De Novo Lipogenesis Maintains Vascular Homeostasis through Endothelial Nitric-oxide Synthase (eNOS) Palmitoylation*‡

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Endothelial dysfunction leads to lethal vascular complications in diabetes and related metabolic disorders. Here, we demonstrate that de novo lipogenesis, an insulin-dependent process driven by the multifunctional enzyme fatty-acid synthase (FAS), maintains endothelial function by targeting endothelial nitric-oxide synthase (eNOS) to the plasma membrane. In mice with endothelial inactivation of FAS (FASTie mice), eNOS membrane content and activity were decreased. eNOS and FAS were physically associated; eNOS palmitoylation was decreased in FAS-deficient cells, and incorporation of labeled carbon into eNOS-associated palmitate was FAS-dependent. FASTie mice manifested a proinflammatory state reflected as increases in vascular permeability, endothelial inflammatory markers, leukocyte migration, and susceptibility to LPS-induced death that was reversed with an NO donor. FAS-deficient endothelial cells showed deficient migratory capacity, and angiogenesis was decreased in FASTie mice subjected to hindlimb ischemia. Insulin induced FAS in endothelial cells freshly isolated from humans, and eNOS palmitoylation was decreased in mice with insulin-deficient or insulin-resistant diabetes. Thus, disrupting eNOS bioavailability through impaired lipogenesis identifies a novel mechanism coordinating nutritional status and tissue repair that may contribute to diabetic vascular disease.

Damage to blood vessels is inextricably linked with diabetes. Microvascular disease causes visual loss, renal failure, and neuropathy, and macrovascular disease (atherosclerotic coronary disease, stroke, and peripheral arterial disease) causes premature death. Macro- and microvascular complications characterize both type 1 (insulin-deficient) and type 2 (insulin-resistant with hyperinsulinemia) diabetes. Hyperglycemia is a biomarker of blood vessel disease (1), and glucose lowering delays microvascular complications (2, 3), but this therapy does not prevent progression of established microvascular (4) or macrovascular disease (5, 6). In addition to improving glycemia, optimal approaches to complications may require manipulating processes that are mostly obscure.

One such process is endothelial lipid metabolism. The endothelium controls tissue access from the circulation and regulates vascular functions, including inflammation and angiogenesis. Endothelial dysfunction, largely due to defects in eNOS, is characteristic of diabetes and probably promotes vascular disease (7, 8). Circulating fatty acids interfere with eNOS and increase inflammation (9, 10), and insulin resistance appears to have similar effects by increasing fatty acid oxidation (11), but little is known about how fuels are partitioned inside endothelial cells. A critical pathway for fuel flow is de novo lipogenesis, the generation of fatty acids from simple sugars, which requires FAS (12, 13). One might predict that de novo lipogenesis would be irrelevant to endothelial cells, which are continually bathed in fatty acids, the product of the FAS reaction.

Here, we report that endothelial FAS is dynamically regulated by the diabetic milieu; its presence is unexpectedly required for normal palmitoylation of eNOS, and its absence results in chronic inflammation and defective angiogenesis. Our observations suggest a relationship between FAS and eNOS that may contribute to vascular complications in diabetics and related metabolic disorders.

**EXPERIMENTAL PROCEDURES**

Mice, Humans, and Reagents—For experiments involving mice, the Washington University Animal Studies Committee-approved protocols were followed. Insulin deficiency was generated by injecting male C57BL/6 mice with streptozotocin (150 mg/kg). db/db mice and Tek-cre (Tie2-Cre) mice were obtained from The Jackson Laboratory. FASTie animals and littermate controls at 3–5 months of age were studied.

3 The abbreviations used are: eNOS, endothelial nitric-oxide synthase; FAS, fatty-acid synthase; MLEC, murine lung endothelial cell; STZ, streptozotocin; HUVEC, human umbilical vein endothelial cell; HA, hydroxylamine; SNP, sodium nitroprusside; F, forward; R, reverse; IP, immunoprecipitation.
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Human venous endothelial cells were isolated by J-wire biopsy from the brachial veins of three healthy volunteers who provided informed consent in a protocol approved by the Boston University Institutional Review Board. These freshly isolated cells were incubated in the presence or absence of 100 nM insulin for 6 h, and FAS was then imaged by immunofluorescence microscopy and quantified by image processing (14). The same assay was used for human aortic endothelial cells (Lonza).

MURINE LUNG ENDOTHELIAL CELLS (MLECS) WERE ISOLATED FROM TISSUE DIGESTED FOR 20 min with collagenase (1.5 mg/ml). This mixture was filtered, pelleted, and incubated with magnetic beads coated with anti-CD31 (eBioscience). Adherent cells were cultured in EGM-2 media (Lonza). Anti-eNOS antibody was from BD Biosciences, the FAS antibody from Abcam, the caveolin-1 antibody from Santa Cruz Biotechnology, and the actin antibody from Sigma. LPS was also purchased from Sigma.

Membrane Preparations—Mouse lung tissues or cells were lysed in hypotonic buffer (10 mM HEPES, pH 8.0, 15 mM KCl, 2 mM MgCl2, 0.1 mM EDTA with protease inhibitor tablet from Roche Applied Science) and subjected to a low speed spin for 15 min, and then the supernatant was ultracentrifuged at 65,000 rpm for 60 min to pellet light membrane fractions that were resuspended in membrane lysis buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40). For density separation, cells were homogenized and layered onto discontinuous sucrose gradients (40, 30, and 5%), separated by ultracentrifugation overnight, and then fractions harvested and assayed by Western blotting.

