Excessive secretion of IL-8 by skeletal muscle in type 2 diabetes impairs tube growth: potential role of PI3K and the Tie2 receptor

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Obesity and type 2 diabetes (T2D) represent major health problems accompanying a Westernized lifestyle. Crucial features of T2D are insulin resistance and impaired glucose disposal in muscle (11). Importantly, skeletal muscle capillarization mediate the supply of oxygen, substrates, hormones, and inflammatory cells from the circulation (20). Inadequate capillarization of skeletal muscle (SkM) may lead to relative hypoxia and an inflammatory response as well as decreased metabolism. Indeed, it has been demonstrated that insulin resistance is associated with lower skeletal muscle capillarization (40, 47).

In skeletal muscle, both myocytes and capillaries undergo rapid remodeling in the face of changing patterns of contractile work, environmental stresses, neurohormonal stimuli, or pathological conditions (50, 64, 65). Morphological changes in muscle, particularly the size of muscle cells and capillary density (CD), are associated with changes in fasting insulin concentrations and glucose tolerance (26, 33). In support of this concept, we and others (40, 42, 43) have shown that CD is reduced in skeletal muscle from obese T2D subjects and that both CD and insulin action are improved with thiazolidinedione treatment (43).

Over the past decade, evidence that skeletal muscle is a secretory tissue, releasing factors termed myokines, has accumulated (52). Several of these myokines, IL-8, GROα, and IL15, while acting as modulators of inflammation (2, 52), are also known to be proangiogenic factors (12, 15, 52). Multiple lines of evidence support associations between circulating levels of IL-8, GROα, and IL-15 and insulin resistance (44, 48, 52, 67). Of more direct relevance is that our laboratory and others found recently that insulin-resistant human myotubes secreted higher levels of IL-8, GROα, and IL-15 compared with normal myotubes (3, 8).

In the current report, we sought to investigate the seeming discrepancy between elevated secretion of multiple proangiogenic factors by T2D myotubes and reduced CD in skeletal muscle from T2D subjects. As a surrogate for angiogenesis in muscle, we employed human umbilical vein endothelial cells (HUVEC), which form capillary tube-like structures in culture. Selected results in HUVEC were verified by monitoring capillary outgrowth from skeletal muscle explants. Understanding the roles of IL-8, GROα, and IL-15 in regulating angiogenesis and the signaling pathways involved could shed light on their ability to modulate vascularization and ultimately the metabolic function of skeletal muscle in T2D.

METHODS

Subjects. Muscle samples were obtained from 13 nondiabetic (ND) and 12 T2D subjects. The designation as T2D was dependent on an existing clinical diagnosis. Subjects with T2D were considered to be in good control, and antidiabetic medication use was stable for ≥3 mo before biopsy. The majority of the subjects with T2D were receiving metformin either alone or in combination with glyburide or glipizide. Clinical characteristics of the subjects are presented in Table 1. The Committees on Human Investigation of the University of California San Diego and Veterans Affairs San Diego Healthcare System approved the experimental protocol. Informed written consent was obtained from all subjects after explanation of the protocol.

Cell culture and muscle tissue. HUVEC (Lonza, Walkersville, MD) were maintained in defined endothelial growth medium-2 (EGM-2) supplemented with 2% fetal calf serum (FCS). Skeletal muscle tissue explant and cell cultures were established from muscle tissue obtained by needle biopsy of the vastus lateralis.
Protein extraction and immunoblotting. HUVEC were treated with recombinant (r) IL-8 in the presence of serum-free EGM-2 for 15–60 min (acute exposure). For chronic exposure experiments, HUVEC were treated with rIL-8 or CM from myotubes for 24–72 h with or without anti IL-8-neutralizing antibodies (R & D Systems) or the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 (Sigma, St. Louis, MO) and then lysed in extraction buffer (7, 38). Frozen muscle tissue was homogenized with a Polytron at half speed and lysed in extraction buffer, as described previously (6).

HUVEC and muscle tissue protein extracts were resolved in 10 and 12% SDS-PAGE, respectively, under reducing conditions, transferred to nitrocellulose membranes, and blocked with Odyssey block (LI-COR Biosciences, Lincoln, NE). HUVEC samples were incubated with one of the following antibodies, all obtained from Cell Signaling Technology (Danvers, MA): Tie2, capase-3, Akt, phospho-Akt (Ser473), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), focal adhesion kinase (FAK), or phospho-FAK (Tyr925). Muscle tissue samples were incubated with anti-IL-8 antibody (R & D Systems, Minneapolis MN). Secondary IRDye antibodies were obtained from LI-COR Biosciences. Detection and quantification of band intensity was performed using the Odyssey Infrared Imaging System and Image Studio analysis software (version 3.1.4).

