Circulating Mononuclear Cells in the Obese Are in a Proinflammatory State

Husam Ghanim, PhD; Ahmad Aljada, PhD; Deborah Hofmeyer, BSc; Tufail Syed, MD; Priya Mohanty, MD; Paresh Dandona, MD, PhD

Background—In view of the increase in plasma concentrations of proinflammatory mediators tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) in obesity, we investigated whether peripheral blood mononuclear cells (MNC) from obese subjects are in a proinflammatory state.

Methods and Results—MNC were prepared from fasting blood samples of obese (n=16; body mass index [BMI]=37.7±5.0 kg/m²) and normal-weight control (n=16; BMI=23.8±1.9 kg/m²) subjects. Nuclear factor κB (NF-κB) binding to DNA in nuclear extracts was elevated (P<0.05) and the inhibitor of NFκB-β (IκB-β) was significantly lower (P<0.001) in the obese group. Reverse transcription–polymerase chain reaction revealed elevated levels of migration inhibitor factor (MIF), IL-6, TNF-α, and matrix metalloproteinase-9 (MMP-9) mRNA expression in the obese subjects (P<0.05). Plasma concentrations of MIF, IL-6, TNF-α, MMP-9, and CRP were also significantly higher. Plasma glucose, insulin, and free fatty acids (FFAs) were measured, and homeostasis model assessment of insulin resistance (HOMA-IR) was calculated. Plasma FFA concentration related significantly to BMI, IL-6, and TNF-α mRNA expression and plasma CRP levels but not to HOMA-IR. On the other hand, the inflammatory mediators were significantly related to BMI and HOMA-IR.

Conclusions—These data show (1) for the first time that MNC in obesity are in a proinflammatory state with an increase in oxidative stress and inflammatory mediators; (2) that plasma FFAs are a modulator of inflammation; and (3) that insulin resistance is a function of inflammatory mediators. (Circulation. 2004;110:1564-1571.)

Key Words: obesity ■ inflammation ■ monocytes

It has been shown previously that obesity is associated with an increase in oxidative stress and with an elevation in the tissue expression and plasma concentrations of the proinflammatory cytokine tumor necrosis factor-α (TNF-α). An elevation of interleukin-6 (IL-6) and other proinflammatory mediators and markers such as C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1), an antifibrinolytic, has also been demonstrated in the obese. Proinflammatory mediators like TNF-α have also been shown to be increased in animal models of obesity in both adipose tissue and plasma. Macronutrient intake has been shown to induce oxidative stress and proinflammatory changes in plasma and peripheral blood mononuclear cells (MNC) of normal subjects. These changes may last 3 to 4 hours after macronutrient intake. It is thus possible that obesity is a pro-oxidative and proinflammatory state resulting from chronically increased macronutrient intake. It is also relevant that caloric restriction in the obese and fasting in normal subjects result in a reduction in oxidative stress and inflammatory mediators. Although there is an increase in the expression of proinflammatory cytokines in the adipose tissue of the obese, there has hitherto been no information on the state of inflammatory changes in MNC in the obese. This is especially important because MNC are known to migrate to the arterial wall to form foam cells in atherosclerotic lesions and into adipose tissue to activate adipocytes into producing proinflammatory cytokines like TNF-α and IL-6.

Clearly, therefore, we need to determine the status of MNC in relation to inflammatory changes. Inflammation at the cellular level can be described as an increase in the proinflammatory transcription factor nuclear factor κB (NF-κB) in the nucleus and with a concomitant decrease in its inhibitors IκB-α and/or IκB-β. In view of the association of inflammation and oxidative stress in obesity, we embarked on a study of (1) NF-κB binding in the nucleus, as a measure of NF-κB activity as a proinflammatory transcription factor; (2) p65 and p50 expression as an indication of the quantity of intranuclear NF-κB; (3) IκB and its isoforms as markers of NF-κB inhibition; and (4) expression level of some of the proinflammatory genes that are regulated by NF-κB as an indication of increased transcriptional activity as well as binding to dem-
onstrate the inflammatory status of MNC. The plasma concentration of these factors was also measured. In addition, we examined p47phox, a key protein of NADPH oxidase, the enzyme responsible for the formation of superoxide radical from molecular O2. p47phox serves as an index of NADPH oxidase expression and has been shown to increase after oxidative expression and has been shown to increase after a fasting.1,13,14,19

The hypothesis for this study was that obesity is associated with (1) an increase in NF-κB binding and p65 (Rel-A) expression in the nucleus because Rel-A is the activator of transcription; (2) a fall in IκB in the cytosol; (3) an increase in proinflammatory gene expression in MNC from the obese compared with controls; and (4) a parallel increase in plasma concentration of proinflammatory cytokines and mediators.

