14S,21R-Dihydroxydocosahexaenoic Acid Remedies Impaired Healing and Mesenchymal Stem Cell Functions in Diabetic Wounds*

Received for publication, January 16, 2010, and in revised form, October 26, 2010 Published, JBC Papers in Press, November 26, 2010, DOI 10.1074/jbc.M110.100388

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Lipid-derived molecules also may play important roles as mediators in wound healing and angiogenesis. As shown from our lipidomic analysis, the essential fatty acid docosahexaenoic acid (DHA)2 is a relatively abundant endogenous lipid component in blood and in wounded full-thickness skin. DHA ameliorates complications of diabetes (3) and significantly promotes wound healing (4). Resolvins, neuroprotectins/protectins, and maresins are lipid mediators (LMs) that are generated naturally from essential ω-3 fatty acids, DHA, and eicosapentaenoic acid, during acute inflammation and have potent anti-inflammatory functions (5–9). Their discovery established a new avenue toward unraveling the mechanisms behind the beneficial effects of DHA. Neuroprotectin/protectin D1 was found to enhance corneal wound healing (10). Recently, we identified 14S,21R-dihydroxydocosahexaenoic acid (14S,21R-diHDHA), a novel endogenous lipid mediator derived from DHA, in skin wounds that is capable of promoting wound healing and angiogenesis (11).

However, diabetic hyperglycemia and the concomitant oxidative stress damage DNA, proteins, and lipids in various tissues resulting in dysfunction of cells and enzyme systems involved in wound healing and LM biosynthesis (12–15). Therefore, diabetic complications are likely to dysregulate the biosynthesis of DHA-derived LMs in wound healing. Treatments that counteract this dysregulation may improve diabetic wound healing.

Recent studies have used bone marrow-derived mesenchymal stem cells (MSCs) to promote wound healing (16–18). MSC transplants promote wound healing and angiogenesis by producing paracrine angiogenic cytokines (2, 19–21) and by possibly differentiating into skin cells (2, 19–24). Treating diabetics with their own MSCs can avoid side effects, including a graft versus host response associated with nonautologous transplantation. However, diabetes also impairs MSC pro-healing functions (24).

To date there are no reports regarding LM formation or the effects of LM either in diabetic wound healing and angiogenesis or in diabetes-impaired MSC functions. To identify dys-

* This work was supported by National Institutes of Health Grant 1R01DK087800 (to S. H.) and by the Start-up Fund from the Neuroscience Center of Excellence, LSUHSC-NO (to S. H.).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

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regulation in the formation of DHA-derived LMs in diabetic mice, we have conducted LC-MS/MS-based lipidomic studies (25) of wounded skin from diabetic db/db and nondiabetic db/+ mice. Here, we describe the identification of a novel DHA-derived mediator 14S,21R-diHDHA, which is suppressed in wounds of diabetic db/db mice and 12/15 (or l-12)-lipoxigenase gene knock-out mice (12/15-LOX is most related to human 15-LOX type-1) (26). We demonstrate that 14S,21R-diHDHA restored wound healing and angiogenesis in diabetic mice as well as the pro-healing functions of db/db MSCs and key cellular processes of angiogenesis. It activates the p38 MAPK but does not affect the ERK1/2, although signaling through both p38 MAPK and ERK1/2 is critical for wound healing and associated angiogenesis (27, 28).

EXPERIMENTAL PROCEDURES

Studies were blinded. Animal protocols were approved by the Institutional Animal Care and Use Committee and Institutional Review Board of Louisiana State University Health Sciences Center, New Orleans.

Mice—Diabetic db/db (BKS.Cg-m+/+leprdb/l) and nondiabetic db/+ mice (10-week-old, female) (Jackson Laboratory, Bar Harbor, ME) were used when blood glucose was 22–35 mm for db/db mice and 5–10 mm for db/+ mice, as measured by a glucometer. 12/15-LOX (Alox15) or l-12-LOX gene knock-out (12/15-LOX-KO) and C57Bl/6J congenic control mice (10 weeks, female) were purchased from The Jackson Laboratory. The KO mice do not express the 12/15-(or l-12)LOX and have a C57BL/6J genetic background (29–32).

Isolation and Identification of DMVECs from db/db and db/+ Mice—This was done as described previously (33) and as detailed in supplemental Fig. S1. The final DMVECs were 95% CD31+ and VE-cadherin+. The cellular identity of the DMVECs was confirmed by immunocytochemical analysis, which demonstrated their purity and expression of the endothelial markers CD31, VE-cadherin, von Willebrand factor, and Tie-2 (supplemental Fig. S1A).

Isolation and Identification of MSCs from Mice—MSCs were isolated as published previously (22) and described in supplemental Fig. S1. To verify the identity of the MSCs, we first confirmed their differentiation ability according to a previous report (18). The isolated MSCs exhibited a spindle-shaped morphology (supplemental Fig. S1B1). After culturing the MSCs for 10 days in osteogenic or adipocytic differentiation media, the cells differentiated into the osteoblastic (supplemental Fig. S1Bii) or adipocytic lineages (supplemental Fig. S1Biiv), respectively.

