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Palmitic acid induces IP-10 expression in human macrophages via NF-κB activation

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Abstract

It is now recognized that cross-talk between adipocytes and adipose tissue stromal cells such as macrophages contributes to local and systemic inflammation. One factor from adipocytes that may participate in this interaction and that is frequently elevated in inflammatory conditions such as obesity, insulin resistance, and type 2 diabetes is free fatty acids (FFA). To investigate the potential for FFA to enhance macrophage inflammation, we exposed U937 macrophages to physiological levels (150 μM) of FFA. Palmitic acid (PA), the predominant saturated FFA released from adipose tissue, but not unsaturated FFA, induced an ~6-fold ($p < 0.05$) increase in IP-10 gene expression (and 2- to 4-fold increases in IL-8, MCP-1, COX-2, and MIG). PA also induced an ~2-fold increase ($p < 0.05$) in active NF-κB, and two structurally distinct NF-κB inhibitors effectively blocked PA-induced IP-10 gene expression. Conditioned medium from PA-treated cells increased lymphocyte migration 41% ($p < 0.05$) which was significantly reduced by IP-10-neutralizing antibody. These results suggest that elevated concentrations of PA commonly present in obese and insulin resistant individuals can increase NF-κB-mediated expression of IP-10 in macrophages. These events in turn may lead to an increasing feed-forward loop of chronic inflammation.

Keywords: Macrophages; Free fatty acids; Inflammation; Obesity; NF-κB; IP-10; Palmitic acid

Obesity is characterized by adipose tissue inflammation, and many of the pro-inflammatory factors secreted from adipose tissue derive from stromal cells such as macrophages [1]. In addition, monocytes/macrophages newly recruited to adipose tissue appear to demonstrate greater production of pro-inflammatory factors and decreased expression of protective factors than more quiescent resident macrophages [2]. The importance of macrophages to adipose tissue inflammation, as well as to local and systemic insulin resistance, has been demonstrated in several recent animal studies [1,3,4].

This pro-inflammatory interaction between macrophages and adipocytes in adipose tissue may also be relevant for vascular disease and may help explain the increased CVD risk with obesity. Adipose tissue inflammation appears to contribute to systemic inflammation, and specifically to enhanced monocyte activation. Recent studies also suggest that perivascular fat inflammation is associated with, and perhaps contributes to, vascular disease [5]. Therefore, it is becoming clear that both adipocytes and macrophages may contribute to the chronic inflammatory processes in adipose tissue [1,4,6,7] and that this interaction may exacerbate a variety of chronic inflammatory conditions.

We recently reported that treatment of 3T3-L1 adipocytes with conditioned medium from RAW264.7 macrophages resulted in the upregulation of several pro-inflammatory factors including free fatty acids (FFA) [8]. The present study examined the hypothesis that FFA may in turn feed back to modulate the inflammatory state of *human* macrophages. While saturated FFAs have been

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reported to have unique pro-inflammatory effects compared to unsaturated FFAs in several murine models, possibly via the endotoxin receptor, TLR4 [9], these effects have not been carefully studied in human cells. In fact, some studies have noted significant differences in TLR signaling between murine and human macrophages [10]. Our own preliminary data suggested that one such difference is that human, but not murine, macrophages substantially upregulate IP-10 expression in response to TLR4-mediated stimuli such as LPS.

There is growing recognition that the chemokine IP-10 plays important roles in chronic inflammatory conditions such as atherosclerosis [11], but the potential roles for FFA-induced IP-10 in these conditions have not been previously examined. We therefore examined the effects of physiological levels of saturated fatty acids on production of IP-10 and other pro-inflammatory factors by human macrophages.

Materials and methods

Reagents. The proteasome inhibitor MG132 (1 μM) was from Biomol (Plymouth Meeting, PA), and Pyrolidinedithiocarbamate (PDTC; 25 μM) was from Sigma (St. Louis, MO). Human TNF-α and a monoclonal anti-human blocking antibody to IP-10 was purchased from R&D Systems (Minneapolis, MN). Recombinant human IP-10 protein was obtained from RDI (Flanders, NJ). All reagents not otherwise specified were from Sigma (St. Louis, MO).

Cells and cell culture. Human U937 monocytes were obtained from the American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 (Invitrogen, Gaithersburg, MD) supplemented as recommended by the vendor. Monocytes were differentiated into macrophages using 12 ng/mL phorbol ester overnight. Lymphocytes were isolated from the blood of healthy volunteers by gradient centrifugation [12] and purified by overnight growth in RPMI containing 10% human serum.

