14*S*,21*R*-dihydroxy-docosa-hexaenoic Acid Treatment Enhances Mesenchymal Stem Cell Amelioration of Renal Ischemia/Reperfusion Injury

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Bone marrow mesenchymal stem cells (MSCs) have shown potential to improve treatment of renal failure. The prohealing functions of MSCs have been found to be enhanced by treatment with the lipid mediator, 14*S*,21*R*-dihydroxy-docosa-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoic acid (14*S*,21*R*-diHDHA). In this article, using a murine model of renal ischemia/reperfusion (I/R) injury, we found that treatment with 14*S*,21*R*-diHDHA enhanced MSC amelioration of renal I/R injury. Treated MSCs more efficiently inhibited I/R-induced elevation of serum creatinine levels, reduced renal tubular cell death, and inhibited infiltration of neutrophils, macrophages, and dendritic cells in kidneys. Conditioned medium from treated MSCs reduced the generation of tumor necrosis factor- α and reactive oxygen species by macrophages under I/R conditions. Infusion of treated MSCs more efficiently reduced I/R-damage to renal histological structures compared with untreated MSCs (injury score: 7.9 ± 0.4 vs. 10.5 ± 0.5). Treated MSCs were resistant to apoptosis *in vivo* when transplanted under capsules of I/R-injured kidneys (active caspase-3+ MSCs: $4.2\% \pm 2.8\%$ vs. $11.7\% \pm 2.4\%$ of control) and *in vitro* when cultured under I/R conditions. Treatment with 14*S*,21*R*-diHDHA promoted viability of MSCs through a mechanism involving activation of the phosphoinositide 3-kinase -Akt signaling pathway. Additionally, treatment of MSCs with 14*S*,21*R*-diHDHA promoted secretion of renotrophic hepatocyte growth factor and insulin growth factor-1. Similar results were obtained when 14*S*,21*R*-diHDHA was used to inhibit apoptosis of human MSCs (hMSCs) and to increase the generation of renotrophic cytokines from hMSCs. These findings provide a lead for new strategies in the treatment of acute kidney injury with MSCs.

Introduction

BONE MARROW MESENCHYMAL stem cells (MSCs) have shown potential for improving the treatment of several incurable diseases through their ability to self-renew and differentiate into mesenchymal and non-mesenchymal cells [1–4]. In particular, MSCs can ameliorate renal ischemia/reperfusion (I/R) injury [5–10]. It has been reported that MSCs repair renal damage by differentiating into tubular epithelial cells [11], mesangial cells [12], endothelial cells [13], and podocytes [14], and by producing renotrophic cytokines and growth factors [15–19]. For example, MSCs can produce insulin growth factor-1 (IGF-1) and hepatocyte growth factor (HGF) [15], both of which are renal-protective or renotrophic factors [18,19].

Renal I/R injury induces secretion of inflammatory cytokines, chemokines, and other factors, which recruit neutrophils, macrophages, and dendritic cells (DCs) to the kidneys [20,21]. Inflammatory leukocytes generate more inflammatory cytokines, amplify the renal injury, and contribute to

tubular cell apoptosis and necrosis [20]. The renal reparative manifestation of MSCs involves their anti-inflammatory functions [9,15]. Studies show that MSC can suppress macrophage trans-differentiation to inflammatory phenotypes [22], inhibit DC maturation and migration, and halt inflammatory cytokine generation from leukocytes [22–24]. HGF is immunoregulatory and reduces inflammatory cytokine generation from DCs [25]. IGF-1 blocks not only the early onset of apoptosis, but also inflammation and accompanying tissue injury [26]. Although MSCs possess the capability to ameliorate acute kidney injury, the low viability of transplanted cells in I/R injured organs remains an important limitation for MSC therapy [2,27]. Reactive oxygen species (ROS) and inflammatory cytokines generated in I/R are the probable source of damage to the grafted MSCs [8,20]. Several remedial approaches have been recommended to overcome MSC apoptosis [2], including ischemic or cytokine preconditioning and *ex vivo* genetic modification of stem cells. For example, melatonin increases survival of MSCs and accelerates kidney recovery from acute kidney injury [8].

Kallikrein-modified MSCs exhibit enhanced renotrophic functions [9]. Thus, pretreating MSCs before transplantation has the potential to improve MSC viability and efficacy in the new, potentially inhospitable, niche in the tissue.

Docosahexaenoic acid (DHA) is an essential omega-3 fatty acid present in blood and kidneys [28]. Resolvin D series, neuroprotectin/protectins, and maresins are potent antiinflammatory lipid mediators (LMs) naturally generated from DHA during inflammation and/or resolution of inflammation [29–33]. In particular, resolvin D1 and protectin D1 have been reported to mitigate I/R injury in mice, reduce leukocyte infiltration to kidneys, and reduce generation of tumor necrosis factor- α (TNF- α) [28,34]. Neuroprotectin/protectin D1 has been reported to protect retinal epithelial cells and neuronal-glial cells from apoptosis induced by oxidative stress [35,36]. The discovery of these LMs suggested that the mechanism is responsible for the beneficial effects of DHA, and opened up directions to further delineate the mechanisms.

Recently, we identified a new DHA-derived LM, 14S,21R-dihydroxy-docosa4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acid (14S,21R-diHDHA), which promotes wound healing [37,38]. We also found that this LM stimulates augmented secretion of vascular endothelial growth factors by MSCs and promotes enhancement of wound healing [37]. However, the specific effects of DHA-derived LMs on renotrophic functions of MSCs have not been reported. The objective of this study was to test the possibility that treatment of MSCs with 14S,21R-diHDHA would more efficiently ameliorate renal I/R injury, and if true, to delineate the mechanisms mediating the effects of 14S,21R-diHDHA. The experiments were conducted by using an established murine renal I/R model [28,39] and in vitro cell culture of murine MSCs (mMSCs) and macrophages. To enhance the human relevance of this study and provide preclinical data, we also used human MSCs (hMSCs) in vitro to confirm results obtained with mMSCs.

