Palmitate- and lipopolysaccharide-activated macrophages evoke contrasting insulin responses in muscle cells

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Samokhvalov V, Bilan PJ, Schertzer JD, Antonescu CN, Klip A. Palmitate- and lipopolysaccharide-activated macrophages evoke contrasting insulin responses in muscle cells. Am J Physiol Endocrinol Metab 296: E37–E46, 2009. First published October 7, 2008; doi:10.1152/ajpendo.90667.2008.—Factors secreted by macrophages contribute to whole body insulin resistance, acting in part on adipose tissue. Muscle is the major tissue for glucose disposal, but how macrophage-derived factors impact skeletal muscle glucose uptake is unknown, or whether the macrophage environment influences this response. We hypothesized that conditioned medium from macrophages pretreated with palmitate or LPS would directly affect insulin action and glucose uptake in muscle cells. L6-GLUT4myc myoblasts were exposed to conditioned medium from RAW 264.7 macrophages pretreated with palmitate or LPS. Conditioned medium from palmitate-treated RAW 264.7 macrophages inhibited myoblast insulin-stimulated glucose uptake, GLUT4 translocation, and Akt phosphorylation while activating JNK p38 MAPK, decreasing IkBα, and elevating inflammation markers. Surprisingly, and opposite to its effects on adipose cells, conditioned medium from LPS-treated macrophages stimulated myoblast insulin-stimulated glucose uptake, GLUT4 translocation, and Akt phosphorylation without affecting stress kinases or inflammation indexes. This medium had markedly elevated IL-10 levels, and IL-10, alone, potentiated insulin action in myoblasts and partly reversed the insulin resistance imparted by medium from palmitate-treated macrophages. IL-10 neutralizing antibodies blunted the positive influence of LPS macrophage-conditioned medium. We conclude that myoblasts and adipocytes respond differently to cytokines. Furthermore, depending on their environment, macrophages negatively or positively influence muscle cells. Macrophages exposed to palmitate produce a mixture of proinflammatory cytokines that reduce insulin action in muscle cells; conversely, LPS-activated macrophages increase insulin action, likely via IL-10. Macrophages may be an integral element in glucose homeostasis in vivo, relaying effects of circulating factors to skeletal muscle.

INSULIN RESISTANCE, particularly diminished insulin responsiveness of skeletal muscle, is a major factor in the development of type 2 diabetes (15). Obesity is frequently accompanied by insulin resistance, possibly through free fatty acids (FFA) and cytokines released by the overgrowing adipose tissue. Insulin resistance in skeletal muscle arises from reduced insulin signaling via insulin receptor substrate-1 (IRS-1) to the Akt axis and impaired gain in GLUT4 glucose transporters at the cell surface (2, 44). Major discoveries of the past few years ascribe the origin of obesity-related cytokines not only to adipose tissue but also to macrophages, which migrate to actively remodeling adipose tissue (20, 32, 33, 48, 49). Adipose tissue macrophages can be activated to release cytokines such as TNFα, IL-1β, and IL-6, known to cause insulin resistance in adipose tissue through activation of stress pathways characterized by IKKβ, JNK1/2, and ERK1/2. These pathways, in turn, cause chemokine synthesis and release by the adipose cells and insulin resistance of IRS-1 to the Akt axis within them (1, 8, 29). Insulin resistance in adipocytes results in increased lipolysis and consequent FFA release to the circulation (15).

In contrast to the ample documentation of low-grade inflammation in adipose tissue accompanying obesity and ensuing insulin resistance, it is less clear whether and how the full complement of macrophage-derived factors affects skeletal muscle. As well, it is debated whether macrophages migrate to skeletal muscle in the obese animal (16, 27) or whether circulating cytokines or FFA are major culprits of muscle insulin resistance. Indeed, both factors can reduce insulin signaling when individually added to skeletal muscle preparations. Specifically, TNFα alone reduces Akt signaling (3, 30, 43), and peripherally infused lipid mixtures reduce IRS-1 phosphorylation and content in muscle tissue (13). Inside muscle fibers, FFA are processed to form fatty acyl-CoA, ceramides, diacylglycerol, and triglycerides, with concomitant inhibition of insulin signaling initiated at the levels of IRS-1 (37) or Akt (17, 42). Thus, skeletal muscle insulin resistance arises from impaired lipid metabolism in adipose tissue, but the individual contribution and interplay of FFA and macrophage-derived factors require further study.