We also analyzed the MSCs by flow cytometry as in Ref. 18 and confirmed that more than 95% of cells expressed MSC marker Sca-1 (supplemental Fig. S1Biv) (18, 19, 22).

Splinted Excisional Wound Healing Model—This model was established as described previously (34) with minor modification. Briefly, paired 5-mm circular, full-thickness wounds were made symmetrically along the midline on the dorsal skin of mice. For each wound on a diabetic db/db mouse, 14S,21R-diHDHA in DMEM (50 ng/wound), MSCs (db/db or db/+), 10⁶ cells/wound), or 14S,21R-diHDHA (50 ng) plus MSCs (db/db, 10⁶ cells) was applied to the wound bed (10 μl) and injected intradermally at four points (10 μl/site) distributed evenly near the wound edge (50 μl total/wound). A donut-shaped silicone splint was adhered around the wound to prevent skin contraction and to allow wounds to heal through re-epithelialization and granulation (34). This model closely parallels human wound healing (34). In another experiment, wounds were imposed to the skin of db/+ mice, and 14S,21R-diHDHA in DMEM (50 ng/wound), MSCs (db/+), 10⁶ cells/wound), or 14S,21R-diHDHA (50 ng) plus MSCs (db/+), 10⁶ cells) was applied to the wound bed and wound edge. Skin wounds were also generated in 12/15-LOX-KO and control C57/6J mice and collected for the analysis of lipids.

Sample Preparation and LC-UV-MS/MS Analysis of Lipid Mediators—Prostaglandin F₂α-d₄, DHA, DHA-d₄, 5S,6R-di-hydroxyeicosatetraenoic acid (HETE), 5S,6S-diHETE, and porcine l-12-LOX were supplied by Cayman Chemicals (Ann Arbor, MI). Neuroprotectin-D1/protectin-D1/(NDP1/1) and its isomers prepared by total organic synthesis were provided by Dr. Nicolas G. Bazan, Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, and by Dr. Charles N. Serhan, Brigham and Women’s Hospital, Harvard Medical School. Adam’s catalyst (PtO₂) was from Sigma.

Lipid samples and standards were handled under dimmed light, stored in −80 °C freezer with head space of the containing vials purged by argon gas, and sealed tightly. The aqueous solutions of DHA and DHA derivatives were freshly prepared on water–ice and used right away. Wounded skin was collected immediately after the mice were sacrificed, soaked in ice-cold acetone, chilled immediately with liquid nitrogen, and stored in −80 °C freezer. Within 12 h of storage at −80 °C after collection, each tissue sample was extracted with ice-cold methanol three times by homogenization and sonication on ice. The acetone and methanol for the extraction contained 0.1% butylated hydroxytoluene to suppress auto-oxidation. The extracts for each sample were pooled together and purified via C18 solid-phase extraction and then analyzed using a LC-UV-MS/MS (LTQ, Thermo, Waltham, MA). The deuterated internal standards added to samples at the time of collection for quantification of interested components were prostaglandin F₂α-d₄ (20 ng), 14S,21R-diHDHA-d₁₀ (10 ng), 14S-HDHA-d₁₀ (10 ng), and DHA-d₁₀ (20 ng) (35–38). The LC unit was equipped with a chiral column (Chiralpak IA, 150 × 2.1 mm × 5 μm) (Chiral Technologies, West Chester, PA). The mobile phase had a flow rate of 0.2 ml/min, eluted as B (methanol/H₂O/acetic acid = 27:73:0.01) from 0 to 1 min, ramped from B/methanol (40:60) to B/methanol (20:80) by 50 min, then ramped to methanol by 55 min, and kept as methanol. DHA, DHA-d₁₀, 14S-HDHA, 14S-HDHA-d₁₀, 14S,21R-diHDHA, or 14S,21R-diHDHA-d₁₀ from the effluent of the chiral LC coupled to the mass spectrometer was analyzed on the basis of selected MS/MS ion chromatograms at mass to charge ratio (m/z) 191 of MS/MS 327, m/z 196 of MS/MS 332, m/z 205 of MS/MS 343, m/z 205 of MS/MS 348, m/z 253 of MS/MS 359, or m/z 253 of MS/MS 363, respectively. The amount of DHA, 14S-HDHA, or 14S,21R-diHDHA in each sample was then quantified based on its LC peak area ratio relative to that of its deuterated internal standard DHA-d₁₀, 14S-HDHA-d₁₀, or 14S,21R-diHDHA-d₁₀, respectively (39).
and granulation tissue area after hematoxylin and eosin (H&E) staining. Results were expressed as decreased percentage of relative epithelial gap compared with control [(relative epithelial gap in treatment − relative epithelial gap in control)/relative epithelial gap in control × 100%] and increased percentage of granulation tissue area compared with control (granulation tissue area in treatment − granulation tissue area in control)/granulation tissue area in control × 100%). Wound vascularity, as determined by CD31 positive area per field/total wound bed area per field × 100% (vascularity), was assessed by staining vessel endothelial cells with monoclonal rat anti-mouse CD31 antibody (BD Biosciences) followed by anti-rat IgG horseradish peroxidase (HRP) detection kits (BD Biosciences) and then by hematoxylin for nuclei. Images were captured using a microscope. Results are depicted as increased percentage of wound vascularity compared with control (% vascularity in treatment − % vascularity in control)/% vascularity in control × 100%.