Materials and Methods

Mice

We used 10-week-old female C57BL/6J mice (Jackson Lab). 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. Studies were conducted in a blinded fashion.

Cell cultures

mMSCs (from C57BL/6J mice) and hMSCs were provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White Hospital through a grant from the National Institutes of Health National Center for Research Resources (Grant No. P40RR017447, Temple, TX). More than 95% of mMSCs were positive for Sca-1 and CD29, and hMSC purity was confirmed by the expression of CD90 and CD105 (>95%). Both mMSCs and hMSCs exhibited adipogenic and chondrogenic differentiation ability and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% mesenchymal supplement (Stem Cell Technologies). Cells in passages 7 through 10 were used for experiments. The murine

macrophage cell line, RAW264.7 (American Type Culture Collection) was cultured in RPMI1640 containing 10% fetal bovine serum (FBS).

Generation, separation, and analysis of 14S,21R-diHDHA and its biosynthetic intermediates

14S/R-hydroxy-DHA (14S/R-HDHA) was from Cayman Chemical. 14S-HDHA was separated from 14S/R-HDHA by using chiral liquid chromatography-photodiode array ultraviolet detector-linear ion-trap tandem mass spectrometry (LC-UV-MS/MS) as described next. 14S,21R-diHDHA was prepared by incubating 14S-HDHA (50 μ g) with a human cytochrome P450 enzyme kit (BioCatalytics) at 30°C for 12 h [37]. Lipid was extracted for analysis as described [28,37]. For separation or analysis, each sample was subjected to LC-UV-MS/MS on a mass spectrometer (LTQ-XL; Thermo) equipped with a chiral column (Chiralpak-IA, 150 mm $\times$ 2.1 mm $\times$ 5 μ m; Chiral Technologies). The mobile phase gradient was as previously described [37]. The purified 14S,21R-diHDHA was determined to be $\geq$ 95% pure by chiral LC-UV-MS/MS [37].

Renal I/R injury model and MSC transplantation

Renal I/R injury was conducted as previously described [28] with some modifications. Briefly, mice were anesthetized by an intraperitoneal injection of ketamine/xylazine, and body temperature was maintained with a thermostatically controlled heating pad (37°C). A dorsal skin incision was made, and the left renal pedicle was clamped by using an atraumatic vascular micro clamp to occlude artery and vein. After 30 min, the clamp was removed, and the muscle and skin were closed. The right kidney was subjected to the same procedures. For mMSC infusion, cells were cultured in DMEM containing 10% FBS with or without 250 nM 14S,21R-diHDHA for 12 h and collected by trypsinization (0.25% trypsin/0.53 mM ethylenediaminetetraacetic acid; Invitrogen). The cells were labeled with PKH26 red fluorescence cell linker following the company's protocol (Sigma-Aldrich). Labeling efficacy was >98%. Viability, evaluated by trypan blue exclusion, was >98%. The MSCs (10^6) were washed and suspended in 100 μ L DMEM and injected into the tail vein after I/R. I/R control mice were subjected to the same I/R procedures and DMEM injection. Sham-operated control mice underwent the surgical procedures and DMEM injection without clamping of the renal pedicles.

Histology

Kidneys were collected at days 1 and 3 post-I/R and perfused with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde. Each kidney was sagittally cut into 2 parts; one part was used to prepare paraffin sections, and the other was used for cryosections. Sections were sagittally cut. The 3 μ m paraffin sections were stained with hematoxylin-eosin, and tubular damage was graded as described [40,41]. Briefly, 6 morphologic patterns were analyzed by light microscopy: (1) apical cytoplasm vacuolization; (2) tubular necrosis; (3) tubular dilatation; (4) cell detachment; (5) brush border integrity; and (6) denuded basement membrane. Morphologic changes were scored on a 5-point scale: 1, no

abnormality; 2, mild lesions affecting 10% or less of the kidney specimen; 3, lesions affecting 25% of the specimen; 4, lesions affecting 50% of the specimen; and 5, lesions affecting 75% or more of the specimen. Ten nonoverlapping high-power fields (hpf) were randomly chosen from the cortical and corticomedullary junction areas of each section (400 $\times$ magnification), and a total score calculated by adding the individual scores of each hpf was used to obtain a mean score per hpf for each mouse.

Immunocytochemistry

Cryosections (5 μ m) of kidneys were blocked with normal goat serum (1% in PBS) for 30 min at room temperature. Sections were then incubated with the following primary antibodies: rat anti-mouse Gr-1 (to detect neutrophils; BD Biosciences), rat anti-mouse F4/80 (to detect macrophages; Santa Cruz Biotechnology), American hamster anti-mouse CD11c (eBioscience) and rabbit anti-mouse MHCII (Santa Cruz Biotechnology) (to detect DCs), and rabbit anti-mouse active caspase-3 (to detect renal cell apoptosis; BD Biosciences). Primary antibodies were followed by incubation with the secondary antibodies: Cy3-labeled goat anti-rat immunoglobulin G (IgG), fluorescein isothiocyanate (FITC)-labeled goat anti-American hamster IgG, Cy5-, or FITC-labeled goat anti-rabbit IgG (Invitrogen). Normal rat IgG, rabbit IgG, and American hamster IgG (Santa Cruz Biotechnology) were used as isotype control. Nuclei were counterstained with Hoechst 33342. Positive cells were counted in ten nonoverlapping hpf (200 $\times$ magnification) from the cortical and corticomedullary junction areas of each section. The mean number of positive cells per hpf for each mouse was calculated.

