coaddition of antioxidants such as glutathione or butylated hydroxytoluene, by inhibition of ROS generation by NADPH oxidase inhibitors, and by inhibition of protein kinase C, a recognized stimulator of NADPH oxidase. Monocytes exposed to NEFA also demonstrated a significant increase in CD11b message expression. Stimulation of monocyte adhesion to endothelial cells by NEFA was inhibited by addition of neutralizing antibodies to either CD11b or CD18. Finally, surface expression of CD11b increased significantly on monocytes as measured by flow cytometry, after their incubation with NEFA.

Conclusion—These studies indicate that elevated concentrations of NEFA may enhance integrin facilitated monocyte adhesion to endothelial cells and these effects appear mediated, in part, through activation of NADPH oxidase and oxidative stress. (Arterioscler Thromb Vasc Biol. 2006;26:514-519.)

Key Words: atherosclerosis ■ integrins ■ monocytes ■ nonesterified fatty acids ■ reactive oxygen species

Numerous studies have suggested that a variety of metabolic abnormalities, including elevated levels of nonesterified free fatty acids, may contribute to the development of atherosclerosis.1–4 Elevated plasma levels of nonesterified fatty acids (NEFA) are commonly present in individuals with insulin resistance or type 2 diabetes, and it has been suggested that these chronically elevated levels of NEFA may contribute to the development of atherosclerosis in these conditions, in part, through their direct effects on vascular cells.5,6

There is evidence that elevated levels of free fatty acids may contribute to both activation of inflammatory cells and their adhesion to endothelium. For example, elevation of plasma free fatty acids in healthy subjects by lipid infusion of a synthetic mixture of triglycerides induced reactive oxygen species (ROS) generation in mononuclear cells and polymorphonuclear leukocytes.7 These cells also demonstrated increased expression of a variety of proinflammatory cytokines including macrophage migration inhibitor factor, possibly a result of upregulation of the NF-κB p65 subunit protein in nuclear homogenates.7 Monocyte firm adhesion to endothelial cells requires interaction of activated β2 (CD11/CD18) or other integrins, such as β1 (CD49d/CD29), with intercellular adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule (VCAM)-1 on the surface of endothelial cells.8,9 Recent data suggest that brief (5 to 10 minutes) exposure to oleic acid (80 μmol/L) increases cell surface expression and binding activity of CD11b on neutrophils and promotes their adherence to human umbilical vein endothelial cells.10

However, little is known about the effects of chronically elevated levels of NEFA on monocyte activation and how this influences their subsequent adhesion to the vascular endothelium. Although some individual fatty acids have been shown to stimulate and even occasionally inhibit activation of mononuclear cells, less is known about the role of elevation of fatty acids in their typical distribution present in serum on monocyte activation and firm adhesion to the vascular endothelium. In the present study, we demonstrate that longer-term exposure of monocytes to elevated physiological concentrations of NEFA enhances their adhesion to endothelial cells in vitro and that this process is mediated through activation of NADPH oxidase, increased ROS generation and enhanced expression of the integrin CD11b.
Materials and Methods

Materials
All free fatty acids used in the experiment were purchased from Sigma Chemical Company (St Louis, Mo). Protein kinase C inhibitors, calphostin C and GF 109203 X, NADPH oxidase inhibitors, diphenyleneiodonium chloride (DPI), and apocynin were from Bi- omol Research laboratory Inc. (Plymouth Meeting, Pa).

Preparation of Fatty Acids Solutions
Palmitic and stearic acids were dissolved in heated (90°C) ethanol as a 20 mmol/L solution. Linoleic, oleic, arachidonic, docosahexaenoic, and eicosapentaenoic acids were first dissolved in a mixture of 5% of bovine serum albumin (BSA) and 30% ethanol in phosphate-buffered saline, gently rotated at 37°C for 4 hours to form fatty acid and BSA complexes. For experiments, the fatty acid solutions were combined with BSA and fetal bovine serum (FBS) to a final NEFA to albumin molar ratio of 2.5:1.

