Elevation of Free Fatty Acids Induces Inflammation and Impairs Vascular Reactivity in Healthy Subjects

Devjit Tripathy, Priya Mohanty, Sandeep Dhindsa, Tufail Syed, Husam Ghanim, Ahmad Aljada, and Paresh Dandona

To test the possible acute proinflammatory effects of fatty acids, we induced an increase in plasma free fatty acid (FFA) concentrations after a lipid and heparin infusion for 4 h in 10 healthy subjects. We determined the nuclear factor-kB (NF-kB) binding activity in mononuclear cells (MNCs), the p65 subunit of NF-kB, reactive oxygen species (ROS) generation by MNC, and polymorphonuclear leukocytes (PMN). Brachial artery reactivity, using postsischemic flow-mediated dilation, was also measured. NF-kB binding activity in the MNC nuclear extracts increased to 163 ± 17% and 144 ± 14% as compared with basal levels at 2 and 4 h (P < 0.005) and remained elevated (P < 0.05) at 6 h (2 h after cessation of lipid infusion). NF-kB p65 subunit protein expression in MNC homogenates also increased at 2, 4, and 6 h (P < 0.05). ROS generation by PMNs increased significantly at 2 and 4 h (P < 0.005), whereas that by MNCs increased at 4 h (P < 0.05). Plasma macrophage migration inhibition factor increased at 2 (P < 0.05) and 4 h (P < 0.005), respectively, and declined to baseline at 6 h. The postsischemic flow-mediated dilation of brachial artery decreased from 6.3 ± 1.1% at baseline to 4.3 ± 1.9% and 2.7 ± 2.1% (P < 0.01) at 2, 4, and 6 h, respectively. We conclude that an increase in FFA concentration induces oxidative stress and has a proinflammatory effect; it also impairs postsischemic flow-mediated vasodilation of the brachial artery. Diabetes 52:2882–2887, 2003

Studies in humans as well as rodents have consistently demonstrated that an experimental elevation in free fatty acid (FFA) concentrations in healthy subjects reduces the insulin-stimulated glucose uptake (1,2). In addition, elevated FFA concentrations lead to impairment in endothelium-dependent (3) and insulin-mediated vasodilation as a result of decrease in nitric oxide (NO) production (4). The mechanisms by which FFA induces insulin resistance, however, are not clear.

Recently, anti-inflammatory agents have been shown to prevent fat-induced insulin resistance in rodents, thereby suggesting the involvement of inflammatory pathways in the pathogenesis of fat-induced insulin resistance (5,6). Inflammation is activated by the proinflammatory transcription factor nuclear factor-kB (NF-kB), usually a heterodimer of p65 (Rel A) and p50 proteins. NF-kB is located in the cytosol, where it is bound to inhibitor kB (IkB). Inflammatory signals, including endotoxin and proinflammatory cytokines, cause phosphorylation and ubiquitination of IkB, thus liberating and activating NF-kB. This allows NF-kB to translocate to the nucleus and to activate transcription of genes that are involved in the inflammatory response, such as proinflammatory cytokines, adhesion molecules, and enzymes generating reactive oxygen species (ROS). We have previously shown that thiazolidinediones (troglitazone and rosiglitazone) exert a significant anti-inflammatory effect in obese subjects and patients with type 2 diabetes (7,8) while also reducing plasma FFA concentrations.

It has recently been shown that inflammatory mechanisms may mediate insulin resistance. Obesity is associated with an increase in the proinflammatory mediators such as tumor necrosis factor-α (TNF-α) in both animal models and humans (9,10). Plasma concentration of TNF-α as well as several indexes of oxidative stress are increased in the obese and fall with weight loss (11). Weight loss is known to restore insulin sensitivity. Thus, inflammation may contribute to insulin resistance. Indeed, it has recently been shown that the inhibition of IkB kinase-β (IKK-β) by the classical anti-inflammatory drug aspirin may significantly increase the sensitivity to insulin in the fat/fa Zucker rat (6). IKK-β is the kinase that phosphorylates IkB, thus allowing NF-kB to translocate into the nucleus and to induce expression of proinflammatory genes.

In the present study, we hypothesized that an increase in FFA concentration in normal, healthy subjects leads to 1) activation of proinflammatory NF-kB and an increase in ROS generation by leukocytes and 2) an increase in proinflammatory mediators in plasma, including the macrophage migration inhibitory factor (MIF).


diabetes @ 2003}

From the Division of Endocrinology, Diabetes and Metabolism, State University of New York and Kaleida Health, Buffalo, New York.