Creatinine analysis

Blood was collected from the tail vein or by heart puncture at days 1 and 3 post-I/R. Serum creatinine concentration (a quantitative marker of renal failure) was determined with a creatinine enzymatic assay kit (Fisher Scientific).

Study of MSC apoptosis in vivo

Most grafted MSCs in organs die within a few days [2]. A few intravenously infused MSCs are retained in the kidneys [27,42], which makes it difficult to quantitatively study the effects of 14S,21R-diHDHA on MSC apoptosis in kidneys. Therefore, we transplanted MSCs under the kidney capsule following previously reported procedures [43]. Briefly, C57BL/6J MSCs were treated with 250 nM 14S,21R-diHDHA or vehicle control for 12 h and were then labeled with PKH26 red fluorescence cell linker. Renal I/R injury was conducted in C57BL/6J mice as described in "Renal I/R injury model and MSCs transplantation." A nick was generated on the kidney capsule by scratching with a 23-gauge needle. PKH26-labeled MSCs (2×10^6) in 5 μ L Matrigel (Growth factor reduced, BD Biosciences) were immediately transplanted through the nick underneath the capsule by using a 50 μ L syringe with a flat needle. The nick was sealed by low-heat cautery (SouthPointe Surgical). Muscle and skin were closed. Kidneys were collected at days 1 and 3 post-I/R and prepared for cryosections for active caspase-3 immunostaining.

In vitro I/R model

An in vitro I/R model was established as previously described [44–47] with some modifications. MSCs were cultured in DMEM in a hypoxia chamber (95% N₂, 5% CO₂) for 16 h at 37°C. The cells were then transferred to normal conditions (5% CO₂, 95% air) for 4 h. For inducing cell apoptosis, 800 μ M H₂O₂ was added into culture medium during reperfusion procedure in some experiments.

Terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick-end labeling, and DNA fragment analysis

To evaluate apoptosis, MSCs (5×10^4) were pretreated with 14S,21R-diHDHA for 12 h and subjected to the in vitro I/R procedure (with H₂O₂). The terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick-end labeling (TUNEL) assay was conducted by using a kit from Roche Applied Science according to the manufacturer's instructions. TUNEL was not used for renal sections obtained from in vivo experiments, because TUNEL staining of renal sections is known to produce false positive apoptosis results [48]. Nuclei were counterstained with Hoechst 33342. DNA fragments were analyzed with an enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science).

Phosphoinositide 3-kinase—Akt signaling

Phosphoinositide 3-kinase (PI3K) activity was analyzed by ELISA (Echelon Biosciences, Inc.) [45]. Since H₂O₂ can affect the analysis of PI3K activity, only hypoxia conditions (95% N₂, 5% CO₂) were used when analyzing PI3K activity. Phosphorylation of Akt was measured by Western-blot as previously described [49]. Briefly, quiescent MSCs (10^6) were obtained by culturing cells in DMEM for 12 h. Cells were then treated with or without 250 nM 14S,21R-diHDHA under hypoxia conditions for 10 min, 2 h and 12 h, respectively. For analysis of PI3K activity, cells were lysed with NP-40 (Sigma-Aldrich), and PI3K was precipitated by using a PI3K p85 α antibody and protein A-agarose (Santa Cruz). PI3K catalysis of the substrate PIP₂ into PIP₃ was then determined. For Western-blot, cells were lysed with RIPA buffer (Sigma-Aldrich) for analysis of expression of P-Akt (phosphorylated forms) and Akt (nonphosphorylated forms). Western blot was performed as described [37] with minor modifications. Briefly, 30 μ g of total proteins from each lysed sample were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4%–15% Tris-HCl gels (Bio-Rad). The electrotransferred protein bands were stained with primary antibody for P-Akt (Ser473) (Cell Signaling Technology), followed by fluorescent-labeled secondary antibody (LI-COR). Bands were quantified by using an Odyssey Imaging System (LI-COR). Blots were stripped and reprobed with antibody for Akt (Cell Signaling Technology). Anti- β Actin antibody (Sigma-Aldrich) was used as an internal control.

Viability assay

After pretreatment with 14S,21R-diHDHA, or with the PI3K inhibitor NVP-BEZ235 (Fisher Scientific), or with both 14S,21R-diHDHA and NVP-BEZ235 for 12 h, MSCs (5×10^4) were subjected to in vitro I/R procedures (with H₂O₂) and

then incubated with 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) for 3 h at 37°C. The amount of blue formazan dye formed from MTT was proportional to the number of live cells. The optical densities were measured at 540 nm with a reference wavelength of 630 nm by a microplate reader (Molecular Devices) and used as an index of cell viability.

Cytokine and ROS generation analysis

Quiescent mMSCs or hMSCs (5×10^4) were treated with 14S,21R-diHDHA (250 nM) in DMEM (under hypoxia condition) or RPMI1640 for 12 h. The culture media were collected and used as MSC-conditioned media. RAW264.7 macrophages were cultured in mMSC-conditioned media (RPMI1640) for 3 h. The media were replaced with fresh RPMI1640 without glucose, and macrophages were subjected to I/R procedure (without H_2O_2). Supernatants were collected and used for analysis of TNF- α and interleukin-1 β (IL-1 β) secreted by macrophages. ROS generation in macrophages was assessed by using the probe 2,7-dichlorofluorescein diacetate (DCFH-DA, 10 μ M; Sigma). After I/R (without H_2O_2) treatment, macrophages were cultured with DCFH-DA in RPMI-1640 for 30 min, and DCFH-DA fluorescence was measured by using an excitation wavelength of 480 nm and an emission wavelength of 535 nm [50]. HGF and IGF-1 in MSC conditioned media (DMEM, under hypoxia condition) were analyzed by ELISA kits obtained from RayBiotech and R&D Systems, respectively.

