Palmitate-induced inflammatory pathways in human adipose microvascular endothelial cells promote monocyte adhesion and impair insulin transcytosis

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Obesity is associated with chronic, low-grade inflammation; microvascular endothelium; insulin; fatty acids obesity like insulin resistance and atherosclerosis but the underlying mechanisms and therapeutic potential remain unclear (14). Muscle and adipose tissue from obese humans show an elevated presence of immune cells (18) that originate from the bone marrow and cross the endothelial layer of capillaries and venules to reach the tissues where they differentiate into macrophages (19). Monocyte chemoattractant protein-1 (MCP1) and other chemokines, as well as nucleotides are recognized chemoattractants for monocytes in obesity-associated inflammation (36), but the infiltration of immune cells requires expression of adhesion molecules at the surface of endothelial cells to promote their adhesion and transmigration. This interaction is carried out at the microvascular endothelium, by members of the immunoglobulin superfamily (intercellular adhesion molecules ICAM-1, ICAM-2, ICAM-3, and vascular cell adhesion molecule VCAM-1) and selectins (E and P) that recognize their cognate ligands on the leukocyte surface. Although the process of adhesion, rolling, and transmigration of leukocytes has been well described (40), the status of immune cell transmigration in obesogenic environments is unclear (39).

Microvascular blood vessels are structurally and functionally distinct from those in larger vessels (1). Large arteries regulate blood flow and its distribution, whereas capillaries and post-capillary venules supply muscle fibers and adipose cells with insulin and nutrients and are the favored site for immune cell transmigration (51). While fatty acid-induced inflammation in cells from macrovessels such as umbilical vein (HUVEC) or aorta (HAEC) is recognized (22, 49), essentially nothing is known about the effects of a high-fat environment on human microvascular endothelia, especially on cells from relevant metabolic tissue beds such as skeletal muscle or adipose tissue.

In parallel to provoking inflammation, high-fat feeding causes insulin resistance (42). Saturated fatty acids are major contributors to this process, as they directly impair insulin sensitivity in adipocytes and muscle cells in culture through lipotoxic and proinflammatory effects (6). In vivo, however, the regulation of insulin sensitivity involves complex integrated responses involving multi-organ cross-talk. In particular, and often underestimated, is the contribution of the microvasculature to insulin resistance, by regulating the delivery of insulin to its target organs (26), specifically to muscle and adipose tissue. Several studies suggest that insulin delivery to tissues is a critical determinant of insulin-mediated glucose uptake, since insulin injected intravenously equilibrates rapidly...
within plasma but only slowly within the skeletal muscle interstitium (13). Indeed, following an intravenous insulin injection, the time course of insulin-dependent Akt phosphorylation in muscle parallels the rise in interstitial insulin but not that of plasma insulin, revealing that the microvascular endothelium is a rate-limiting step in insulin action (26). Moreover, the access of insulin to its target tissues is markedly diminished in high-fat-fed dogs (16, 27) and in diabetic humans (47), suggesting that endothelial cells play a crucial role in regulating insulin-induced glucose uptake. The delivery of proteins across the microvascular endothelium can in principle occur between (paracellular) or through (transcytosis) endothelial cells (3). Importantly the endothelium lining the microvasculature of muscle and adipose tissue is continuous in nature, with circumferential tight junctions and no gaps between cells. Accordingly, we recently demonstrated that insulin crosses a confluent monolayer of human adipose microvascular endothelial cells (HAMEC) specifically by transcytosis, involving endocytosis via clathrin-coated pits (4). However, it is unknown whether and how saturated fatty acids and inflammation affect this process.

Here, we explore whether saturated or unsaturated fatty acids activate a proinflammatory response in HAMEC and its possible consequences on two major endothelial cell functions: monocyte adhesion and insulin transcytosis. Strikingly, whereas palmitate treatment increased monocyte adhesion and transmigration across HAMEC, it reduced insulin transcytosis. Potentially, these responses to saturated fat environments could contribute to elevated immune cell infiltration and reduced insulin delivery to muscle and fat tissues during obesity.