In vitro angiogenesis assay. Measurements of tube formation by HUVEC were achieved using an in vitro assay (13). Matrigel (BD Bioscience, San Jose, CA) was added to 96-well plates (50 μl/well) and incubated at 37°C for 60 min. HUVEC were cultured in EGM-2 containing 2% FCS and rIL-8, rGROα, and/or rIL-15 (all recombinant proteins are human low endotoxin from R & D Systems), media alone, or CM from myotubes for 24–72 h and then seeded onto matrigel at 10,000 cells/well. Each experiment was repeated at least three times in quadruplicate. In control studies, the extent of tube formation with EGM-2-2% FCS was minor and with serum free α-MEM containing 0.1% BSA was negligible. In some experiments, anti IL-8-neutralizing antibodies (R & D Systems) or a PI3K inhibitor (LY-294002; Sigma) were added. Different numbers of cells were seeded for treatment with inhibitors and CM (5,000 cells/well) or rIL-8 (7,500 cells/well), permitting more accurate detection of altered tube formation distinct from that induced by varying concentrations of IL-8.

Quantification of vessel formation in cultured cells. Phase contrast images were captured on a Nikon TS100 microscope with a ×4 objective and then analyzed using the NIH ImageJ program. The readout for this assay is the formation of capillary-like tubes. A tube is defined as a closed network unit or an intact loop. Results were obtained by counting the number of tubes in a field (13, 41, 60). For statistical analysis, the average number of tubes per 500-μm² field for three fields for each condition out of three independent wells for each experiment (3 × 3 = 9 fields) was calculated (unless otherwise noted).

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>n (F/M)</th>
<th>Age, yr</th>
<th>BMI, kg/m²</th>
<th>Fasting Glucose, mM</th>
<th>Fasting Insulin, pM</th>
<th>HOMA-IR</th>
<th>Serum</th>
<th>Secreted</th>
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<tr>
<td>ND</td>
<td>13 (1/12)</td>
<td>55.2 ± 3.5</td>
<td>27.7 ± 1.2</td>
<td>5.0 ± 0.2</td>
<td>30 ± 6</td>
<td>0.8 ± 0.3</td>
<td>7.83 ± 0.507</td>
<td>543 ± 179</td>
</tr>
<tr>
<td>T2D</td>
<td>12 (2/10)</td>
<td>60.4 ± 2.7</td>
<td>31.6 ± 1.9</td>
<td>8.8 ± 1.2</td>
<td>114 ± 24</td>
<td>6.1 ± 1.5</td>
<td>8.65 ± 3.66</td>
<td>6,351 ± 883</td>
</tr>
<tr>
<td>P value</td>
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Values are means ± SD. F, females; M, males; ND, nondiabetic; T2D, type 2 diabetic; HOMA-IR, homeostatic assessment of insulin resistance.

Assay of ex vivo angiogenesis and quantification of capillary sprout formation. To test the physiological relevance of observations made in HUVEC, similar experiments were performed using SKM tissue explants. The assay was performed by employing established protocols (21, 27, 53). Briefly, freshly harvested human SKM was cut into ∼0.5-mm³ pieces, which were then embedded individually in wells of a 24-well plate in growth factor-depleted matrigel (280 μl/well) and cultured in EGM-2-MV. A portion of the tissue explants was treated with rIL-8. Media and treatments were replaced every other day. Routinely, three independent explants per subject were embedded. Each explant was analyzed for capillary number by light microscopy and images captured with a ×4 objective. The readout for this assay is the formation of capillary sprouts. A capillary is defined as a branching structure of at least three cells connected to each other in a linear manner (53). The existence of capillaries at the periphery of the growth area was quantified by three separate observers (from each explant) using the NIH Imagej program. In some experiments, anti IL-8-neutralizing antibodies were added. It has been demonstrated that >90% of sprouting cells from the explants express endothelial cell markers and thus represent angiogenic growth (21, 27, 53).

Determination of IL-8 level. Determination of IL-8 level in serum and myotube CM was performed using Miliplex Map kits (Millipore), using a BioPlex instrument (Bio-Rad, Hercules, CA). The sensitivity of the assay for IL-8 is 0.2 pg/ml, whereas inter- and intra-assay CVs are 12 and 7%, respectively.

For direct comparison of levels of IL-8 in myotube CM and CM from SKM explants, both CM were collected after 24 h, and IL-8 levels were determined using the DuoSet ELISA kit for human IL-8 (R & D Systems). The sensitivity of the assay is 31.3 pg/ml, whereas inter- and intra-assay CVs are 7.9 and 6.1%, respectively.

LDH assay. HUVEC on matrigel were treated with rIL-8 in the presence of EGM-2-2% FCS for 24–72 h. Media were collected, centrifuged, and stored at −80°C. LDH release into the media was quantified using an in vitro toxicology assay kit (Sigma).

