Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action

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1Central Arkansas Veterans Healthcare System, 2Division of Endocrinology, Department of Medicine, and 3Department of Pediatrics and Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, Arkansas; 4Department of Biostatistics, University of Wisconsin, Milwaukee, Wisconsin; and 5College of Health Sciences, University of Kentucky, Lexington, Kentucky

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Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action. Am J Physiol Endocrinol Metab 296: E1300–E1310, 2009. First published March 31, 2009; doi:10.1152/ajpendo.90885.2008.—Obesity is characterized by adipose tissue expansion as well as macrophage infiltration of adipose tissue. This results in an increase in circulating inflammatory cytokines and nonesterified fatty acids, factors that cause skeletal muscle insulin resistance. Whether obesity also results in skeletal muscle inflammation is not known. In this study, we quantified macrophages immunohistochemically in vastus lateralis biopsies from eight obese and eight lean subjects. Our study demonstrates that macrophages infiltrate skeletal muscle in obesity, and we developed an in vitro system to study this mechanistically. Myoblasts were isolated from vastus lateralis biopsies and differentiated in culture. Coculture of differentiated human myotubes with macrophages in the presence of palmitic acid, to mimic an obese environment, revealed that macrophages in the presence of palmitic acid synergistically augment cytokine and chemokine expression in myotubes, decrease IkB-α protein expression, increase phosphorylated JNK, decrease phosphorylated Akt, and increase markers of muscle atrophy. These results suggest that macrophages alter the inflammatory state of muscle cells in an obese milieu, inhibiting insulin signaling. Thus in obesity both adipose tissue and skeletal muscle inflammation may contribute to insulin resistance.

Obesity is a prominent cause of insulin resistance. Although obesity involves an expansion of adipose tissue, peripheral insulin resistance involves a defect in muscle glucose transport, and the precise mechanism for obesity-induced insulin resistance has not been fully elucidated. Among the adipose tissue products that may induce muscle insulin resistance are an increase in circulating nonesterified fatty acids (NEFA) (5, 7, 28, 34) and an increase in adipose tissue-derived cytokines (17, 63). The adipose tissue of obese animals and humans is characterized by the accumulation of macrophages (20, 67, 68), resulting in the secretion of tumor necrosis factor (TNF)-α, interleukins (ILs), and other inflammatory cytokines, all of which circulate and contribute to insulin resistance. The degree to which an inflammatory state in other organs contributes to insulin resistance in obesity is relatively unexplored; however, macrophage infiltration is also found in pancreatic islets and liver of insulin-resistant subjects (22, 35, 44). Although macrophage infiltration associated with obesity and insulin resistance has not been described in human muscle, it was recently reported that the subpopulation of macrophages that infiltrates adipose tissue of high-fat-fed mice also increases in muscle (43). In muscle, macrophages are required for muscle hypertrophy during modified loading (21) and accumulate after damage, where they play both a phagocytic role during degeneration and a reparative role during regeneration dependent on their activation state (2, 12, 62), possibly explaining some of the conflicting results obtained from in vitro studies on the influence of macrophages on muscle cell function. Macrophages have been shown to damage muscle cells (42, 66) or promote proliferation and survival of myogenic precursor cells (11, 14, 39, 50). Moreover, depending on the method of activation, macrophages have different effects on insulin responses in muscle cells (51). Whether macrophage infiltration in muscle contributes to the development of insulin resistance is unknown.

In addition to macrophage infiltration, the other characterized link between obesity and muscle insulin resistance is the release of NEFA from adipose tissue, and a number of potential mechanisms for the induction of insulin resistance by NEFA have been described (3). Elevated plasma NEFA is associated with increased muscle lipids, including triglycerides, as well as diacylglycerol (DAG), ceramides, and long-chain acyl-coenzyme A, that activate kinases such as protein kinase C (PKC) (28, 69, 72) and c-Jun-NH2-terminal kinase (JNK) (27, 61). These kinases impair insulin signaling by increasing the inhibitory serine phosphorylation of insulin receptor substrates (IRS) (1, 18) and thus decreasing phosphatidylinositol 3-kinase (PI3K) activity and Akt phosphorylation. NEFA also increase oxidative stress and activate the NF-κB pathway in muscle, interfering with insulin signaling (59). More recently, it has been shown that NEFA can also activate the JNK/AP-1 and the IKK-β/IκB-α/NF-κB pathways in muscle via the toll-like receptors TLR-2 and -4 (49, 57, 58). Activation of AP-1 and NF-κB are also demonstrated to lead to muscle atrophy (25, 40, 71).