Address correspondence and reprint requests to Paresh Dandona, MD, PhD, Diabetes-Endocrinology Center of Western New York, 3 Gates Circle, Buffalo, NY 14209. E-mail: pdandona@kaleidahealth.org.

Received for publication 5 March 2003 and accepted in revised form 12 September 2003.

CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; ICAM, intracellular adhesion molecule; IkB, inhibitor kB; IKK-β, IkB kinase-β; MCP-1, monocyte chemotactant protein; MIF, macrophage migration inhibitory factor; MNC, mononuclear cell; NF-kB, nuclear factor-kB; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; sICAM, soluble ICAM; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-α.

© 2003 by the American Diabetes Association.

RESEARCH DESIGN AND METHODS

Participants. Ten healthy volunteers (eight men and two women; age, 28.2 ± 4.1 years; BMI, 24.8 ± 4.7 kg/m²) participated in the study (Table 1). All participants had normal glucose tolerance, were not on any medications, had normal blood pressure, and had normal fasting lipid profiles. All volunteers...
TABLE 1
Clinical characteristic of the subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male/female</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Pulse</th>
<th>BP (mmHg)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>HDL cholesterol (mg/dl)</th>
<th>LDL cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>8/2</td>
<td>28.2 ± 4.1</td>
<td>24.8 ± 4.7</td>
<td>78 ± 6</td>
<td>114 ± 13</td>
<td>156 ± 47.4</td>
<td>75.6 ± 16</td>
<td>39.7 ± 8.6</td>
<td>114 ± 30.2</td>
</tr>
</tbody>
</table>

Data are mean ± SD.

TABLE 2
Clinical characteristic of the subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male/female</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Pulse</th>
<th>BP (mmHg)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>HDL cholesterol (mg/dl)</th>
<th>LDL cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>8/2</td>
<td>28.2 ± 4.1</td>
<td>24.8 ± 4.7</td>
<td>78 ± 6</td>
<td>114 ± 13</td>
<td>156 ± 47.4</td>
<td>75.6 ± 16</td>
<td>39.7 ± 8.6</td>
<td>114 ± 30.2</td>
</tr>
</tbody>
</table>

Data are mean ± SD.

gave their written, informed consent, and the study protocol was approved by the Human Research Committee of the State University of New York at Buffalo.

Protocol. Participants came to the clinic research center at 8.00 a.m., after an overnight 12-h fast. A catheter was inserted into the antecubital vein. A baseline blood sample was obtained, and a triglyceride emulsion (Liposyn, 10%, Abbott Laboratories, North Chicago, IL) and heparin (0.2 units · kg⁻¹·h⁻¹; 1) were infused at a rate of 50 ml/h for 4 h. We used a lower concentration of Liposyn compared with most earlier studies because infusion of 20% Liposyn resulted in lipemic serum where separation of MNCs and PMNs was not possible. The triglyceride infusion was stopped after 4 h, and blood samples were collected at 0, 2, 4, and 6 h. Vascular reactivity was also measured at 0, 2, 4, and 6 h. On two different occasions, seven subjects participated in two other studies in which 0.9% normal physiologic saline or 5% dextrose was infused for 4 h and samples were collected at 0, 2, 4, and 6 h.

MNC and PMN isolation. Blood samples were collected in Na₂EDTA as an anticoagulant. A total of 3.5 ml of the anticoagulated blood sample was carefully layered over 3.5 ml of the PMN isolation medium (Robbins Scientific, Sunnyvale, CA). Samples were centrifuged at 450g in a swing-out rotor for 30 min at 22°C. At the end of the centrifugation, two bands separated out at the top of the red blood cell pellet. The top band consists of MNC, and the bottom band of PMN. The MNC band was harvested with a Pasteur pipette, repeatedly washed with Hanks’ balanced salt solution, and reconstituted to a concentration of 4 × 10⁶ cells/ml in Hanks’ balanced salt solution. This method provides yields >90% pure MNC suspension.