Glucose and lactate assay. HUVEC CM were collected as described for LDH assay. Glucose and lactate content was quantified using a dual glucose/lactate analyzer (YSI, Yellow Springs, OH).

Data analysis. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA). Between-group comparisons were evaluated by independent group t-test if the data were normally distributed and with a Mann-Whitney test for nonnormally distributed data. Within group comparisons (treatment effects) were evaluated by paired t-test. For results that were not normally distributed, data were log-transformed for statistical analysis and then back-transformed and reported in original units as means ± SD. Statistical significance was accepted as P < 0.05. The number of individual determinations for each measurement is indicated in the figure legends.

RESULTS

Effects of myotube-CM on tube formation. To explore the paracrine effects of skeletal muscle on tube formation by endothelial cells, HUVEC were cocultured with CM generated by fully differentiated myotubes derived from T2D and ND subjects, and the tube number was determined. Compared with
the untreated control, CM from both ND and T2D myotubes induced tube formation by HUVEC. However, after 24 h of treatment, HUVEC that were incubated with CM from T2D myotubes (T2D-CM) exhibited a significantly lower number of tubes compared with those exposed to CM from ND myotubes (ND-CM; \( P < 0.0001 \)) (Fig. 1, A and B). The difference between groups was sustained over 48 and 72 h of treatment (both \( P < 0.0001 \)).

Effects of exogenous myokines on tube formation. We and others have reported recently that insulin-resistant myotubes secrete higher levels of IL-8, IL-15 and GRO\( \alpha \), as well as other myokines, compared with cells from healthy subjects (3, 8). To
explore whether the higher levels of these myokines are involved in the impaired tube formation by HUVEC after incubation with T2D-CM, a mixture of exogenous rIL-8, rGROα, and rIL-15 was added to HUVEC in concentrations equal to the averages of those found in T2D-CM and ND-CM from myotubes isolated from a larger cohort of subjects (T2D-IL-8 = 3,280 ± 2,182 and ND-IL-8 = 2,071 ± 1,842 pg/ml, \( P < 0.05 \); GROα = 4,620 ± 2,799 and 1,859 ± 1,396 pg/ml, \( P < 0.001 \); and IL-15 = 1.07 ± 0.71 vs. 0.6 ± .040 pg/ml) (8). After 24 h of treatment with the mixture equivalent to T2D-CM, HUVEC

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Fig. 2. Effects of combined or individual exogenous myokines on HUVEC tube formation. Tube formation after incubation with a mixture of recombinant (r)IL-8, rGROα, and rIL-15 (A) or rIL-15 (C), rGROα (E), and IL-8 (G) in concentrations equivalent to ND-CM (a–c) or T2D-CM (d–f) for 24 (a and d), 48 (b and e), or 72 h (c and f). Quantification of tube formation induced by rIL-8 + rGROα + rIL15 (B), rIL-15 (D), rGROα (F), or rIL-8 (H) in concentrations equivalent to ND-CM (black bars) or T2D-CM (open bars). Means ± SD; \( n = 3 \). ***\( P < 0.005 \) and **\( P < 0.01 \), T2D vs. ND.
exhibited a significantly lower number of tubes compared with the ND-CM mixture ($P < 0.0001$; Fig. 2, A and B). Similar effects were observed after 48- ($P < 0.0005$) and 72-h ($P < 0.01$) treatment. Thus, a combination of IL-8, IL-15, and GROα was able to recapitulate the effects of myotube CM on tube formation, including the difference between ND and T2D-CM. Next, we examined the effects of individual myokines. No significant differences between groups were obtained after addition of rIL-15 (Fig. 2, C and D) or rGROα (Fig. 2, E and F) in concentrations equivalent to ND-CM or T2D-CM. However, when rIL-8 was added to HUVEC, we found that after 24 h of treatment the higher concentration of IL-8 (T2D level) resulted in a significantly lower number of tubes compared with the ND level ($P < 0.0001$; Fig. 2, G and H). Similar results were obtained after 48 and 72 h (both $P < 0.0001$). These results suggest that the higher levels of IL-8 secreted from T2D myotubes might contribute to lower capillarization in T2D skeletal muscle.

Dose dependency of IL-8 regulation of angiogenesis. The levels of exogenous IL-8 that exerted differential effects on endothelial capillarization replicated the average values in ND and T2D-CM. The dose dependency of these actions was investigated in greater detail by adding rIL-8 to HUVEC over a concentration range (207–10,350 pg/ml) representing the lowest to highest levels found in myotube CM (8). A clear biphasic dose dependency of rIL-8 on angiogenesis was seen after 24 h of treatment (Fig. 3). Low concentrations of IL-8 (207–2.071 pg/ml) significantly increased the number of tubes formed by HUVEC over control ($P < 0.0001$), whereas higher concentrations (3.280–10,350 pg/ml) impaired tube formation ($P < 0.0001$ vs. 2.071 pg/ml). Similar results were obtained after 48 and 72 h (Fig. 3). Thus, the transition from optimal stimulation to impairment of tube formation occurs over the range represented by the difference between secretion from ND and T2D myotubes.