In this study, we show that muscle macrophage number is increased with obesity and insulin resistance. To study mechanistically the effect of macrophages on muscle cells within an obese environment, we developed an in vitro culture system in

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which differentiated human myotubes were cocultured with macrophages in the presence of the NEFA palmitic acid. Effects on pro- and anti-inflammatory myotube gene expression and the effect on insulin signaling were quantified. Our results demonstrate that NEFA synergize with macrophages to exacerbate the inflammatory state of muscle cells, resulting in impaired insulin signaling and increased expression of markers of muscle atrophy. Thus macrophage infiltration into muscle, coupled with elevated NEFA, may represent another manifestation of the inflammatory environment of obesity-associated insulin resistance.

MATERIALS AND METHODS

Human subjects and muscle biopsies. Generally healthy, sedentary, nondiabetic human subjects were recruited by local advertisement. All subjects signed informed consent forms under protocols that were approved by the local institutional review board. The studies were performed at the University of Arkansas for Medical Sciences/Central Arkansas Veterans Healthcare System General Clinical Research Center. Twenty-three subjects were included in this study. Subjects were between 20 and 60 yr old, and they were not involved in regular moderate or vigorous physical activity. Subjects were not taking any anti-inflammatory medications. Insulin sensitivity (SI) was measured from vastus lateralis muscle under local anesthesia in fasting conditions. Muscle biopsy specimens were obtained for the presence of macrophages (described below) in 16 subjects (8 obese and 8 lean). The obese group had mean body mass index (BMI) of 36.9 ± 0.87 kg/m² and SI of 2.1 ± 0.02, significantly different from the lean group, with mean BMI of 23.8 ± 0.09 kg/m² and SI of 1.8 ± 0.01, significantly different from the lean group, with mean BMI of 23.8 ± 0.09 kg/m² and SI of 0.87 ± 0.01.

Isolation and culture of myoblasts from human muscle biopsies. Myoblasts were isolated and enriched from the cell suspension (connective tissue or gaps due to sectioning artifacts), the nonfiber area was subtracted from the total area of the field. Additionally, care was taken to avoid recounting fibers in adjacent fields. Collectively, 1,000–1,400 fibers were counted per subject. Macrophage abundance is expressed in two different ways: macrophage content in muscle biopsy (% of muscle fibers) and the number of macrophages per cross-sectional area of muscle.

Isolation and culture of myotubes from human muscle biopsies. Myoblasts were isolated from the biopsied tissue with modifications of a previous method (46). Briefly, the biopsied tissue weighing between 0.05 and 0.2 g was collected and stored in cold Dulbecco’s phosphate-buffered saline without calcium and magnesium (PBS-CMF, Invitrogen, Carlsbad, CA), 1% glucose, 10 U/ml penicillin, and 100 μg/ml streptomycin overnight at 4°C. On the day of cell isolation, the tissue was washed with basal F-10 medium (Hyclone, Logan, UT) and any obvious clots and fat tissue were removed. The muscle tissue was digested in a 1:1 mix of collagenase D (Boehringer Mannheim, Indianapolis, IN) and Dispase II (Boehringer Mannheim), minced, and then incubated at 37°C for 30 min with frequent mixing. After incubation, the digested tissue was filtered through a 70-μm filter and washed down with the PBS-glucose medium described above. The filtered cell suspension was centrifuged at 900 g for 6 min at 10°C and resuspended in RPMI medium (Invitrogen) containing 1% FBS (HyClone). Myoblasts were isolated and enriched from the cell suspension by using 5.1H11 antibody (46)-coated magnetic beads, with the CELLection Pan Mouse IgG kit (Dynal Biotech), according to the manufacturer’s instructions. The isolated myoblasts were plated and grown in a 1:1 mix of MRC-5 conditioned medium and Ham’s F-10 growth medium containing 20% FBS until the first passage, followed by growth in Ham’s F-10 growth medium containing 20% FBS. Medium was changed every 3 days. Stocks were made at the second or third passage. Myoblast cultures were examined for MyoD staining by immunocytochemistry. Myoblasts that were 90% or greater MyoD positive were used in experiments, and myoblasts between passages 4 and 5 were used for experiments in 6- or 12-well plates. When the cells were 90% confluent, they were induced to differentiate by culturing in α-MEM medium containing 5 mM glutamine, 1 mg/ml penicillin-streptomycin, and 2% FBS. The myotubes used in this study were derived from eight different subjects, who were representative of the larger group of subjects. After induction of differentiation and passage in culture, no differences in gene expression were apparent between the subjects with extremes of BMI and hence the myotube cultures were examined together for all further analysis.