Methods

Subjects

Two groups (n=16 each) of normal-weight controls (body mass index [BMI]=23.8±1.9 kg/m2) and healthy nondiabetic obese (BMI=37.7±5.0 kg/m2) female subjects were recruited for this study. The human research internal review board of the State University of New York at Buffalo approved the study protocol. Written informed consent was obtained from all subjects. All subjects had normal fasting glucose levels and were on no medication known to influence the inflammatory pathway. The subjects’ demographic data are summarized in Table 1.

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<tr>
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<th>Lean Subjects</th>
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<tr>
<td>n</td>
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<td>16</td>
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<tr>
<td>BMI</td>
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<td>43.2±8.6</td>
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<tr>
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<tr>
<td>Diastolic BP, mm Hg</td>
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<td>Glucose, mg/dL</td>
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<td>83.7±8.2</td>
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<td>Insulin, mU/mL</td>
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<td>16.73±6.34*</td>
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<td>HOMA-IR</td>
<td>0.74±0.5</td>
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<td>FFA, mmol/L</td>
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<td>Cholesterol, mg/dL</td>
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<td>Triglycerides, mg/mL</td>
<td>62.4±33.0</td>
<td>121.3±63.7*</td>
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</table>

Values are mean±SD.

*P<0.05.

TABLE 1. Demographic Data of Subjects

Plasma Insulin, Glucose, and Free Fatty Acid Measurements and Homeostasis Model Assessment of Insulin Resistance Calculation

Insulin was measured from fasting plasma samples with the use of an enzyme-linked immunoassay kit (Diagnostics Systems Laboratories, Inc). Glucose was measured in whole blood by YSI glucose analyzer. Free fatty acid (FFA) concentration was measured with the Half-Micro Calorimetric Kit from Roche Diagnostic. Homeostasis model assessment of insulin resistance (HOMA-IR), an index of insulin resistance, was calculated according to Matthews et al21 with the use of the following

\[
\text{HOMA-IR} = \frac{\text{Fasting Insulin Concentration (\mu U/ml)}}{22.5} \times \text{Fasting Glucose Concentration (mmol/L)}
\]

MNC Isolation

Fasting blood samples were collected in Na-EDTA as an anticoagulant from all subjects. Four milliliters of blood sample was layered carefully over 3.5 mL of PMN medium (Robbins Scientific Corp). The upper layer containing the MNC was harvested and washed with Hank’s balanced salt solution and then with PBS.

NF-κB DNA Binding Activity

Nuclear NF-κB DNA binding activity was measured by electrophoretic mobility shift assay (EMSA) as described previously.22 The specificity of the bands was confirmed by supershifting these bands with specific antibodies against Rel-A (p65) and p50 (Santa Cruz Biotecnology) and by competition with cold oligonucleotides (Figure 1A).

Western Blotting

MNC total cell lysates were prepared as described previously.22 Polyclonal or monoclonal antibodies against p47phox, Rel-A subunit (Transduction Labs), p50 subunit (SantaCruz Biotechnology), IκB-α (Upstate Biotechnology), IκB-β (Rockland), and actin (SantaCruz Biotechnology) were used. Densitometry was performed with the use of Bio-Rad molecular analyst software, and all values were corrected for loading with actin.

Total RNA Isolation and Real-Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method. One microgram of total RNA was reverse transcribed with the use of Advantage RT-for-PCR Kit (Clontech). Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed with the use of Cepheid Smart Cycler, in which 2 μL cDNA, 10 μL Sybergreen Master mix (Qiagen), and 0.5 μL of 20 μmol/L gene-specific primers (Life Technologies) (Table 2) were used. The specificity and the size of the PCR products were tested by adding a melt curve at the end of the amplifications and by running it on a 2% agarose gel. All values were normalized to 18S expression.

Plasma Concentrations of Proinflammatory Mediators

Concentrations of migration inhibitor factor (MIF), IL-6, TNF-α, and matrix metalloproteinase-9 (MMP-9) (total) were measured in plasma with ELISA kits (R&D Systems). CRP plasma level was measured with the use of ELISA kit from Alpha Diagnostic International.

Statistical Analysis

Statistical analysis was performed with the use of SigmaStat software (Jandel Scientific). All data are expressed as mean±SE of arbitrary units, and percent change is calculated from the means. Statistical analysis was performed with the use of unpaired t test. Correlation analysis was performed with Spearman rank order correlation. The sample size was 26, which represents the number of samples from both obese and normal-weight subjects that were analyzed for all parameters.