Statistical analysis

Results were analyzed by 1-way analysis of variance (ANOVA) followed by Fisher's least significant difference *post-hoc* test and expressed as mean $\pm$ standard error of the mean. 2-way ANOVA was used for the analysis of creatinine, neutrophils, macrophages, and DCs in kidneys, as well as apoptotic MSCs in kidneys. A *P* value less than 0.05 was considered significant.

Results

14S,21R-diHDHA treatment promotes the capacity of mMSCs to mitigate loss of kidney function and histological structures impaired by I/R injury

To determine whether 14S,21R-diHDHA-treated MSCs carry this compound when infused, we analyzed its level in MSCs by LC-MS/MS. 14S,21R-diHDHA was not detectable in MSC pellets after its treatment of MSCs for 12 h (Fig. 1A). To determine the effect of 14S,21R-diHDHA treatment on mMSC amelioration of I/R injury, mice were subjected to I/R injury followed by mMSC infusion, and renal function was assessed by serum creatinine level. We found that I/R injury increased serum creatinine compared with sham at day 1 (1.2 ± 0.06 vs. 0.35 ± 0.04 mg/dL, $P < 0.01$) and day 3 (1.08 ± 0.14 vs. 0.35 ± 0.04 mg/dL, $P < 0.05$) post-I/R (Fig. 1B), which is in agreement with previous reports [8,51]. Mice transplanted with mMSCs exhibited lower serum creatinine levels than the I/R control at day 1 (0.98 ± 0.06 mg/dL, $P = 0.08$) and day 3 (0.6 ± 0.05 mg/dL, $P < 0.05$) post-I/R, which is also consistent with a previous report [8]. However, 14S,21R-diHDHA-treated mMSCs were more effective than

untreated mMSCs in reducing serum creatinine (0.8 ± 0.09 mg/dL, $P = 0.07$ at day 1 and 0.3 ± 0.05 mg/dL, $P < 0.01$ at day 3 post-I/R (Fig. 1B).

Histologic analysis of the kidneys at day 3 post-I/R revealed considerable tubular damage of the cortical and corticomedullary junction areas with apical cytoplasm vacuolization, tubular necrosis, tubular dilatation, cell detachment, loss of brush border, and denuded basement membrane, thus resulting in a higher injury score (17.3 ± 0.2) than in sham controls (6.5 ± 0.3 , $P < 0.001$; Fig. 1C, D). Infusion of untreated MSCs after I/R reduced the injury score to 10.5 ± 0.5 ($P < 0.001$), but infusion of 14S,21R-diHDHA-treated MSCs further reduced the tubular damage score compared with the untreated MSC group (7.9 ± 0.4 , $P < 0.001$; Fig. 1C, D), thus demonstrating that 14S,21R-diHDHA improved MSC amelioration of I/R-induced tubular damage.

14S,21R-diHDHA treatment of mMSCs ameliorates kidney I/R injury through anti-apoptotic and anti-inflammatory mechanisms

Apoptosis is a critical mechanism for renal lesions in I/R injury [5,8,9]. MSC infusion attenuates renal cell apoptosis, thereby protecting kidneys [5,8,9]. Thus, we evaluated whether 14S,21R-diHDHA treatment increased MSC renal protection by enhancing MSC anti-apoptotic actions. Consistent with a previous report [8], more apoptotic (active caspase-3+) cells were detected in I/R-injured kidneys than in kidneys of sham controls (200.6 ± 12.8 vs. 30.4 ± 2.5 active caspase-3+ cells/hpf, $P < 0.001$; Fig. 2). As expected, MSC treatment reduced the number of apoptotic cells (100.8 ± 8.8 active caspase-3+ cells/hpf, $P < 0.001$; Fig. 2); moreover, 14S,21R-diHDHA-treated MSCs reduced the number of apoptotic cells in the kidneys (72.2 ± 6.8 active caspase-3+ cells/hpf, $P < 0.01$) compared with non-treated MSCs (Fig. 2), thus demonstrating that 14S,21R-diHDHA treatment enhanced the anti-apoptotic action of MSCs.

Inflammation caused by leukocytes plays an important role in I/R injury [20]. To investigate whether anti-inflammatory mechanisms were involved in the enhancement of MSC actions, we assessed neutrophil, macrophage, and DC infiltration of kidneys at days 1 and 3 post-I/R. The number of infiltrated neutrophils was clearly higher in the cortical and corticomedullary junction areas of I/R-injured kidneys at day 1 post-I/R than in kidneys of sham controls (11.8 ± 2.4 vs. 1.6 ± 0.5 neutrophils/hpf, $P < 0.001$; Fig. 3A, B). Transplanted MSCs attenuated neutrophil infiltration (6.7 ± 1.0 neutrophils/hpf, $P < 0.05$) compared with the I/R control. More strikingly, treatment with 14S,21R-diHDHA increased the effect of MSCs on neutrophil infiltration at day 1 post-I/R (3.0 ± 0.4 neutrophils/hpf, $P < 0.001$; Fig. 3A, B). Similarly, massive macrophage infiltration was observed in the cortical and corticomedullary junction regions of I/R-injured kidneys at day 3 post-I/R, whereas a few macrophages were observed in the sham controls (57.1 ± 4.4 vs. 2.5 ± 0.8 macrophages/hpf, $P < 0.001$; Fig. 3A, C). In MSC-treated mice, the number of macrophages in I/R-injured kidneys was clearly reduced compared with I/R controls (29.0 ± 2.3 macrophages/hpf, $P < 0.001$; Fig. 3A, C). Treatment with 14S,21R-diHDHA improved the anti-inflammatory action of MSCs at day 3 post-I/R (17.8 ± 2.1 macrophages/hpf, $P < 0.001$; Fig. 3A, C), although no difference was found

