An Advanced Glycation End Product (AGE)-Receptor for AGES (RAGE) Axis Restores Adipogenic Potential of Senescent Preadipocytes through Modulation of p53 Protein Function*

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The impaired adipogenic potential of senescent preadipocytes is a hallmark of adipose aging and aging-related adipose dysfunction. Although advanced glycation end products (AGEs) derived from both foods and endogenous nonenzymatic glycation and AGE-associated signaling pathways are known to play a key role in aging and its related diseases, the role of AGES in adipose aging remains elusive. We show a novel pro-adipogenic function of AGEs in replicative senescent preadipocytes and mouse embryonic fibroblasts, as well as primary preadipocytes isolated from aged mice. Using glycated bovine serum albumin (BSA) as a model protein of AGEs, we found that glycated BSA restores the impaired adipogenic potential of senescent preadipocytes in vitro and ex vivo. However, glycated BSA showed no effect on adipogenesis in nesnenscent preadipocytes. The AGE-induced receptor for AGE (RAGE) expression is required for the pro-adipogenic function of AGES in senescent preadipocytes. RAGE is required for impairment of p53 expression and p53 function in regulating p21 expression in senescent preadipocytes. We also observed a direct binding between RAGE and p53 in senescent preadipocytes. Taken together, our findings reveal a novel pro-adipogenic function of the AGE-RAGE axis in p53-regulated adipogenesis of senescent preadipocytes, providing new insights into aging-dependent adiposity by diet-driven and/or endogenous glycated proteins.

Preadipocytes from aged adipose tissue display impaired adipogenic potential and increased pro-inflammatory cytokine secretion (1, 2). This is accompanied by cellular senescence of preadipocytes as evidenced by increased levels of senescence-associated β-galactosidase (SA-β-gal)2 activity, reactive oxygen species (ROS), and p53, a tumor suppressor protein, expression (3), suggesting a positive correlation between senescence of preadipocytes and adipose dysfunction. Analogous to aged preadipocytes, the preadipocytes isolated from obese animals and humans appear to exhibit senescence-like phenotypes and impaired adipogenic potential with elevated sensitivity to inflammatory cytokine-induced macrophage-like phenotypes (3–7). Although understanding the precise role of senescent preadipocytes in adipose aging still warrants further investigation, facilitated conversion of senescent preadipocytes to adipocytes appears to contribute to adipose development during aging.

AGEs are a heterogeneous group of macromolecules that are formed by nonenzymatic glycation of proteins, lipids, or nucleic acids (7). Although exogenous AGES are mainly generated during tobacco smoking and the Maillard reaction (a nonenzymatic browning reaction between amino acids and reducing sugars mostly in heat-processed foods), endogenous AGES are formed through the interaction between proteins and glucose derivatives, such as methylglyoxal and glyoxal in circulation. Endogenous AGES are virtually formed in all tissues, particularly during hyperglycemic conditions (8). Excess AGE intake and chronic accumulation of AGE-related glycated proteins in tissues are suggested to further potentiate the aging process resulting in impaired mitochondrial function and decreased life span in Caenorhabditis elegans and mice (9–11). Glycated proteins are largely known to interact with an AGE-interacting pattern recognition receptor known as RAGE. An AGE-RAGE axis contributes to aging-associated inflammation, oxidative stress, and the development of some of the chronic diseases.

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such as atherosclerosis (12), diabetes complications, and Alzheimer disease (13). In addition, AGEs in high fat diets are shown to potentiate high fat diet-induced metabolic and inflammatory disorders such as obesity, insulin resistance, and cardiac disease (14, 15). Conversely, reduction of AGE intake (9, 10), inhibition of AGE formation (15, 16), or alleviation of RAGE expression and function (17–20) was suggested to be an effective approach to attenuate AGE-RAGE axis-associated metabolic and inflammatory disorders. However, the precise role of the AGE-RAGE axis in adipose tissue development during aging is unknown.