Results

Plasma Glucose, Insulin, and FFA Concentrations and HOMA-IR

Mean plasma glucose concentrations were similar in both groups. Insulin concentration was significantly higher in the obese group (P<0.01). HOMA-IR index was 0.74±0.5 in the lean group and 3.15±2.0 in the obese subjects. FFA concentration was significantly higher (P<0.05) in obese subjects compared with lean subjects (Table 1).
NF-κB DNA Binding Activity
The DNA binding of the transcriptionally active NF-κB form (p65/p50) was significantly higher by 28.3% (P<0.05) in MNC of obese subjects compared with lean subjects (Figure 1B, 1C).

Rel-A, p50, IκB-α, and IκB-β Protein Levels
Expression of the Rel-A (p65) subunit of the NF-κB complex was higher by 37% in the nuclear homogenates of the obese group compared with the control group (Figure 2A, 2B), but this difference was not statistically significant. There was no difference in Rel-A quantity in the total cell lysate between the 2 groups (data not shown). Expression of the other component of the transcriptionally active NF-κB complex, the p50 subunit, was also similar in the nuclear extracts of the 2 groups. The inhibitory subunits of NF-κB were measured in the total cell homogenate and showed a moderate but not significant increase in IκB-α but a significant decrease in IκB-β by 48% (P<0.001) in the obese group (Figure 2C, 2D).

RNA Expression of NF-κB and IκB Subunits
The obese subjects had similar expression levels of Rel-A and IκB-β compared with the control subjects (Figure 3). However, the mRNA expression level of the p105 subunit, which represents the precursor of p50, was higher by 100% (P=0.012) and IκB-α mRNA levels were higher by 76% (P=0.01) in the obese group (Figure 3).

NADPH Oxidase p47phox Protein Content
As measured in the total cell lysate by Western blotting, there was no difference in basal p47phox protein quantity between the 2 groups (data not shown).

NF-κB–Regulated Gene Expression Levels
NF-κB regulates the expression of many proinflammatory genes, including cytokines, chemokines, adhesion molecules, and others. Real-time RT-PCR revealed elevated levels of mRNA of NF-κB–regulated proinflammatory genes in the obese group. MIF mRNA expression was higher by 60% (P=0.031), IL-6 by 129% (P=0.002), TNF-α by 72% (P=0.04), and MMP-9 by 91% in the obese group (Figure 4).

Plasma Concentrations of Proinflammatory Mediators
Circulating levels of MIF were significantly higher by 158% (3.337±2.432 versus 1.295±0.880 ng/mL; P<0.001), IL-6 by 124% (3.308±2.381 versus 1.47±1.124 pg/mL; P<0.001), TNF-α by 31% (3.613±1.804 versus 2.729±0.942 pg/mL; P=0.014), MMP-9 by 78% (469.5±194.7 versus 263.9±179.6 ng/mL; P<0.001), and CRP by 304% (3012±770 versus 745±613 ng/mL; P<0.001) in obese subjects compared with normal subjects (Figure 5).

Correlation Analysis
BMI correlated significantly with HOMA-IR, NF-κB binding, plasma IL-6, FFA, and CRP levels and with TNF-α, IL-6, and MIF mRNA expression. HOMA-IR was also significantly related to NF-κB binding activity, plasma MIF, and CRP levels and with IL-6 mRNA expression. There was a strong trend toward correlation between HOMA-IR and plasma levels of IL-6 (P=0.055). There were significant correlations between NF-κB binding and plasma CRP levels, IL-6 mRNA, and MIF mRNA expression. There were significant correlations between FFA concentrations and BMI, IL-6, and plasma CRP levels. All correlation coefficients (r) and probability values for these correlations are summarized in Table 3.
Discussion

Our data show clearly that obesity is associated with an increase in NF-κB binding in the nucleus and a decrease in the inhibitory IκB in the MNC, consistent with the proinflammatory state of obesity. The significance of an increase in intranuclear NF-κB binding is consistent with the increase in the transcription of proinflammatory genes that are activated by NF-κB and for which there are NF-κB binding sites on their respective promoters.23–25 It is of interest that NF-κB binding was significantly correlated to BMI and HOMA-IR, an index of insulin resistance. The increased transcriptional activity of TNF-α, IL-6, MIF, and MMP-9 genes, as reflected in increased mRNA levels, is consistent with increased intranuclear NF-κB binding and also with the known proinflammatory state of obesity. Reinforcing this concept was the significant correlation between NF-κB binding and mRNA levels for IL-6 and MIF. Increased mRNA levels of proinflammatory cytokines were associated with increased plasma concentration of the cytokines MMP-9 and CRP, although mRNA and plasma levels of these cytokines were not significantly related. There are significant correlations between many inflammatory mediators and HOMA-IR, supporting a possible role of these mediators in the development of insulin resistance.