Free fatty acid preparation. Sodium salts of palmitic acid (PA), dodecanoic (lauric) acid, oleic acid, linoleic acid, eicosapentaenoic acid, and arachidonic acid were from lots demonstrated to be >99% pure by gas chromatography and were tested for endotoxin contamination using the Pyrogent assay kit (Cambrex, Gaithersburg, MD); only lots containing <5 pg endotoxin per mole FFA were used on cells. FFAs were dissolved to 40 mM in 0.1 N NaOH 70% ethanol at 70 °C. We have found that this solvent neutralizes >10 ng/mL endotoxin (unpublished data). Fatty acids were purged with nitrogen gas and stored in small aliquots at −80 °C. Aliquots were used within four weeks and freeze-thawed no more than twice.

Exposure of cells to free fatty acids. FFA preparations were thawed and briefly warmed immediately before addition to cell culture media. FFA were added to final concentrations of 50, 100, and 150 mM in RPMI 1640 media (containing 10% FBS, 0.3 mg/mL L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) and allowed to complex with the albumin present in the FBS at 37 °C for 1 h. The final FFA/albumin molar ratio was approximately 2:1. For individual experiments, U937 macrophages were treated in media containing 0–150 μM FFA, and were subsequently maintained under standard cell culture conditions for 4–72 h.

Generation of conditioned media and cytokine protein quantification. Macrophages were maintained under control conditions or treated with 150 μM PA for 48 h, washed and subsequently incubated in fresh, PA-free media for 6–48 h. Conditioned media were collected from these cultures at the appropriate time points, then aliquoted and snap-frozen to −80 °C. Aliquots were thawed only once prior to analysis or use on cultured cells. IP-10 protein concentrations in conditioned cell culture media were analyzed by ELISA (R&D Systems) following manufacturer's instructions. Samples outside the range of the standard curve were diluted in ELISA sample buffer and re-analyzed.

Measurement of NF-κB activation. Following treatment of cells with PA for the indicated time period, cells were fractionated to extract nuclear and cytoplasmic proteins using the hypotonic lysis method of Berg et al. [13]. Equal amounts of protein from each lysate were analyzed for NF-κB activation using a TransAM™ NF-κB p65 assay kit (Active Motif, Carlsbad, CA) following manufacturer's instructions. Phosphorylation of IκBα in the cytoplasmic fraction was analyzed by Western blot, using standard laboratory methods and a specific monoclonal antibody against phosphorylated IκBα (clone B9; Santa Cruz Biotechnology, Santa Cruz, CA).

Lymphocyte migration assay. Primary human lymphocyte migration was measured using Micro Chemotaxis Transwell plates (polycarbonate membrane, pore size 5.0 μm; Costar, Corning, NY). PA-free conditioned media from macrophages were prepared as described above in *Generation of conditioned media and cytokine protein quantification*. Cell migration was stimulated under standard cell culture conditions with various conditioned media (described below in Results), and cells that had migrated through the membrane after 90 min were counted using a Coulter counter.

Reverse transcription-PCR and real-time quantitative PCR analysis. Total RNA was isolated, converted to cDNA, and analyzed by real-time quantitative PCR using SYBR Green chemistry (qPCR) with reagents and equipment from Bio-Rad (Hercules, CA). Primer pairs were: IP-10 sense, AGGAACCTCCAGTCTCAGCA; IP-10 antisense, CAAAATTGGC TTGCAGGAAT and 18 s sense, GGACTTCGAGCAAGAGATGG; 18 s antisense, AGCACTGTGTTGGCGTACAG. Quantification of gene expression was performed by the δδCt method [14].

Statistics and data analysis. Data are expressed as means ± SD. Data were analyzed by ANOVA using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Experiments demonstrating a difference between groups by ANOVA were subsequently analyzed by post hoc *Z*-statistics (corrected for multiple comparisons) when experiments compared samples that had been normalized to controls (e.g., real-time PCR), or by Bonferroni's *t*-tests when experiments compared different means (e.g., migration assays). All experiments were repeated a minimum of three times. Results were considered significant at $p < 0.05$.

Results

Expression of pro-inflammatory cytokine mRNA following treatment of macrophages with FFAs

We first exposed U937 macrophages to the major individual FFAs found in human plasma (Fig. 1) at a concentration of 150 μM (except arachidonic acid, which was toxic to macrophages above 50 μM). The saturated FFAs palmitic and lauric acid induced a 6-fold increase in IP-10 mRNA after 48 h. In contrast, the unsaturated FFAs either reduced IP-10 mRNA levels or had no significant effect. We further investigated the effects of palmitic acid (PA) because it is the most abundant saturated FFA in human tissue and plasma.

PA treatment of macrophages induces time- and concentration-dependent increases in IP-10 mRNA

Macrophages exposed to 150 μM PA for time periods of 6–72 h demonstrated increased IP-10 expression in a time-dependent manner, reaching a maximum at 48 h, but remaining elevated for at least 72 h (Fig. 2A). Expression of several other pro-inflammatory genes was also examined