NOS Assays—eNOS activity in lysates was assayed using [3H]arginine and other reagents provided by Cayman Chemicals. Exports were incubated at room temperature for 30 min in reaction buffer (1 mM NADPH, 0.02 μCi of [3H]arginine, 0.6 mM CaCl2, 25 mM Tris-HCl, pH 7.4, 3 mM BH4, 1 μM flavin adenine dinucleotide, 1 μM flavin adenine mononucleotide) and then stopped with 50 mM HEPES, pH 5.5, + 5 mM EDTA, and unreacted arginine was removed with a resin, and radiolabeled citrulline in the effluent was quantitated. Activity in intact cells was assayed by addition of ionomycin (1 μM) and [3H]arginine (0.75 μCi/well) and then harvesting the cells 30 min later to measure radiolabeled citrulline. As a confirmatory assay of NO production, nitrite accumulation in media was measured using a 2-mm ISO-NPO sensor (World Precision Instruments). Cells were stimulated with acetylcholine (1 μM) for 1 h followed by supernatant collection and determination of nitrite content that was based on a calibration curve of varying concentrations of a standard nitrite solution generated for each assay.

FAS Knockdown—FAS-specific (5′-gaucacuggagcaucagcag) or scrambled (5′-aacucucgguauuccacguc) siRNA molecules provided by Invitrogen as Stealth RNAi™ oligonucleotides were transfected into HUVECs or COS-7 cells with Lipofectamine-2000 (Invitrogen). For the COS-7 experiments, the siRNA oligonucleotides were transfected 1 day before the cells were transfected with the eNOS expression vector. The efficiency of FAS knockdown in cultured cells was verified by Western blotting 48–72 h after the transfection.

Acyl Biotin Switch Assay—Tissues or cells were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) with 10 mM N-ethylmaleimide and sonicated on ice, and Triton X-100 (to 1.7%) and CHAPS (to 1.0%) were added, and the mixture was rotated in a cold room for 1 h. Following a chloroform/methanol step to precipitate proteins, samples were aliquoted to two tubes and resuspended in buffer containing N-6-(b-aminomido)hexyl)-3′-(2′-pyridylidithio)-propionamide-biotin (1 μM) and Triton X-100 (0.2%) in the presence or absence of hydroxylamine (HA). After 1 h, proteins were again precipitated with chloroform/methanol, suspended in buffer, including 0.1% SDS, and clarified, and then streptavidin-agarose beads were added to the supernatant. After 1.5 h at room temperature, biotin-labeled proteins were eluted for 15 min at 37°C with buffer containing 0.1% SDS, 0.2% Triton X-100, and 1% β-mercaptoethanol. Samples were pooled, TCA-precipitated, and Western blotted for eNOS and caveolin-1. For some COS-7 experiments, samples were subjected to eNOS immunoprecipitation and detected with streptavidin-HRP (Sigma).

Metabolic Labeling of eNOS-associated Fatty Acids—Cells were labeled with [14C]acetate, treated with lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM NaVO4 protease inhibitor mixture), and subjected to eNOS immunoprecipitation and Western blotting. eNOS bands were excised and subjected to alkaline hydrolysis at 37°C overnight, and then tolune extracts of the hydrolysate were evaporated, re-dissolved in ethanol, and analyzed by C-18 reverse phase thin layer chromatography. Migrations of [14C]palmitate, [14C]myristate, and [14C]acetate were included as standards.

Extravasation Assays and Lectin Whole Mount Staining—For animal studies, mustard oil (5% allyl-isothiocyanate in mineral oil; Sigma) was applied topically on two occasions 15 min apart to one ear of an anesthetized mouse following the administration of Evans blue dye (30 mg/kg). The other ear was treated with mineral oil. Thirty min after the second application, the vasculature was fixed by perfusion with 1% paraformaldehyde. Dye was extracted from dried tissues with formamide at 65°C for 24 h, and quantified at 610 nm. For culture studies, confluent endothelial monolayers on inserts were placed in 24-well tissue culture wells. Minimum Eagle's medium with 1% BSA was placed in the luminal (insert) and abluminal (well) chambers. LPS was added to the luminal chamber, and then 4 h later Evans blue albumin was added to the same chamber to a concentration of 0.45%. After 15 min, abluminal media were assayed at 610 nm for dye. For vascular lectin staining, mustard oil was applied topically, and then 20 min later animals were perfused with 1% paraformaldehyde, 0.5% glutaraldehyde followed by FITC-conjugated Ricinus communis I (Vector Laboratories), 500 μg/mouse. The skin was dissected from the cartilage as a whole mount and subjected to fluorescence microscopy.

Migration, Ring, and Hindlimb Ischemia Assays—Confluent cells were serum-starved overnight; sterile pipettes were used to scratch cultures; fresh media were added, and images were...
recorded over time. For ring assays, thoracic aortas were dissected free of adventitia, cut into 1–1.5-mm segments, placed in Matrigel (BD Biosciences)-coated wells, and covered with endothelial cell growth medium (EGM-2). Media were replaced every other day, and images were captured and analyzed using ImageJ software. For hindlimb studies, the femoral artery was ligated and transected as described previously (15). Perfusion was quantified by laser Doppler imaging (Perimed) and tissue ischemia scored as described previously (16).

Bone Marrow Transplantation—C57BL/6 male mice bearing CD45.1 were lethally irradiated and then infused with marrow harvested from wild type (Wt) or FASTie (littermates generated from seven backcrosses of our model into the pure C57BL/6 background) mice bearing CD45.2. Two months later, marrow cells were analyzed by flow cytometry (anti-CD45.1-APC, anti-CD45.2-PE, and anti-CD11b-FITC from eBioscience).

Other Assays—Serum chemistries, blood pressures, and quantitative RT-PCR assays were performed as described previously (17, 18). Primers for Fas and the invariant control Rpl32 were described previously (17), and others included the following: Ccl2 (MCP-1), F 5'-aggtgccccaagaagctga and R 5'-aggtctgagccatctctct; Vcam-1, F 5'-tgccagcataaattcaccatg and R 5'-ttctgtgaggtgttgctaca; Icam-1, F 5'-tccgtgtaggtgacagt and R 5'-gaccggagctgaaaatgttg; Sele (E-Selectin), F 5'-agctacccatggaacacgac and R 5'-cgcaagttctccagctgtt; and Nos3 (eNOS), F 5'-caacgctaccagaggatt and R 5'-tcctgtgaaagaagcttg.