METHODS

Reagents. Sulfo-N-succinimidyl oleate (SSO) and cardamonin were from Cedarlane (Burlington, ON, Canada) and Takara242 from Millipore (Etohioke, ON, Canada). Neutralizing antibody for intercellular adhesion molecule 1 (ICAM-1; CD54, BBA4) was from R&D Systems (Minneapolis, MN). All other chemicals were from Sigma-Aldrich (St. Louis, MO). siRNA oligonucleotides against Toll-like receptor 4 (TLR4) were purchased from Life Technologies (Burlington, ON, Canada), siRNA for ICAM-1, E-selectin (SELE), and MYD88 were purchased from Qiagen (Valencia, CA).

Palmitate preparation. Palmitate or palmitoleate (P9767 and P9417, Sigma-Aldrich) stock solutions (200 mM) were prepared in 50% ethanol by heating at 50°C. Fatty acid-free, low-endotoxin BSA (A8806, Sigma-Aldrich) was dissolved in serum-free α-MEM to 10.5%. Fatty acid stocks were diluted 25× in the BSA solution and conjugated under agitation at 40°C for 2 h. This solution (lipid/BSA ratio 5:1) was further diluted in culture medium. Palmitate and palmitoleate solutions thus coupled to BSA are denoted as PA and PO, respectively.

Cell culture, viability. HAMEC were purchased from ScienCell (Carlsbad, CA). Primary human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC) were purchased from Lonza (Allendale, NJ). Endothelial cells were cultured in EGM-MV medium (Lonza, Allendale, NJ) in an incubator at 37°C and 5% CO2. Only cells from passages 4–9 were used. L6 muscle cells were maintained in α-MEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. Differentiation was induced by switching confluent cells to α-MEM with 2% FBS for 4–5 days. Cellular viability was assessed from Trypan blue uptake and LDH activity (Cytotoxicity Detection Kit; Roche Applied Science, Indianapolis, IN).

siRNA transfection. HAMEC were seeded at 60% confluence, and transfection of siRNA was performed using Lipofectamine RNAiMax reagent according to the manufacturer’s protocol with slight modification. In brief, 24 h after seeding, cells were transfected with 100 nM siRNA for 5 h and then changed to regular medium for 24 h followed by a second round of siRNA transfection. Cells were used for experiments 24 h thereafter, and knockdown was compared with scramble siRNA sequences.

RNA isolation and qPCR. All reagents were from Life Technologies (Carlsbad, CA). RNA was isolated using TRIzol and cDNA synthesized using the SuperScript VILO cDNA kit. Ten nanograms per reaction was used for RT-qPCR using predesigned Taqman probes. HPRT1 and ABT1 expression was not affected by any of the treatments, and they were therefore considered to be suitable housekeeping genes. qPCR were performed for 40 cycles (95°C for 1 s, 60°C for 20 s), and the relative quantities of each mRNA were calculated using the comparative ΔΔCT method.

Fatty acid uptake. To calculate disappearance of fatty acids from the medium, nonesterified fatty acids were quantified in the media of fatty acid-incubated myotubes by use of the NEFA-HR kit (Wako Chemicals, Richmond, VA). Briefly, cells were seeded in a 96-well plate, L6 were differentiated as described above, and HAMEC were grown to confluence. Cells were treated in 100 μl of medium supplemented with fatty acids for 18 h. Uptake was indirectly estimated from the fatty acid content in the supernatant at the beginning and end of the incubation.