In addition, the possibility that FFA impinge on macrophages to compound release of cytokines and thereby affect skeletal muscle requires exploration. Emerging studies support this possibility, as saturated FFA themselves can promote inflammatory responses in resident macrophages of omental adipose depots through interaction with Toll-like receptor (TLR)2 and TLR4, which are otherwise receptors participating in the innate immune response (20, 27, 35, 38). Moreover, fatty acids promote release of proinflammatory cytokines from RAW 264.7 (RAW) macrophages and bone marrow-derived macrophages (27). Therefore, FFA could be considered an important factor in the genesis and/or propagation of chronic inflammation beyond adipose tissue (15).

Interestingly, it was recently shown that conditioned medium from fatty acid-activated bone marrow-derived macrophages reduced glucose uptake in muscle cells (16, 27). However, exactly how saturated FFA-activated macrophages downregulate insulin action in skeletal muscle cells remains largely unknown. Moreover, the contribution to muscle insulin resistance of factors from macrophages activated by classical bacterial cues remains to be explored. Lipopolysaccharide (LPS) is
a wall component of Gram-negative bacteria and a well-known activator of the TLR4 signaling and stimulator of proinflammatory responses in macrophages. Serum LPS levels are elevated in type 2 diabetes and high-fat feeding in rodents (4, 9), and direct LPS administration can render skeletal muscle resistant to insulin (4, 6). In addition, exposing adipocytes to conditioned medium from LPS-treated macrophages impaired insulin-stimulated glucose uptake through reduction in Akt signaling and GLUT4 expression and elevation in GLUT1 (24). However, exactly how LPS-activated macrophages affect insulin action in skeletal muscle cells has not been defined.

In the present study, we hypothesize that macrophage-derived factors uniquely affect insulin action in muscle cells and that this response may be influenced by the macrophage environment. To this end, we compared and contrasted the effects of conditioned medium from FFA-treated or LPS-treated RAW macrophages on insulin-stimulated glucose uptake, GLUT4 translocation, insulin signals, and markers of inflammation in L6 muscle cells. We report that conditioned medium from FFA-treated macrophages induced insulin resistance at the levels of GLUT4 translocation, glucose uptake, and Akt signaling, but conditioned medium from LPS-treated macrophages (10 ng/ml, 24 h) unexpectedly improved insulin responsiveness of all parameters tested. This response contrasted with its inhibitory action on insulin action on adipose cells. The positive effect of conditioned medium from LPS-treated macrophages could be causally attributed to the presence of the anti-inflammatory cytokine IL-10 in the medium from LPS-treated macrophages. Beyond showing muscle-specific reactions to macrophage-derived factors, the results support the participation of the macrophage as a relay in the action of fatty acids on muscle cells and further lend support to the use of IL-10 in antagonizing inflammation induced-insulin resistance.

MATERIALS AND METHODS

Materials and reagents. Monoclonal (9E10) and polyclonal (A-14) anti-myc antibodies were from Santa Cruz Biotechnology. Antibodies to phospho-Akt (Ser473), phospho-JNK (Thr183/Tyr185), phospho-ERK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-STAT3 (Tyr705), and IκBα were from Cell Signaling Technology. LPS from E. coli, o-phenylenediamine dihydrochloride (OPD reagent), sodium palmitate, fatty acid-free BSA, and protease inhibitor cocktail were from Sigma-Aldrich. Rat IL-10 and IL-10-neutralizing antibodies were from R & D Systems.

Cell cultures. L6-GLUT4myc rat myoblasts stably expressing GLUT4 with an exofacial myc epitope (L6-GLUT4myc) (47) and RAW macrophages were separately grown in α-MEM supplemented with 1% antibiotic-antimycotic and 10% fetal bovine serum. 3T3-L1 fibroblasts were differentiated into adipocytes as described earlier (46) and used 10 days after differentiation.