Specificity of IL-8 action on angiogenesis. The specificity of IL-8’s effects on endothelial capillarization was examined by the use of IL-8-neutralizing antibodies. ND or T2D-CM levels of rIL-8 were added to HUVEC with and without anti-IL-8 Ab. Addition of 4 μg/ml anti-IL-8 antibody to T2D-IL-8 significantly increased tube number ($P < 0.01$) over T2D-IL-8 alone. Conversely, the same concentration of neutralizing antibody significantly impaired the induction of tube formation by ND-IL-8 ($P < 0.005$). The addition of a higher concentration of IL-8-neutralizing antibody (20 μg/ml) to the system inhibited the angiogenesis induced by either concentration of rIL-8 (both $P < 0.005$; Fig. 4, A and B). The addition of a nonrelevant antibody had no effect on angiogenesis induced by rIL-8 (Fig. 4, A and B).

The importance of endogenous IL-8 on the effects of myotube-CM on angiogenesis was also examined with IL-8-neutralizing antibodies. The addition of 4 μg/ml anti-IL-8 antibody significantly elevated tube formation by HUVEC incubated with T2D-CM ($P < 0.0001$), whereas it reduced the number of tubes induced by ND-CM ($P < 0.0001$). Addition of a nonrelevant antibody did not affect the angiogenesis regulated by CM (Fig. 4, C and D).

IL-8 and HUVEC viability. The ability of high levels of IL-8 to impair tube formation could be attributed to lower stimulation of angiogenesis or to induction of cell damage and death, leading to the loss of existing capillaries (rarefaction). To address the second possibility, measurements of cell viability and metabolism were monitored in HUVEC under similar conditions. After 24–72 h of treatment there were no differences between HUVEC exposed to ND-IL-8 or T2D-IL-8 with regard to cleavage of caspase-3 (Fig. 5A), release of LDH into the media (Fig. 5B), or glucose (Fig. 5C) and lactate (Fig. 5D) concentrations in the media. The data suggest that impaired tube formation by endothelial cells exposed to T2D levels of IL-8 is presumably not an outcome of major changes in HUVEC metabolism or viability but rather specific disruption of events essential to triggering and maintaining angiogenesis.

IL-8 activation of angiogenic signaling. Multiple signaling pathways are involved during induction of angiogenesis by IL-8, including PI3K/Akt, mitogen-activated protein kinases (MAPKs), and FAK/Src. Some pathways trigger tube formation, whereas others mediate tube stabilization (61). To address the possibility that different concentrations of IL-8 might initiate a bifurcation of signaling, HUVEC were treated with ND and T2D-IL-8, and proteins were extracted for the evaluation of protein phosphorylation. Differences were noted in the responses to the two levels of IL-8. Phosphorylation of FAK (Tyr397) in HUVEC was rapidly (15 min) stimulated by ND-IL-8 (Fig. 6A, a and b, and 6B) and was higher than T2D-IL-8 ($P < 0.01$). The effect was transient, since at the 30- and 60-min time points there were no significant differences in FAK phosphorylation between the two concentrations.

A distinctly different behavior was observed regarding phosphorylation (Thr202/Tyr204) of ERK1/2 (Fig. 6A, c and d, and C). At 15 min of treatment, phosphorylation was higher in response to T2D-IL-8 ($P < 0.05$). The response to T2D-IL-8 was rapidly lost so that by 30 min phosphorylation was higher in cells treated with ND-IL-8 ($P < 0.01$).

A similar pattern was seen for IL-8’s effects on Akt phosphorylation (Ser473). The response to T2D-IL-8 was rapid (Fig. 6A, e and f, and D), and 15-min treatment was greater than that of ND-IL-8 ($P < 0.005$) and highly transient. Stimulation of Akt phosphorylation by ND-IL-8 was slower, significantly higher than control at 30 min and sustained (Fig. 6A, e and f, and D), such that the response to ND-IL-8 was higher than that of T2D-IL-8 after both 30 ($P < 0.005$) and 60 min ($P < 0.05$).
Role of PI3K in IL-8 regulation of angiogenesis. The addition of the PI3K inhibitor LY-294002 (3 μM) reduced tube formation in response to both ND-IL-8 (P < 0.0001) and T2D-IL-8 (P < 0.01). However, we found that the inhibition of tube formation by LY-294002 was greater in HUVEC treated with ND-IL-8 compared with T2D-IL-8 (P < 0.0001). Similar inhibitory effects were obtained with 15 μM LY-294002 (both P < 0.0001), and the inhibition by 15 μM LY-294002 was also greater in HUVEC treated with ND-IL-8 compared with T2D-IL-8 (P < 0.0001; Fig. 7, A and B).