RESULTS

Regulation of Endothelial FAS by Insulin—FAS is regulated by insulin in major lipogenic tissues like liver and white adipose tissue (19, 20), but the effects of insulin and diabetes on endothelial FAS are not established. FAS protein was decreased in endothelium-rich tissues from mice with streptozotocin (STZ)-induced diabetes (an insulin-deficient model) and db/db mice (an insulin-resistant model), each with blood glucose values in excess of 300 mg/dl, as compared with controls (Fig. 1A). Cultured human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells demonstrated a time-dependent induction of FAS protein with 100 nM insulin (data not shown). Similar induction was seen in HUVECs using 1 nM insulin concentrations, and FAS induction using 0.2 or 0.5 nM insulin was abolished following shRNA-mediated knockdown of the insulin receptor (data not shown). Because HUVECs and human aortic endothelial cells may display a phenotype reflecting adaptation to chronic cell culture, we also demonstrated that FAS is in-
duced by insulin using freshly isolated human endothelial cells (Fig. 1B). Given the effects of diabetes and insulin on endothelial FAS, we inactivated FAS at this site to assess the potential role of de novo lipogenesis in vascular function.

**Inactivation of Endothelial FAS Decreases eNOS Bioavailability**—Global FAS deficiency is embryonically lethal (21), but tissue-specific FAS disruption is feasible with animals carrying loxP sites flanking Fasn exons 4–8 (17). These mice were bred with mice expressing Cre recombinase under the control of the Tie-2 promoter known to drive expression in endothelial and hematopoietic cells (22) to generate FASTie mice. Adult FASTie mice were fertile and appeared normal. Murine lung endothelial cells (MLECs) from FASTie mice demonstrated the expected rearrangement of the Fasn gene and decreased FAS mRNA (Fig. 1C). FAS protein was decreased in lung and aorta of FASTie mice (Fig. 1D), as well as in MLECs isolated from FASTie mice (Fig. 1E). Serum glucose, fatty acids, cholesterol, and triglycerides were normal (Table 1). FAS protein was also decreased in bone marrow (see Fig. 8B, below) but levels of white blood cells, red blood cells, platelets, and hemoglobin were indistinguishable from wild type littermates (Table 1).

E NOS protein abundance (Fig. 2A) as well as mRNA (data not shown) were the same in FASTie and WT mouse lungs using unfractonated whole cell lysates, mirroring the lack of change in whole cell eNOS abundance in diabetes models (Fig. 1A). However, isolated membrane fractions showed decreased eNOS protein (Fig. 2B) and decreased calcium-dependent enzyme activity (Fig. 2C) in FASTie compared with WT mice. eNOS knock-out mice have hypertension (23), and FASTie mice had mild increases in both systolic and diastolic pressures compared with WT (Fig. 2D), suggesting decreased NO bioavailability in vivo.

To confirm that eNOS effects were due to FAS deficiency and not some aberration of the mouse model, we studied HUVECs and eNOS-transfected COS-7 cells. Chemical inhibition of FAS with cerulenin in HUVECs decreased membrane localization of eNOS (Fig. 2E). In the same cell type, siRNA-mediated knockdown of FAS also resulted in less membrane-associated eNOS (data not shown). Cerulenin-treated HUVECs had decreased ionomycin-stimulated eNOS enzyme activity using whole cells (Fig. 2F), but no difference was detected using homogenized cell lysates (data not shown), consistent with an effect of FAS on eNOS membrane localization. To validate the eNOS activity findings, FAS was inhibited either chemically with cerulenin (Fig. 2G) or genetically using siRNA (Fig. 2H) followed by treatment with acetylsalicylic acid to stimulate NO production, which was decreased with both strategies for FAS inhibition. siRNA-mediated knockdown of FAS in COS-7 cells subsequently transfected with an eNOS expression vector decreased eNOS membrane localization as shown in Fig. 2J (arrow) with Western blotting to demonstrate the FAS and eNOS effects shown in Fig. 2J. As with genetic inhibition, chemical inhibition of FAS with cerulenin in COS-7 cells resulted in a redistribution of eNOS from the plasma membrane (arrow) to the cytoplasm (Fig. 2K). This effect could not be rescued with exogenous palmitate complexed with albumin (data not shown). These results suggest that endothelial FAS maintains normal eNOS activity through a post-translational process involving membrane localization.

It is conceivable that the lack of FAS-derived lipids could alter membrane composition to affect eNOS localization. However, lipid composition of HUVECs (reflecting mostly membrane lipids under these conditions) was not affected by FAS knockdown as follows: nonesterified fatty acids 2.30 ± 0.15 pmol/mg control versus 2.16 ± 0.15 knockdown (p = 0.52); phospholipid 10.35 ± 0.65 μg/mg control versus 9.84 ± 0.88 knockdown (p = 0.67); cholesterol 2.74 ± 0.28 μg/mg control versus 2.30 ± 0.23 knockdown (p = 0.29). It is also possible that FAS disruption might alter cell membrane components such as caveolae required for eNOS targeting. However, FAS deficiency did not affect caveolin-1 or β-catenin localization as determined by immunofluorescence (data not shown). In addition, density gradient centrifugation demonstrated no effect of FAS knockdown on migration of caveolae as determined by Western blotting for caveolin-1 (data not shown).