Macrophage activation with LPS. RAW macrophages were cultured for 24 h in α-MEM (10 ml per 10-cm-diameter dish) in the absence or presence of 10 ng/ml LPS. Because of the reversibility of macrophage activation by LPS (empirically determined), LPS was not removed prior to collecting the conditioned medium. Conditioned media from untreated macrophages (CM) or LPS-treated macrophages (CM-LPS) were centrifuged (7 min, 3,500 g) and used to incubate L6-GLUT4myc myoblasts or 3T3-L1 adipocytes for 24 h, followed by outcomes analyses (glucose uptake, surface GLUT4myc levels, kinase profiles, or cytokine production). As control, L6-GLUT4myc myoblasts were directly treated with 10 ng/ml LPS. Where indicated, IL-10-neutralizing antibodies (100 ng/ml) were added to CM-LPS for 1 h at 37°C prior to addition to L6-GLUT4myc cultures.

Macrophage activation with palmitate. A 5 mM palmitate-10% BSA stock solution was prepared as described (12). RAW macrophages were treated with BSA alone or with 500 µM palmitate/1% BSA in culture medium for 6 h and then washed several times with PBS, and fresh medium was added. After 12 h, the media were collected, centrifuged (7 min, 3,500 g), and used to incubate L6-GLUT4myc myoblasts for 24 h. Given the intermediate washes, no residual palmitate is expected to be present in conditioned medium from macrophages previously treated with palmitate-BSA (CM-P).

Cell lyses and immunoblotting. Confluent myoblasts in 10-cm-diameter dishes were treated with RM, CM, CM-P, or CM-LPS and immediately processed for SDS-PAGE and immunoblotting as described (28).

mRNA extraction and RT-PCR. RNA was extracted from myoblasts by guanidine isothiocyanate-phenol-chloroform (TRIZol, Invitrogen). Semiquantitative reverse transcriptase (RT)-PCR was performed on 200 ng of total RNA using a Qiagen One-Step RT-PCR kit (Qiagen) as follows: reverse transcription (50°C, 30 min), heat inactivation (95°C, 15 min), denaturation (94°C, 30 s), annealing (56°C, 30 s), and extension (72°C, 60 s). RT-PCR products were subjected to 2.5% agarose gel electrophoresis in TAE buffer, ethidium bromide stained, and photographed under ultraviolet light. The number of PCR cycles was titrated for each gene-specific primer pair target to ensure linearity.

2-deoxy-[3H]glucose uptake and cell surface GLUT4myc. L6-GLUT4myc myoblasts grown in 24-well plates and serum starved for 3–5 h were treated without or with insulin (100 nM, 20 min). 2-Deoxyglucose uptake was measured as described in Ref. 28, and cell surface GLUT4myc was measured as described in Refs. 45 and 47.

Cytokine determination. CM-P and CM-LPS were centrifuged (7 min, 3,500 g). Supernatants were analyzed by ELISA for mouse IL-6, TNFα, and IL-10 (ALPCO Immunoassays, Salem, NH).

Statistical analysis. Results are presented as means ± SE. Statistical analysis used ANOVA with a Bonferroni post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Effect of macrophage-conditioned media on glucose uptake and GLUT4 translocation in myoblasts. A first objective of this study was to compare the effects that pretreatment of macrophages with a saturated fatty acid or their classical activator LPS has on insulin action in L6-GLUT4myc myoblasts. In untreated myoblasts incubated in RM, insulin increased glucose uptake by 75 ± 9% (P < 0.05; Fig. 1A) and GLUT4myc translocation by 68 ± 5% (P < 0.05; Fig. 1B). Incubating myoblasts with CM had no effect on either basal glucose uptake or surface GLUT4myc levels or on their subsequent stimulation by insulin. On the other hand, in myoblasts incubated with CM-P, insulin-stimulated glucose uptake was reduced by 24 ± 2% (P < 0.05; Fig. 1A) and the gain in surface GLUT4myc by 26 ± 2% (P < 0.05; Fig. 1B) without any effect on the basal glucose uptake or surface GLUT4myc. These effects were likely due to diminished activation of intracellular insulin-dependent signals, since exposure of L6 myoblasts to CM-P reduced insulin-stimulated phosphorylation of Akt on Ser73 by 28 ± 3% (P < 0.05; Fig. 2A).