Preparation of MSC-conditioned Media—Quiescent db/db MSCs (3 × 10⁵ cells) were prepared by starvation in DMEM containing high glucose (25 mM) and 0.5% FBS (Invitrogen) for 12 h. The cells were then cultured in DMEM containing high glucose (25 mM) without or with 14S,21R-diHDHA (100 nM) for 24 h. MSCs from non-diabetic db/+ mice were cultured similarly in the absence of 14S,21R-diHDHA. After centrifugation at 3,000 × g for 15 min at 4 °C, the supernatant became “MSC-conditioned medium.”

DMVEC Migration—Quiescent db/db and db/+ DMVECs at 80% confluence were resuspended in DMEM containing 25 mM (for db/db cells) or 5 mM (for db/+ cells) glucose and added to the upper chamber of a 24-transwell plate (8-μm pore, BD Biosciences) at 1 × 10⁴ cells/well. DMEM containing high glucose (25 mM) or low glucose (5 mM) in the presence or absence of 14S,21R-diHDHA (100 nM) or MSC-conditioned media were added to the lower chamber. The cells were allowed to migrate for 4 h at 37 °C in 5% CO₂. Migrated cells, which were attached to the undersides of membranes, were stained with Giemsa. The images were captured by a microscope. Results were expressed as increased percentage of migrated DMVECs compared with control using the equation ((migrated DMVECs per field in treatment − migrated DMVECs per field in control)/migrated DMVECs per field in control × 100%).

DMVEC Vasculature Formation—Quiescent db/db and db/+ DMVECs were cultured on Matrigel (BD Biosciences) in DMEM containing high glucose (25 mM, for db/db cells) or low glucose (5 mM, for db/+ cells) in the presence or absence of 14S,21R-diHDHA (100 nM) or MSC-conditioned media for 24 h at 37 °C in 5% CO₂. The vasculature length was measured using a microscope and ImageJ1.40 software (National Institutes of Health). Results were expressed as increased percentage of vasculature length compared with control using the equation (vasculature length per field in treatment − vasculature length per field in control)/vasculature length per field in control × 100%).

Bio-Plex Protein Array—Quiescent db/db DMVECs (3 × 10⁶ cells) were cultured in DMEM containing high glucose (25 mM) in the presence or absence of 14S,21R-diHDHA for
14S,21R-Dihydroxy-DHA for Diabetic Wound Healing

**FIGURE 1.** Formation of 14S,21R-diHDHA was suppressed in skin wounds in diabetic db/db mice compared with nondiabetic db/+ mice. Wounded skin was harvested from mice immediately following sacrifice at 24 h. The supernatants were collected. VEGF in db/db DMVEC supernatants or MSC-conditioned media was quantified by the Bio-Plex Protein array kit (Bio-Rad).

**Western Blot**—14S,21R-diHDHA (50 ng/wound) was immediately injected into wound bed and edge in db/+ and db/db mice after wounds were made. 15 min later, wounds were collected for analysis of expressions of P-p38 and p38. Quiescent db/db DMVECs or db/db MSCs were incubated in DMEM with or without 14S,21R-diHDHA (100 nM) for 10–120 min. Cells were lysed for analysis of expression of P-p38 and P-ERK1/2 (phosphorylated forms) as well as p38 and ERK1 (nonphosphorylated forms). Western blot was performed as described previously (27, 28) with minor modification. Briefly, 30 μg of total proteins from each lysed sample was resolved by SDS-PAGE on 4–15% Tris-HCl gels (Bio-Rad). The electrotransferred protein bands were stained by primary antibodies for P-p38 or P-ERK1/2 (BD Biosciences), followed by fluorescent-labeled secondary antibodies (LI-COR, Lincoln, NB), and finally quantified using an Odyssey Imaging System (LI-COR). When necessary, blots were stripped and reprobed.

**Results**—Results were analyzed by one-way ANOVA analysis of variance followed by Fisher’s LSD post hoc comparison and expressed as means ± S.E. A value of p < 0.05 was considered significant.