FIG. 1. 14S,21R-diHDHA-treated mMSCs mitigate deterioration of renal function and histological structures after renal ischemia/reperfusion injury. After I/R, mice received Dulbecco's modified Eagle's medium, PKH26-labeled mMSCs, or 14S,21R-diHDHA-treated PKH26-labeled mMSCs. **(A)** 14S,21R-diHDHA was not detectable in the MSC pellets after its treatment of MSCs, because there was no peak of this compound in the liquid chromatography-linear ion-trap tandem mass spectrometry selective reaction monitoring chromatogram (m/z 359 > 253). **(B)** Serum creatinine (days 1 and 3; $n=6$). **(C)** Hematoxylineosin-stained kidney (day 3; magnification, $\times 400$; scale bar = 50 μ m). Arrowheads show glomeruli. **(D)** Tubular damage ($n=6$). Results are expressed as mean $\pm$ SEM. * $P < 0.05$ versus sham, † $P < 0.05$ versus I/R control, ‡ $P < 0.05$ versus MSCs. 14S,21R-diHDHA, 14S,21R-dihydroxy-docosa-4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acid; I/R, ischemia/reperfusion; mMSCs, murine mesenchymal stem cells; SEM, standard error of the mean.

between mice transplanted with 14S,21R-diHDHA-treated MSCs and nontreated MSC controls at day 1 post-I/R ($P=0.34$; Fig. 3C). During I/R injury, TNF- α causes an influx of pro-inflammatory DCs into kidneys and contributes to parenchymal damage [52]. CD11c+MHCII+ DCs were increased in I/R injured kidneys (Fig. 3A, D). However, MSC infusion considerably decreased DC influx compared with I/R control at day 1 post-I/R (9.6 ± 1.1 vs. 20.1 ± 2.9 DCs/hpf, $P < 0.001$) and day 3 post-I/R (7.0 ± 0.9 vs. 14.0 ± 1.2 DCs/hpf, $P < 0.001$) (Fig. 3A, D). MSCs treated with 14S,21R-diHDHA further decreased infiltrated DCs in kidneys compared with nontreated MSCs at day 1 ($P < 0.05$) and day 3 ($P < 0.05$) post-I/R, thus suggesting that 14S,21R-diHDHA treatment improved the anti-inflammatory action of MSCs (Fig. 3A, D).

14S,21R-diHDHA treatment inhibits apoptosis of MSCs in vivo

Since treatment with 14S,21R-diHDHA enhanced the ability of MSCs to ameliorate I/R injury (Fig. 1), coupled with the fact that increased MSC survival is associated with enhanced MSC promotion of kidney recovery from I/R

injury [8,9], we hypothesized that treatment with 14S,21R-diHDHA inhibited apoptosis of MSCs. To test this hypothesis, we assessed apoptosis of PKH26-labeled MSCs transplanted under kidney capsule. Nearly $11.7\% \pm 2.4\%$ of grafted MSCs at day 3 post-I/R were active caspase-3+ and underwent apoptosis (Fig. 4). However, treatment with 14S,21R-diHDHA decreased apoptosis of transplanted MSCs as shown by decreased active caspase-3+ cells ($4.2\% \pm 2.8\%$, $P < 0.05$; Fig. 4), thus suggesting that 14S,21R-diHDHA inhibits apoptosis.

Enhanced MSC survival after 14S,21R-diHDHA treatment involves PI3K activation

To confirm the action of 14S,21R-diHDHA on viability and apoptosis of MSCs in vivo in kidneys, and to explore possible mechanisms, we subjected mMSCs to the in vitro I/R procedure, that is, hypoxia followed by H_2O_2 [8,45]. Results of TUNEL analysis revealed that apoptosis induced by in vitro I/R was reduced by 14S,21R-diHDHA treatment (non-I/R control: 0.4 ± 0.2 TUNEL+ cells/hpf; I/R control: 20 ± 1.8 TUNEL+ cells/hpf; 250 nM 14S,21R-diHDHA:

FIG. 2. 14S,21R-diHDHA treatment enhances anti-apoptosis action of mMSCs in kidneys after renal ischemia/reperfusion injury. Immunostaining against active caspase-3 was performed at day 3 post-I/R. **(A)** Representative kidney sections (magnification, $\times 200$; scale bar = 50 μ m). **(B)** Active caspase-3+ apoptotic cells (cells/hpf). Results are expressed as mean $\pm$ SEM, $n = 6$. * $P < 0.05$ versus sham, † $P < 0.05$ versus I/R control, ‡ $P < 0.05$ versus MSCs.

13.4 ± 0.9 TUNEL+ cells/hpf, $P < 0.01$ compared with I/R control; Fig. 5A, B).