Here, we found that exogenous AGEs were able to restore the adipogenic potential of replicative senescent preadipocytes, whereas AGEs showed no effect on adipogenesis of young preadipocytes. This is through AGE-induced RAGE expression and its interaction with p53, thereby abrogating senescence-associated p53 function in preadipocytes. These results reveal a novel pro-adipogenic function of AGES in adipose aging in which the AGE-RAGE axis modulates the function of senescence-induced p53, resulting in restoring the impaired adipogenic potential of aged adipose tissue.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Bovine serum albumin (BSA) as well as methylglyoxal (MG) (21) were purchased from MP Biomedicals, and glyceraldehyde (GA) was from Sigma. Insulin, dexamethasone, 3-isobutyl-1-methylxanthine, propidium iodide, and RNase A were purchased from Sigma. Fetal bovine serum (FBS) was purchased from PAA (Dartmouth, MA). Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, and sodium pyruvate were from VWR (Radnor, PA). TRIZol® reagent and SuperScriptII were purchased from Invitrogen. A protein assay kit was obtained from Bio-Rad.

**AGE Preparation**—Twenty mg/ml of BSA dissolved in 0.2% (w/v) Na₂N containing phosphate-buffered saline (PBS) was incubated with 100 mg/ml MG or 100 mg/ml GA at 37 °C for 6 days to generate MG-BSA or GA-BSA, respectively. The glycated BSA (i.e. MG-GSA and GA-BSA) was then filtered through a filter with a pore size of 0.22 μm followed by dialysis at 4 °C against water to remove any free form of MG or GA. The glycated BSA was then aliquoted and stored at −20 °C.

**Preadipocyte Culture and Differentiation**—3T3-L1 preadipocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured and differentiated as described previously (22). Replicative senescence of 3T3-L1 preadipocytes was introduced by performing a serial passaging of 70–80% confluent cells. To determine the effect of BSA or glycated BSA on adipogenesis of 3T3-L1 cells, BSA, MG-, or GA-BSA was either added during the course of adipogenesis or at day −2 to day 0 prior to the initiation of adipogenesis. In a RAGE neutralization study, 10 μg/ml anti-RAGE antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added to the differentiating 3T3-L1 cells from day 0 to day 2. To examine the effect of inhibition of p53 function on adipogenesis of senescent 3T3-L1 preadipocytes, cells were pretreated with the indicated concentrations of pifithrin-α (Calbiochem) and BSA or glycated BSA at day −3 to day −2 and day −2 to day 0, respectively, prior to the initiation of adipogenesis. The lipids accumulated in differentiated preadipocytes at day 8 were stained with Oil Red O (ORO) as described previously (22, 23).

**Mouse Embryo Fibroblasts (MEFs), Primary Stromal Vascular (SV) Cell Culture, and Animal Study**—MEFs purchased from ATCC were cultured in 10% FBS/DMEM with 1% penicillin/streptomycin. Passage numbers of 1–2 and 7–10 were considered as LP and HP, respectively. The cells were differentiated to adipocytes as described previously (24). Inguinal and epididymal adipose tissues were dissected from 6-week-old and 1-year-old chow diet-fed male C57BL/6N mice for the study in Fig. 1H and 14-week-old chow diet-fed or high fat diet (60% calories from fat)-fed mice for the studies in Fig. 1I. Twelve-week-old and 1-year-old chow diet-fed male C57BL/6N mice were used for the studies in Fig. 3, C and D. All procedures were approved by Purdue University Animal Care and Use Committee. Primary adipose tissues isolated from these mice were digested with collagenase to isolate the SV fraction as described previously (25). The SV fraction containing primary preadipocytes was cultured in 10% FBS/DMEM, and subjected to adipogenesiss in 10% FBS/DMEM containing 1 μg/ml insulin, 5 μm dexamethasone, 0.5 mm 3-isobutyl-1-methylxanthine, and 2.5 μg/ml rosiglitazone for 3 days, followed by incubation in 10% FBS/DMEM supplemented with 1 μg/ml insulin for 5 days.