The ability of PI3K to mediate the biphasic effects of myotube-CM on angiogenesis was also examined with LY-294002. LY-294002 (3 μM) reduced tube formation in response to both ND-CM (P < 0.0001) and T2D-CM (P < 0.05). However, we found that the inhibition of tube formation by LY-294002 was greater in HUVEC treated with ND-CM compared with T2D-CM (P < 0.01). Similar inhibitory effects were obtained with 15 μM LY-294002 (P < 0.0001 for ND-CM and P < 0.01 for T2D-CM), as the inhibition by 15 μM LY-294002 was again greater in HUVEC treated with ND-CM compared with T2D-CM (P < 0.01) (Figs. 6D and 7C).

IL-8 regulation of Tie2 receptor expression is mediated via PI3K. The Tie2 receptor is a known promoter of angiogenesis (18). Because certain cytokines have been shown to modulate the expression level of Tie2, we asked whether the dose dependency of signals mediated by IL-8 involved regulation of Tie2. HUVEC were treated with ND- and T2D-IL-8, and the protein expression of Tie2 was determined. The expression of Tie2 was increased after 24 h of incubation at either concentration (Fig. 8A). However, after 48 h with T2D-IL-8, Tie2 expression was lower compared with treatment with ND-IL-8.
Similar effects were detected under physiological conditions since, after 48 h of incubation with T2D-CM, Tie2 expression in HUVEC cells was lower compared with treatment with ND-CM (\(P < 0.01\); Fig. 8B). Moreover, the addition of 4 \(\mu g/ml\) anti-IL-8 antibody to HUVEC incubated with ND-CM decreased the level of Tie2, whereas the same treatment of HUVEC incubated with T2D-CM increased the level of Tie2, just as was seen for tube formation. The differences between ND-CM and T2D-CM were significant (\(P < 0.01\)).

The addition of 20 \(\mu g/ml\) anti-IL-8 antibody to HUVEC incubated with ND-CM or T2D-CM decreased the level of Tie2, yet the level of Tie2 in T2D-CM was significantly higher than that in ND-CM (\(P < 0.05\); Fig. 8C and D).

Next, we explored whether the involvement of PI3K in angiogenesis might include regulation of Tie2 expression. The addition of LY-294002 (3 \(\mu M\)) reduced Tie2 expression in response to ND-IL-8 after 48 h (\(P < 0.05\)), whereas it did not alter the expression in response to T2D-IL-8 (Fig. 8, E and F). A higher dose of LY-294002 (15 \(\mu M\)) significantly reduced Tie2 expression in response to both ND- (\(P < 0.005\)) and T2D-IL-8 (\(P < 0.005\)). However, we found that the reduction of Tie2 content induced by LY-294002 was significantly greater in HUVEC treated with ND-IL-8 compared with T2D-IL-8 (\(P < 0.01\); Fig. 8, E and F).

Physiological role of endogenous IL-8 in regulating SkM vascularization. The fundamental assumption on which the current work is based is that elevated IL-8 secretion by T2D myotubes is consistent with elevated IL-8 levels in SkM tissue.

To test the validity of this assumption, we compared the content of IL-8 in skeletal muscle from ND and T2D subjects. Similarly to what was found in CM from T2D myotubes, we observed significantly higher IL-8 content in T2D SkM compared with tissue from ND subjects (\(P < 0.05\); Fig. 9). IL-8 is known to exist in multimeric forms (19, 63). Interestingly, the distribution between multimers appears to differ between serum and SkM tissue (Fig. 9A). SkM explants produce and release IL-8. In fact, over 24 h, explants secrete ~110–120% as much IL-8 as myotubes from the same subjects when normalized to tissue or cell protein (not shown). Consistent with the higher IL-8 content of SkM tissue from T2D subjects (Fig. 9), levels of IL-8 secreted from the T2D myotubes included in this specific cohort are also higher than those released by ND myotubes (Table 1). Meanwhile, circulating levels of IL-8 in the same subjects did not differ between ND and T2D subjects and are orders of magnitude lower than what is released into muscle (Table 1).