Measurement of ROS generation. Respiratory burst activity of PMNs and MNCs was measured by detection of superoxide radical via chemiluminescence (12). A total of 500 ml of PMNs or MNCs (2 × 10⁵ cells/ml) was measured by detection of superoxide radical via chemiluminescence. A total of 500 ml of PMNs or MNCs was measured by chemiluminescence. We have further established that in our assay system, there is a 78% inhibition of chemiluminescence by superoxide dismutase (assay is 6%). Half of the immunoprecipitated IKK-β complex was incubated in the kinase buffer with 3 μg of the substrate for 30 min at 37°C. The reaction was stopped by boiling the samples for 5 min in 2× SDS loading buffer. Samples were resolved by 10% SDS-PAGE and autoradiography. The remaining half of the immunoprecipitated samples were run on a separate 10% SDS-PAGE and transferred to the IKK-β antibody to check for equality of loading.

Plasma C-reactive protein, MIF, TNF-α, soluble intracellular adhesion molecule-1, thiobarbituric acid reactive substances, and monocyte chemoattractant protein-1 measurements. Plasma MIF, monocyte chemoattractant protein-1 (MCP-1), soluble intracellular adhesion molecule (s-ICAM-1), and TNF-α were assayed with enzyme-linked immunosorbent assay (ELISA) kits from R&D systems (Minneapolis, MN). C-reactive protein (CRP) ELISA kit was purchased from Diagnostic Systems Laboratories (Webster, TX). Thiobarbituric acid reactive substances (TBARS) were measured using the method described by Yagi et al. (16).

Assessment of brachial arterial reactivity. Brachial artery diameter was measured by an Acuson 128XP/10 high-resolution ultrasonograph with a 7.5-MHz linear array transducer as previously described (17). The forearm was compressed 40 mm above the systolic blood pressure for 5 min, and brachial artery diameter was recorded at 15 s and again at 45 to 60 s after ischemia. Vascular reactivity was assessed at 0, 2, 4, and 6 h.

Statistical analysis. Statistical analysis was carried out using SigmaStat software (Jandel Scientific, San Rafael, CA). All data are expressed as mean ± SD. Analysis was carried out with Kruskal-Wallis ANOVA on ranks. Dunnett’s method was used for multiple comparison procedures. Data for ROS generation by MNCs, PMNs, and MIF were logarithmically transformed. Wilcoxon signed rank test was used to compare endothelium-dependent vasodilation.

RESULTS
Plasma FFA, triglycerides, and insulin concentrations. Plasma FFA concentration rose from 352 ± 62 μmol/l to 621 ± 59 μmol/l at 2 h, 732 ± 75 μmol/l at 4 h (P < 0.005), and was 345 ± 42 μmol/l at 6 h. Plasma triglycerides concentration rose from 75 ± 16 mg/dl to 139 ± 42 mg/dl at 2 h, 120 ± 53 mg/dl at 4 h, and declined toward baseline to 85 ± 27 mg/dl at 6 h. Plasma insulin and glucose concentrations did not change significantly (Table 2).

ROS generation by PMNs and MNCs. The mean logROS generation by PMNs in the fasting state (basal) was 4.39 ± 0.25 mV. It increased to 5.13 ± 0.25 mV (P < 0.05; Fig. 1) to 2 h and to 5.12 ± 0.25 at 4 h (P < 0.05). It declined to 4.69 ± 0.25 at 6 h. The mean logROS generation by MNCs
in the fasting state was 4.97 ± 0.26 mV; it increased to a peak of 5.63 ± 0.26 mV at 4 h (P < 0.05). The protein expression of p47phox subunit of NADPH oxidase in MNC homogenates, however, did not change during the infusion (data not shown).

**Plasma TBARS concentrations and TBARS/triglycerides ratio.** Plasma TBARS concentrations increased significantly at 2 h (P < 0.05); the increases at 4 and 6 h were not significant. When expressed as a ratio of TBARS to triglycerides, the changes after lipid infusion were not significant (Table 2).

**NF-κB binding activity, cellular p65 (Rel A), IκB and phosphorylated IκB protein content, and IKK-α and IKK-β.** NF-κB binding activity in MNCs increased to 163 ± 17% (P < 0.005) and 144 ± 2% (P < 0.05) above the basal levels (100%) at 2 and 4 h, respectively (P < 0.005), and remained elevated at 6 h (154 ± 15%; P < 0.05; Fig. 2A). NF-κB p65 subunit protein quantity in MNC homogenates increased at 2, 4, and 6 h (246 ± 1%, 343 ± 165%, and 223 ± 37% above the basal; P < 0.05; Fig. 2B). Neither total IκB protein expression nor phosphorylated IκB protein expression in MNC homogenates changed significantly after the lipid infusion. Also, IKK-α and IKK-β expression in the MNCs did not change after the lipid infusion.