In stark contrast to CM-P, CM-LPS potentiated insulin-stimulated glucose uptake and GLUT4myc translocation in myoblasts (Fig. 1). Specifically, 24-h incubation with CM-LPS increased insulin-stimulated glucose uptake and GLUT4myc translocation by 28 ± 3 and 32 ± 4%, respectively (P < 0.05;
There was no effect of CM-LPS on basal glucose uptake or surface GLUT4 myc levels. CM-LPS also potentiated Akt phosphorylation in response to insulin (Fig. 2). These observations suggest that CM-P and CM-LPS contain different sets of macrophage-derived factors, which evoke completely distinct effects on insulin action in myoblasts.

It was recently documented that CM from J774 macrophages incubated with 0.1 ng/ml LPS for 16 h disrupts insulin signaling in 3T3-L1 adipocytes (24). Since the CM-LPS used in our study exerted an opposite effect on skeletal muscle cells, we tested whether it would still impair insulin action in 3T3-L1 adipocytes. Incubation of 3T3-L1 adipocytes with CM or CM-LPS significantly reduced the ability of insulin to stimulate glucose uptake, an effect mediated primarily through an increase in basal glucose uptake (Supplemental Fig. 1). As well, insulin-stimulated Akt phosphorylation in adipocytes was diminished by either CM or CM-LPS (Supplemental Fig. 1). These responses in 3T3-L1 adipocytes are in agreement with their responses to CM or CM-LPS derived from J774 macrophages (24), even though different concentrations of LPS were used in each case. Hence, our results highlight the divergent responses of muscle and fat cells.

It is important to stress that direct addition of 10 ng/ml LPS to L6-GLUT4myc myoblasts for 24 h had no effect on basal or insulin-stimulated glucose uptake, GLUT4myc cell surface density (Supplemental Fig. 2, A and B), or Akt phosphorylation nor on phosphorylation of stress kinases.

Effects of macrophage-conditioned media on inflammatory signals and stress kinases in myoblasts. Macrophages can elicit inflammatory or anti-inflammatory action in target tissues. Given the divergent effects of CM-P and CM-LPS on insulin signaling and action in myoblasts, we explored whether either medium evoked inflammatory responses in these cells. Compared with RM, CM did not alter the protein level of the inflammation marker IkBα nor the phosphorylation status of JNK, ERK, or p38 MAPK (Fig. 3). However, CM-P induced a reduction in IkBα and elevated the phosphorylation of JNK and p38 MAPK but not ERK (Fig. 3). In contrast, CM-LPS did not activate any of these markers of inflammatory signaling (Fig. 3). Hence, the selective inflammatory response of the myoblasts to CM-P correlates with the ensuing insulin resistance, and conversely, CM-LPS failed to elicit either an inflammatory response or insulin resistance in muscle cells.

Characterization of macrophage-secreted factors. The striking contrast between the effects of CM-P and CM-LPS on inflammatory indexes and insulin action in L6-GLUT4myc myoblasts suggests that each may contain distinct macrophage-derived factors. Macrophages secrete numerous cytokines, but only few are known to modulate insulin action. Among these, the proinflammatory TNFα and IL-6 stand out, as they cause insulin resistance in adipose and hepatic cells and muscle cells (19, 21, 51). Among the anti-inflammatory cytokines, IL-10 stands out, since it can oppose the negative effects of TNFα on growth factor signaling, and IL-10 has beneficial effects on insulin-regulated glucose homeostasis (21). We therefore determined the levels of these cytokines in the macrophage-conditioned media used in this study. CM-P was found to contain markedly elevated levels of TNFα and IL-6 (11.4 ± 1.5- and 5.3 ± 0.7-fold, respectively, P < 0.05 vs. CM),
whereas IL-10 was only modestly elevated (1.8 ± 0.2-fold, P < 0.05). In contrast, CM-LPS showed robust elevation in the anti-inflammatory cytokine IL-10 (16.2 ± 1.5-fold, P < 0.05; Fig. 4), along with the expected elevated levels of TNF-α and IL-6 (10.3 ± 1.2- and 2.1 ± 0.3-fold, respectively, P < 0.05).

LPS and palmitate differentially affect RAW macrophage polarization. Activated macrophages can be broadly categorized into two different states, M1, or proinflammatory, and alternatively M2, or anti-inflammatory (25). The enzymes iNOS and arginase control the production of NO and are used as markers of M1 and M2 polarization, respectively. Treatment of RAW macrophages with LPS or palmitate elevated the expression of iNOS (Fig. 5A), but, in striking contrast, LPS significantly elevated arginase expression compared with palmitate treatment (Fig. 5B). These results suggest that LPS polarizes RAW macrophages toward M2.