Although the HUVEC line is widely accepted as a model system for the study of angiogenesis (5, 11), to establish the physiological relevance of our in vitro observations on the paracrine function of IL-8 as a regulator of SkM vascularization, we extended our studies to an ex vivo system. ND and T2D-SkM explants were embedded in matrigel and exposed to endogenous and exogenous IL-8. The absolute total level of endogenous and exogenous IL-8 the tissue was exposed to replicated the range of values in ND and T2D-CM. A clear biphasic dose dependency of IL-8 on capillary outgrowth, a
and induction of an insulin-resistant state (66). Previously, our regulated by differentiation (50), electrical stimulation (49), DISCUSSION

IL-8-neutralizing antibody (20 SkM. Moreover, the addition of a high concentration of the T2D-like levels reduced the response in both ND- and T2D-results. Supplementing the media with exogenous IL-8 to higher concentrations (both (Fig. 10). Low (ND) concentration of endogenous IL-8 signif- int by 10.220.33.2 on June 25, 2017 http://ajpendo.physiology.org/ Downloaded from

marker of angiogenesis, was seen after 6 days of treatment (Fig. 10). Low (ND) concentration of endogenous IL-8 significantly increased the number of capillaries over T2D-like higher concentrations (both $P < 0.0001$), similar to the in vitro results. Supplementing the media with exogenous IL-8 to T2D-like levels reduced the response in both ND- and T2D-SkM. Moreover, the addition of a high concentration of the IL-8-neutralizing antibody (20 µg/ml) to T2D-SkM decreased capillary outgrowth ($P < 0.0001$) (Fig. 10B).

**DISCUSSION**

Following on the observation that exercise induces a large release of IL-6 from SkM tissue (59), considerable attention has been paid to the ability of skeletal muscle to act as an endocrine organ (reviewed in Ref. 52). Studies in cultured SkM cells have shown that myokine expression and secretion are regulated by differentiation (50), electrical stimulation (49), and induction of an insulin-resistant state (66). Previously, our group found that T2D myotubes released higher levels of TNFα. Recently we and others found the same to be true for additional factors (3, 8). Among those factors are IL-8, GROα, and IL-15 that, in addition to their immunomodulatory function, are known modulators of angiogenesis (12, 15, 52). Given that T2D is associated with disordered vascularization (31) and with reduced CD in skeletal muscle specifically (26, 33, 40, 43), we wished to explore the potential paradox between a T2D-related increase in proangiogenic factors and reduced vascularization, with both occurring in skeletal muscle.

Our initial observation was that factors secreted by T2D myotubes in CM could induce in HUVEC a behavior similar to that seen in vivo in T2D subjects, where tube formation is reduced compared with nondiabetics. Several lines of evidence indicate that IL-8 is the primary factor responsible for the reduced tube formation and capillary outgrowth under T2D conditions. One is that rIL-8 in HUVEC cells displayed a biphasic dose response for its angiogenic effects over the range of levels found in CM from T2D and ND myotubes, with high levels (T2D-IL-8) impairing it; neither IL-15 nor GROα displayed similar effects. Second, an IL-8-neutralizing antibody was able to influence tube formation induced by both exogenous IL-8 and CM, essentially retracing the IL-8 dose-response curve (Fig. 6) from higher to lower concentrations.

Angiogenesis is a multistep process that requires EC activation, migration, proliferation, and tube formation (58). The HUVEC tube formation assay as we employed it does not distinguish between these individual processes, nor does it reveal rarefaction of existing tubes. The same is true for following capillary outgrowth from SkM explants. However, our control studies show that the viability of HUVEC is maintained regardless of the concentration of IL-8 to which they are exposed and that reduction of tube formation at T2D levels of IL-8 is a specific response, suggesting that under these conditions it is angiogenesis that is the primary process being regulated.

IL-8 is a member of the CXC chemokine family that has been shown to regulate endothelial cell migration, proliferation, and angiogenesis (39) as well as induce neutrophil migration (61). Previous studies demonstrated a biphasic dose dependency for the ability of IL-8 to induce migration of multiple cell types, including HUVEC (4, 9, 14, 17, 22, 55, 56). Because one of the key steps in angiogenesis is migration of endothelial cells (46), these results are consistent with the biphasic nature of the IL-8 effect on angiogenesis reported here. IL-8 signaling to these various responses is mediated by the PI3K/Akt (22, 35), MAPK, and FAK (17, 34) pathways. A dose dependency for IL-8 is present at the level of FAK, where levels of IL-8 that stimulate cell migration result in a different pattern of FAK phosphorylation and subcellular localization than higher concentrations that attenuate migration (17). We found the same thing to be true with regard to FAK phosphorylation (Fig. 5). The importance of PI3K in IL-8-stimulated angiogenic signaling is emphasized by our results showing the dose dependency of Akt phosphorylation and the effects of PI3K inhibition on IL-8-induced tube formation and Tie2 receptor expression. The differences between ND- and T2D-IL-8 on FAK and Akt phosphorylation and angiogenesis would be examples of hormesis, where low doses of an agent prove stimulating, whereas higher doses can be inhibitory or even toxic (24, 57). A similar behavior was observed with PC-3P cells overexpressing IL-8; when transplanted into mice they