**SA-β-gal Staining**—3T3-L1 cells fixed with 3.7% formaldehyde overnight at 4 °C were incubated with freshly prepared staining buffer (pH 6.0, 150 mm NaCl, 2 mm MgCl₂, 40 mm citric acid, 5 mm sodium phosphate, 5 mm K₂Fe(CN)₆, and 5 mm K₃Fe(18)₆) containing 1 mg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) (IBI Scientific, New Haven, CT) for 18 h at 37 °C. At the end of the incubation, cells were rinsed with PBS and were photographed under bright field (26). SA-β-gal-positive cells were quantified by Quantity One 4.5 software.

**Measurement of Reactive Oxygen Species**—Levels of intracellular ROS in LP and HP preadipocytes were measured by loading the cells with 40 μm 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Cyanam Chemical) for 30 min at 37 °C. Intracellular DCFH-DA oxidized to fluorescent 2',7'-dichlorodihydrofluorescein was monitored by the fluorescence microscope (Leica Microsystems). For quantification of the intracellular 2',7'-dichlorodihydrofluorescein, cells grown in a 96-well plate incubated with DCFH-DA for 30 min were subjected to a fluorescence microplate reader (SpectraMax Gemini EM, Molecular Devices) at an excitation wavelength of 485 nm and emission of 535 nm.

**Immunoblotting**—3T3-L1 cells were subjected to immunoblot analysis as described previously (22). Primary antibodies used in the study include phospho-Akt (Ser(P)-473) and total Akt (Epitomics, Burlingame, CA), carboxymethyl lysine (CML) (R&D system, Minneapolis, MN), p21 (Genscript, Scotch Plains, NJ), phospho-p53 (Ser-15) (Cell Signaling, Beverly, MA), RAGE, p53, β-actin, and α-tubulin (Santa Cruz Biotechnology), and secondary HRP-conjugated mouse and rabbit antibodies (The Jackson Laboratory, Bar Harbor, ME). Protein bands were detected with Pierce ECL Plus Western blotting reagents by autoradiography. Film was scanned and processed using the ImageJ software, version 1.45s, from National Institutes of Health, for quantification of protein band intensity by normalization to the band intensity of β-actin or α-tubulin.
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Immunofluorescence—3T3-L1 preadipocytes were seeded on coverslips overnight and then treated with or without glycated BSA for 24 h. The cells were fixed with 3.7% formaldehyde for 30 min and permeabilized in 0.2% Triton-contained PBS for 20 min. One percent BSA-contained PBST solution (0.1% Tween 20 in PBS) was used for blocking, and the cells were incubated with RAGE antibody (1:50). Primary antibody binding was detected using a fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology). 4′-6-Diamidino-2-phenylindole (DAPI) (Calbiochem) was used for nuclear staining. RAGE and DAPI signals from the cells were visualized by LSM 710 confocal microscopy (Carl Zeiss, Inc.).

Immunoprecipitation—To examine the interaction between RAGE and p53, 3T3-L1 cells treated with BSA or glycated BSA were lysed with radioimmunoprecipitation assay buffer. RAGE and p53 in cell lysates were incubated with 2 µg of anti-RAGE and anti-p53 antibody, respectively, or control IgG at 4 °C for 1 h, followed by immunoprecipitation with protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) for 30 min at 4 °C. The beads were washed with cold PBS four times and boiled with the sample buffer (60 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromphenol blue, and 0.1% dithiothreitol) for SDS-PAGE and immunoblot analysis with the indicated antibodies.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed as described previously (27) with some modifications. 3T3-L1 cells were cross-linked with formaldehyde at 1.5% in PBS for 15 min and neutralized with 125 mM glycine. The nuclei extracted from these cells were subjected to sonication to produce the chromatin fragments from 200 to 1,000 bp 5′. The beads were washed with cold PBS four times and boiled with the sample buffer (60 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromphenol blue, and 0.1% dithiothreitol) for SDS-PAGE and immunoblot analysis with the indicated antibodies.