CM-P and CM-LPS upregulate expression of MCP-1 in L6 myoblasts. We next examined whether the differential alteration in inflammatory indexes in myoblasts incubated with CM-P and CM-LPS would correlate with changes in production of cytokines from the myoblasts themselves, creating a possible iterative cycle that might impact on insulin action. The results in Fig. 6 reveal that treatment with CM-P modestly enhanced the mRNA expression of TNF-α (1.5 ± 0.2-fold) and monocyte chemoattractant protein-1 (MCP-1) (1.8 ± 0.1-fold) in the myoblasts without affecting IL-6 mRNA. In contrast, CM-LPS exerted a much more potent upregulation of MCP-1 (8.9 ± 1.1 fold vs. 1.8 ± 0.1 for CM-P), mildly decreased the expression of TNF-α (21 ± 2%), and elevated IL-6 expression (1.6 ± 0.2-fold) in the myoblasts (Fig. 6). Notably, there were no changes in IL-10 expression within myoblasts in either condition (Fig. 6). Similarly, numerous other anti- and proinflammatory cytokines and their respective receptors (TNFR1, IL-6R, IL-10R, gp130, CCR2, and IL-4R) remained unaltered.
in L6 myoblasts after treatment with either CM-P or CM-LPS (Supplemental Fig. 3).

**IL-10 mimics the insulin-sensitizing effects of CM-LPS in myoblasts.** The finding that IL-10 was elevated in CM-LPS and a recent report that this cytokine stimulates insulin action in adipocytes (23) prompted us to test whether IL-10 could be a factor mediating the positive influence of CM-LPS on insulin action in L6 myoblasts. To this end, we explored the effects that direct addition of recombinant IL-10 or the depletion of endogenous IL-10 from CM-LPS would have on insulin action and glucose uptake in L6-GLUT4myc myoblasts. First, incubation with 60 ng/ml IL-10 recapitulated the potentiation of insulin-stimulated glucose uptake and GLUT4myc translocation and an increase in phosphorylation of Akt in myoblasts that had been caused by CM-LPS. Specifically, IL-10 increased insulin-stimulated glucose uptake and GLUT4myc translocation by 38 ± 5 and 31 ± 4%, respectively, compared with RM (P < 0.05; Figs. 7A and 6B). Furthermore, incubation of myoblasts with IL-10 potentiated insulin-stimulated Akt phosphorylation at Ser473 (Fig. 7C). However, the cytokine did not affect glucose uptake or Akt phosphorylation in the absence of insulin, although it activated its cognate signaling via Tyr705 in STAT3 (Fig. 7D), demonstrating a distinct action from that reported in adipocytes (23).

Doses of IL-10 lower than 20 ng/ml did not enhance insulin-stimulated glucose uptake (results not shown). It is possible that the recombinant interleukin is less potent than the macrophage-produced one, whether through posttranslational modifications, protein folding, or interaction with additional proteins present in CM-LPS.

Given the qualitative similarity between the direct action of recombinant IL-10 and the effect of CM-LPS on insulin action in myoblasts, we explored whether IL-10 might mediate some of the actions of CM-LPS. This was tested by depleting IL-10 from CM-LPS. Addition of an IL-10-neutralizing antibody (100 ng/ml) to CM-LPS prior to addition to L6-GLUT4myc myoblasts partially blocked the potentiation by CM-LPS of insulin-stimulated glucose uptake and surface GLUT4myc, so that the increase above the insulin-stimulated CM control was no longer statistically significant (Figs. 7C and 6D). The fact that CM-LPS depleted of IL-10 does not cause insulin resistance suggests that the levels of TNFα present are insufficient...
or are counteracted by other anti-inflammatory cytokines present in CM-LPS.