PI3K-Akt signaling regulates cell survival; therefore, its activation may enhance MSC viability and protect against apoptosis [53]. For this reason, we investigated the effect of 14S,21R-diHDHA on PI3K activation in MSCs. We found that PI3K activity was rapidly induced in mMSCs by 14S,21R-diHDHA treatment ($P < 0.01$; Fig. 5C). Akt plays an important role in controlling cell survival and apoptosis [53,54] through phosphorylating and inactivating several targets, including Bcl-2 homology domain 3-only protein (BAD), Forkhead box transcription factor, class O (FOXO), and caspase-9 [55]. Treatment with 14S,21R-diHDHA triggered phosphorylation of Akt (Fig. 5D). Results of MTT analysis showed that in vitro I/R reduced MSC viability, but 14S,21R-diHDHA treatment restored viability in a dose-dependent manner (Fig. 5E). Conversely, the increase in MSC viability mediated by 14S,21R-diHDHA was diminished by blocking PI3K activation (Fig. 5E). Taken together, these results indicate that treatment with 14S,21R-diHDHA enhances viability and inhibits apoptosis of MSCs, at least in part, by activating the PI3K-Akt signaling pathway.

To determine whether these findings are applicable to hMSCs, we evaluated the effect of 14S,21R-diHDHA treatment on hMSC viability and apoptosis under the same in vitro I/R conditions. As predicted, 14S,21R-diHDHA treatment reduced hMSC apoptosis (Fig. 6A, B) and DNA fragmentation (Fig. 6C). Additionally, 14S,21R-diHDHA activated the PI3K (Fig. 6D) and increased hMSC viability (Fig. 6E).

14S,21R-diHDHA treatment enhances mMSC anti-inflammatory function in vitro by suppressing the generation of inflammatory cytokines and ROS in RAW264.7 macrophages

Acute kidney injury has been shown to induce macrophages to produce inflammatory cytokines and ROS that play crucial roles in inflammation and injury processes of kidneys [20,28]. In order to determine whether 14S,21R-diHDHA treatment enhances MSC anti-inflammatory function, we studied the effect of 14S,21R-diHDHA-treated MSCs on the generation of inflammatory cytokines and ROS by RAW264.7 macrophages under I/R conditions. Although 14S,21R-diHDHA-treated MSCs had no effect on IL-1 β generation (Fig. 7A), MSC conditioned media reduced the generation of TNF- α (67.1 ± 1.4 vs. 104.0 ± 3.8 pg/mL, $P < 0.01$) from RAW264.7 macrophages compared with I/R controls (Fig. 7B). When 14S,21R-diHDHA-treated MSC conditioned medium was used, the level of TNF- α was further decreased to 43.1 ± 2.8 pg/mL ($P < 0.01$ compared with MSC conditioned medium; Fig. 7B). ROS generation in RAW264.7 macrophages can be induced by I/R treatment. However, MSCs reduced ROS levels in RAW264.7 cells ($P < 0.05$), and 14S,21R-diHDHA treated MSCs further decreased ROS induction by I/R treatment ($P < 0.01$; Fig. 7C). These results suggest that 14S,21R-diHDHA treatment enhances MSC anti-inflammatory function by reducing the capacity of macrophages to generate inflammatory cytokines and ROS.

14S,21R-diHDHA treatment enhances mMSC and hMSC secretion of renoprotrophic growth factors

MSCs generate an array of cytokines and growth factors [15,56] that protect kidneys from injuries [15–17,57]. Therefore, we postulated that 14S,21R-diHDHA treatment enhanced the beneficial effects of MSCs by augmenting their secretion of renoprotrophic factors. We found that 14S,21R-diHDHA treatment increased the amount of the renoprotrophic growth factors IGF-1 and HGF secreted by mMSCs into the cell culture medium (IGF-1: 15.3 ± 1.0 vs. 10.7 ± 1.5 pg/mL, $P < 0.05$; HGF: 405.3 ± 10.6 vs. 277.5 ± 2.9 pg/mL, $P < 0.01$; Fig. 8A, B). Treatment with 14S,21R-diHDHA also augmented the generation of HGF and IGF-1 by hMSCs (IGF-1: 1982.7 ± 194.3 vs. 1208.0 ± 115.4 pg/mL, $P < 0.05$; HGF: 36.5 ± 2.6 vs. 18.7 ± 1.7 pg/mL, $P < 0.001$; Fig. 8C, D).

Discussion

Compared with untreated MSCs, 14S,21R-diHDHA-treated MSCs more efficiently improved renal function by suppressing I/R-induced elevation of serum creatinine (Fig. 1), restoring I/R-damaged renal histological structures, and decreasing tubular cell apoptosis (Fig. 2).

FIG. 3. 14S,21R-diHDHA treatment promotes anti-inflammatory action of mMSCs after renal ischemia/reperfusion injury. Immunostaining against Gr-1, F4/80, CD11c, and MHCII were performed on kidney sections collected at days 1 and 3 post-I/R. **(A)** Representative kidney sections showing Gr-1+ neutrophils, F4/80+ macrophages, and CD11c+MHCII+ DCs (arrows) at day 1 or 3 post-I/R (magnification, $\times 200$; scale bar = $50\ \mu\text{m}$). Quantification of Gr 1+ neutrophils **(B)**, F4/80+ macrophages **(C)**, and CD11c+ MHCII+ DCs **(D)** in kidney. Results are expressed as mean $\pm$ SEM, $n=6$. * $P<0.05$ versus sham, $^{\dagger}P<0.05$ versus I/R control, $^{\ddagger}P<0.05$ versus MSCs. Color images available online at www.liebertonline.com/scd