Cell Culture Conditions and Experiments
Human monocytic leukemia (THP-1) cells (American Type Tissue Collection, Manassas, Va) were grown in RPMI 1640 medium containing 10% heat-inactivated FBS and other supplements at 37°C in 5% CO₂. Human peripheral monocytes were isolated from blood of healthy donors as described previously and cultured in RPMI 1640 medium containing 10% human AB serum in ultra-low attachment plates (Corning Inc, Corning, NY). Porcine aortic endothelial cells were grown in medium199 (M-199) medium supplemented with 15% FBS, bovine insulin, and bovine brain extract. Human aortic endothelial cells were grown in endothelial cell growth medium (Cell Applications Inc, San Diego, Calif). Unless stated otherwise, a mixture of NEFA reflecting typical plasma composition (13% stearic acid, 31% palmitic acid, 32% oleic acid, 20% linoleic acid, 2% arachidonic acid, 1% docosahexaenoic acid, and 1% eicosapentaenoic acid) was added to THP-1 cells or human peripheral monocytes in supplemented RPMI 1640 medium for up to 48 hours. Initial cell-free experiments with this NEFA-BSA mixture demonstrated that levels of thiobarbituric acid-reactive substances (as a measure of oxidation) did not increase during the typical 2-day experiments in RPMI 1640 medium plus 10% FBS. Specifically, medium with 200 μmol/L NEFA consistently yielded malondialdehyde equivalents <1.35 mmol/mmol of NEFA, barely above that found for medium alone, or <10% of that of copper oxidized NEFA (as a positive control). Similar results were obtained when oxidation of the NEFA-BSA mixture was measured by conjugated dienes.

Assessment of ROS Formation
ROS formation in THP-1 cells treated with or without NEFA was measured using a chemiluminescence probe L-012 (Wako Chemical, Eugene, Ore), pre-incubated with anti-CD11b monoclonal antibody (R&D Systems, Minneapolis, Minn), and anti-CD18 monoclonal antibody (Immunotech, Marseille Cedex, France), or a nonspecific monoclonal antibody and then added to confluent endothelial cells in 24-well plates. The plate was rotated at 64 RPM for 30 minutes at room temperature. Untreated monocytes were washed off, and the fluorescence of remaining adherent cells was measured by a Wallac Victor2 plate reader using an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

RNA Isolation and Real-Time Polymerase Chain Reaction
Total RNA was isolated from THP-1 cells or human peripheral monocytes using Trizol RNA isolation reagent (Invitrogen, Carlsbad, Calif) and treated with DNase I. Complement DNA was synthesized using cDNA synthesis kit of BioRad. Real-time polymerase chain reaction (PCR) was performed using iQ SYBR Supermix and analyzed by an iCycler iQ Real-Time Detection System (BioRad, Inc., Richmond, Calif). The mRNA levels of CD11b, CD11a, and CD11c were normalized to levels of cell 18s rRNA. The sequences of real-time PCR primers used were human CD11a forward 5’-TCATCATCGCTCA-TATCG-3’, reverse 5’-CAGTCCTTGTCCTCTA-CTG-3’; human CD11b forward 5’-GGGAAATGGCAAAGAATGTA-3’, reverse 5’-CTGCGTGTGC-TGTTTTTGGT-3’; human CD11c forward 5’-ATGCCAAATGG-GGTT-GGATTA-3’, reverse 5’-CTCCAGACCGAGGTAGCTC-3’; human VLA4 (CD49d) forward 5’-ATAATGCTGAAGA-TTTGGG-3’, reverse 5’-CAGACTTGGCTTGGTACAG-3’. Flow Cytometry
THP-1 cells or human peripheral monocytes treated with NEFA for 2 days were washed with DPBS containing 1% BSA and 0.01% sodium azide. Fc receptors were blocked by incubation of the monocytes in DPBS containing 2% human serum for 30 minutes at room temperature. Phycoerythrin-conjugated mouse anti-human CD11b monoclonal antibody or nonspecific mouse IgG (PD Pharmingen, San Diego, Calif) was added to the cell suspensions and incubated at room temperature for 30 minutes. Cells were then washed and fixed with 1% of paraformaldehyde in DPBS. Flow cytometry was performed on a Becton Dickinson FACS Cytometer.