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![Image](97x569 to 151x598)

![Image](230x415 to 304x496)

![Image](230x626 to 304x706)

![Image](232x523 to 302x600)

![Image](243x620)

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![Image](316x54)
Fig. 7. Effect of phosphatidylinositol 3-kinase (PI3K) inhibition with LY-294002 on HUVEC tube formation induced by rIL-8. A: tube formation after incubation with rIL-8 in concentrations equivalent to ND-CM (a–c) or T2D-CM (d–f) for 24 h with 0 (a and d), 3 (b and e), or 15 µM LY-294002 (c and f). B: quantification of tube formation. Control (black bars) or 3 (open bars) or 15 µM LY-294002 (striped bars). C: tube formation after incubation with ND-CM (a–c) or T2D-CM (d–f) for 24 h with 0 (a and d), 3 (b and e), or 15 µM LY-294002 (c and f). D: quantification of tube formation. Control (black bars) or 3 (open bars) or 15 µM LY-294002 (striped bars). Means ± SD; n = 6 for ND-CM and n = 4 for T2D-CM, with each experiment repeated 3 independent times. ***P < 0.005, LY-294002 vs. control; **P < 0.01, LY-294002 vs. control; *P < 0.05, LY-294002 vs. control.

Fig. 8. IL-8-regulated Tie2 expression is mediated via PI3K. A: rIL-8 in concentrations equivalent to ND-CM or T2D-CM was added to HUVEC for 24–48 h. Representative Western blot for Tie2. B: ND-CM or T2D-CM was added to HUVEC for 48 h. Representative Western blot for Tie2. C: ND-CM (2.5 µg; top) or T2D-CM (13 µg; bottom) was added to HUVEC for 48 h with 0, 4, or 20 µg/ml α-IL-8. Representative Western blot for Tie2. Line between lanes at top denotes rearrangement to place lanes in the appropriate order; space indicates that images are from 2 separate gels containing samples from the same experiment run at the same time. D: quantification of Western blots; 4 (open bars) and 20 µg/ml α-IL-8 (striped bars). Results are % control of each condition (ND-CM and T2D-CM). E: rIL-8 in concentrations equivalent to ND-CM or T2D-CM was added for 48 h, without or with 3 or 15 µM LY-294002. F: quantification of Western blots; control (black bars) or 3 (open bars) or 15 µM LY-294002 (striped bars). Means ± SD; n = 6 in A, n = 2 in B, n = 2 in C, and n = 2 in E. ***P < 0.005, LY-294002 vs. control; *P < 0.05, LY-294002 vs. control; **P < 0.01, 4 µg/ml α-IL-8 ND-CM vs. 4 µg/ml α-IL-8 T2D-CM; *P < 0.05, 20 µg/ml α-IL-8 ND-CM vs. 20 µg/ml α-IL-8 T2D-CM.
generated tumors with high microvessel density, except for the clones expressing and secreting the highest amounts of IL-8. In that case, tumor microvessel density was actually reduced (25).

Tie2 is a receptor tyrosine kinase highly expressed in endothelial cells that modulates the transition between remodeling blood vessels and a mature, stable vasculature (18). Indeed, mice deficient in Tie2 display multiple vascular defects (28, 37). We found that under conditions where tube formation was optimal, such as treatment with ND-CM or ND-IL-8, Tie2 expression was highest, suggesting that downregulation of Tie2 is one way that elevated levels of IL-8 reduce angiogenesis. That would be consistent with the ability of the IL-8-neutralizing antibody to improve both Tie2 expression and tube formation in the presence of high levels of IL-8 as well as with studies in mice with a deficiency in the p110α/H9251 subunit of PI3K that found both diminished Tie2 expression and vascular defects similar to those in mice defective in the Tie2 signaling pathway (28, 37). However, regulation of Tie2 expression may be a more distal event in this process, possibly sustaining lower angiogenesis, as it is possible for angiogenesis in the presence of T2D-IL-8 to be reduced (at 24 h), whereas Tie2 expression is still normal (Fig. 8).

What might be the physiological relevance of ND and T2D myotubes secreting different amounts of IL-8? Several groups have shown that circulating levels of IL-8 are elevated in T2D subjects (16, 67), although we did not observe that in our cohort (Table 1). Although the concentration range over which IL-8 transitions from pro- to antiangiogenic effects on HUVEC reflects the levels secreted from ND and T2D myotubes, these concentrations are several orders of magnitude greater than what is seen in vivo. It is possible that the effects observed in vitro do not translate to the in vivo situation, although our finding that IL-8 expression and secretion were correlated with capillary number in our in vitro models suggests that this may be the case.