**IL-10 reduces the deleterious effect of CM-P on insulin action in L6 myoblasts.** As seen in Fig. 1, CM-P effectively inhibited insulin action in myoblasts, whereas CM-LPS potentiated it. Given the much higher levels of IL-10 in CM-LPS than in CM-P, we next explored whether addition of IL-10 to CM-P would overcome the inhibitory action that CM-P exerted on myoblasts. To this end, 60 ng/ml IL-10 was added to CM-P and then presented to L6-GLUT4myc myoblasts for 24 h, as usual. Figure 8 shows that addition of IL-10 partially prevented CM-P from disrupting insulin action on glucose uptake and GLUT4myc translocation in myoblasts such that the decrease below the insulin-stimulated CM control was no longer statistically significant. This result suggests that IL-10 not only exerts insulin-sensitizing effects but can also protect cells against the insulin resistance induced by proinflammatory cytokines.

**Fig. 6.** Gene expression of cytokines by L6-GLUT4myc myoblasts assessed by semiquantitative RT-PCR. L6-GLUT4myc myoblasts were incubated as described in Fig. 1. Immediately thereafter, total RNA was isolated for RT-PCR. Agarose gels of PCR products were imaged and quantified with the GelDoc-It Imaging System and ImageJ software. Illustrated are the PCR product levels for TNFα (A), IL-6 (B), MCP-1 (C), and IL-10 (D) for the indicated conditions. Data are means ± SE band intensity relative to ribosomal 18S mRNA of 3 experiments. *P < 0.05 relative to RM or CM.

**Fig. 7.** Exogenously added IL-10 potentiates insulin action in myoblasts and IL-10 neutralizing antibody partly reverses the enhanced insulin action induced by CM-LPS. L6-GLUT4myc myoblasts were treated with RM or RM supplemented with 60 ng/ml IL-10 for 24 h. Following 2 h of serum starvation in the continued absence or presence of IL-10, myoblasts were stimulated or not with insulin (100 nM, 20 min) and then assayed for 2-DG uptake (A) cell surface density of GLUT4myc (B) immunoblotting for phospho-Akt (Ser473; C), and immunoblotting for phospho-STAT3 (Tyr705; D). E and F: L6-GLUT4myc myoblasts were incubated for 24 h with RM, CM, CM-LPS, or CM-LPS preincubated with IL-10 neutralizing antibody (+nAb, 100 ng/ml, 1 h, 37°C). Following 2 h of serum starvation, myoblasts were stimulated or not with insulin (100 nM, 20 min). Data are presented as means ± SE of 3 experiments. *P < 0.05 relative to RM-basal, #P < 0.05 relative to CM-insulin. NS, not significantly different from CM-insulin.
DISCUSSION

There is growing evidence that secreted factors from the resident macrophages of adipose tissue contribute to the insulin resistance of adipocytes in obesity or type 2 diabetes (10, 18, 33). Recently, there has been growing appreciation of the role that FFA play in propagating this behavior and of the ability of FFA to signal through TLRs (27, 36). However, there is poor understanding of the effects of macrophage-derived products on muscle cells, a major cell type involved in insulin action. Here, we collected conditioned medium from macrophages exposed to two distinct stimuli, palmitate and the activator of the innate immune response, LPS, and compared their subsequent impact on insulin action in cultured skeletal muscle cells. The results showed an unexpected dichotomy in the responses by muscle cells to these conditioned media. Opposite to observations made in adipocytes, CM-LPS enhanced insulin-stimulated glucose uptake, GLUT4 translocation, and Akt phosphorylation in L6 myoblasts. In contrast, and following stimulated glucose uptake, GLUT4 translocation, and Akt phosphorylation in L6 myoblasts. In contrast, and following stimulated glucose uptake, GLUT4 translocation, and Akt phosphorylation in L6 myoblasts.

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IL-10 contributes to the positive cross talk. Additional factors present in CM-LPS may contribute to this effect. Although IL-6 (10–100 ng/ml, 2 h) reportedly increases basal and insulin-stimulated glucose uptake in L6 cells (5), we have not observed any change in either basal or insulin-stimulated glucose uptake in L6-GLUT4myc cells upon prolonged treatment (10 ng/ml, 18 h) with IL-6 (Ref. 31). We further show here that IL-6 neutralizing antibodies did not reduce the enhanced response of insulin-stimulated glucose uptake in these cells treated with CM-LPS for 24 h, arguing that this cytokine is not required for the potentiation of insulin action (Supplemental Fig. 4).

Regarding the cytokine(s) involved in the negative cross talk, it is unlikely that TNFα or IL-6 may be individually responsible for this action, given our previous observations that each of them either elevated the basal levels of glucose uptake and surface GLUT4 or very modestly stimulated insulin action in the same L6 muscle cells system (31).