**FAS Is Required for eNOS Palmitoylation**—Given the apparent relationship between FAS and eNOS, we sought to determine whether these proteins interact physically. When extracts from eNOS-transfected COS-7 cells were immunoprecipitated with an antibody to eNOS, FAS was detected in the pellet (Fig. 3A, top panels). When the same extracts were

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**TABLE 1**

| Serum chemistries and complete blood counts in wild type and FASTie mice |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Data are presented as means ± S.E. for 3–5 mice per genotype for chemistries at 3 months of age, 12–13 mice per genotype for chemistries at 10 months of age, and 4–5 mice per genotype for CBC results. There were no differences between genotypes. The following abbreviations are used: HGB, hemoglobin; PLAT, platelet count; thsn, thousand; mill, million. |
| 3 months of age | Triglycerides | Cholesterol | Free fatty acids | Glucose |
|----------------|---------------|-------------|-----------------|--------|
| mg/dl           | mg/dl        | mmol/liter  | mg/dl           |
| Wild type       | 63 ± 22       | 84 ± 27     | 0.178 ± 0.047   | 184 ± 38 |
| FASTie          | 63 ± 24       | 97 ± 28     | 0.195 ± 0.068   | 170 ± 26 |
| 10 months of age | Triglycerides | Cholesterol | Free fatty acids | Glucose |
|----------------|---------------|-------------|-----------------|--------|
| mg/dl           | mg/dl        | mmol/liter  | mg/dl           |
| Wild type       | 92 ± 3        | 106 ± 4     | 0.751 ± 0.087   | 154 ± 6  |
| FASTie          | 86 ± 6        | 100 ± 6     | 0.772 ± 0.074   | 156 ± 6  |
| 3 months of age | WBC           | RBC         | HGB             | PLAT |
| thsn/mm³        | mill/mm³     | g/dl        | thsn/mm³        |
| Wild type       | 7.8 ± 1.7     | 9.6 ± 0.4   | 15.2 ± 0.2      | 848 ± 13 |
| FASTie          | 9.1 ± 1.9     | 9.5 ± 0.4   | 15.4 ± 0.3      | 888 ± 84 |

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immunoprecipitated with an antibody to FAS, eNOS was detected in the pellet (Fig. 3A, middle panels). Using HUVECs (that intrinsically express both FAS and eNOS), immunoprecipitation using an antibody to eNOS yielded pellets containing FAS (Fig. 3B). Extracts from WT lung tissue immunoprecipitated with eNOS antibody contained FAS (Fig. 3C, left panels). eNOS was detected in the pellets from WT aorta immunoprecipitated with FAS antibody, but not when extracts from FASTie aorta were immunoprecipitated with FAS antibody (Fig. 3C, right panels).

The major product of FAS is palmitate, and eNOS is palmitoylated on cysteine residues, a lipid modification required for targeting eNOS to caveolae (24). To determine whether FAS deficiency decreases palmitoylated eNOS, we utilized a "biotin switch" assay that exchanges palmitoyl moieties for biotin (25). Protein extracts from lungs of either wild type or FASTie mice were treated with N-ethylmaleimide to block free thiol groups and prevent their subsequent biotinylation. HA cleavage of the thioester bond between fatty acids and cysteine residues generated nonlipidated cysteines that were biotinyl-labeled. Biotinylated proteins, including eNOS, were selected using streptavidin-agarose, and eNOS was detected by Western blotting. Palmitoylated eNOS (bands in extracts subjected to HA cleavage in Fig. 3D) was diminished in FASTie as compared with WT mice. Palmitoylated eNOS was also decreased in extracts from WT mice subjected to a prolonged fast (Fig. 3E), a condition that decreases FAS expression, and in extracts from mice treated with C75 (Fig. 3F), an FAS inhibitor. FAS deficiency did not alter palmitoylation of caveolin-1 (Fig. 3D–F), which interacts with eNOS (26). To confirm these observations in another system, siRNA-mediated knockdown of FAS followed by expression of eNOS was performed in COS-7 cells, and the biotin switch procedure was repeated, and this time employing immunoprecipitation with an eNOS antibody. Palmitoylated eNOS (detected with labeled streptavidin in extracts subjected to

**FIGURE 2. eNOS bioavailability in the setting of FAS deficiency.** A, Western blotting of eNOS and FAS in unfractionated whole cell lysates of lungs from wild type and FASTie mice. B, Western blotting of eNOS and caveolin-1 in fractionated membrane extracts of lungs. C, NOS enzyme activity (assayed using radiolabeled arginine) in membrane fractions from wild type and FASTie mice (n = 3 for each genotype). D, systolic (SBP) and diastolic (DBP) blood pressures in conscious mice by the tail cuff method. E, eNOS immunofluorescence in HUVECs treated with either vehicle (DMSO) or cerulenin (Ceru) for 24 h. F, NOS enzyme activity (assayed using radiolabeled arginine) in intact HUVECs treated with either vehicle (DMSO) or cerulenin for 24 h followed by ionomycin treatment for 30 min (n = 4 for each bar). G, NOS enzyme activity (assayed by stimulating with acetylcholine and quantifying nitrite release into the supernatant) in HUVECs treated with vehicle (DMSO) or cerulenin (Ceru) for 24 h. H, NOS enzyme activity assayed as in G in HUVECs subjected to FAS siRNA knockdown (n = 12–13 for each). I, COS-7 cells transfected with eNOS were subjected to FAS siRNA knockdown of FAS, and then eNOS (green) and FAS (red) were detected by immunofluorescence. Arrow points to the plasma membrane. J, Western blotting of the cells from I to confirm the lack of eNOS membrane localization with FAS knockdown. K, immunofluorescence of eNOS in COS cells treated with vehicle (DMSO) or cerulenin for 24 h. Con, control. * indicates p < 0.05; scale bar, 50 μm.
HA cleavage in Fig. 3G) was diminished with FAS deficiency. Inhibition of FAS activity with cerulenin in the same eNOS-reconstituted cell model showed similar effects on palmitoylated eNOS (data not shown). These findings suggest that FAS participates in S-acylation of eNOS. However, because palmitoylated eNOS was diminished but not eliminated in FASTie mice, it is possible that sources of palmitate other than FAS can contribute to eNOS palmitoylation.