Statistical Analysis
All data are represented as mean ± SE. The means and standard error were determined from ≥3 experiments for each data point. ANOVA and unpaired t test were used for comparisons between groups. A value of P<0.05 was considered significant.

Results
Effects of NEFA on Monocyte Adhesion
We first tested the effects of increasing incubation time (0 to 72 hours) of THP-1 cells with 200 μmol/L of the NEFA mixture on subsequent monocyte adhesion to endothelial cells. Monocyte adhesion increased at 24 hours and reached a maximum at 48 hours (Figure 1A). Lipopolysaccharide (LPS) in the experimental medium containing 200 μmol/L of NEFA was below the limit of detection of LPS (5 pg/mL), indicating that LPS contamination was not a cause of increased monocyte adhesion. Experiments conducted with the addition of polymyxin B, an antibiotic that binds to the lipid A portion of LPS and inhibits LPS activity, yielded similar results and confirmed this conclusion. This effect of NEFA was concentration-related, with increasing amounts of the NEFA mixture leading to increased monocyte adhesion (Figure 1B). Cell toxicity, as assessed by trypan blue staining was minimal (and similar to vehicle only cells) at these NEFA concentrations, but began to induce cell toxicity at higher levels (data not shown). Subsequent experiments with THP-1 cells were therefore performed with 200 μmol/L of combined NEFA mixture. We also used human peripheral monocytes to validate the physiological relevance of the experiments. Treatment of these monocytes with 200 μmol/L of NEFA for as little as 16
hours also increased monocyte adhesion to endothelial cells nearly 2.5-fold over monocytes treated with vehicle only (P<0.05). Interestingly, at a NEFA:BSA molar ratio of 2:1, human monocytes demonstrated no toxicity over this time frame at even greater higher concentrations of NEFA. Moreover, there was a clear graded increase in monocyte adhesion with higher concentrations of NEFA. At 400 μmol/L of NEFA, there was a nearly 4-fold increase in monocyte adhesion (Figure 1B, inset).

Role of Oxidative Stress and Monocyte Adhesion
Because NEFA have been demonstrated to induce generation of ROS in some cell types, we assessed whether this process may mediate induction of monocyte adhesion. As shown in Figure 2A, NEFA stimulation of ROS formation increased up to 48 hours and remained modestly increased at 72 hours. Increasing concentrations of NEFA generated a progressive increase in ROS formation, reaching a maximum at 200 μmol/L. Thus, the overall pattern of ROS generation (time, concentration dependency, and level of increase) in response to NEFA mixture was very similar to that seen for monocyte adhesion.

To further evaluate the role of NEFA-induced production of oxidative stress in enhanced monocyte adherence, we tested the effects of several antioxidants on production of ROS and stimulation of monocyte adherence. As demonstrated in the Table, both glutathione and butylated hydroxytoluene (BHT) reduced ROS production by nearly 50%,

### Table: Effects of Antioxidants and Inhibitors of NADPH Oxidase and Protein Kinase C on NEFA-Stimulated Monocyte Adhesion to Endothelial Cells and Generation of ROS