**RESULTS**

**Suppressed Formation of 14S,21R-diHDHA in Skin Wounds of Diabetic Mice**—To determine whether diabetes dysregulates DHA-derived LM formation in skin wounds, we first conducted LC-UV-MS/MS based mediator-lipidomic studies of wounded skin from diabetic db/db and nondiabetic db/+ mice. Two diHDHA chromatographic peaks (I and II) of ion m/z 253 from MS/MS spectra of peaks I and II (Fig. 1A) possessed ions at m/z 359 [M – H]−, 341 [M – H-H2O]−, 323 [M – H2O]−, 297 [M – CO2-H2O]−, and 279 [M – CO2-2H2O]− that were consistent with the molecular weight of 360, one carboxyl (for loss of one CO2 (44 atomic units)), and two hydroxyls (for 2H2O loss);
ions m/z 205, 233, 189 [233 − CO2]−, and 161 [205 − CO2]− indicated a hydroxyl at C14. Another hydroxyl at the C21 position was indicated by ions m/z 315 [M − H-CH(O)CH3]−, 271 [M − H2CO2-CH(O)CH3]−, and 253 [M − H2O-CO2-CH(O)CH3]− that involved C20-C21 cleavage in molecular ion m/z 359 [M − H]− and loss of CH(O)CH3 (44 atomic units) derived from “−H(OH)C21-H22 terminal” group of the molecular ion m/z 359 [M − H]− (insets, 5th panel of Fig. 1A). Additionally, ion m/z 315 could be [M − H-CH2O]−, which was generated by losing CO2 (44 atomic units) from molecular ion m/z 359 [M − H]−; and it further transformed to m/z 271 [M − H2CO2-CH(O)CH3]− and 253 [M − H-H2O-CO2-CH(O)CH3]− by the cleavage equivalent to what occurred in the formation of m/z 315 [M − H-CH(O)CH3]− from molecular ion m/z 359 [M − H]− and the loss of CH(O)CH3 (see MS/MS fragmentation pathways in insets of the 5th panel of Fig. 1A). As an α-cleavage to 21-hydroxy, C20-C21 cleavage generated MS/MS ions m/z 271 [M − H2CO2-CH(O)CH3]− and 253 [M − H2O-CO2-CH(O)CH3]− that are the fingerprints showing 21-hydroxy diHDHAs. In brief, these structural diagnostic ions indicate that peaks I and II are 14,21-diHDHA (Fig. 1A), namely 14,21-diHDHA existed in wounds (Fig. 1A, top).

The LC-MS/MS spectrum of H12-14S,21R-diHDHA prepared from hydrogenation of 14S,21R-diHDHA showed molecular ion m/z 371 [M − H] and ions m/z 353 [M − H-H2O]− and 335 [M − H2-2H2O]−, the 14-hydroxy was demonstrated by MS/MS ions m/z 157, 211, and 197 [241-CO2]−. The 21-hydroxy was demonstrated by m/z 325, 323 [325-2H], 307 [325-H2O]−, and 281 [325-CO2]−. These data are consistent with the structure of H12-14S,21R-diHDHA, consequently support the 14,21-diHDHA structure (supplemental Fig. S2E). The ChiralPak-IA-based LC condition above is able to separate chromatographically four 14,21-diHDHA diastereomers, including 14S,21R-diHDHA and 14S,21S-diHDHA generated from 14S-HDA by P450 (Fig. 1A, 3rd panel); and 14R,21R-diHDHA and 14R,21S-diHDHA generated from 14R-HDA by P450 (Fig. 1A, 4th panel); as well as an enantiomeric pair of 14S-HDA and 14R-HDA. The order of elution for 14,21-diHDHAs is based on the following observations: diastereomer 55S,6R-dihydroxyeicosatetraenoic acid (HETE) eluted before 55S,6diHETE with same double bond geometry; diastereomer 10R,17S-dihydroxy-NPD1 eluted before 10S,17R-dihydroxy-NPD1; and 10S,17R-dihydroxy-NPD1 eluted before 10S,17S-dihydroxy-NPD1 (the structures of NPD1 or PD1 stereoisomers were already elucidated and presented by Serhan et al. (44)) (supplemental Fig. S2F). The elution order is from 14R,21R-diHDHA (the earliest), 14R,21S-diHDHA, 14S,21R-diHDHA, to 14S,21S-diHDHA (the latest), which is the same as that for Chiralpak-AD-RH LC chiral column (11) except that 14R,21S-diHDHA and 14S,21R-diHDHA overlapped for the latter. The MS/MS spectra and chiral LC retention times for peaks I and II of wound tissue matched those for 14S,21R-diHDHA and 14S,21S-diHDHA, respectively, generated from 14S-hydroxy-DHA by P450 enzyme (Fig. 1A) (11). Therefore, peaks I and II from wounds are 14S,21R-diHDHA and 14S,21S-diHDHA. Their amount ratio was 9.5:1 based on their peak areas of the selected ion chromatogram at m/z 359 for wounds of nondiabetic mice.