**IKK and IKK-α kinase activity in IKK immunoprecipitates.** To rule out the possibility that the bioactivity might be altered even in the absence of a change in the expression of IKK-α and IKK-β contents, we performed the bioactivity assays. Neither IKK-α nor IKK-β enzymatic activity changed significantly after lipid infusion. IKK activity in MNCs was 91 ± 13% and 109 ± 37% of the basal levels (100%) at 2 and 4 h, respectively (NS), and tended to decline to 80 ± 26% at 6 h (NS). IKK-α activity in MNCs was 108 ± 12%, 116 ± 27%, and 80 ± 15% of the basal levels (100%) at 2, 4, and 6 h, respectively (NS). Correspondingly, the phosphorylated IκB-α did not change significantly: 121 ± 18, 111 ± 29, and 103 ± 18% of baseline at 2, 4, and 6 h, respectively (NS) after lipid infusion.

**Plasma MIF, CRP, MCP-1, sICAM-1, and TNF-α concentrations.** Plasma MIF concentration increased from 0.56 pg/ml (100%) at baseline to 7.69 ± 0.94 pg/ml (Table 2). Neither CRP, MCP-1, sICAM-1, nor TNF-α concentration changed significantly after lipid infusion. The postischemic flow-mediated dilation at 0 h was 6.3 ± 1.1% of the basal. It decreased significantly to 3.7 ± 1.5% at 2 h (P < 0.05), to 2.1 ± 1.9% at 4 h (P < 0.05; Fig. 4), and to 4.7 ± 2.1% at 6 h (P < 0.05; Fig. 4).

**FIG. 1. ROS generation by PMNs before and after lipid infusion at 2, 4, and 6 h. Note that ROS generation increased significantly at 2 and 4 h.**

<table>
<thead>
<tr>
<th>Glucose (mg/dl)</th>
<th>0</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>78.7</td>
<td>80.8</td>
<td>80.7</td>
<td>82.8</td>
</tr>
<tr>
<td>Insulin (mU/l)</td>
<td>11.3</td>
<td>8.7</td>
<td>8.4</td>
<td>7.3</td>
</tr>
<tr>
<td>FFA (μmol/l)</td>
<td>352</td>
<td>621</td>
<td>732</td>
<td>345</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>75</td>
<td>139</td>
<td>120</td>
<td>88</td>
</tr>
<tr>
<td>TBARS (μmol/l)</td>
<td>1.46</td>
<td>1.77</td>
<td>1.54</td>
<td>1.56</td>
</tr>
<tr>
<td>TBARS (μmol/l)/triglycerides (mmol/l)</td>
<td>0.85</td>
<td>0.95</td>
<td>1.35</td>
<td>1.91</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>124</td>
<td>113.8</td>
<td>114.5</td>
<td>114.5</td>
</tr>
<tr>
<td>ICAM (ng/ml)</td>
<td>221</td>
<td>214</td>
<td>216</td>
<td>210</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>3.0</td>
<td>3.6</td>
<td>3.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Log MIF (pg/ml)</td>
<td>7.15</td>
<td>7.69</td>
<td>7.99</td>
<td>7.58</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. *P < 0.005 baseline vs. 4 h; †P < 0.05, 2-h and 4-h values vs. the baseline; ‡P < 0.05, baseline vs. 2-h values.

**TABLE 2**

<table>
<thead>
<tr>
<th>Glucose (mg/dl)</th>
<th>Insulin (mU/l)</th>
<th>FFA (μmol/l)</th>
<th>Triglycerides (mg/dl)</th>
<th>TBARS (μmol/l)</th>
<th>TBARS (μmol/l)/triglycerides (mmol/l)</th>
<th>MCP-1 (pg/ml)</th>
<th>ICAM (ng/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>Log MIF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>78.7 ± 2.3</td>
<td>11.3 ± 6.5</td>
<td>352 ± 62</td>
<td>75 ± 16</td>
<td>1.46 ± 0.4</td>
<td>0.85 ± 0.2</td>
<td>124 ± 33</td>
<td>221 ± 36</td>
<td>3.0 ± 0.9</td>
<td>7.15 ± 0.56</td>
</tr>
</tbody>
</table>

Plasma concentrations of glucose, insulin, FFAs, triglycerides, TBARS, and cytokines.
NS. Normal saline or a 5% dextrose infusion did not cause change in any of the above parameters.