Lentiviral Transduction—A lentivirus-based shRNA transfer plasmid (pLKO.1) was a gift from Dr. Xiaodi Liu (Purdue University). All the procedures were described previously (29). The targeting sequence of mouse RAGE (GenBank™ accession number NM_07425) is GGG AGA GAG GTC AAC AAG CCA. To generate lentivirus, 293T cells were co-incubated with 4 µg of pLKO.1-RAGE, 4 µg of pH′(-CMV-R8.20vpr, and 2 µg of pH′(-CMV-VSV-G using Lipofectamine 2000 reagents (Invitrogen). 24 h post-transfection, viruses were then harvested every 12 h until 72 h. The harvested media containing virus particles were filtered through a 0.45-µm pore size filter and centrifuged at 170,000 rpm for 90 min. 3T3-L1 cells were infected with the viral pellets in the presence of 10 µg/ml Polybrene. After 24 h of viral transduction, cells were selected with 1 mg/ml puromycin for at least 3 days.

Statistical Analysis—All data are presented as means ± S.E. Statistical analysis was performed using SAS 9.0 software. One-way analysis of variance was used to determine significance of treatment effect and interactions. Significant differences between group means were assessed by Dunnett’s multiple comparison. \( p < 0.05 \) was considered significant.

RESULTS

RAGE Expression Positively Correlates with Replicative Senescence-impaired Adipogenesis in Vitro, Adipose Tissue Aging, and Obesity—Preadipocytes in adipose tissue from aged animals and humans have been shown to have impaired adipogenic potency (1, 30). Because continuous cell division is known to promote replicative senescence of cells in vitro (31–33), we first examined the effect of replicative senescence on the adipogenic ability of 3T3-L1 murine preadipocytes and their cellular
delayed cell cycle progress during the mitotic clonal expansion of adipogenesis (data not shown). Moreover, these HP cells displayed a significant increase in SA-β-gal activity (Fig. 1C) and mRNA levels of p53 and IL-6 (Fig. 1D) compared with those from LP preadipocytes. Replicative senescence of 3T3-L1 preadipocytes is associated with enhanced ROS production (Fig. 1E) and impaired insulin-induced Akt phosphorylation (Fig. 1F). These results collectively suggested that a serial passage in 3T3-L1 preadipocytes yields senescent characteristics with impaired adipogenic potential in vivo. The RAGE gene has been reported to be expressed in various tissues such as mouse bone marrow in an aging-dependent manner (34). In addition, aging is reported to be positively associated with accumulation of AGEs. AGEs have the intrinsic fluorescence (i.e. pentosidine) and are chemically characterized by modification of lysine residues primarily to CML (35, 36). To further examine whether the AGE-RAGE axis is associated with adipose aging, we next measured the levels of intracellular AGEs, as judged by detecting the presence of CML-modified proteins and/or RAGE in senescent adipocytes and adipose tissue isolated from aged or obese mice. Differentiated HP senescent preadipocytes exhibited a higher level of RAGE than that in LP adipocytes with reduced levels of adiponectin and PPARγ compared with those in LP adipocytes (Fig. 1G). Similarly, elevated levels of the CML-modified proteins RAGE and p53 were also observed in the inguinal fat pads isolated from 1-year-old mice compared with those in 6-week-old chow-fed mice (Fig. 1H). Moreover, high fat diet-induced obese mice displayed elevated levels of CML-modified proteins, RAGE and ap2, an adipocyte marker protein, in epididymal fat pads compared with those in lean mice (Fig. 1I), as well as RAGE mRNA (Fig. 1J). Overall, our results indicate that a serial passage in 3T3-L1 preadipocytes is associated with development of senescent characteristics. Furthermore, adipose aging and obesity appear to be correlated with an elevated level of AGE-RAGE interaction.