The formation of 14S,21R-diHDHA and its biosynthetic precursor 14S-HDA (11) was reduced, although the reduction of 14S-HDA was modest, and 14S,21S-diHDHA was under the detection limit in wounds of diabetic mice compared with nondiabetic control animals (Fig. 1, A and B) based on LC-MS/MS quantification using deuterated internal standard of each compound. The DHA level was slightly reduced, but it was not significant. We also analyzed the skin wounds of 12/15-LOX-KO mice and controls. It was observed that the formation of 14S-HDA was significantly reduced, and 14S,21R-diHDHA was found in the wounds of wild-type controls but was undetectable in the 12/15-LOX-KO mice, although DHA levels were not significantly different (Fig. 1B, right panel). This implicates that 12/15-LOX participates in the formation of 14S,21R-diHDHA and 14S-HDA.

14S,21R-diHDHA Remedies Diabetic Wound Healing and Vascularization—Because 14S,21R-diHDHA formation was decreased in diabetic wounds, we were motivated to study whether administration of exogenous of 14S,21R-diHDHA could enhance wound healing. 14S,21R-diHDHA was applied into the wounds of db/db mice. Interestingly, the administration of 14S,21R-diHDHA promoted wound healing significantly (Fig. 2). In comparison with vehicle control, 14S,21R-diHDHA accelerated re-epithelialization and promoted granulation formation. Analysis of hematoxylin and eosin-stained cryosections of wounds collected at day 8 post-wounding revealed that the relative epithelial gap was decreased 36.2% (Fig. 2B, left panel), whereas the granulation tissue area was increased 60.7% (Fig. 2B, right panel). Because impaired vascularization or angiogenesis is a critical hallmark of nonhealing wounds in diabetes (45, 46), we investigated whether 14S,21R-diHDHA could promote vascularization during wound healing in diabetic mice. The data demonstrate that wounds from db/db mice treated with 14S,21R-diHDHA exhibited a 41.9% increase in vascularity compared with control animals at day 8 post-wounding (Fig. 2C). When 14S,21R-diHDHA was applied on wounds of db/+ mice, the same effects on re-epithelialization, granulation formation, and vascularization were observed (supplemental Figs. S4 and S5). We further used in vitro models of DMVEC migration and vasculature formation to investigate the action of 14S,21R-diHDHA on the cellular processes of angiogenesis. In simulated diabetic hyperglycemia (25 mM glucose in medium) (45, 46), 14S,21R-diHDHA promoted the cellular processes of angiogenesis through significantly enhancing db/db DMVEC migration 45.5% (Fig. 3A) and vasculature formation 40.8% (Fig. 3B). Moreover, diabetic db/db DMVECs treated with 14S,21R-diHDHA produced more vascular endothelial growth factor (VEGF), a potent angiogenic factor (47, 48), than control-treated cells (Fig. 3C). We also compared migration and vasculature formation between db/db DMVECs and db/+ DMVECs and found db/+ DMVECs could form a longer tube than db/db DMVECs without any treatment, but no difference was observed for migration. In addition, 14S,21R-diHDHA could also promote db/+ DMVEC migration and vasculature formation (supplemental Fig. S6).
14S,21R-Dihydroxy-DHA for Diabetic Wound Healing

14S,21R-diHDHA Acts Together with db/db MSCs to Promote Wound Healing—Previous reports have demonstrated that MSCs from diabetic donors are less efficient at promoting wound healing (2). To determine whether 14S,21R-diHDHA and db/db MSCs together could enhance wound healing in db/db mice better than either one alone, we administered 14S,21R-diHDHA (50 ng/wound) along with db/db MSC transplantation (10^6 cells/wound). This treatment resulted in a decrease in the relative epithelial gap of 75.2% (Fig. 4, A and B) and an increase in granulation tissue area of 107.8% (Fig. 4, A and C) at day 8 post-wounding. In particular, 14S,21R-diHDHA combined with db/db MSCs was more effective than db/db MSCs alone (Fig. 4, A–C) or 14S,21R-diHDHA alone (Fig. 2, A and B) in accelerating wound healing. In addition, administration of 14S,21R-diHDHA with db/db MSCs was as effective as db/+ MSCs alone (Fig. 4, A–C). Treatment with db/db and db/+ MSCs promoted re-epithelialization with decreased percentages of the relative epithelial gap of 25.6 and 61.7%, respectively, as well as increased percentages of the total granulation area by 52.0 and 82.4%, respectively, compared with control (Fig. 4, A–C). Indeed, db/db diabetic MSCs are less efficient than db/+ nondiabetic MSCs in promoting wound healing, indicating impaired wound healing due to diabetes. Therefore, 14S,21R-diHDHA coacts with db/db MSCs to promote diabetic wound healing and compensates for the diabetes-impaired pro-healing functions of db/db MSCs. In addition, we also confirmed that 14S,21R-diHDHA coacts with db/+ MSCs to promote wound healing on db/+ mice (supplemental Fig. S4).