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Fig. 9. IL-8 content in SkM tissue from ND and T2D subjects. A: representative Western blot for IL-8. SkM biopsies from multiple ND and T2D subjects were extracted and immunoblotted for IL-8 content. rIL-8 and serum were included as positive controls. Line between lanes denotes removal of lane from the original blot image due to the presence of extraneous sample. B: quantification of Western blots; ND subjects (black bar) and T2D subjects (open bar). Means ± SD; n = 3 for ND subjects and n = 4 for T2D subjects. *P < 0.05 ND vs. T2D.

Fig. 10. Effects of ND- and T2D [IL-8] on capillary outgrowth from ND- and T2D-SkM explants. A and B: quantification of capillary number from SkM explants from ND (A) or T2D (B) subjects embedded in matrigel for 6 days and exposed to different levels of exogenous rIL-8. The final concentration of IL-8 results from addition of endogenous and exogenous IL-8 that replicated the range of values in ND (black bars) or T2D (open bars) and high-T2D (checkered bars) myotube CM. In some experiments, 20 μg/ml α-IL-8 (vertically striped bars) or 20 μg/ml nonrelevant antibody (horizontally striped bars) was added. Means ± SD; ND-AT, n = 3; T2D-AT, n = 4. Results are percent of [T2D IL-8 final] for 3 fields of at least 3 different explants/subject. C: representative images of SkM tissue explants from a ND subject after incubation with and without rIL-8 in concentrations that replicated the average values in ND (a) and T2D-CM (b). ***P < 0.005, 0 vs. 1.209 exogenous IL-8 in ND-SkM; #P < 0.05 1.209 vs. 3.627 exogenous IL-8 in ND-SkM; **P < 0.005 0 vs. 2.418 exogenous IL-8 in T2D-SkM; ***P < 0.005 0 vs. α-IL-8 in T2D-SkM.
those seen in the circulation (Table 1). We propose that secretion from myotubes is more reflective of the IL-8 content within SkM tissue, a supposition supported by the data presented in Fig. 9, where SkM from T2D subjects does contain more IL-8 than ND, and by the fact that myotubes and SkM explants secrete similar amounts of IL-8. That would be consistent with other instances of dissociation between localized and circulating IL-8 levels. For example, after exercise, both IL-8 mRNA and protein are increased in muscle, whereas plasma levels are unchanged (1, 57). Similarly, interstitial levels of IL-8 in skeletal muscle of subjects with polymyalgia rheumatica are considerably higher than those in the plasma of the same subjects (32).

Several limitations of the current work merit mention. One is that myotube CM represents a highly complex mixture of factors, and in focusing on IL-8, IL-15, and GROα, we could be neglecting a number of other factors, including pro- and antiangiogenic factors, that might contribute to the ability of T2D-CM to suppress angiogenesis. A second concern is that tube formation by HUVEC, albeit a widely accepted model for angiogenesis (36, 55, 62), might not be fully reflective of the behavior of endothelial cells in skeletal muscle. One way to address these concerns is to study the actual target tissue, human SkM, in an ex vivo context. We found that SkM explants behaved similarly to muscle tissue with regard to their angiogenic response to varying levels of IL-8, validating our primary experimental system.

The observation that in diabetes the nature of vascularization varies between tissues, elevated in the eye and impaired in skeletal muscle, has been termed the angiogenic paradox (reviewed in Ref. 10). This behavior reflects tissue-specific interactions between endothelial cells and the local microenvironment. In the current report, we focused on the microenvironment in SkM and demonstrated that diabetes-related dose-dependent effects of myotube CM on angiogenesis were mediated specifically, at least in part, by IL-8 and were not due to cell damage but rather through influencing tube production and maintenance. Moreover, we showed that this dose dependency of IL-8 is observable in the PI3K and FAK pathways, with PI3K regulating Tie2 expression.

Resistance to the ability of insulin to stimulate glucose uptake and disposal by SkM in T2D occurs at several levels. Most distally is impaired translocation of GLUT4 to the myotube surface, resulting in reduced glucose transport (reviewed in Ref. 45). Proximal to the myotube is defective recruitment of existing capillaries (reviewed in Ref. 29). Prior to that is a T2D-related deficit of capillaries available for recruitment (40, 43). Our results show that elevated levels of IL-8 secretion are an intrinsic property of T2D skeletal muscle and myotubes specifically, acting to create a microenvironment that reduces CD, ultimately limiting the availability of substrates, including glucose, and exacerbating impaired muscle glucose disposal in T2D.

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DISCLOSURES

No potential conflicts of interest relevant to this article are reported.

AUTHOR CONTRIBUTIONS

Y.A.L. researched data and wrote manuscript. T.P.C. researched data, contributed to discussion and reviewed/edited manuscript. S.R.M., S.A.P. and R.R. H. contributed to discussion and reviewed/edited manuscript.

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