DISCUSSION

We have shown for the first time that an increase in plasma FFA concentrations acutely causes an increase in the intranuclear NF-κB binding activity and p65 expression in circulating MNCs, as well as ROS generation by MNCs and PMNs. Clearly, therefore, an increase in FFA concentration is associated with the induction of proinflammatory changes and oxidative stress. The infusion of physiological saline or a low dose of dextrose into control subjects did not produce any changes in ROS generation (18). Our data provide the first link between FFA and an increase in NF-κB, a cardinal step in the induction of inflammation. In addition, our data demonstrate that an increase in FFA also induces an increase in MIF, a proinflammatory cytokine secreted by monocytes and adrenocorticotrophs in the pituitary gland (19,20).

Because elevated FFA concentrations have been shown previously to inhibit glucose uptake by insulin responsive organs such as the skeletal muscle, it has been suggested that FFA may be the major mediators of insulin resistance in insulin-resistant states (21). Recent observations have shown that an increase in FFA causes the induction of protein kinase-θ, which in turn inhibits phosphatidylinositol 3-kinase, the key enzyme that mediates the action of insulin in skeletal muscles (22).

It is noteworthy that Itani et al. (23) have recently shown that elevation of FFA leads to decreased IkB-α in the skeletal muscles of healthy subjects. However, no data on NF-κB were provided in that study.
Although our study has demonstrated an increase in NF-κB, no change was observed in IκB protein quantity. Because phosphorylation of IκB causes a dissociation of NF-κB from IκB, we also measured phosphorylated IκB, which did not alter either. Also, the IKK-α and IKK-β did not change after the lipid infusion. To investigate further the possibility that the IKK bioactivity might have been altered without the expression of IKK-α and IKK-β, we performed the enzymatic activity assays, and again there was no significant change with the lipid infusion. It is possible, therefore, that the activation of NF-κB in MNCs may be through a mechanism independent of IκB phosphorylation and ubiquitination, possibly through a direct increase in the expression of p65 (RelA), which was impressive at 300% when compared with the basal (100%). This could lead to an increase in NF-κB. These data are similar to our preliminary data on the effect of oral fat intake; it also induces an increase in ROS generation and p65 expression without a change in IκB.

The observation that FFA increase induces NF-κB binding is relevant to the action of thiazolidinediones, which reduce FFA concentrations and have profound anti-inflammatory effects while enhancing insulin sensitivity in humans in vivo (24). Consistent with these observations is the recent finding that insulin is an anti-inflammatory hormone that also suppresses plasma FFA concentrations and NF-κB, the key proinflammatory transcription factor (18).

Our study showed a strong stimulatory effect of FFA on plasma levels of MIF. Macrophages contain a significant amount of preformed MIF within intracellular pools that can be rapidly released on stimulation. It inhibits the ability of glucocorticoids to induce IκB synthesis in lipopolysaccharide-stimulated MNCs, thus promoting the translocation of NF-κB into the nucleus (25). MIF also promotes TNF-α expression and recently has been shown to be associated with insulin resistance (26,27). It is interesting that plasma TNF-α levels did not change in our study. This may be related to the fact that de novo mRNA generation and synthesis of TNF-α is required before secretion is observed. Also, MIF secretion from macrophages requires a lesser lipopolysaccharide stimulus than for TNF-α. It is possible that FFA infusion for a prolonged period or at a higher concentration of FFA could have resulted in an induction of plasma cytokines other than MIF.

Endothelium-dependent flow-mediated vasodilation of the brachial artery was significantly impaired within 2 h of

![FIG. 3. Changes in plasma MIF before and after lipid infusion at 2, 4, and 6 h. Note that MIF concentrations increased significantly at 2 (P < 0.05) and 4 (P < 0.005) h.](image)

![FIG. 4. Changes in the postischemic dilation in brachial artery. Results are expressed as percentage change over the baseline artery diameter after 5 min of distal occlusion. Note that it was significantly decreased after 2 and 4 h (P < 0.05).](image)
lipid infusion and persisted for 4 h. Our results are consistent with the previous observations of Steinberg et al. (3,4,28), who also demonstrated impaired acetylcholine-induced NO-dependent lower limb blood flow within 2 h of lipid infusion. The increased formation of ROS including superoxide may quench NO released from phagocytes in small volumes of whole blood. 

In conclusion, an increase in FFA concentration in plasma results in an acute increase in the intranuclear and total cellular NF-κB and ROS generation with a significant increase in MIF. These actions indicate a proinflammatory effect in association with an increase in oxidative stress. These effects provide a potential link between inflammation and insulin resistance.

REFERENCES