14S,21R-diHDHA Remedies Diabetes-impaired Functions of db/db MSCs and Acts Synergistically with db/db MSCs in Promoting Angiogenesis—We further investigated whether 14S,21R-diHDHA would improve the function of db/db MSCs in promoting diabetes-impaired angiogenesis. The angiogenic functions of db/db MSCs were impaired compared with db/+ MSCs even though db/db MSCs increased wound vascularity 78.5% (versus 130.1% for db/+ MSCs) relative to...
the DMEM control (Fig. 5, A and B). The administration of db/db MSCs and 14S,21R-dihydroxy-DHA together to the wounds of db/db mice increased wound vascularity by 205.0%, which was significantly more than the summation of the enhancement percentages by db/db MSCs alone (78.5%) (Fig. 5B) and 14S,21R-dihydroxy-DHA alone (41.9%) (Fig. 2B), indicating a synergistic effect. Therefore, 14S,21R-dihydroxy-DHA remedies diabetes-impaired MSC functions and acts synergistically with MSCs to promote vascularization or angiogenesis in wounds of db/db mice. The synergistic promoting effect of 14S,21R-dihydroxy-DHA and db/+ MSCs on angiogenesis was also obtained on db/+ mice (supplemental Fig. S5).

Endothelial cell migration and vasculature formation, the key cellular processes of angiogenesis were studied under simulated hyperglycemia to delineate the action of 14S,21R-dihydroxy-DHA on diabetes-impaired angiogenic functions of db/db MSCs (Fig. 5, C–F). High glucose (25 mM) MSC-conditioned media were prepared by culturing db/db MSCs in the presence or absence of 14S,21R-dihydroxy-DHA and by culturing db/+ MSCs in the absence of 14S,21R-dihydroxy-DHA. The media were used to assess db/db DMVEC migration and vasculature formation (Fig. 5, C–F). Media conditioned by MSCs from db/db and db/+ mice increased db/db DMVEC migration 32.0 and 81.6%, respectively, and enhanced vasculature formation 43.4 and 83.3%, respectively, compared with controls (i.e. without MSC-conditioned media) (Fig. 5, C–F). As expected, medium from db/db MSCs without 14S,21R-dihydroxy-DHA treatment exhibited significantly less db/db DMVEC migration and vasculature formation (Fig. 5, C and D). 14S,21R-dihydroxy-DHA acted together with db/db MSCs to promote vascularization or angiogenesis in wounds of db/db mice (supplemental Fig. S5).
lature formation compared with db/+ MSCs, demonstrating impaired angiogenesis. When the medium from 14S,21R-diHDHA-treated db/db MSCs was used, db/db DMVEC migration and vasculature formation were enhanced 98.5 and 101.7%, respectively, relative to the vehicle control (Fig. 5, C–F). These results were greater than the combined increased percentages induced by db/db MSCs alone (32.0% for migration and 43.4% for vasculature formation) (Fig. 5, C–F) and 14S,21R-diHDHA alone (45.5% for migration and 40.8% for vasculature formation) (Fig. 3, A and B). These data indicate that 14S,21R-diHDHA rescues the impaired angiogenic functions of db/db MSCs in promotion of DMVEC migration and vasculature formation.

MSCs secrete angiogenic cytokines such as VEGF to promote angiogenesis (22). As such, we hypothesized that 14S,21R-diHDHA promoted angiogenic functions of db/db MSCs partly as a result of increased angiogenic cytokine production by the cells. To test this, VEGF levels were quantified in media from db/db MSCs, 14S,21R-diHDHA-treated db/db MSCs, and db/+ MSCs. The data demonstrate that greater amounts of VEGF were released by db/+ MSCs than by db/db MSCs (Fig. 5G), consistent with impaired angiogenesis in db/db MSCs. This impairment was restored by 14S,21R-diHDHA treatment, which induced db/db MSCs to secrete significantly more VEGF under simulated hyperglycemia (Fig. 5G). Taken together, these results suggest that 14S,21R-diHDHA can rescue the functions of db/db MSCs or synergize with db/db MSCs to promote angiogenesis by rescuing db/db MSC paracrine angiogenic functions.

14S,21R-diHDHA Activates the p38 but Not ERK1/2 Signaling Pathway—Activation of MAPK pathways is essential in wound healing and associated angiogenesis (28, 48, 49). Therefore, we sought to determine whether 14S,21R-diHDHA treatment led to activation of the MAPKs. Our results showed that 14S,21R-diHDHA treatment triggered phosphorylation of p38 in db/db DMVECs and MSCs, but had no effect on phosphorylation of ERK1/2. The ratio of phosphorylated p38 in db/db DMVECs and MSCs treated with 14S,21R-diHDHA remained higher than that of the control for at least 120 min (Fig. 6, A and B). In addition, 14S,21R-diHDHA treatment increased the levels of phosphorylated p38 in wounds of db/+ and db/db mice, and there was no significantly different levels of phosphorylated p38 in wounds of db/+ and db/db mice (Fig. 6, C and D).