THP-1 macrophages. THP-1 cells, a human monocyte cell line, were maintained in RPMI medium with 10% FBS and 1% penicillin-streptomycin. To obtain macrophages, cells were plated at 1.4 × 10^6 cells/100-mm culture dish, in Macrophage-SFM (Invitrogen) with 1% penicillin-streptomycin and 250 nM phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma) for 2 h, to differentiate the THP-1 monocytes to macrophages. The TPA-treated macrophages were then cocultured with the myotubes as described below.

MRC-5 fibroblasts. MRC-5 cells, a human fibroblast cell line, were grown in Dulbecco’s modified Eagle’s medium (DMEM), 1 g/l glucose (Mediatech, Manassas, VA), containing 10% FBS and 1% penicillin-streptomycin. MRC-5 fibroblasts were cocultured with myotubes as described below.

Myotube–fibroblast and myotube–macrophage cocultures. Myotubes differentiated for 48–72 h were cultured alone, cocultured with THP-1 macrophages, or, as a control, cocultured with MRC-5 fibroblasts. For cocultures, TPA-differentiated THP-1 macrophages obtained as described above were gently scraped, counted with a Trypan blue, and plated at 1.8–2.0 × 10^6 cells on inserts accompanying the complete six-well plate. Thus the macrophages were plated at 20–25% of the confluent numbers of myotubes (7–9 × 10^5), comparable to the abundance of macrophages observed in the muscle from obese subjects. The macrophages were allowed to settle and adhere for 12 h before the coculture was started. All coculture experiments were performed for 48 h. Cocultures were treated with 0.2 mM palmitic acid for 24 h before collection of samples for RNA or for 48 h for cell lysates for protein. The myotubes were grown on plates, and the THP-1 macrophages or MRC-5 fibroblasts were grown on inserts. A more detailed account of the cocultures is described in the supplemental methods for this article. After coculture, the cells from the inserts and plate wells were collected separately with RNA lysis buffer. Medium from myotube cultures and the mytohe cocultures with macrophages or fibroblasts were collected and stored at −70°C until used for assay of cytokines by ELISA.
For palmitic acid treatment of cultures, palmitic acid (Sigma) was conjugated with fatty acid-free bovine serum albumin (BSA; MP Biochemicals, Irvine, CA) in a ratio of 2.5:1 as described previously (54). Briefly, palmitic acid was dissolved in ethanol and mixed with fatty acid-free BSA solution (MP Biochemicals) made in α-MEM medium (Invitrogen). The palmitic acid-BSA mix was conjugated at 45°C for 20 min until homogeneous, along with a control that contained BSA with the same volume of ethanol as used for palmitic acid. The BSA-conjugated palmitic acid was then diluted to the required concentration in the culture medium of the cells tested.

Total RNA isolation and real-time RT-PCR. Total RNA from differentiated myotubes, THP-1 macrophages, and MRC-5 fibroblasts was obtained with the RNAqueous kit (Ambion) per the manufacturer’s instructions. The quantity and quality of the isolated RNA were determined by Agilent 2100 Bioanalyzer (Palo Alto, CA). The real-time RT-PCR was performed as described previously (64). The primers used are described in the supplemental methods for this article.

Western blotting. Myotubes were cocultured in the presence or absence of palmitic acid (0.2 mM) for 48 h as described above. After coculture, the myotubes were washed with PBS and stimulated with 0, 1, or 10 nM insulin (Novolin, NovoNordisk, Princeton, NJ) for 20 min at 37°C as indicated and then harvested with M-Per mammalian protein extraction reagent (Pierce, Rockford IL) containing protease inhibitor cocktail mix (1:100) and phosphatase inhibitor cocktail 1 and 2 (1:100) (Sigma).

Thirty micrograms of protein was electrophoresed in 4–15% SDS polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membrane at 100 V for 1 h at 4°C. Membranes were blocked for 30 min with Casein Blocker (Pierce) and immunoblotted with phospho-Akt (Ser473), Akt, or IκB-α antibodies (Cell Signaling) or blocked with Blotto B for 1 h and immunoblotted with phospho-JNK (Thr183 and Tyr185) or JNK antibody (Santa Cruz) overnight at 4°C with gentle rocking. All blots were stripped and reacted with β-actin antibody (Santa Cruz) for 30 min at room temperature. After washing, the blots were incubated for 1 h at room temperature with goat anti-rabbit secondary Ig (Pierce) or goat anti-mouse secondary Ig (Pierce) appropriately. Bands were visualized with SuperSignal West Dura Extended Duration substrate (Pierce), followed by exposure to X-ray film. Densitometric analysis was performed with ImageQuant software (Molecular Dynamics), and results are expressed as arbitrary densitometric value normalized to β-actin.