<table>
<thead>
<tr>
<th>Pharmacological Agent</th>
<th>Concentration</th>
<th>Percent of NEFA-Induced Adhesion</th>
<th>Percent of NEFA-Induced ROS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
<td>10 mmol/L</td>
<td>52±5*</td>
<td>40±10*</td>
</tr>
<tr>
<td>BHT</td>
<td>20 μmol/L</td>
<td>43±*</td>
<td>55±4*</td>
</tr>
<tr>
<td>Diethyl maleate</td>
<td>0.125 mmol/L</td>
<td>234±*</td>
<td>205±24†</td>
</tr>
<tr>
<td>DPI</td>
<td>1 μmol/L</td>
<td>37±6*</td>
<td>51±3*</td>
</tr>
<tr>
<td>Apocynin</td>
<td>30 μmol/L</td>
<td>58±10*</td>
<td>31±9*</td>
</tr>
<tr>
<td>Calphostin C</td>
<td>0.25 μmol/L</td>
<td>32±7†</td>
<td>18±7†</td>
</tr>
<tr>
<td>GF109203X</td>
<td>1 μmol/L</td>
<td>55±10†</td>
<td>28±1†</td>
</tr>
</tbody>
</table>

*P<0.05. Antioxidant or NADPH oxidase inhibitors treatment effect vs NEFA treatment alone by unpaired t test.
†P<0.05. Glutathione-depletion or protein kinase C inhibitors effect vs NEFA treatment alone by unpaired t test.

Mean of at least 3 independent experiments. Whereas the reagents glutathione, butylated hydroxytoluene (BHT), diethyl maleate, diphenyleneiodonium (DPI), and apocynin were present along with NEFA for the full 2 days of incubation, calphostin C and GF109203X were only added for the final 24 hours.

Figure 1. Effect of NEFA treatment on adhesion of THP-1 cells to endothelial cells. A, THP-1 cells were treated with 200 μmol/L NEFA in RPMI 1640 medium containing 10% FBS and 0.2% BSA for up to 72 hours, washed, and then added to endothelial cells for measurement of adhesion (see Methods). B, THP-1 cells were treated with 0, 50, 100, or 200 μmol/L NEFA in RPMI 1640 medium containing 10% FBS and 0.2% BSA for 48 hours. Insert, Concentration-dependent effects of 16 hours of NEFA treatment on adhesion of human peripheral monocytes. Data are the mean of 3 independent experiments. *P<0.05, compared with vehicle-treated control, determined by ANOVA and subsequent Dunnett’s test.

Figure 2. Effect of NEFA treatment on generation of reactive oxygen species (ROS) by THP-1 cells. A, Measurement of ROS (see Methods) produced by THP-1 cell treated with 200 μmol/L of NEFA in RPMI 1640 medium containing 10% FBS and 0.2% BSA for 4, 24, 48, and 72 hours. B, THP-1 cells were treated with 0, 50, 100, or 200 μmol/L NEFA in RPMI 1640 medium containing 10% FBS and 0.2% BSA for 48 hours. Data are mean of 3 independent experiments. *P<0.05, compared with vehicle-treated control, determined by ANOVA and subsequent Dunnett’s test.
approaching levels present in unstimulated cells. Concurrent with this effect on ROS, both agents reduced monocyte adherence to near control levels. In contrast, when THP-1 cells were treated with diethyl maleate, an agent known to reduce intracellular concentrations of glutathione, in combination with NEFA, both ROS and monocyte adherence were increased beyond that seen with NEFA addition alone. We next assessed potential sources of increased ROS production in NEFA-treated cells by use of selective inhibitors of ROS. Whereas oxypurinol (xanthine oxidase inhibitor) and L-N- monomethyl arginine (nitric oxide inhibitor) and inhibitors of the mitochondrial electron transport complex II did not effectively reduce NEFA-stimulated ROS and monocyte adhesion (data not shown), 2 structurally distinct inhibitors of NADPH oxidase, DPI and apocynin, significantly reduced both of these processes (Table). Because it has been recognized that an important stimulator of NADPH oxidase and ROS generation in inflammatory cells is PKC, 2 different general inhibitors of PKC, calphostin C and GF109203 X, were tested and effectively reduced NEFA-stimulated ROS and monocyte adhesion (Table).