Macrophages as a relay of fatty acids in the pathway leading to insulin resistance. Circulating FFA are elevated in obesity and type 2 diabetes, and FFA induce insulin resistance in skeletal muscle. A large body of work has demonstrated that FFA can directly affect insulin action in muscle through increased lipid accumulation and metabolism (26, 37, 42). Acyl-CoA enters the sphingolipid or triacylglyceride metabolic pathways, thereby elevating ceramides or diacylglycerols, and both lipid metabolites have been firmly linked to reduced insulin signaling at the levels of IRS-1 and/or Akt in skeletal muscle (for review see Refs. 37 and 42). More recently, FFA-induced insulin resistance has been functionally linked to activation of proinflammatory stress response in macrophages residing in adipose tissue. Interestingly, we found that CM-P stimulated JNK and p38 MAPK phosphorylation and elevated the expression of the proinflammatory cytokines TNFα and IL-6 in the target myoblasts. As such, the cocktail of secreted factors from FFA-treated macrophages propagates the stress response even in muscle cells. TNFα and IL-6 are proinflammatory cytokines that have both negative and positive effects on insulin-stimulated glucose uptake in muscle, depending on time of exposure and dosage (5, 31). The relatively higher amounts of IL-10 in CM-LPS compared with CM-P might counteract the relatively lower amounts of TNFα and IL-6 and other proinflammatory cytokines present in CM-LPS compared with CM-P. Future studies should define their combined contribution and whether phospho-JNK is responsible for the insulin resistance elicited by CM-P. Regarding the latter, exposure of C2C12 muscle cells to IL-10 diminished the stimulation of JNK phosphorylation by TNFα or IL-1β (40, 41).

LPS and palmitate induce opposite polarization of RAW macrophages. Macrophage activation varies with stimulus and environment and can be broadly divided into two polarization states, M1 and M2. M1 cells produce high levels of proinflammatory cytokines and display iNOS ac-
activation. By contrast, M2 polarized macrophages have enhanced secretion of anti-inflammatory cytokines and express arginase (23, 25). In the present study, treating RAW macrophages with palmitate and LPS significantly increased iNOS mRNA, but LPS also increased expression of arginase mRNA. These distinctive marker profiles suggest that LPS shifts RAW macrophages toward the M2 status. Alternative activation of macrophages toward M2 status might be a means to combat the proinflammatory signals from macrophages that cause insulin resistance in vivo.

**Divergent actions of CM-LPS in muscle and fat cells.** The finding that CM-LPS from RAW macrophages potentiated, rather than inhibited, insulin action in muscle cells was unexpected, since pretreatment of 3T3-L1 adipocytes with media from J774 macrophages exposed to much lower concentration of LPS caused effects consistent with insulin resistance. Specifically, basal glucose uptake was elevated so the fold response of insulin-stimulated glucose uptake was diminished, and Akt phosphorylation on Ser473 was significantly blunted (24). In contrast, we found that insulin-dependent stimulation of glucose uptake, GLUT4 translocation, and Akt phosphorylation were all enhanced in L6 muscle cells pretreated with CM-LPS for 24 h, without any change in JNK, p38 MAPK, and ERK and phosphorylation status. These observations suggest that the different doses of LPS used affect macrophages distinctly, that LPS elicited release of different factors from RAW and J774 macrophages, or that muscle and adipose cells respond differently to such media. The fact that CM and CM-LPS from RAW macrophages had inhibitory action on glucose uptake in 3T3-L1 adipocytes (Supplemental Fig. 1) while potentiating insulin action in L6 muscle cells argues that muscle and fat cells react in unique ways to a given mixture of cytokines. The cell-autonomous differences could pertain to the responses to IL-10 (see below) and/or to TNFα and IL-6. In fact, whereas adipocytes are highly sensitive to TNFα, skeletal muscle cells are highly resistant to the negative effects of this cytokine (11). In our previous study (31), we demonstrated that high doses of TNFα (10–50 ng/ml, 18 h) stimulated basal glucose uptake without affecting cell proliferation or mortality of L6-Glut4myc myoblasts. At similar times of treatment and doses, others have shown that TNFα modestly inhibits insulin-stimulated glucose uptake in L6 muscle cells or primary cultured myocytes (3, 39, 50). Given the relative resistance of muscle cells to deleterious actions of TNFα, the IL-10 present in CM-LPS may exert an anti-inflammatory effect that may override negative effects of the relatively low amounts of TNFα present in the CM-LPS. In contrast, the high sensitivity of adipocytes to TNFα may overwhelm the potentially positive influence of IL-10. Finally, it was unexpected to find that treatment with CM-LPS induced expression of MCP-1 in L6 greater than did treatment with CM-P. In spite of the downregulation of TNFα mRNA expression by CM-LPS in L6 myoblasts, the impact of MCP-1 on insulin action could be important. Skeletal muscle cells are sensitive to MCP-1 and this polypeptide can cause insulin resistance in these cells at physiological concentrations (34). However, that study did not analyze the interplay between MCP-1 and other cytokines, expected to take place in CM-P or CM-LPS. The harmful effect of MCP-1 might be overwhelmed by the presence of anti-inflammatory cytokines found in CM-LPS such as IL-10.