Uptake of radiolabeled palmitate was also used to measure rates of fatty acid uptake. [14C]palmitate was prepared in 10% BSA (2.6 μCi in 162 μl BSA) and dissolved 1:40 in α-MEM supplemented with 2% FBS. Cells seeded in 12-well plates were treated with 500 μl (0.2 μCi/well) for 0.5, 1, 2, and 4 h. Cells were washed with α-MEM-FBS and then with PBS, detached using 500 μl of trypsin, and transferred into glass tubes containing 3 ml of methanol-chloroform (2:1). Lipids were extracted by adding 1 ml of chloroform and 1 ml of NaCl (1 M), 30 s of vortex agitation, and centrifugation (1,500 rpm for 20 min). The lower layer containing the lipids was transferred into a glass tube, solvents were evaporated under nitrogen, and residue was reconstituted in 2 ml of chloroform. Radioactivity was counted in 200 μl of this extract by use of a Packard TRI-CARB 2900TR liquid scintillation counter (Packard, Meriden, CT) with a detector efficiency of 45.81%. Radioactivity is expressed as nCi/mg protein.

Cell metabolism analysis. Oxygen consumption rate (OCR) was measured using a Seahorse XF296 analyzer (Seahorse Bioscience, Billerica, MA). L6 and HAMEC grown in Seahorse 96-well cell culture plates were differentiated as described above and then equilibrated for 1 h in XF Base Medium supplemented with 1 mM glucose and 0.2 mM fatty acids. Baseline measurements were performed for 1 h, and then oligomycin (1 μM, 20 min) and FCCP (1 μM, 20 min) were added sequentially, followed by rotenone and antimycin A (1 μM, 20 min).

Immunohistochemistry. HAMEC were grown to confluent monolayer and then treated for 18 h with 0.2 mM PA, PO, or the BSA vehicle. Cells were washed with PBS, fixed in 4% PFA for 20 min on ice, quenched with 0.1 M glycine for 10 min, and then blocked with 3% BSA for 30 min at room temperature. Cell surface ICAM-1 was detected with mouse anti-ICAM-1 antibody (1:100, R&D Systems) and donkey anti-mouse antibody conjugated to Cy3 (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescence images were taken with a Leica spinning-disk confocal microscope using Velocity software (PerkinElmer, Waltham, MA). Each field was quantified by ImageJ software (NIH, Bethesda, MD).

Monocyte adhesion assay. HAMEC grown in 96-well plates until confluence were treated with fatty acids for 18 h. THP1 monocytes (106 cells/ml) were resuspended with LPS (200 ng/ml for 3 h) and loaded with calcein (2 μg/ml for 30 min). Monocytes were then washed with PBS and suspended in endothelial media at 106 cells/ml, and 100 μl was added on top of endothelial cells. The plate was

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Table 1. Viability of HAMEC treated with fatty acids

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<th>Treatment</th>
<th>BSA</th>
<th>PA</th>
<th>PO</th>
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<tr>
<td>Trypan blue-negative cells (% total)</td>
<td>94.7 ± 5.1</td>
<td>95.5 ± 3.6</td>
<td>93.9 ± 6.1</td>
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<tr>
<td>LDH release (% total)</td>
<td>1.40 ± 2.05</td>
<td>0.27 ± 0.45</td>
<td>1.48 ± 2.16</td>
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Potential cytotoxicity caused by fatty acid treatment was estimated through Trypan blue uptake and LDH release after exposing primary endothelial cells from human adipose tissue microvasculature (HAMEC) to 0.2 mM BSA, palmitate (PA), and palmitoleate (PO) for 18 h. LDH results are normalized to the total LDH released after lysing the cells with 0.1 % Triton X-100. Results are expressed as means ± SD; n = 3.

incubated for 45 min at 37°C and then washed three times with PBS, and fluorescence was measured using a microplate reader (excitation/emission: 495/515 nm).