**Effects of NEFA on β2 Integrin Expression, Activation, and Function**

Because β1 (VLA4) and β2 (CD11a, b and c) integrins expression on the surface of monocytes are critical determinants of monocyte adherence, we explored the effects of NEFA treatment on synthesis and expression of these molecules. We first compared CD11a, 11b, and 11c and VLA4 mRNA levels by real-time PCR before and after exposure to the 200 μmol/L NEFA mixture for 2 days. Whereas mRNA levels for CD11a, CD11c, and VLA4 did not change, levels of CD11b increased by ≈2.5-fold (Figure 3A). This increase in CD11b mRNA in response to incubation with increased levels of NEFA could be inhibited by the cotreatment of cells with antioxidants, such as glutathione or inhibitors of NADPH oxidase (DPI and apocynin). The expression level of CD11b mRNA was even higher for human peripheral monocytes after exposure to the 400 μmol/L NEFA mixture for 16 hours (5.6±1.1-fold compared with vehicle-treated cells, P<0.05).

Surface expression of CD11b in THP-1 cells was also increased after exposure to NEFA as assessed by flow cytometry (Figure 3B). There was ≈67% increase in the fraction of THP-1 cells expressing CD11b (P<0.05) after NEFA treatment for 2 days compared with vehicle-only–treated cells. Similarly, there was a 31% increase in the proportion of human peripheral monocytes expressing CD11b and a 39% increase of mean fluorescence of CD11b (P<0.05) after only 16 hours treatment with 400 μmol/L NEFA compared with vehicle-only–treated cells.

**Studies to Demonstrate the Importance of Enhanced β2 Expression to NEFA Induced Monocyte Adhesion**

In preliminary experiments, we demonstrated that monocytes incubated with the NEFA mixture for 2 days bound in increased numbers to ICAM-1–coated plates compared with vehicle-only–treated cells, consistent with the notion that β2 integrins, a counter-ligand for ICAM-1, may be important for the enhanced monocyte binding to endothelial cells. To further demonstrate a functional role of β2 integrin expression in NEFA-induced adherence of monocytes, we tested whether adherence of NEFA-stimulated THP-1 cells to endothelial cells could be blocked by neutralizing antibodies to the common beta chain (CD 18) of the β2 integrin complex or to the CD11b protein. As shown in Figure 4, incubation with either of these blocking antibodies, but not with a nonspecific control (IgG) antibody, reduced NEFA-induced monocyte adhesion to endothelial cells (Figure 4A). Similar results were observed with experiments using human peripheral monocytes (Figure 4B). Consistent with these observations, we also demonstrated that a blocking antibody to ICAM-1 reduced NEFA-stimulated THP-1 cell binding to endothelial cells by >50% (data not shown). In contrast to β2
Elevated levels of glucose and triglycerides have been suggested as potential mediators of these events. However, there is increasing evidence that elevated levels of NEFA may have numerous proinflammatory effects on vascular cells in subjects with insulin resistance and diabetes. The goals of this study were to test whether elevated levels of NEFA could contribute to the enhanced adhesion of monocytes to endothelial cells and to ascertain the mechanism by which NEFA may achieve this effect.

We first demonstrated that exposure of monocytes to a physiological mixture of NEFA for 48 hours led to maximum monocyte adhesion; adhesion increased in a concentration-related fashion. This is the first report to our knowledge of increased monocyte adhesion resulting from prolonged exposure to a physiological mixture of fatty acids. Although NEFA-treated monocytes showed increased adhesion to unstimulated endothelial cells, pretreatment of endothelial cells with LPS greatly enhanced monocyte binding as has been previously reported. This indicates that one consequence of prolonged exposure of monocytes to NEFA may be to prime these cells to bind to activated endothelial cells. This may be particularly relevant for the development of atherosclerosis where monocyte accumulation is enhanced at sites of vascular inflammation, where upregulation of a variety of adhesion molecules occurs. Monocyte firm adhesion usually requires interaction of integrins such as the β2 integrin Mac-1 (CD11/CD18) with ICAM-1 on the surface of endothelial cells. Our studies indicate that NEFA stimulates the expression of both message and protein for CD11b (Figure 4), and this contributes to the increased binding of monocytes to LPS-stimulated endothelial cells. This is supported by fact that blocking antibodies to CD11b or CD18 as well as to ICAM-1 inhibited monocyte binding to endothelial cells. Although the β1 integrin (VLA4) did not appear important in NEFA-induced monocyte adhesion to endothelial cells, we cannot exclude the possibility that this important integrin may also play a role under flow conditions.