FIG. 4. 14S,21R-diHDHA treatment decreases apoptosis of mMSCs in kidneys after renal ischemia/reperfusion injury. PKH26-labeled mMSCs in matrigel were transferred under kidney capsules of mice immediately after I/R. Kidneys were collected at days 1 and 3 post-I/R and prepared for cryosections that were then subjected to active caspase-3 staining. **(A)** Apoptotic PKH26-labeled mMSCs (yellow stained appearance resulting from the overlap of red PKH26+ labeling of MSCs and green apoptosis marker of active caspase-3+) in cryosections of kidney. **(B)** Quantification of apoptotic PKH26-labeled mMSCs in kidney: number of active caspase-3+ PKH26-labeled mMSCs/100 PKH26-labeled mMSCs ($n=15$). Magnification, $\times 200$. Scale bar = 50 μ m. Results are expressed as mean $\pm$ SEM. * $P < 0.05$ compared with MSC control without treatment. Color images available online at www.liebertonline.com/scd

Tubular cell damage is an important characteristic of acute kidney injury, and the elevation of serum creatinine indicates impaired renal functions [6–9,15]. Apoptosis is an early event in renal I/R injury that interacts with the subsequent inflammation and tissue injury. Our data demonstrate that 14S,21R-diHDHA treatment improves the capacity of transplanted MSCs to ameliorate I/R injury. The observed effects were not likely mediated by direct transfer of free 14S,21R-diHDHA, because this LM was not detectable in MSC pellets after treatment for 12 h (Fig. 1A), and MSCs were re-suspended in fresh 14S,21R-diHDHA-free DMEM before infusion. Although 14S,21R-diHDHA was not detectable in renal tissue by LC-MS/MS, it is known to promote wound healing [37,38,58], and its potential use as a therapeutic alone in treating I/R injury should be assessed in addition to being used as a promoter of MSC renoprotective functions under the scope of this report.

Both animal studies and clinical studies have demonstrated the safety, feasibility, and efficacy of MSC-based therapies for repair of acute kidney injury [6–9,15]. However, the mechanisms by which MSCs ameliorate I/R injury are not clear. Several studies have demonstrated the capacity of MSC to differentiate into kidney cells [11–14]. Controversial studies indicated that the grafted MSCs were not the predominant cellular components involved in the regeneration of injured tubuli [7,59], and it has been postulated that, rather than differentiation, cell fusion accounts for these cells [7,59–61]. Recent studies have ascribed reparative effects of MSCs to their endocrine/paracrine functions [15–19]. MSCs

can generate different cytokines and growth factors that are important to injury recovery [17,57,62, 63], including renoprotective factors IGF-1 and HGF (Fig. 8). We found that 14S,21R-diHDHA promoted generation of these two growth factors by MSCs (Fig. 8).

Renal ischemia and reperfusion results in leukocyte infiltration and activation, which play crucial roles in kidney inflammation and injury [28]. Treatment with 14S,21R-diHDHA enhanced the capacity of MSCs to suppress neutrophil, macrophage, and DC infiltration in kidneys post I/R (Fig. 3), which parallels the enhanced capacity of 14S,21R-diHDHA-treated MSCs to inhibit renal tubular cell death in kidneys (Figs. 1 and 2). Depletion of macrophages during acute kidney injury reduces renal inflammation and protects kidneys against ischemic injury [28,64]. Conditioned medium from 14S,21R-diHDHA-treated MSCs is more efficient at inhibiting the generation of TNF- α and ROS by macrophages than is conditioned medium from untreated MSCs (Fig. 7), which suggests that 14S,21R-diHDHA treatment promotes production of anti-inflammatory mediators by MSCs in I/R injury.

The low viability of transplanted MSCs in I/R injured organs remains an important limitation for MSC therapy [2,27]. The enhancement of MSC renoprotective functions after 14S,21R-diHDHA treatment is likely mediated in part by the promotion of MSC survival, as suggested by the reduced rate of apoptosis of MSCs grafted into I/R injured kidneys, an inhospitable environment for MSC survival (Fig. 4). This notion was further supported by *in vitro* experiments

FIG. 5. 14S,21R-diHDHA treatment reduces apoptosis of mMSCs and promotes viability of mMSCs via a mechanism involving activation of PI3K-Akt. After in vitro I/R treatment (Ischemia: 95% N₂, 5% CO₂, reperfusion: 5% CO₂, 95% air and treated with 800 μM H₂O₂), mMSC apoptosis, viability, or PI3K-Akt signaling (under hypoxia: 95% N₂, 5%CO₂) was analyzed. (A) Representative micrographs of TUNEL stained apoptotic mMSCs. (B) Quantification mMSC apoptosis: the number of TUNEL+ apoptotic cells/hpf ($n=15$; magnification, $\times 200$; scale bar=50 μm). (C) PI3K activity of mMSCs ($n=3$). (D) mMSC lysates were analyzed by western blot. P-Akt quantities relative to Akt were measured based on optical densities of the gel bands, and normalized to that of hypoxia control ($n=4$). (E) mMSC viability was decreased by PI3K inhibition. The mMSCs were pretreated with 14S,21R-diHDHA, PI3K inhibitor NVP-BEZ235, or 14S,21R-diHDHA + NVP-BEZ235 and then subjected to in vitro simulated I/R treatment. Cell viability was analyzed by MTT ($n=6$). Results are expressed as mean $\pm$ SEM. * $P<0.05$ versus non-I/R. † $P<0.05$ versus I/R or hypoxia controls. ‡ $P<0.05$ for the marked comparison. TUNEL, terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick-end labeling; PI3K, phosphoinositide 3-kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

showing that 14S,21R-diHDHA treatment increased viability and reduced apoptosis of MSCs (Fig. 5).