Monocyte transmigration assay. HAMEC were grown to confluence onto transwells (6.5 mm in diameter, 8 μm pore, Costar). The monolayer was then treated with BSA or 0.2 mM PA or PO in serum-free culture medium for 18 h. For the chemotaxis assay, 1 × 10⁶ THP-1 cells/ml were incubated with 200 ng/ml LPS at 37°C for 3 h and then washed with PBS and resuspended in RPMI 1640. 1 × 10⁶ THP-1 cells in 100 μl of RPMI 1640 were added to the upper chamber, and 600 μl of RPMI 1640 with or without 10 μM ATP was added to the lower chamber. The assembly was incubated at 37°C, 5% CO₂ for 3 h. THP-1 cells migrating into the lower chamber were counted manually using a hemocytometer.

Total internal reflection fluorescence microscopy. Visualization of insulin arriving at the ventral membrane was performed by total internal reflection fluorescence microscopy (TIRF) microscopy using a method developed by our laboratories (4). Briefly, HAMEC were treated with 500 nM Alexa fluor 568-conjugated insulin for 10 min at 4°C to allow insulin to bind to its receptor, but prevent internalization. Excess insulin was then washed off with PBS. Samples were then imaged using a ×150/1.45NA objective on a heated stand (37°C) in RPMI 1640 media supplemented with HEPES buffer using an Olympus cell TIRF motorized multicolor module mounted on an Olympus IX81 microscope (Olympus, Hamburg, Germany). Images (150 per cell) were taken at 10 FPS. Transcytosis events were quantified in an automated and blinded fashion using custom-written matlab scripts, as described in Ref. 4. Briefly, fluorescent insulin-laden vesicles were identified using a local background subtraction followed by binary thresholding. The movement of individual vesicles was tracked frame-to-frame, and those undergoing fusion were identified by detection of vesicles displaying highly confined movement (“docked” vesicles) (9) and which underwent a rapid decrease in fluorescence over the final frames of the vesicle track.

Insulin uptake. HAMEC were seeded onto glass coverslips and allowed to grow to confluence. Cells were incubated with 50 nM FITC-tagged insulin (I3661, Sigma) for 15 min at 37°C to allow insulin to internalize, as reported earlier (4). Cells were then washed with PBS to remove noninternalized insulin and fixed with 4% PFA for 20 min. Remaining PFA was quenched with 0.3 M glycine for 10 min. Nuclei were stained with DAPI for 10 min. Coverslips were covered with microscope mounting medium (Dako, Carpinteria, CA) supplemented with DAPI (1 μg/ml) and imaged using an Olympus IX81 spinning disc confocal microscope with a ×60/1.35 NA oil immersion objective with settings kept constant between conditions. Fluorescence intensity was assessed by ImageJ (NIH, Bethesda, MD).

Transwell assay of insulin transcytosis. HAMEC were grown to confluence on 12-well transwells with a membrane pore size of 0.4 μm (Costar 3460, Corning, NY). Cells were incubated with 500 nM biotinylated insulin (I2258, Sigma) at 4°C for 10 min to allow binding to the cell surface but not internalization. Unbound insulin was excess insulin was then washed off with PBS. Samples were then imaged using a ×150/1.45NA objective on a heated stand (37°C) in RPMI 1640 media supplemented with HEPES buffer using an Olympus cell TIRF motorized multicolor module mounted on an Olympus IX81 microscope (Olympus, Hamburg, Germany). Images (150 per cell) were taken at 10 FPS. Transcytosis events were quantified in an automated and blinded fashion using custom-written matlab scripts, as described in Ref. 4. Briefly, fluorescent insulin-laden vesicles were identified using a local background subtraction followed by binary thresholding. The movement of individual vesicles was tracked frame-to-frame, and those undergoing fusion were identified by detection of vesicles displaying highly confined movement (“docked” vesicles) (9) and which underwent a rapid decrease in fluorescence over the final frames of the vesicle track.