To demonstrate FAS-dependent palmitoylation directly, control and FAS siRNA knockdown COS cells were metabolically labeled with [14C]acetate followed by immunoprecipitation of eNOS and isolation of eNOS-associated fatty acids by thin layer chromatography. A representative distribution of counts in fractions relative to standards is shown. The inset shows a Western blot of the cells from this experiment to verify the FAS knockdown and that equivalent amounts of eNOS were immunoprecipitated before being analyzed for radioactive carbon content.

FIGURE 3. FAS interacts with eNOS, and its deficiency decreases eNOS palmitoylation. A, COS-7 cells transfected with or without eNOS were immunoprecipitated with anti-eNOS antibody (top panels), anti-FAS antibody (middle panels), or subjected to preparation of total lysates without IP (bottom panels). Western blotting was performed using the antibodies indicated to the left of each gel. B, HUVEC lysates were immunoprecipitated with anti-eNOS or control antibody and then immunoblotted with anti-FAS or anti-eNOS antibodies. C, co-IP using lung (left panel) and aortic (right panel) tissue from mice. Lung tissue was subjected to IP with anti-eNOS or control antibody and then detected with anti-FAS or anti-eNOS antibodies. Aortic tissue from wild type and FASTie mice was subjected to IP with anti-eNOS or anti-FAS antibody and then immunoblotted with anti-eNOS antibody. D–F, acyl biotin switch assay of palmitoylated proteins shown in the upper panels and blotting of total lysates in the lower panels. HA dependence indicates endogenous labeling of acylated residues by biotin that is subsequently captured with an affinity column. Intensity of the Western blot signal reflects the degree of acylation. Extracts were blotted for eNOS and caveolin-1 (Cav-1). D, wild type and FASTie tissues were compared. E, tissues from wild type mice in the fed and fasting state were compared. F, tissues from wild type mice treated with vehicle (DMSO) or the FAS inhibitor C75 were compared. G, acyl biotin switch assay in eNOS-transfected COS-7 cells subjected to FAS siRNA knockdown. eNOS was immunoprecipitated and biotinylated residues were detected with streptavidin-HRP (top gel). eNOS blotting is shown in the bottom gel. H, endogenous carbon labeling of eNOS. COS-7 cells were treated as described in G, incubated with [14C]acetate, and then eNOS-associated fatty acids were analyzed by thin layer chromatography. A representative distribution of counts in fractions relative to standards is shown. The inset shows a Western blot of the cells from this experiment to verify the FAS knockdown and that equivalent amounts of eNOS were immunoprecipitated before being analyzed for radioactive carbon content. I, biotin switch assay of HUVECs subjected to FAS siRNA knockdown. J, biotin switch assay of HUVECs treated with 100 nM insulin for 12 h. Cont., control.
To address the possibility of a nonspecific interaction between FAS and eNOS, we generated a series of HA-tagged eNOS deletion mutants, expressed these peptides in COS cells, and then subjected extracts from these cells to immunoprecipitation with an FAS antibody. Truncated proteins missing the eNOS oxygenase domain were unable to interact with FAS in co-IP assays (Fig. 4A). These results suggest that the interaction between FAS and eNOS is specific, occurring at a site that remains to be definitively identified.

To determine whether the FAS-eNOS interaction is dependent on palmitoylation, we confirmed that the N-terminal 500 residues of eNOS interact with FAS but the N-terminal 150 residues (containing the palmitoylation sites) do not (Fig. 4B). These results are consistent with the concept that palmitoylation is not related to the FAS interaction but could be confounded by the possibility of protein misfolding. To address the issue of palmitoylation directly, we performed FAS IP with mouse lung and subjected the pellet to the biotin switch assay. Palmitoylated eNOS was detected in the pellet (Fig. 4C) indicating that FAS interacts with acylated eNOS.

We mutated the cysteines at residues 15 and 26 (required for palmitoylation) of eNOS to serines and transfected this HA-tagged C15S and C26S eNOS or wild type eNOS into COS cells. FAS IP brought down both the wild type and mutant eNOS (Fig. 4D), suggesting that palmitoylation is not required for the FAS-eNOS interaction.

To estimate what percentage of cellular FAS protein associates with eNOS, FAS protein present after immunoprecipitation with lung endothelial cells was compared with FAS protein detected in defined dilutions of total cellular extracts by Western blotting. In three separate experiments quantified by densitometry, 0.45 ± 0.15% of total FAS protein was associated with eNOS (blots not shown).

**FASTie Mice Manifest a Proinflammatory State**—Given the role of endothelial cells in mediating permeability, we infected MLECs from FAS floxed mice with a Cre adenovirus. Cells cultured in transwell inserts were treated with LPS, and leakage of Evans blue-albumin complexes from the luminal chamber to the abluminal chamber was quantified. In cells documented to have decreased FAS mRNA expression (Fig. 5A), there was more leakage reflecting increased permeability (Fig. 5B). If this phenotype is related to decreased eNOS bioavailability, the product of the eNOS reaction should be capable of reversing the effects of FAS deficiency. Using MLECs from