Cytokine assays. TNF-α secreted into medium was quantified with the TNF-α human ultrasensitive ELISA kit (Biosource) according to the manufacturer’s instructions. Secreted IL-10 and IL-6 were quantified with the IL-10 Hs kit and the IL-6 kit (R&D Systems), respectively, according to the manufacturer’s instructions.

Statistics. Comparisons were made with nonparametric Wilcoxon rank test for paired tests. The association of macrophage numbers with BMI and S1, respectively, was described with Pearson’s correlation coefficients. S1 values were transformed to natural logarithm to attain approximate normality before analysis. Data are presented as means ± SE, or medians and ranges when the change in response to experimental treatment was very high. Statistical significance was set at P ≤ 0.05, with no adjustment for multiple comparisons.

RESULTS

Increased macrophage abundance in skeletal muscle of obese insulin-resistant human subjects. Obesity is a state of chronic inflammation associated with macrophage infiltration in adipose tissue. To determine whether obesity is also associated with macrophage infiltration in skeletal muscle, CD68, a cell surface marker of monocytes/macrophages, was quantified immunohistochemically in vastus lateralis biopsies from eight obese and eight lean subjects (Fig. 1, A–C). BMI ranged from 20 to 28 kg/m² in the lean group and from 33 to 41 kg/m² in the obese group. As expected, S1 was lower in the obese group (range 1.49–2.78 × 10⁻⁴ min⁻¹ μU⁻¹ ml⁻¹) compared with the lean group (range 2.65–13.64 × 10⁻⁴ min⁻¹ μU⁻¹ ml⁻¹). The correlation coefficient of S1 versus BMI in the subjects examined was r = 0.79 (P < 0.001, n = 16). As shown in Fig. 1C, muscle from obese subjects contained approximately twofold more macrophages compared with their lean counterparts. Furthermore, as seen in Fig. 1, D and E, the number of macrophages, expressed as a percentage of total myofibers, was positively associated with BMI and negatively associated with S1 (r = 0.81, P < 0.001 and r = 0.83, P < 0.001, respectively). No significant difference in fiber cross-sectional area was apparent between the lean and obese subjects (data not shown). The presence of the significantly higher number of macrophages in muscle from obese subjects and the correlations with BMI and S1 suggest that macrophage infiltration of muscle is a component of the obese insulin-resistant state, much like the well-characterized inflammation of adipose tissue.

Macrophages in presence of palmitic acid synergistically increase cytokine gene expression in myotubes. To understand mechanistically the influence of macrophages on muscle inflammatory state in an obese environment, we studied the influence of macrophages in the presence of the NEFA palmitic acid on muscle cell cytokine gene expression. Myoblasts were isolated from skeletal muscle biopsies from eight subjects and differentiated in vitro to myotubes. Treatment of myotubes alone with palmitic acid caused an increase in the expression of proinflammatory cytokine genes (TNF-α, IL-1β) and pleiotropic (IL-6) cytokine genes (Fig. 2). The expression of the anti-inflammatory gene IL-10 was unchanged, while IL-1 receptor antagonist (IL-1RA) tended to increase with palmitic acid but did not reach significance. We next determined whether macrophages also play a role in regulating muscle cell cytokine gene expression. To address this, myotubes were cocultured with THP-1-derived macrophages in the presence or absence of palmitic acid. As a control for the specific influence of macrophages, myoblasts were also cocultured with a human fibroblast cell line, MRC-5. Myotubes cocultured with macrophages increased the expression of the pro- and anti-inflammatory cytokine genes 4- to 18-fold compared with those cocultured with fibroblasts (Fig. 3A). Exposure of myotubes to both palmitic acid and macrophages resulted in a synergistic up-regulation of the proinflammatory cytokine genes TNF-α, IL-6, and IL-1β, whereas the effect was additive for the anti-inflammatory genes IL-10 and IL-1RA (Fig. 3B). For example, in the case of myotube TNF-α expression, there was a 2-fold increase by palmitic acid, 15-fold by macrophage coculture, and 500-fold in the presence of both palmitic acid and macrophages. Treatment of myotubes with macrophage conditioned medium in the presence of palmitic acid (Supplemental Fig. S1) showed a smaller induction of myotube cytokine gene expression, which was still apparent even after significant dilution (Supplemental Fig. S2), suggesting that factors derived from macrophages activated by palmitic acid are very effective in altering myotube gene expression.

Because macrophages were found to infiltrate skeletal muscle in obese subjects, we examined the myotubes for expression of macrophage chemoattractant protein-1 (MCP-1), a known recruiter of macrophages. As shown in Fig. 3C, palmi-
tic acid had relatively little effect on myotube MCP-1 in the presence of fibroblasts. However, myotube MCP-1 gene expression was increased 4.5-fold by coculture with macrophages and was increased 10-fold in myotubes cocultured with macrophages in the presence of palmitic acid compared with myotubes cocultured with fibroblasts. Although less robust, similar effects were observed with conditioned medium (Supplemental Fig. S1). Thus the combination of palmitic acid plus macrophage coculture synergistically augmented myotube MCP-1 expression, which would then be expected to result in the recruitment of more macrophages to muscle.