Fig. 1. Palmitate induces a proinflammatory response in human endothelial cells. Primary human endothelial cells from adipose microvasculature (HAMEC: A and B), umbilical vein (HUVEC: C and D), and aorta (HAEC: E and F) were exposed to 0.2 mM palmitate (PA), palmitoleate (PO), or BSA vehicle for 18 h. Gene expression of inflammatory cytokines and immune receptors was measured by RT-qPCR as described in METHODS and normalized to BSA-treated cells. Results are means ± SE; n ≥ 4. *P < 0.05, **P < 0.01, ***P < 0.001 vs. BSA control.

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washed off with PBS, and then medium at 37°C was added to the transwells to initiate insulin internalization. After 30 min, the bottom chamber was sampled using ELISA for biotinylated-insulin. Briefly, samples were incubated on a streptavidin-coated plate for 1 h (Life Technologies, Carlsbad, CA). The plate was then incubated with an anti-insulin antibody for 1 h [AbD Serotec 7F8 (E6E5), Raleigh, NC]. Thereafter, HRP-conjugated secondary antibody to mouse IgG (Cell Signaling no. 7076S; Whitby, ON, Canada) was added for 1 h, followed by addition of TMB substrate (Life Technologies) for 30 min and inactivation with H2SO4 (2 M). Optical density at 450 nm was determined with a spectrophotometric plate reader.

Statistical analysis. Analyses were performed using Prism software (GraphPad Software, San Diego, CA). The effects of inhibitors were analyzed by two-way ANOVA, followed by adequate Sidak post hoc tests. One-way ANOVA was used to test differences between groups with equal variances. Statistical significance was set at $P < 0.05$.

**RESULTS**

Palmitic acid is the most abundant saturated fatty acid in the Western diet and a major constituent of the blood nonesterified fatty acids (38). The total free fatty acid concentration in serum can reach 0.8 mM in obese individuals, of which roughly 25% is palmitate (45, 52). We consequently used 0.2 mM as a physiological concentration to treat HAMEC in vitro that would emulate endothelial exposure to fatty acid exposure in vivo.

The fatty acid stock solutions contain 8 mM fatty acids and 1.6 mM BSA, while the culture medium (5% fetal bovine serum) contains 0.06 mM albumin. After dilution (40-fold), the final molar ratio fatty acid to total albumin in the culture medium is 3:1. This ratio does not surpass the binding capacity of the fatty acid to the albumin.

**Fig. 2.** Palmitate induces expression of adhesion molecules in HAMEC. HAMEC were treated with 0.2 mM PA, PO, or BSA vehicle for 18 h. 

A: gene expression of adhesion molecules was measured by RT-qPCR as described in METHODS. Results are means ± SE; $n = 6$. *$P < 0.05$ vs. control. 

B and C: Western blot was performed on whole cell lysate using antibodies against intercellular adhesion molecule 1 (ICAM-1) and E-selectin (SELE). Quantification relative to the β-actin loading control (means ± SE, $n = 4$). *$P < 0.05$ vs. control and representative blots are shown. 

D: cells were stained for surface ICAM-1 using antibodies against the extracellular portions of ICAM along with DAPI nuclear stain. Representative images are shown for X-Y and Z-stack projections. White arrow points to ICAM-1 localization at the apical cell surface of the HAMEC.
of albumin, as each molecule of albumin contains six high-energy binding sites for fatty acids (43), and it helps reduce the amount of residual fatty acid-free BSA that may have nonspecific effects such as depleting cholesterol from membranes (34). At this concentration and ratio, neither palmitate, palmitolate, nor BSA affected LDH release or Trypan blue uptake by HAMEC (Table 1).

Palmitate, but not palmitolate, activates a proinflammatory response in microvascular endothelial cells. Saturated fatty acids can activate proinflammatory pathways in immune and nonimmune cells, leading to the expression and release of cytokines and chemokines (17). Overnight exposure (18 h) of nonimmune cells, leading to the expression and release of cytokine and chemokine activity (17). Overnight exposure (18 h) of HAMEC to the saturated fatty acid PA, but not to the mono-unsaturated PO, induced gene expression of the proinflammatory cytokines IL6 and IL8 (Fig. 1A) and of the immune receptor TLR2 (Fig. 1B). A qualitatively similar response was also observed in endothelial cells from large vessels, HUVEC and HAEC (Fig. 1, C–F), consistent with a number of previous reports (35, 49). These data demonstrate that endothelial cells, including those derived from microvessels, can activate proinflammatory pathways in response to palmitate.