Activation of PI3K-Akt signaling pathway is crucial to many aspects of cell growth and survival in adverse physiologic and pathologic conditions. PI3K activates Akt kinase, which directly binds and inactivates BAD and subsequently blocks the functions of proapoptotic proteins [65]. Akt can directly phosphorylate FOXOs and inhibit their ability to induce the expression of death genes, subsequently resulting in the inhibition of the activation of caspase-9 [55]. Many approaches to enhancing MSC viability involve activating the PI3K-Akt signaling pathway [2]. Overexpression of PI3K in cardiomyocytes significantly enhanced their survival on subsequent exposure to hypoxia [66]. Akt gene modified stem cells exhibited enhanced survival after engraftment into

the infarcted heart [67]. We found that 14S,21R-diHDHA treatment activated the PI3K-Akt signaling pathway in MSCs, and that inhibition of PI3K activity in MSCs suppressed the capacity of 14S,21R-diHDHA treatment to promote MSC viability (Figs. 5 and 6), thus suggesting that the mechanism by which 14S,21R-diHDHA treatment promotes MSC survival involves, or is mediated through, the PI3K-Akt signaling pathway.

Differences between hMSCs and mMSCs were previously reported. For example, hMSCs, but not mMSCs, possess antimicrobial properties against a range of clinically relevant bacteria through their ability to up-regulate the expression of indoleamine 2,3-dioxygenase [68]. Additionally, mMSCs, but not hMSCs, express inducible nitric oxide synthase [68], and it has been shown that hMSCs and mMSCs express different

FIG. 6. 14S,21R-diHDHA treatment decreases apoptosis of hMSCs, increases their viability, and increases activation of PI3K. After 14S,21R-diHDHA pretreatment, hMSCs were subjected to simulated I/R (with H_2O_2) or hypoxia. (A) Apoptotic cells were stained by TUNEL and (B) quantified: number of TUNEL+ apoptotic cells/hpf ($n=15$, magnification, $\times 200$. Scale bar = $50 \mu m$). (C) DNA fragment was analyzed by ELISA kit ($n=4$). (D) PI3K activity. Cells were treated with 14S,21R-diHDHA under hypoxia conditions. Activity of PI3K was analyzed by ELISA ($n=3$). (E) Cell viability was analyzed by MTT ($n=3$). Results are presented as mean $\pm$ SEM. * $P < 0.05$ versus non-I/R control. $^\dagger P < 0.05$ versus I/R control or hypoxia control. hMSCs, human MSCs; ELISA, enzyme-linked immunosorbent assay.

FIG. 7. 14S,21R-diHDHA treatment enhances anti-inflammatory functions of mMSC in vitro by suppressing the generation of TNF- α and ROS from RAW264.7 macrophages. RAW264.7 macrophages were treated with mMSC conditioned medium for 3 h. Medium was replaced with fresh RPMI1640 without glucose, and macrophages were subjected to I/R treatment (Ischemia: 95% N_2 , 5% CO_2 , Reperfusion: 5% CO_2 , 95% air without H_2O_2). (A) IL-1 β and (B) TNF- α secretion from RAW264.7 cells ($n=4$). (C) ROS generation in cells was assessed by using the probe, 2,7-dichlorofluorescein diacetate ($n=4$). Results are expressed as mean $\pm$ SEM. * $P < 0.05$ versus normal control, $^\dagger P < 0.05$ versus I/R control, $^\ddagger P < 0.05$ versus MSCs. IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor- α ; ROS, reactive oxygen species.

FIG. 8. 14S,21R-diHDHA treatment increases the production of IGF-1 and HGF by mMSCs and hMSCs in vitro. mMSCs or hMSCs (5×10^4) were treated with 4S,21R-diHDHA (250 nM) under hypoxia condition (95% N_2 , 5% CO_2) for 12 h. The level of IGF-1 or HGF in 2 culture supernatants was analyzed by ELISA. (A) IGF-1 and (B) HGF secretion from mMSCs. (C) IGF-1 and (D) HGF secretion from hMSCs ($n=4$). Results are presented as mean $\pm$ SEM. * $P < 0.05$ versus control. IGF-1, insulin growth factor-1; HGF, hepatocyte growth factor.

FIG. 9. Mechanisms involved in 14S,21R-diHDHA-induced improvement in the capacity of MSCs to mitigate I/R injury. This improvement includes inhibiting renal tubular cell apoptosis and leukocyte infiltration and inflammatory cytokine generation. The mechanisms by which 14S,21R-diHDHA improve MSC renal protective functions involve promotion of MSC survival, and generation of renotrophic factors. PI3K-Akt signaling participates in the enhancement of MSC survival.

chemokine receptors [69]. In view of these known differences between hMSCs and mMSCs, we intended to determine whether the results obtained from 14S,21R-diHDHA-treated mMSCs could be confirmed in hMSCs. In this regard, our results demonstrating that 14S,21R-diHDHA treatment inhibited apoptosis of hMSCs (Fig. 6) and promoted renotrophic cytokine generation (Fig. 8) enhanced the potential of using 14S,21R-diHDHA-treated hMSCs in treatment of human acute kidney injury. As summarized in Fig. 9, the LM 14S,21R-diHDHA improves MSC mitigation of renal I/R injury in both renal functions and histological structures. This improvement includes inhibiting renal tubular cell apoptosis and leukocyte infiltration and inflammatory cytokine generation. The mechanisms by which 14S,21R-diHDHA improves MSC renal protective functions involve promoting MSC survival and generation of renotrophic factors. The PI3K-Akt signaling pathway plays a role in 14S,21R-diHDHA promotion of MSC survival. Pretreatment of MSCs with 14S,21R-diHDHA, or related LMs or structural mimics, may offer a new clinical strategy for improved treatment of acute kidney injury.

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