DISCUSSION

The reduced formation of the novel reparative lipid mediator, 14S,21R-diHDHA, in cutaneous wounds of db/db diabetic mice indicates that diabetes suppresses the formation of this molecule (Fig. 1). Platelet (P)12-LOX, the enzyme counted for 12-LOX-activity of platelets (31, 50, 51), or l-12-LOX (5) converts DHA to 14S-hydroperoxy-DHA (14S-hydroperoxy-DHA), which is reduced to 14S-HDHA in tissues and further transformed to 14S,21R-diHDHA by the P450 enzyme (11). The formation of 14S-HDHA, the intermediate in 14S,21R-diHDHA biosynthetic pathways and a marker of 12-LOX activity (11), was also modestly reduced in diabetic wounds (Fig. 1B, middle panel). These results indicate that critical 12-LOX-related 14S-hydroxylation for biosynthesis of 14S,21R-diHDHA in wounds could be impaired in diabetes. The hyperglycemia and excessive oxidative stress in diabetic wounds appears to have impaired the DHA 14S-hydroxylation system, which reduced 14S,21R-diHDHA biosynthetic pathways and a marker of 12-LOX-related 14S-hydroxylation (Scheme 1).

It has been reported that the knock-out of 12/15 (or l-12)LOX gene from mice reduced DHA 14-hydroxylation by peritoneal macrophages by >95% (5). There was no report before to study of the effect of 12/15-LOX knock-out on DHA.
14S,21R-Dihydroxy-DHA for Diabetic Wound Healing

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SCHEME 1. Diabetes reduces formation of 14S,21R-diHDHA in cutaneous wounds and application of exogenous 14S,21R-diHDHA counteracts the diabetic impairment on healing, angiogenesis, and associated functions of mesenchymal stem cells. Treatment with 14S,21R-diHDHA rescues healing, angiogenesis, and associated MSC functions that are impaired in diabetes. 14S,21R-diHDHA restores the diabetes-impaired cellular processes of angiogenesis and paracrine functions of MSCs, including endothelial cell (EC) migration, vasculature formation, and production of VEGF (autocrine and paracrine). This lipid mediator activates the p38 MAPK pathway in endothelial cells and MSCs. 14S,21R-diHDHA may also act directly to enhance re-epithelialization and granulation tissue formation in wound healing.

14S,21R-Dihydroxy-DHA in skin wounds. Our results showed that 12/15-LOX knock-out reduced the formation of 14S-HDHA and 14S,21R-diHDHA in the murine skin wounds (Fig. 1B). The portion of 14S-HDHA that was still formed in 12/15-LOX-KO skin may result from P-12-LOX which is known to efficiently participate DHA 14S-hydroxylation and exist in murine skin (51–53). Therefore, 12-LOX plays a role in producing 14S-HDHA in skin wounds (Fig. 1). Additionally P-12-LOX is likely to participate in DHA 14S-hydroxylation. The levels of 14S-HDHA and 14S,21R-diHDHA in the skin wounds of db/+ mice were quite different from those of C57BL/6 mice (Fig. 1B), which may be due to the distinct phenotypic difference between two strains (strain information, The Jackson Laboratory).

In murine skin wounds, there are other lipoxigenases, including epidermis-type LOX3, 12R-LOX, and 8-LOX as well as 5-LOX from recruited leukocytes (26, 30, 31, 50, 53–59). However, LOX3, 12R-LOX, 8-LOX, or 5-LOX is unlikely to catalyze DHA 14S-hydroxylation in skin wounds based on the study conducted by other scientists (52) and ourselves. Therefore, 1- and P-12-LOXs could be the major 12-LOXs responsible in 14S-hydroxylation for 14S,21R-diHDHA formation. Diabetes could affect 1- and/or P-12-LOX in skin wounds, which is of our interest for future study.

21-Hydroxyl in 14,21-diHDHAs is a ω-1-hydroxyl, thus it is very likely to be generated by specific P450, such as 2E1 (60) or other P450s. P450 CYP1A1, 2B6/7, 2E1, 3A4/7, and 3A5 were identified at protein level and found to possess catalytic activities in skin (61); and many other P450s were found in skin at mRNA level (61). It is known that CYP2E1 generates 19-hydroxyeicosatetraenoic acid, an ω-1 hydroxylation product from arachidonic acid substrate (60); therefore, it is likely to participate in the ω-1 hydroxylation of 14S-HDHA substrate for the formation of 14S,21R-diHDHA in the skin (60).

Moreover, treating wounds with 14S,21R-diHDHA improved healing as well as vascularization or angiogenesis in diabetic mice (Fig. 2). Treatment with 14S,21R-diHDHA also rescued the key cellular processes of healing-associated angiogenesis: DMVEC transmigration and vasculature formation (Fig. 3). Angiogenesis is critical for growing new vessels that are essential for optimal healing (1). Endothelial cells in the vascular network adjacent to wounds migrate, proliferate, and undergo vascularization and neovessel formation within the granulation tissue (62). Diabetes impairs angiogenesis and causes microcirculatory deficiencies in skin wounds (1, 2). Thus, new approaches toward ameliorating impaired angiogenesis would significantly contribute to efforts to develop better treatments for diabetic wounds (2, 63). 14S,21R-diHDHA represents a new lead for this approach. The promotion of re-epithelialization and granulation tissue formation by 14S,21R-diHDHA in wounds suggests that this lipid mediator may also enhance other cellular processes of healing that involve epithelial cells and fibroblast cells (Scheme 1) (64).