Macrophages and palmitic acid increase secretion of cytokines. To examine whether the increased myotube cytokine gene expression was complemented by an increase in cytokine secretion, we examined representative pro- and anti-inflammatory cytokines in the medium by ELISA. Results showed that the changes in cytokine transcript levels of myotubes described in the cocultures above were also reflected in the increased cytokine concentration in the medium (Fig. 4). The concentration of the proinflammatory cytokine TNF-α (Fig. 4A) increased 400-fold on coculture with macrophages compared with coculture with fibroblasts, while IL-6 (Fig. 4B) and IL-10 (Fig. 4C) showed 10- to 15-fold increases. Palmitic acid had relatively little effect on TNF-α or IL-10 secretion in the presence of fibroblasts but had a small but significant effect on IL-6, consistent with the mRNA results. However, in the presence of macrophages, palmitic acid increased cytokine secretion of TNF-α, IL-6, and IL-10 in the medium.

During coculture, cytokines are likely to be derived from both myotubes and macrophages. Hence, we examined cytokine transcript levels in the fibroblasts/macrophages that were cocultured with myotubes, in the presence or absence of palmitic acid for TNF-α, IL-6, and IL-10 (Supplemental Fig. S3, A, B, and C, respectively). When cultured with myotubes, macrophages treated with palmitic acid showed no increase in either TNF-α or IL-10 gene expression and only a small increase in IL-6, suggesting that the robust increase in secreted cytokines following combined palmitic acid and macrophage coculture with myotubes was derived from the dramatic increase in myotube cytokine gene expression.

Potential mediators of cytokine gene expression in myotubes in response to palmitic acid and macrophages. NEFA activate TLR-4 and TLR-2 in muscle (49, 57, 58), resulting in increased NF-κB and JNK signal transduction that may mediate the
observed increase in muscle cytokine expression. To determine whether the expression of TLR-4 and TLR-2 is affected by palmitic acid alone or by the combination of macrophages and palmitic acid, we examined the mRNA levels of TLR-4 and TLR-2 in myotubes. As seen in Fig. 5A, myotubes cocultured with fibroblasts or macrophages expressed similar levels of TLR-4 mRNA. Palmitic acid treatment upregulated TLR-4 gene expression by approximately twofold in the cocultures of myotubes with fibroblasts or macrophages. However, unlike TLR-4, myotubes cocultured with macrophages significantly increased the expression of the TLR-2 gene compared with coculture with fibroblasts (Fig. 5A). Furthermore, myotubes cocultured with macrophages did not show an effect in response to palmitic acid treatment, while coculture of myotubes with fibroblasts showed a slight but significant increase in TLR-2 gene expression (Fig. 5A). These results suggest that altered expression of the TLRs may contribute to the cytokine response in myotubes.

The TLRs, as well as cytokines, signal through both the JNK/AP-1 and IKK-β/IκB-α/NF-κB pathways. Hence, we examined protein levels of IκB-α and phospho-JNK in our coculture system. The effects of palmitic acid treatment and macrophage coculture on IκB-α and phospho-JNK are shown in Fig. 5B. Addition of palmitic acid to myotubes cocultured with fibroblasts showed no change in IκB-α, nor did coculture with macrophages. However, macrophages in the presence of palmitic acid induced significant downregulation of IκB-α in myotubes, suggesting that NF-κB was activated. Figure 5C illustrates the effect of palmitic acid and macrophage coculture on phosphorylation of JNK. Both the presence of macrophages and the addition of palmitic acid increased JNK phosphorylation, and the combination of palmitic acid and macrophages resulted in the highest level of JNK phosphorylation. Thus both signaling pathways likely mediate the combined effects of macrophages and NEFA on muscle inflammatory state.

Dampened insulin-dependent Akt phosphorylation in myotubes with palmitic acid and macrophage coculture. The dramatic increase in cytokine expression and activation of downstream signaling pathways suggested that coculture with macrophages, particularly in the presence of palmitic acid, would impair insulin action in myotubes. To test this hypothesis, a downstream insulin signaling target was analyzed. As shown by the representative Western blot in Fig. 6A, the addition of insulin to myotube cultures resulted in a dose-dependent increase in Akt phosphorylation. When the myotubes were incubated with palmitic acid, Akt phosphorylation was dampened at all insulin concentrations, with no change in Akt protein abundance, and similar effects were observed with macrophage coculture. The combination of palmitic acid and macrophage coculture resulted in overall blunting of Akt phosphorylation in response to insulin, although the cells were still responsive. These data are quantified in Fig. 6B. At 10 nM insulin, palmitic acid in the presence of fibroblasts reduced Akt phosphorylation by 35.6 ± 14.7%. Macrophage coculture had a quantitatively similar effect, and reduced Akt phosphorylation by 25.4 ± 7.9%. The combination of palmitic acid and macrophage coculture reduced Akt phosphorylation by 81.4 ± 16.9%, consistent with a synergistic effect on insulin action.