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**FIGURE 4. FAS interacts with some eNOS deletion mutants and with eNOS regardless of palmitoylation status.** A, schematic representation of the functional domains in eNOS is shown at upper left. A series of HA-tagged, truncated eNOS constructs (upper right) were expressed in COS cells. Lysates were immunoprecipitated using an anti-FAS antibody followed by Western blotting with eNOS fragments identified by HA-positive bands (middle panel, long exposure and short exposure to visualize the 1–500 band in lane 5). Truncated proteins missing the oxygenase domain (residues 150–500) in lanes 6 (residues 1–150) and 8 (residues 500–1204) did not co-IP with FAS. Western blotting of FAS (above long exposure) and HA (below long exposure) are also shown to demonstrate appropriate input for each lane. HC, heavy chain; LC, light chain; IB, immunoblot. B, expanded view of a repeat analysis of COS cells transfected with the 1–500 and 1–150 eNOS mutants and subjected to FAS and HA IP. The 1–500 but not the 1–150 mutant (containing palmitoylation sites) interacts with FAS. C, mouse lung extracts were immunoprecipitated with an FAS antibody, and then the immunoprecipitate was subjected to the biotin switch assay for S-acylated proteins. Hydroxylamine-dependent detection of eNOS reflects palmitoylated eNOS in the FAS IP. D, cysteines at residues 15 and 26 of full-length eNOS were mutated to serines (that cannot undergo S-acylation); mutagenesis was confirmed by sequencing, and then HA-tagged WT and mutated (C15S,C26S) eNOS molecules were expressed in COS cells. FAS IP was performed, and the pellet was subjected to blotting for HA to detect eNOS (left panel). HA blotting of input protein for the IP is shown on the right panel.
FASTie mice (Fig. 5C) or FAS-deficient HUVECs (Fig. 5D) cultured in transwell inserts and challenged with LPS, treatment with the nitric oxide donor sodium nitroprusside (SNP, 10 μM) 1 h before the assay diminished in vitro permeability. In FAS-deficient HUVECs, electrical resistance was decreased following LPS treatment (Fig. 5E), suggesting that the hyperpermeability phenotype is due in part to paracellular effects.

To confirm these observations in mice, we assayed blood vessel permeability following application of mustard oil to the ear. With this treatment, tissue extravasation of Evans blue-albumin complexes (Fig. 6A) and focal binding of a lectin (R. communis agglutinin I) to exposed basement membranes of ear blood vessels (Fig. 6B) were greater in FASTie compared with WT mice. Treatment of FASTie mice with a single dose of SNP (2 g/g) decreased mustard oil-induced tissue leakiness (Fig. 6C) and lectin binding (Fig. 6D) in ear vessels. Collectively, these data suggest that endothelial FAS loss causes vascular hyperpermeability by disrupting eNOS.

Hyperpermeability may reflect the presence of an enhanced inflammatory response. To determine whether defective eNOS signaling with FAS deficiency is proinflammatory, MLECs were treated with LPS (100 ng/ml) and assayed for expression of adhesion molecules. Message levels encoded by Icam-1, Clec2, Sele, and Vcam1 were higher in FASTie as compared with WT cells (Fig. 6E). To provide a functional correlate of this observation, in vitro adhesion was assayed using MLECs and fluorescence-labeled THP-1 (monocyte-like) cells. More adherent monocytes were found with FASTie as compared with WT endothelial cells (data not shown). Consistent with these in vitro observations, administration of low dose LPS (7 mg/kg) to mice caused greater inflammation in FASTie lungs as evidenced by inflammatory cells migrating from the vasculature to alveolar spaces (Fig. 6F). Myeloperoxidase (a neutrophil marker) was increased in FASTie compared with WT tissues after low dose LPS (data not shown).

FASTie mice were more likely than WT mice to die following low dose LPS (Fig. 6G), consistent with a proinflammatory phenotype. However, these findings could also be viewed as due to the lack of an appropriate inflammatory response. Just as a nitric oxide donor rescued the permeability phenotype (Fig. 6C), susceptibility to LPS-induced mortality was rescued by a single dose of SNP (2 μg/g) 4 h before LPS challenge (Fig. 6G). Because Tie2 Cre is also expressed in bone marrow, FAS effects in inflammatory cells might confound these results. Complete blood counts were normal in FASTie mice (Table 1), but FAS protein was decreased in FASTie as compared with control bone marrow (see Fig. 8 below). Therefore, we transplanted WT mice with WT or FASTie bone marrow (see Fig. 8 below). WT mice transplanted with FASTie marrow did not die after 7 mg/kg LPS (Fig. 6H), suggesting that the endotoxin-susceptibility phenotype in FASTie mice is unrelated to any potential effects of FAS on bone marrow cells and instead is due to FAS deficiency at the endothelium.

**FASTie Mice Have Impaired Angiogenesis**—Low grade inflammation in diabetes presumably contributes to vascular complications. One of the most intractable is peripheral vas-
cular disease (27), which leads to limb amputations associated with loss of eNOS integrity and defective angiogenesis (28). Because FAS deficiency disrupts eNOS integrity, we predicted that FASTie mice would have impaired ischemia-induced angiogenesis as occurs in diabetes models (29, 30). To address this possibility, we first determined the in vitro migration capacity of FAS-deficient endothelial cells in “scratch” assays (Fig. 7A). When FAS was knocked down in HUVECs, these cells were less able to decrease the width of a wound induced in culture (by “scratching” with a sterile tip) as compared with control cells (Fig. 7B). To confirm impaired migratory capacity with FAS deficiency, FASTie and WT aortic rings were cultured in an artificial extracellular matrix, and vessel outgrowth was quantified. Compared with control aortas, FASTie aortas had less vessel outgrowth (Fig. 7C and D) with fewer branches (or sprouting, Fig. 7C).

Confirmation of these in vitro findings was provided using a chronic hindlimb ischemia model. The femoral artery was ligated and excised, and blood flow was compared with the sham-operated contralateral limb over the ensuing month by laser Doppler perfusion imaging. WT mice progressively reestablished blood flow (see images from two control mice in the left panel of Fig. 7E), although this effect was impaired in FASTie mice (right panel, Fig. 7E, note the lack of perfusion at 4 weeks on the right side of the image for each FASTie animal). Quantified perfusion was lower in FASTie mice as compared with controls at 2 and 4 weeks (Fig. 7F); the standardized clinical ischemia score was higher (indicating more ischemic damage) in FASTie mice (Fig. 7G), and auto-amputation occurred in ~1/3 of FASTie as compared with none of the control mice (data not shown). Consistent with decreased angiogenesis in the setting of ischemia, capillary density in muscle tissue (assayed by detection of the endothelial marker CD31) supplied by the ligated femoral artery was decreased in FASTie as compared with control mice (Fig. 7H–J).