Palmitate induces expression of adhesion molecules in microvascular endothelial cells. In response to infection or tissue damage in vivo, the endothelial proinflammatory response involves activation and recruitment of circulating immune cells. This occurs through the expression of adhesion molecules at the luminal side of endothelial cells, inducing leukocyte rolling and eventually diapedesis (40). In HAMEC, PA treatment brought about an increase in mRNA expression of SELE (3-fold) and ICAM-1 (3.5-fold) (Fig. 2A). Conversely, PA reduced the expression of platelet endothelial cell adhesion molecule 1 (PECAM-1), whereas no change was detected in...
fatty acids in the supernatant of L6 myotubes (Fig. 3) but did not inhibit (29). SSO significantly prevented the depletion of inflammatory response may be independent of its metabolic effects in nonpermeabilized cells by confocal fluorescence microscopy. Interestingly, only ICAM-1 was significantly elevated at the plasma membrane in response to PA (Fig. 2D). The exclusive localization of ICAM-1 at the apical membrane of HAMEC verifies the polarity of the cell monolayer and the endothelial phenotype of HAMEC cultures. On the other hand, and consistent with the immunoblotting results, SELE detection by immunofluorescence did not increase with PA, and in fact it was detected in only a small number of cells and was not clearly at the cell surface, even when two distinct antibodies were used (data not shown). In contrast, LPS increased the amount of SELE at the surface of HAMEC, indicating that the antibodies used are reliable for immunofluorescence. Hence, although PA increases SELE mRNA levels, these do not translate into frank protein elevation within the time frame analyzed in the majority of the cells in the monolayer.

Gene expression of adhesion molecules requires TLR4→NF-κB signaling but not PA uptake. Many of the deleterious effects of fatty acids have been ascribed to impairment of metabolism due to an overload of nutrient entering cells through CD36 (32). However, the concentration of fatty acids in the supernatant from HAMEC did not change before and after exposure to either PA or PO (Fig. 3A). By comparison, L6 myotubes cultured under the same conditions reduced the fatty acid concentration in the medium by ~55% (Fig. 3B). This differential rate of fatty uptake was confirmed using radiolabeled [14C]palmitate. Over 4 h, HAMEC exhibited a significantly lower rate of [14C]palmitate uptake compared with muscle cells (Fig. 3C). In addition, HAMEC had a very low OCR even when incubated with 0.2 mM PA or PO (Fig. 3D). Moreover, the addition of oligomycin (an inhibitor of the ATP synthase) did not significantly reduce the OCR in HAMEC (Fig. 3E), suggesting that these cells scarcely oxidize fatty acids to produce energy. Collectively, these observations reveal that HAMEC neither accumulate nor utilize a significant amount of fatty acids, and therefore the PA-induced proinflammatory response may be independent of its metabolic effects in these cells. To further confirm the independence of the HAMEC response to PA from fatty acid uptake, cells were incubated with PA in the presence of SSO, a potent CD36 inhibitor (29). SSO significantly prevented the depletion of fatty acids in the supernatant of L6 myotubes (Fig. 3F) but did not affect the PA-induced gene expression of SELE and ICAM-1 (Fig. 3, G and H) or of the cytokines IL-6 and IL-8 in HAMEC (Fig. 3, I and J).