Catabolic effect of palmitic acid and macrophage coculture on myotubes. One of the best-characterized downstream effects of inflammatory cytokine signaling in muscle cells is the upregulation of muscle-specific E3 ubiquitin ligases, muscle atrophy F-box (MAFbx), and muscle ring-finger protein-1 (MuRF-1), promoting muscle wasting (24, 25, 40, 65, 71). Because our in vitro cultures of myotubes showed dramatic...
augmentation of inflammatory cytokines in an obese environment, we examined the potential upregulation of the muscle protein catabolic markers MAFbx and MuRF-1. Figure 7A shows that myotube MAFbx gene expression in the presence of macrophages and palmitic acid was significantly increased compared with myotubes cocultured with either macrophages or fibroblasts. Macrophages alone did not induce a significant increase in expression compared with fibroblasts. Similarly, in the case of MuRF-1 (Fig. 7B), only the combination of macrophages and palmitic acid elicited an increase in myotube MuRF-1 gene expression, although this increase did not reach statistical significance ($P = 0.06$ for myotubes cocultured with macrophages + palmitic acid vs. myotubes cocultured with macrophages and $P < 0.07$ for myotubes cocultured with macrophages + palmitic acid vs. myotubes cocultured with fibroblasts). Thus increased protein degradation in muscle may result from macrophage infiltration during obesity.
DISCUSSION

Obesity is a chronic inflammatory state, and adipose tissue macrophages have a causative role in obesity-induced insulin resistance (18). NEFA efflux and cytokine secretion that ensue from inflamed adipose tissue promote insulin resistance in other insulin-sensitive organs, such as skeletal muscle and liver. We show here that macrophages also accumulate in human skeletal muscle in obesity. Obese subjects have approximately twice as many macrophages in skeletal muscle than their lean counterparts, and muscle macrophage number is in the range of relative abundance of macrophages to adipocytes in adipose tissue from obese subjects associated with insulin resistance (8, 20). Since muscle accounts for most peripheral glucose uptake, which is reflected in the SI, we hypothesized that muscle macrophages exacerbate peripheral insulin resistance by directly altering the phenotype of muscle fibers. To test this hypothesis, we developed an in vitro system in which differentiated myotubes were cocultured with macrophages in the presence of the NEFA palmitic acid to mimic the obese environment. Results demonstrate that both palmitic acid and macrophage secretory products alter myotube function, but in combination they lead to dramatic increases in myotube cytokine/chemokine expression and activation of signaling pathways that interfere with insulin responsiveness.

A limitation of this in vitro system is the use of the human monocyctic cell line THP-1, which was differentiated to macrophages with TPA. Although TPA-treated THP-1 cells have been widely used as a cell line for macrophages, their similarity to muscle-resident macrophages is unknown. THP-1-differentiated macrophages share some morphological properties with peripheral blood monocytes differentiated to macrophages; however, they are unique cell types (33). The THP-1 macrophages in our study did not show sustained induction of cytokines in response to palmitic acid, although induction may have been observed after short-term treatment, as reported for RAW macrophages (51, 58), given that palmitic acid-treated RAW macrophage conditioned media had an effect on myoblasts comparable to that reported here.

Muscle macrophages have been most studied within the context of muscle injury and repair in the mouse, where they have been proposed to mediate progression from an inflammatory response to the development of an environment that promotes myogenic differentiation (2, 62). Although the proinflammatory properties of macrophage infiltration in human muscle following eccentric, damaging exercise have been described (47), other studies suggest that inflammatory cells do not infiltrate human skeletal muscle after damage (37, 38) or may be restricted to individuals with specific genetic haplotypes (19). Although exercise that promotes muscle growth may not change the total number of macrophages in human muscle, the number of alternatively activated macrophages increases, with only a small subpopulation expressing classical, proinflammatory characteristics (48). Results from our in vitro system, which mimicked the obese state by culturing myotubes with classically activated macrophages in the presence of palmitic acid, suggest that macrophage infiltration in muscle during obesity may promote a strong inflammatory response from muscle cells, quite unique from the muscle cell response to macrophages during exercise. Consistent with this idea, recent work suggests that, unlike palmitic acid, LPS-activated macrophages may have a positive effect on muscle cell insulin sensitivity, potentially through preferential induction of IL-10 expression (51).