As expected, plasma FFA concentrations were elevated significantly in obese subjects. FFA concentration was related to BMI but not to HOMA-IR or NF-κB binding. However, it was related to TNF-α and IL-6 mRNA. The absence of a significant correlation between FFA level and HOMA-IR and the presence of a significant correlation with BMI suggest that obesity contributes to increased FFAs but that FFAs may not be directly related to insulin resistance. The significant correlation of NF-κB binding and proinflammatory cytokines with HOMA-IR suggests that these mediators are in fact more directly related to insulin resistance. We have recently shown that the intravenous infusion of triglycerides and heparin into normal subjects to elevate FFA concentration to a level comparable to that observed in obese subjects leads to an

### Table 2. List of Primers Used in Real-Time RT-PCR

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<tr>
<th>Gene</th>
<th>Sense Primers (5’ → 3’)</th>
<th>Anti-Sense Primers (5’ → 3’)</th>
<th>Fragment Length, bp</th>
<th>Annealing Temperature, °C</th>
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<td>Rel-A</td>
<td>CCCCCCTGCTCTGATGGTCAGCT</td>
<td>CTCATCACGCTGTTAGGACGACAGG</td>
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<tr>
<td>p105</td>
<td>CCAACCGCTTGTTAGGACGACAGG</td>
<td>TCTACACGCTCTGTTAGGACGACAGG</td>
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<td>64</td>
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<tr>
<td>IκB-α</td>
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<tr>
<td>IκB-β</td>
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<td>MMP-9</td>
<td>CCAATCTGACAGATGTTAGGG</td>
<td>GGGAGGCTTTAATACACAGGTTAGG</td>
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![Figure 2](http://circ.ahajournals.org/)

Figure 2. Protein quantification of NF-κB subunits and inhibitors by Western blotting. A, Protein content of Rel-A and p50 from nuclear extracts. B, IκB-α and IκB-β from total cell homogenates of 3 lean (L) and obese (O) subjects. C and D, Densitometry of protein content corrected for loading with actin (n=16 vs 16). Rel-A expression in the obese showed a statistical trend toward an increase (P<0.1). *P<0.05.
acute inflammatory and oxidative stress response. Thus, FFAs induce an increase in generation of reactive oxygen species by leukocytes, an increase in intranuclear NF-κB binding, p65 (Rel-A) expression, plasma concentrations of MIF, and abnormalities in vascular reactivity similar to those observed in the obese. It is therefore possible that FFAs exert a proinflammatory effect that in turn leads to interference with insulin signaling. 26

This proinflammatory state may contribute to insulin resistance because TNF-α and possibly other proinflammatory cytokines may interfere with insulin action. TNF-α is known to cause a decrease in insulin receptor tyrosine phosphorylation and an increase in serine phosphorylation of insulin receptor substrate-1, which in turn causes serine phosphorylation of insulin receptor and blocks tyrosine phosphorylation. 27 In endothelial cells, TNF-α causes a reduction in the expression of the insulin receptor itself, in addition to causing a reduction in tyrosine phosphorylation of the insulin receptor. 28 IL-6, on the other hand, induces suppressor of cytokine signaling-3 (SOCS-3), which interferes with insulin signaling at the insulin receptor substrate-1 level. 29 Thus, the inflammatory mediators may contribute to insulin resistance.

The paradoxical increase in IκB-α expression is probably due to the fact that NF-κB binds to the IκB-α gene promoter to activate its transcription, and thus IκB-α expression is enhanced. 30–33 IκB-α is a rapid modulator of NF-κB activity, and thus the cycles of IκB-α induction and the binding of the latter to NF-κB to reduce its transcriptional activity, which is in turn followed by a reduction in IκB-α expression, may result in rapid cycles of inhibition and stimulation of NF-κB transcriptional activity. 24,34–36 IκB-β, which was significantly lower in the obese group, is not regulated by NF-κB, 34,37 and its degradation follows a slower but more persistent pattern compared with that of IκB-α. The reduction in IκB-β probably leads to prolonged activation of NF-κB. Furthermore, stimuli that activate NF-κB may favor the targeting of one inhibitory IκB protein over the other. For example, treatment of cells, such as peritoneal macrophages and 70Z/3 pre-B cells, with lipopolysaccharide or IL-1 leads to the rapid loss followed by the return of IκB-α levels over a 2-hour period. In contrast, after such stimulation, IκB-β levels slowly decrease over 2 hours and remain greatly reduced for >24 hours. 23,38,39 Therefore, it is possible that in a chronic low-grade proinflammatory condition, such as obesity, the degradation of IκB-β might play a major role in NF-κB activation.