Our studies also demonstrate that NEFA-induced generation of ROS may mediate monocyte adhesion to endothelial cells. This was demonstrated by several lines of evidence. First, maximum stimulation of ROS by NEFA occurred after the same duration of exposure and at the same concentration as that of monocyte adhesion (Figure 1). Second, addition of glutathione or BHT, 2 structurally different antioxidants, prevented both production of monocyte ROS and monocyte adhesion (Table). Moreover, depletion of GSH with diethyl maleate before addition of NEFA further increased ROS generation and monocyte adhesion. Third, inhibitors of NADPH oxidase (a major producer of ROS in monocytes), but not those of nitric oxide synthase, xanthine oxidase or the mitochondrial electron transport pathway were shown to be effective inhibitors of monocyte adhesion. These latter experiments also demonstrate that NADPH oxidase appears to be an important and specific source of NEFA induced ROS in monocytes. Our results are consistent with those of previous studies that have indicated that inhibitors of NADPH oxidase, but not various mitochondrial complex inhibitors, inhibit ROS release from THP-1 cells induced by high glucose conditions, and that inhibitors of PKC, a recognized stimulator of NADPH oxidase, also reduced ROS generation and

Discussion
Monocytes from subjects with diabetes have been demonstrated to bind to endothelial cells in greater numbers than monocytes isolated from subjects without diabetes. Both elevated levels of glucose and triglycerides have been sug-
monocyte adherence.18 Finally, the antioxidant GSH and several inhibitors of NADPH oxidase also reduced the mRNA levels of CD11b, thus providing an explanation for the observed reduced monocyte adhesion to endothelial cells under these conditions.

Several other points deserve mention. The concentrations of NEFA used in this study are within physiological levels, suggesting that these in vitro studies may have relevance for in vivo function of monocytes. Consistent with this possibility, the NEFA effects on monocytes were not abolished in the presence of serum. As with THP-1 cells, NEFA stimulation of human peripheral monocytes was concentration-dependent, with adhesion to endothelial cells increasing directly in proportion to the NEFA concentration. These NEFA effects on human peripheral monocytes occurred more rapidly, possibly indicating that some maturation of THP-1 cells was first needed for NEFA-induced THP-1 cell adhesion to endothelial cells.

Although levels of NEFA are increased in individuals with diabetes, similar degrees of elevation are frequently present in individuals with insulin resistance and might be expected to increase adhesiveness of monocytes in individuals with insulin resistance as well as in those with diabetes. Consistent with this notion, insulin resistance, as measured by a direct measure of insulin mediated glucose uptake, was a significant predictor of monocyte adhesion to endothelial cells.19 Although adipose tissue is a major source of serum NEFA, triglyceride-rich lipoproteins may also provide free fatty acids directly to the artery wall. Insulin resistance and type 2 diabetes are associated with increased levels of just such lipoproteins, resulting primarily from increased hepatic secretion of triglyceride-rich very-low-density lipoprotein and exaggerated postprandial hyperlipidemia.20 Of relevance, we have found that THP-1 cells incubated with lipoprotein lipase-treated very-low-density lipoprotein, but not native very-low-density lipoprotein, exhibited a 2-fold increase in both ROS production and adhesion to endothelial cells (unpublished data).

In summary, these studies demonstrate that elevated levels of NEFA, as frequently occurs in conditions of obesity, insulin resistance and type 2 diabetes, may contribute to increased monocyte expression of CD11b and enhance their adhesion to activated endothelial cells. These data provide another example of elevated levels of free fatty acids inducing inflammation and support the concept that modalities that will diminish levels of NEFA or inhibit their intracellular signaling may contribute to reduced atherogenesis in these individuals.

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References
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