When 14S,21R-diHDHA was combined with db/db MSCs and used to treat the wounds of diabetic db/db mice, the combination promoted the crucial processes of wound healing: re-epithelialization and granulation tissue growth (Figs. 2 and 4) (34, 65). Furthermore, 14S,21R-diHDHA was observed to restore diabetes-impaired angiogenic functions of db/db MSCs that promote wound vascularization, as well as db/db DMVEC migration and vasculature formation (Figs. 3 and 5). 14S,21R-diHDHA stimulated db/db DMVECs (Fig. 3C) and MSCs to increase VEGF secretion (Fig. 5G), which is at least partly responsible for accelerated angiogenesis in wound healing (Scheme 1). VEGF is one of the most important angiogenic cytokines (48) and promotes wound healing through enhancing angiogenesis (63). By producing VEGF, DMVECs promote their own angiogenesis processes, and MSCs promote vascularization achieved by endothelial cells and other type cells in wounds; thus DMVECs and MSCs manifest their autocrine and/or paracrine functions of promoting angiogenesis in wound healing (Scheme 1). The promotion of VEGF production represents a molecular mechanism for the proangiogenic functions of 14S,21R-diHDHA in wound healing.

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3 H. Tian, Y. Lu, S. P. Shah, and S. Hong, unpublished data.
and also further indicates that 14S,21R-diHDHA remedies diabetes-impaired pro-angiogenic functions of db/db MSCs.

Treatment of nonhealing wounds in diabetic patients with autologous MSCs is of clinical significance, but its value is restricted by deficient functions of the MSCs due to diabetes. In this regard, being a remedy for diabetes-impaired pro-angiogenic functions of db/db MSCs, 14S,21R-diHDHA is a promising new compound that could clinically restore these impaired MSC functions.

Activation of p38 and/or ERK1/2 is required for normal angiogenesis (27), including endothelial cell migration and vasculature formation (49, 66). p38 and ERK1/2 activation also have important roles in VEGF expression in endothelial cells and MSCs (67, 68). 14S,21R-diHDHA treatment results in immediate phosphorylation of p38 in wounds, db/db DMVECs, and MSCs, but not of ERK1/2, in db/db DMVECs and MSCs (Fig. 6), which suggests that the p38 signaling pathway is involved in 14S,21R-diHDHA promotion of healing and angiogenesis in diabetic wounds and MSC angiogenic functions. The receptor(s) of 14S,21R-diHDHA and mechanisms for 14S,21R-diHDHA activation of p38 signaling pathways are of interest for our future studies.

As summarized in Scheme 1, our studies reveal that diabetes impedes the formation of 14S,21R-diHDHA in wounds. Administration of exogenous 14S,21R-diHDHA rescues diabetes-impaired healing, angiogenesis, and associated MSC functions. These actions of 14S,21R-diHDHA appear to involve the activation of p38-MAPK signaling in endothelial cells and MSCs. This study identifies the novel lipid mediator, 14S,21R-diHDHA, as an important new lead for developing better therapeutics in the treatment of diabetic wounds.

The supplemental Fig. S1 shows the detailed isolation and identification of DMVECs and MSCs from mice. The supplemental Fig. S2 demonstrated that 14S,21R-diHDHA generated from 14S,21R-diHDHA by h-P450 and separated by chiral LC is highly pure, confirmed its structure by LC-MS/MS analysis of its hydrogenated product, and further justified its chiral analysis using the analogous diastereomers. The supplemental Fig. S3 shows that several products are generated by h-P450 from 14S,21R-diHDHA and are separated from 14S,21R-diHDHA by the chiral LC. The supplemental Fig. S4 shows 14S,21R-diHDHA coacts with db/+ MSCs to promote wound healings in db/+ mice. The supplemental Fig. S5 shows the synergistic effect of 14S,21R-diHDHA and db/+ MSCs on angiogenesis in db/+ mice. The supplemental Fig. S6 shows 14S,21R-diHDHA improves the cellular angiogenic processes of db/+ DMVECs.

Acknowledgments—We are very grateful to Dr. Nicolas Bazan (Neuroscience Center of Excellence at Louisiana State University Health Sciences Center) and Dr. Charles N. Serhan (Brigham and Women’s Hospital, Harvard Medical School) for providing authentic synthetic standards of protectin/neuroprotectin D1 and its isomers. We thank Ryan R. Labaden and Dr. Eric C. B. Milner for their expertise in editing and manuscript preparation, and we thank Dr. Ping Zhang and Constance Porretta (Louisiana State University Health Sciences Center Immunology Core) for FACS analysis.

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