Although both IκB-α and IκB-β bind to NF-κB, IκB-β can restrict NF-κB to the cytoplasm more efficiently than IκB-α. IκB-β derives its high affinity toward NF-κB dimers by binding to the nuclear localization signals (NLS) of both of the NF-κB subunits, whereas IκB-α binds to only one NF-κB NLS. The presence of one free NLS in the NF-κB/IκB-α complex allows it to shuttle between the nucleus and the cytoplasm, whereas NF-κB/IκB-β complexes are localized to the cytoplasm of resting cells. 40 It is also known that newly synthesized IκB-α can affect NF-κB binding to κB sites in the nucleus and induce the transport of p65/p50 complexes into the cytoplasm. 31,41 Clearly, this function of IκB-α in obese subjects is either defective or not sufficient to remove p65/p50 from the nucleus. Other factors may also prolong the duration of NF-κB binding, such as increased activity of coactivators such as CREB binding protein (CBP) or decreased deacetylation activity of histone deacetylase 3 (HDAC3), which affect the duration of NF-κB binding in the nucleus. 42 These aspects need further investigation.
The higher expression of the p105 mRNA, the precursor of the p50 subunit, in the obese group is probably due to the fact that the promoter of the p105 gene, like the IκB-α promoter, is actively regulated by NF-κB transcriptional activity as part of its autoregulatory mechanism. It has been demonstrated that the non-transcriptionally active p50/p50 homodimer can compete with the p65/p50 heterodimer for the same binding sites and thus function as an inhibitor of the p65/p50 complex. Therefore, in lean normal subjects, the activated NF-κB terminates its own activity by producing more IκB-α.

**Figure 5.** Plasma concentrations of proinflammatory markers TNF-α, IL-6, MIF, MMP-9, and CRP measured by ELISA in normal and obese groups. *P<0.05.

**Table 3.** Correlation Analysis Results

<table>
<thead>
<tr>
<th></th>
<th>BMI</th>
<th>HOMA-IR</th>
<th>NF-κB EMSA</th>
<th>TNF-α mRNA</th>
<th>IL-6 mRNA</th>
<th>MMP-9 mRNA</th>
<th>MIF mRNA</th>
<th>TNF-α Plasma</th>
<th>IL-6 Plasma</th>
<th>MIF Plasma</th>
<th>CRP Plasma</th>
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Spearman rank order correlation was performed on the combined data from both groups. *P<0.05.
and p50.43,44 In obese subjects, this increase in p105 mRNA was not accompanied by an increase in the p50 protein level in the nucleus. This can be due either to slower translation or processing rate of the p105 to produce the p50 protein or to decreased translocation to the nucleus under the influence of continued stimulation by NF-κB inducers.

Although it is accepted that adipocytes secrete proinflammatory cytokines (adipokines) that probably contribute to increased concentrations of TNF-α, IL-6, and PAI-1 in plasma in states of obesity, the role of the circulating MNC has hitherto not been clear. We have previously shown that MNC respond to macronutrient challenge in normal subjects and the obese by increase in reactive oxygen species generation and NF-κB increase in nuclear fraction. Our present data now provide the first evidence establishing a basal proinflammatory state in MNC in obesity. Our work also raises the possibility that MNC may contribute to the increased concentrations of proinflammatory cytokines in the obese. In addition, there is recent evidence that monocytes/macrophages may also regulate the proinflammatory activity of adipose tissue itself in obesity20,45 because these cells are found in increased numbers in the adipose tissue of obese subjects. At that site they produce proinflammatory cytokines, which in turn activate adipocytes through paracrine activity.

Although fasting insulin concentrations are elevated in the obese, it is not likely that they contribute to the proinflammatory state of MNC. Insulin has been shown to be antiobese, it is not likely that they contribute to the proinflammatory state of MNC. Insulin has been shown to be antiobese, it is not likely that they contribute to the proinflammatory activity of adipose tissue itself in obesity20,45 because these cells are found in increased numbers in the adipose tissue of obese subjects. At that site they produce proinflammatory cytokines, which in turn activate adipocytes through paracrine activity.

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