**IL-10 as a mediator of the insulin-potentiating action of macrophages.** From the above it emerges that muscle cells respond positively to CM-LPS but negatively to CM-P with regard to insulin signaling and GLUT4 translocation. As mentioned, a major difference between these two media is the elevated content of IL-10 in CM-LPS. This observation is reminiscent of the LPS-induced high secretion of IL-10 from alveolar-derived macrophages (7). Moreover, IL-10 could have autocrine effects on macrophages, reducing production of proinflammatory cytokines (14). Here, we show that the IL-10 present in CM-LPS is indeed responsible for part of the positive effect of this medium on muscle cells, since neutralization of IL-10 with specific antibodies blunted the positive effect of CM-LPS on insulin action in muscle cells. In addition, treating muscle cells with IL-10 alone recapitulated the positive effect of CM-LPS on insulin action. Intriguingly, treatment of RAW macrophages with higher doses of LPS (50 or 100 ng/ml, 24 h) than those used in this study lowered the concentration of IL-10 present in CM-LPS (unpublished observation). Conversely, treating RAW macrophages with a lower dose of palmitate used to treat macrophages (250 μM instead of 500 μM) elevated the concentration of IL-10 present in the macrophage medium (unpublished observation). This alerts one to the pliable nature of the cytokine response of macrophages and must be considered when comparing results across studies in the literature.

Interestingly, exogenous IL-10 partly counteracted the deleterious action of CM-P on insulin signaling in muscle cells. On the basis of these observations, we suggest that IL-10 contributes to the insulin-sensitizing properties of CM-LPS. In fact, others have demonstrated that coinfusion of IL-10 (0.5 μg/h) reverses the induction of insulin resistance on whole body glucose turnover and skeletal muscle glucose uptake caused by infusion of triglyceride emulsion (20% wt/vol, Liposyn II) or of IL-6 [0.5 μg/h (21)] using hyperinsulinemic euglycemic clamps. More recently, IL-10 reversed the resistance to IGF-I-stimulated muscle differentiation in muscle cells induced by TNFα. In that study, IL-10 reduced the activation of JNK, and this may be one mechanism by which IL-10 reverses the growth factor-resistant effects of the proinflammatory cytokines (41). Very recently, IL-10 was reported to have strong atheroprotective and antiatherogenic properties (22) and insulin mimetic action in adipocytes, stimulating Akt phosphorylation and glucose uptake (23). Thus, IL-10 has been associated with a beneficial role toward countering insulin resistance.

In summary, we provide proof-of-principle for cross talk from macrophages to skeletal muscle cells, which bears differences with the response of adipocytes to macrophage-derived factors. Depending on the stimulatory factors influencing macrophages, their influence on muscle cells may be positive or negative. IL-10 was found to mediate the positive aspect of the macrophage to muscle cell cross talk, acting as an insulin sensitizer that antagonized the negative effects of macrophage-secreted factors on muscle cells. The prevalence of the positive action of IL-10 may depend on the extent of its secretion and the coexisting levels of other cytokines. Our results further suggest that the well-known capacity of FFAs to induce insulin resistance may include action on macrophages, inducing them to secrete proinflammatory cytokines that will negatively impact on insulin action in skeletal muscle.
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