The independence of the PA response of HAMEC from fatty acid uptake suggested that the fatty acid may be sensed by pathways unrelated to its metabolism. It has been proposed that fatty acids can act on TLRs, leading to activation of the NF-κB transcription factor (41). TLR4 can be selectively inhibited by TAK242 (23), while cardamonin prevents IKK phosphorylation and the consequent NF-κB activation (21). We verified that TAK242 and cardamonin act accordingly in HAMEC, as these inhibitors eliminated the induction of cytokines and adhesion molecules provoked by the canonical TLR4 agonist LPS (data not shown). The increase in ICAM-1 and SELE expression in response to PA was significantly blunted by TAK242 and cardamonin (Fig. 4, A and B). This result suggests that the TLR4→NF-κB pathway is required for the inflammatory response of HAMEC to PA.

PA increased monocyte adhesion and transmigration. Elevated ICAM-1 at the surface of endothelial cells would be expected to promote immune cell adhesion and transmigration. Indeed, PA increased the adhesion of human THP1 monocytes to PA-treated HAMEC compared with BSA- and PO-treated cells (Fig. 5A). In good agreement with this finding, PA also promoted the transmigration of monocytes toward the chemotaxattractant ATP across a HAMEC monolayer in transwell assays (Fig. 5B). Mechanically, the PA-induced monocyte adhesion was likely mediated by the rise in surface levels of ICAM-1, since it was reduced by neutralizing antibodies against this adhesion molecule (Fig. 5C). While these results clearly show that PA did not promote monocyte adhesion in the presence of the neutralizing anti-ICAM-1 antibody (compared with neutralization in BSA-treated cells), the antibody alone unexpectedly increased monocyte adhesion in both PA- and BSA-treated cells. Therefore, as a complementary approach to determine ICAM-1 participation, we used siRNAs to efficiently reduce the levels of ICAM-1 and SELE (Fig. 5D). Silencing of ICAM-1, but not SELE, prevented the PA-induced monocyte adhesion (Fig. 5E).

TLRs couple with adaptor MyD88 to activate the NF-κB transcription factor, and interestingly, silencing of MyD88 also prevented the PA-induced monocyte adhesion (Fig. 5F). Furthermore, inhibition of the TLR4→NF-κB pathway by TAK242 or cardamonin blunted the increase in monocyte adhesion to HAMEC induced by PA (Fig. 5G), consistent with the prevention of ICAM expression observed with these inhibitors. All together, these findings indicate that PA provokes activation of the TLR4→NF-κB pathway in HAMEC, and the consequent expression of ICAM-1 to promote monocyte adhesion and transmigration.

Palmitate impairs insulin transcytosis. As mentioned in the introductory section, local and systemic inflammation is associated with obesity-induced insulin resistance in vivo, and obesity also impairs insulin delivery to metabolic tissues. We therefore examined the possibility that the PA-induced activa-

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**Fig. 4.** Inhibition of the Toll-like receptor (TLR)4→NF-κB pathway prevents PA-induced inflammation. HAMEC were treated with 0.2 mM PA or BSA vehicle for 18 h in the presence of TLR4 inhibitor TAK242 (200 μM) or NF-κB inhibitor cardamonin (CARD, 50 μM). Gene expression of ICAM-1 (A) and SELE (B) was measured by RT-qPCR and normalized to BSA control. Results are means ± SE; n ≥ 5. *P < 0.05.
PA promotes monocyte adhesion and transmigration. HAMEC were treated with 0.2 mM PA or BSA vehicle for 18 h. A: adhesion of THP1 monocytes to HAMEC endothelium was measured as described in METHODS. Data are means ± SE; *P < 0.05. B: transmigration of monocytes was measured in a transwell assay, using ATP as a chemoattractant in the bottom well. Data are means ± SE; n = 3. **P < 0.05. C: adhesion of monocytes to the endothelium was inhibited by neutralizing antibodies (2 μg/ml) directed against ICAM-1. Data are means ± SE; n = 5. *P < 0.05. D: silencing of ICAM-1, SELE, and MyD88 was performed using siRNA and quantified by RT-qPCR. Data are means ± SE; n = 2. E and F: adhesion of THP1 monocytes to HAMEC transfected with siRNA to knock down ICAM-1, SELE, or MyD88. Data are means ± SE; n = 6. *P < 0.05. G: adhesion of monocytes to the endothelium was inhibited by cardamonin (50 μM) and TLR4 inhibitor TAK242 (100 μM). Data are means ± SE; n ≥ 4. *P < 0.05.