Numerous recent studies have examined adipose tissue macrophages. Interestingly, adipose tissue macrophages phenotypically resemble the alternatively activated, anti-inflammatory type, and diet-induced obesity shifts the macrophages to a proinflammatory state (36, 70). Although adipose macrophages clearly alter adipocyte function (64), cytokine production in adipose tissue appears to derive primarily from macrophages, whereas the data from this study demonstrate a considerable increase in expression of muscle-derived cytokines. Although previous studies detected only low levels of inflammatory markers in skeletal muscle by real-time PCR (10), the present study suggests that a relatively small change in the absolute number of proinflammatory macrophages during obesity could be functionally important by altering inflammatory and metabolic properties of muscle cells. However, the relative impor-

Fig. 4. Secreted TNF-α (A), IL-6 (B), and IL-10 (C) in coculture media. Differentiated myotubes were cocultured with macrophages (MMc) or, as a control for the coculture, with fibroblasts (MF) for 48 h. Cocultures were treated without or with (+P) 0.2 mM palmitic acid for 24 h before collection of cell lysates. Media collected after coculture were assayed for TNF-α, IL-6, and IL-10 by ELISA as described in MATERIALS AND METHODS. Data are means ± SE. *P ≤ 0.05 vs. MMc and MF; †P < 0.02 vs. MF.
tance of adipose tissue- versus muscle-derived inflammatory products in the progression of peripheral insulin resistance during obesity remains to be determined.

The expression of cytokines from differentiated human myotubes in response to macrophage coculture and NEFA likely has detrimental effects on muscle cell metabolism through well-characterized downstream signaling pathways. Cytokines such as TNF-α, IL-6, and IL-1β activate both JNK and NF-κB pathways, inducing further expression of inflammatory mediators in muscle cells (56, 63), contributing to impaired glucose

Fig. 5. Effect of macrophages and palmitic acid on potential mediators of inflammation. A: effect of palmitic acid on toll-like receptor TLR-4 and TLR-2 mRNA expression. Differentiated myotubes were cocultured with macrophages (MMc) or, as a control for the coculture, with fibroblasts (MF) for 48 h. Cocultures were treated without or with (+P) 0.2 mM palmitic acid for 24 h before collection of cell lysates. Data are means ± SE. *P < 0.01 vs. MMc, †P < 0.05 vs. MF. B and C: effect of macrophages and palmitic acid on IκB-α (B) and phospho (p)JNK (C) protein expression in myotube cocultures. Representative Western blots of myotube cell lysates after coculture with fibroblasts (F, MF) or macrophages (Mc, MMc) in the absence or presence (+P) of 0.2 mM palmitic acid for 48 h are shown. Bar plots are means ± SE of densitometric analysis of the Western blots normalized to β-actin from myotubes from 6 different subjects. *P ≤ 0.05 MMc+P vs. MMc, **P < 0.03 MMc vs. MF, †P < 0.01 MF+P vs. MF.

Fig. 6. Effect of macrophages and palmitic acid on pAkt (Ser473) protein expression in myotube cocultures. A: representative Western blot of pAktT (Ser473), total Akt, and β-actin in cell lysates from cocultures of myotubes with fibroblasts (F, MF) or macrophages (Mc, MMc) in the absence or presence (+P) of 0.2 mM palmitic acid for 48 h. After coculture, myotubes were treated with 0, 1, or 10 nM insulin for 20 min. B: densitometric analysis of Western blots of myotube cocultures for pAkt (Ser473) normalized to β-actin. Values are means ± SE from myotubes of 8 different subjects. *P < 0.02 MMc vs. MF, **P < 0.02 MMc+P vs. MF, †P = 0.012 MF+P vs. MMc, ‡P < 0.02 MF+P vs. MF.
and fatty acid metabolism, increased insulin resistance, and muscle wasting (25, 40, 65, 71). In coculture, these cytokines may be involved in autocrine activation of the pathways observed in myotubes, evidenced by downregulation of IκB-α and increased phosphorylation of JNK. JNK and NF-κB pathways are also activated by NEFA via pattern recognition TLRs, including TLR-4 (6, 30, 49, 54, 58) and TLR-2 (57), which converge to further upregulate the proinflammatory cytokines in muscle cells. Furthermore, mice lacking TLR-4 are protected from developing fatty acid-induced insulin resistance in muscle (58). In our coculture system, in addition to activating the TLRs, myotube TLR-4 gene expression was increased by palmitic acid, with macrophages showing no specific effect. For TLR-2, palmitic acid and macrophage coculture individually upregulated TLR-2 gene expression in myotubes, with no additive effect of the combined treatment. These results suggest that NEFA, especially when combined with macrophages, contributes to increased inflammatory signaling and amplifies cytokine expression by increasing myotube TLR-4 and/or TLR-2 gene expression. Finally, NEFA metabolites, DAG, and ceramide activate both NF-κB and JNK pathways in muscle cells (41, 53, 61). Thus upregulation of proinflammatory cytokines in combination with NEFA resulted in the most robust activation of the JNK/AP-1 and IκB-β/IκB-α/NF-κB pathways in myotubes in vitro, and would be expected to have the greatest effect on muscle cell metabolism.