FIGURE 6. FASTie mice have endothelial dysfunction and are susceptible to endotoxin. A, Evans blue dye assays of tissue extravasation using mineral and mustard oil application to the ears of wild type and FASTie mice. B, visualization of exposed endothelial basement membrane using FITC-conjugated ricin lectin in ears treated with mustard oil. C and D, Evans blue dye assays and lectin staining following injection with a single dose of sodium nitroprusside (2 μg/g) before mustard oil treatment (n = 5–9 per condition). E, message levels for adhesion molecules over 4 h in MLECs treated with LPS (100 ng/ml) in vitro (n = 3 per genotype). F, representative H&E staining of lung tissues from wild type and FASTie mice 24 h after LPS injection. G, survival curves of mice following LPS (7 mg/kg). p < 0.01 by log rank testing for the comparison between wild type and FASTie mice. H, survival curves of wild type mice transplanted with bone marrow from either wild type or FASTie mice and treated with LPS (7 mg/kg) 2 months following the transplantation.

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transplanted with both WT and FASTie marrow (likely due to radiation exposure), but there was no difference in perfusion recovery or clinical score following femoral artery ligation (Fig. 8, D–F). Thus, loss of FAS at the endothelium impairs reparative angiogenesis.

To implicate the FAS-eNOS palmitoylation pathway in disease, we assessed eNOS palmitoylation in mouse diabetes models. If eNOS-mediated endothelial dysfunction is related to FAS, eNOS palmitoylation should be disrupted in diabetes. Biotin switch assays of lung tissue from animals with STZ (insulin-deficient) diabetes and from db/db mice (insulin-resistant diabetes) showed decreased eNOS palmitoylation (Fig. 9, A and B).

**DISCUSSION**

The current results suggest that FAS is involved in maintaining eNOS integrity, an observation potentially relevant to diabetes syndromes. Endothelial FAS is decreased in diabetes models; it is induced by insulin in freshly isolated human cells, and its inactivation disrupts targeting of eNOS to the plasma membrane by preventing eNOS palmitoylation. The absence of FAS in endothelial cells promotes inflammation and disrupts angiogenesis. In concert with FAS deficiency, diabetic endothelial cells have decreased eNOS palmitoylation. Our findings are schematically depicted in Fig. 9C. Insulin induces FAS, which generates palmitate (16:0, represented as a wavy line), which preferentially S-acylates eNOS to localize NO production to the plasma membrane (left panel of Fig. 9C). In FASTie mice with endothelial deletion of FAS (right panel of Fig. 9C), FAS-derived palmitate is unavailable to modify eNOS. eNOS, no longer tethered to caveolae where sources of tetrahydrobiopterin and arginine are optimized to produce NO, can produce superoxide (31) and contribute to oxidative stress resulting in endothelial dysfunction manifested as inflammation and impaired angiogenesis.

There is precedent for FAS involvement in cellular localization and signaling. Chemical and genetic inactivation of FAS disrupts the membrane localization and activity of the oncogene HER2 (c-erbB-2) in breast and ovarian cancer cell lines (32). Treatment of HUVECs with orlistat, a nonspecific FAS inhibitor also used clinically to treat obesity by inhibiting fat digestion, impairs proliferation in part by preventing the cell surface appearance of the VEGF receptor Flk1/VEGFR2/KDR (33). Overexpression of FAS in immortalized prostate cells leads to palmitoylation of Wnt-1, activation of β-catenin, and tumor formation in nude mice (34). These findings support the notion that de novo lipogenesis mediated by FAS may be involved in signaling events but do not establish how the metabolic regulation of FAS alters physiological responses such as inflammation and angiogenesis.
Our observations indicate that this occurs because endothelial FAS is induced by insulin and necessary for eNOS palmitoylation. eNOS is acylated by palmitate (at two cysteines near the N terminus through a post-translational process) and myristate (at an N-terminal glycine through a cotranslational process), lipid modifications that target eNOS to caveolae (35, 36). Palmitoylation is required for normal eNOS function through this effect on protein localization (24, 37, 38). Consistent with our findings, the association of eNOS with membrane fractions is decreased (with no effect on whole cell content of eNOS) in diabetic kidneys (39). Little is known about palmitoylation, a reversible S-acylation reaction. A palmitoyltransferase associates with eNOS, but substantial palmitoylation remains after knockdown of this transferase (40). FAS may form a complex with an unidentified acyltransferase and eNOS, or FAS itself may contain sufficient acyltransferase activity to palmitoylate eNOS (41).

Why would FAS be the preferred lipid source for eNOS palmitoylation? De novo lipogenesis requires considerable energy, and endothelial cells are continually exposed to palmitate, an abundant circulating fatty acid mostly of dietary origin. However, increasing the availability of circulating fatty acids disrupts eNOS production (9, 10), indicating that fatty acids have context-specific effects on eNOS. Palmitate that S-acylates eNOS is derived from insulin induction of endothelial FAS, reflecting recent access to nutrients because insulin increases in response to feeding. eNOS emerged phylogenetically as fish evolved into amphibians, i.e. as respiration was transitioned from water to air (42). Thus, one of the earliest functions of eNOS may have been to enhance perfusion (through vasodilation and angiogenesis) of peripheral tissues in the setting of regional hypoxia and tissue damage. Both were likely as animals left the nearly weightless aqueous environment and developed limbs (distant from the central oxy-

FIGURE 8. Defective ischemia-induced angiogenesis in FASTie mice is not due to decreased FAS in marrow elements. A and B, characterization of wild type mice after transplantation of marrow from wild type or FASTie mice. The 317-bp band in genomic DNA (A) and Western blotting of FAS in bone marrow cell lysates (B) indicates reconstitution with FASTie marrow. C, cell surface marker analysis of post-transplant mice gated for total white cells or CD11b-positive cells. Reconstitution is demonstrated by presence of the donor marker CD45.2 in recipients. D, laser Doppler imaging for hindlimb perfusion before and after femoral artery ligation in transplanted mice. E, quantification of ischemia by perfusion (n = 6 for each genotype). F, clinical scores of ischemia determined by extent of tissue necrosis (n = 6 for each genotype). BM, bone marrow; n.s., not significant.
gen exchange mechanism) that were subject to trauma. Linking enhanced perfusion and angiogenesis to insulin induction of FAS would be adaptive because palmitoylated eNOS signals adequate nutrition, an important determinant of tissue repair. Coordinating nutritional status, peripheral perfusion, and angiogenesis may have been so important to survival that redundant mechanisms for insulin signaling to eNOS were required. For example, insulin also stimulates eNOS through PI3K/Akt-mediated phosphorylation (43). Loss of Akt1, the major vascular isoform, decreases eNOS, increases permeability, and alters blood vessel maturation (44).