DISCUSSION

High-fat feeding in mice and obesity in humans leads to insulin resistance and promotes infiltration of blood-derived immune cells in the adipose tissue and skeletal muscle. Although these two defects suggest involvement of the microvasculature, it is difficult to dissect the particular role of microvascular endothelial cells in vivo due to the diversity of cells coexisting within metabolic tissues and the heterogeneous nature of the endothelium itself. Since endothelial cells from different tissues exhibit significantly different phenotypic and functional characteristics (1), it is essential to choose the most appropriate model to study physiological responses appropriately. Both monocyte diapedesis and insulin transcytosis occur in the microvasculature, and adipose tissue is a major contributor to the regulation of insulin sensitivity and glucose uptake. In this regard, HAMEC are a superior model compared with cells from the aorta or the umbilical vein to study the delivery of cells and molecules to the parenchyma of metabolic tissues. Using this cell culture paradigm, we demonstrate that micro-
vascular endothelial cells mount a proinflammatory response to saturated fatty acids that affects two of their major functions: monocyte transmigration and insulin transcytosis.

Deleterious effects of saturated fatty acids have been described in many different cell types (25). Long-term exposure to high concentrations of saturated fatty acids has previously been found to trigger apoptosis in cells in culture. In endothelial cells, most studies on viability and lipotoxic effects of saturated fatty acids that affects two of their major functions: monocyte transmigration and insulin transcytosis.

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Another major contribution of the microvasculature to metabolic diseases is its function as a barrier between circulating insulin and its target tissues. Key physiological studies have indeed shown that transfer across the endothelium is a rate-limiting step in insulin availability and its metabolic action (13, 28). Obese subjects have higher lymph insulin concentrations reflecting the muscle interstitial space levels (11), and in dogs fed a high-fat diet, there was a further delay in the time that injected insulin reached the interstitial space (27). These important findings suggest that profound modifications of the vasculature physiology take place during obesity.

In the present study, we demonstrate that insulin transcytosis is impaired by PA but not by PO. Because PA can cause insulin resistance in metabolic relevant cells, we investigated whether the PA inhibition of insulin transcytosis in HAMEC would also involve elements of insulin action. Surprisingly, insulin transcytosis in HAMEC was not affected by silencing either the insulin or the IGF-I receptor (results not shown). Future work will explore the nature of the receptors mediating insulin uptake into microvascular endothelial cells leading to transcytosis, a process we recently reported to occur through clathrin-mediated endocytosis (4). Of course, the PA-provoked inhibition of insulin transcytosis reported herein may reflect a susceptibility of microvesicles to circulating saturated fats, which may contribute to whole body insulin resistance by reducing or delaying insulin delivery to metabolic tissues.

Interestingly, and similarly to the binding of monocytes, the impairment of insulin transcytosis by PA also depends on the activation of cell autonomous proinflammatory pathways, as it was prevented by inhibiting or silencing TLR4. Our findings open new perspectives to decipher the mechanisms involved and identify targets to restore insulin transit. The PA-induced reduction in insulin transcytosis across HAMEC suggests the possibility that functional alterations may occur at the level of the microvasculature during the metabolic syndrome, in addition to the well-documented defects in large vessel vasodilation (25).

In conclusion, using physiologically relevant microvascular cells in culture, we demonstrate that HAMEC mount an inflammatory response to palmitate that impairs insulin traffic while promoting monocyte binding and transmigration. These findings have relevant implications for diabetes and the metabolic syndrome and suggest that endothelial cells could potentially constitute a novel therapeutic target to taper tissue inflammation and restore insulin sensitivity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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