The JNK and IκB-β/IκB-α/NF-κB pathways activate serine kinases (72) that inactivate IRS and thus inhibit Akt, a key mediator of the insulin signaling pathway. Examination of Akt phosphorylation in myotubes demonstrated the synergistic downregulation of phosphorylated Akt protein in the presence of macrophages and palmitic acid, with an apparent dose response to insulin. Thus fatty acids and cytokines activate diverse pathways in concert, potentially contributing to insulin resistance. Furthermore, insulin resistance is shown to accelerate muscle protein degradation (65). In skeletal muscle, the IRS/P13K/Akt signaling pathway is crucial in regulating muscle hypertrophy and atrophy. Activation of the pathway stimulates hypertrophy by increasing muscle protein synthesis. Inhibition of Akt phosphorylation in muscle decreases the phosphorylation of the transcription factor target FOXO1, resulting in its activation and nuclear translocation, and trans-activation of the E3 ubiquitin ligase genes MAFbx and MuRF-1 associated with protein degradation and muscle atrophy (52, 60). Accordingly, the synergistic blunting of phosphorylated Akt seen in the presence of macrophages and palmitic acid resulted in the greatest increase of MAFbx and MuRF-1 gene expression in myotubes, suggesting increased atrophy as seen with insulin resistance.

The chemokine MCP-1 is widely associated with insulin resistance and recruitment of macrophages in adipose tissue (20, 31, 32). In this study, MCP-1 gene expression was induced in myotubes after coculture with macrophages, and expression was further augmented in the presence of palmitic acid. Myoblast-derived MCP-1 has previously been implicated in the recruitment of macrophages to muscle during regeneration (14), and MCP-1 has been localized to human muscle satellite cells and macrophages after damaging exercise, where it has been proposed to play a role in cell-cell communication and adaptation (29). However, MCP-1 was also shown to induce insulin resistance in adipocytes and skeletal muscle cells in vitro (55). Our results suggest that exposure of muscle cells to proinflammatory macrophages in an obese milieu, represented by the addition of palmitic acid, may promote further macrophage infiltration through overexpression of MCP-1, potentially contributing to a cycle of increased macrophage infiltration in muscle during obesity. The increase in MCP-1 may be mediated by TNF-α, which is a known inducer of MCP-1 expression via induction of NF-κB and AP-1 pathways (45). Thus an obese environment may cause a modest increase in proinflammatory cytokines in muscle that stimulates the induction of MCP-1, which then contributes to macrophage recruitment, further increasing cytokines in muscle during obesity.

Along with the proinflammatory cytokines/chemokines in myotubes, a less robust enhancement of the expression of anti-inflammatory cytokines was observed by coculture with macrophages and NEFA. The anti-inflammatory cytokine IL-1RA is an endogenous highly selective antagonist to IL-1 and is upregulated in tandem with IL-1β and IL-1α. IL-10 is an
immunosuppressive and/or anti-inflammatory cytokine and acts by inhibiting cytokine production by monocytes/macrophages and neutrophils (15, 16, 23). As expected, our in vitro data showed the parallel increase of IL-1RA along with IL-1β. In our system, the observed induction of IL-10 was not sufficient to counter the harmful effects of the proinflammatory cytokines (51). An increase in secretion of IL-6 by myoblasts was previously demonstrated in response to soluble factors from activated peripheral blood monocytes that promoted myoblast proliferation (11). The precise role of IL-6 in muscle and insulin action is not clear, since this cytokine may be proinflammatory but also may be beneficial to muscle recovery from exercise. It is likely that the effects of IL-6 on muscle will largely depend on whether the elevation in IL-6 is acute, as in the case of exercise, or more chronic, as with inflammatory diseases of muscle (13, 26).

Our results suggest that overall an inflammatory state may exist in muscle during obesity as a result of macrophage infiltration. The presence of macrophages, coupled with the increase in NEFA from adipose tissue, may augment the expression of inflammatory cytokines from muscle, leading to a decrease in insulin sensitivity and the promotion of muscle atrophy.

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