Although our results suggest that the link between FAS and eNOS is impaired in mouse models of diabetes, establishing a critical role for FAS in diabetic vascular disease would require reversing diabetic vascular dysfunction by rescuing aberrant FAS signaling. FAS has multiple domains, which have been approached pharmacologically to inhibit de novo lipogenesis (45, 46). Endogenous inhibitors also regulate FAS. CEACAM1, through a phosphorylation-dependent mechanism, acutely decreases FAS activity (47). The macrophage-derived protein AIM can be taken up by non-macrophage cells and inhibit FAS activity (48). These findings raise the possibility that interfering with an endogenous endothelial FAS inhibitor could increase FAS activity and promote eNOS palmitoylation. If FAS plays a significant role in protecting the vasculature, such an approach might improve vascular function in metabolic disease.

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REFERENCES
1. Selvin, E., Steffes, M. W., Zhu, H., Matsushita, K., Wagenknecht, L., Pankow, J., Coresh, J., and Brancati, F. L. (2010) N. Engl. J. Med. 362, 800–811
2. DCCT Group (1993) N. Engl. J. Med. 329, 977–986
3. UKPDS Group (1998) Lancet 352, 837–853
4. DCCT/EDIC Writing Team (2003) JAMA 290, 2159–2167
5. Gerstein, H. C., Miller, M. E., Byington, R. P., Goff, D. C., Jr., Bigger, J. T., Buse, J. B., Cushman, W. C., Genuith, S., Ismail-Beigi, F., Grimm, R. H., Jr., Probstfield, J. L., Simons-Morton, D. G., and Friedewald, W. T.
29. Emanuelli, C., Graiani, G., Salis, M. B., Gadau, S., Desortes, E., and Madeddu, P. (2004) *Diabetes* **53**, 1096–1103
30. Schiekofer, S., Galasso, G., Sato, K., Kraus, B. J., and Walsh, K. (2005) *Arterioscler. Thromb. Vasc. Biol.* **25**, 1603–1609
31. Berk, V., Wu, G., Yeh, H. C., Palmer, G., and Tsai, A. L. (2004) *J. Biol. Chem.* **279**, 32243–32251
32. Menendez, J. A., Vellon, L., Mehmii, I., Oza, B. P., Ropero, S., Colomer, R., and Lupu, R. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 10715–10720
33. Browne, C. D., Hindmarsh, E. J., and Smith, J. W. (2006) *FASEB J.* **20**, 2027–2035
34. Fiorentino, M., Zadra, G., Pallescandolo, E., Fedele, G., Bailey, D., Fiore, C., Nguyen, P. L., Migita, T., Zamponi, R. D., Priolo, C., Sharma, C., Xie, W., Hemler, M. E., Mucci, L., Giovannucci, E., Finn, S., and Loda, M. (2008) *Lab. Invest.* **88**, 1340–1348
35. Michel, T. (1999) *Braz. J. Med. Biol. Res.* **32**, 1361–1366
36. Shaul, P. W. (2002) *Annu. Rev. Physiol.* **64**, 749–774
37. Liu, J., García-Cardeña, G., and Sessa, W. C. (1996) *Biochemistry* **35**, 13277–13281
38. Sowa, G., Liu, J., Papapetropoulos, A., Rex-Haffner, M., Hughes, T. E., and Sessa, W. C. (1999) *J. Biol. Chem.* **274**, 22524–22531
39. Komers, R., Schütz, W. E., Reed, J. F., Lindsley, J. N., Oyama, T. T., Buck, D. C., Mader, S. L., and Anderson, S. (2006) *Diabetes* **55**, 1651–1659
40. Fernández-Hernando, C., Fukuta, M., Bernatchez, P. N., Fukuta, Y., Lin, M. I., Bredt, D. S., and Sessa, W. C. (2006) *J. Cell Biol.* **174**, 369–377
41. Ueno, K., and Suzuki, Y. (1997) *J. Biol. Chem.* **272**, 13519–13526
42. Toda, N., and Ayajiki, K. (2006) *Rev. Physiol. Biochem. Pharmacol.* **157**, 31–80
43. Zeng, G., Nystrom, F. H., Ravichandran, L. V., Cong, L. N., Oyama, T. T., Buck, D. C., Mader, S. L., and Anderson, S. (2006) *Diabetes* **55**, 1651–1659
44. Martinez-Villaluenga, C., Rupasinghe, S. G., Schuler, M. A., and Gonzalez-de Mejia, E. (2010) *FEBS J.* **277**, 1481–1493
45. Najjar, S. M., Yang, Y., Fernström, M. A., Lee, S. J., Deangelis, A. M., Rjaily, G. A., Al-Share, Q. Y., Dai, T., Miller, T. A., Ratnam, S., Ruch, R. J., Smith, S., Lin, S. H., Beauchemin, N., and Oyarce, A. M. (2005) *Cell Metab.* **2**, 43–53
46. Kurokawa, J., Ariai, S., Nakashima, K., Nishijima, A., Miyata, K., Ose, R., Morii, M., Kubota, N., Kadowaki, T., Oike, Y., Koga, H., Febbraio, M., Iwana, T., and Miyazaki, T. (2010) *Cell Metab.* **11**, 479–492