Glycated BSA Restores Impaired Adipogenic Potential of Senescent Preadipocytes—To examine the effect of AGEs on the adipogenic potential of senescent preadipocytes, we first exposed senescent preadipocytes to glycated BSA during adipogenesis as depicted in Fig. 2A. Glycated BSA generated by incubation of BSA with glucose derivatives such as MG and GA is fluorescent, a characteristic of AGEs (37), and this has been used as in vitro model of AGEs in many studies (38, 39). Both BSA and glycated BSA, such as MG-BSA and GA-BSA at the concentration of 300 μg/ml, showed no additional effect on adipogenesis of LP preadipocytes as judged by both ORO (Fig. 2A, middle panel) and quantification of ORO stained lipids (Fig. 2B). Consistent with Fig. 1A, senescent HP preadipocytes failed to differentiate when the cells were treated with BSA during 8 days of adipogenesis (Fig. 2A). Interestingly, treatments of senescent HP preadipocytes with 300 μg/ml MG-BSA or GA-BSA resulted in ~2.5- and 3.5-fold induction of intracellular lipid droplet accumulation, respectively, compared with BSA-treated control cells (Fig. 2, A and B). In accordance with increased lipid accumulation, senescent HP preadipocytes differentiated in the presence of MG-BSA or GA-BSA showed dramatically increased levels of adipocyte marker genes such as PPARγ, C/EBPα, adiponectin, FAS, and leptin (Fig. 2C) as well

**FIGURE 1.** Replicative senescence-impaired adipogenic potential of 3T3-L1 preadipocytes and a positive correlation of AGE-RAGE axis with adipose tissue aging and adiposity. A, ORO staining of differentiated 3T3-L1 preadipocytes (Pre) with different passage numbers (e.g. p.5, p.10, and p.15) for 8 days. B, gene expression analysis of adiponectin and PPARγ in differentiated preadipocytes with passage number of ≤5 (LP) and ≥15 (HP) by real-time PCR. C, SA-β-galactosidase activity assay in LP and HP 3T3-L1 preadipocytes as shown in the overall view (top panel) and microscopic image (middle panel, ×100 magnification) and quantification of SA-β-gal-positive cells (bottom panel). D, real-time PCR analysis of mRNA levels of p53 and IL-6 in LP and HP preadipocytes. E, levels of ROS in LP and HP preadipocytes were detected by DCHF-DA assay. The fluorescent signals in these cells were viewed by fluorescence microscopy (top panel, ×100 magnification) and quantitated by a fluorescence microscope reader (bottom panel). F, LP and HP 3T3-L1 preadipocytes were treated with or without 167 μM insulin for 30 or 60 min. The levels of phosphorylated Akt (p-Akt Ser473), total Akt, and β-actin in these cells were analyzed by imunoblot assay. G, RT-PCR analysis of RAGE, adiponectin, and PPARγ in preadipocytes (D0) and differentiated adipocytes (D9) of LP and HP 3T3-L1 cells. H, immunoblot analysis of inguinal fat pad isolated from 6-week-old and 1-year-old chow diet-fed male mice (left panel), and quantification of intensity of the major CML-modified protein (~55 kDa) and RAGE and p53 bands (right panel). I, immunofluorescence analysis of epidermal fat pads isolated from 14-week-old chow diet-fed lean and high fat diet-fed obese male mice (left panel) and quantification of intensity of the major CML-modified protein (~55 kDa) and RAGE and ap2 bands (right panel). J, real-time PCR analysis of RAGE in epididymal fat pads isolated from lean and obese mice. Bars represent mean ± S.E. (n = 3–5). *, p < 0.05; **, p < 0.01; ***, p < 0.001. All experiments were repeated at least 2–3 times.

senescence by measuring the levels of SA-β-gal activity, mRNA levels of p53 and IL-6, and ROS production. Preadipocytes at passage number 15 (high passage (HP)) showed impaired adipogenesis as judged by ORO staining (Fig. 1A) and quantitative analysis of adipocyte marker gene expressions such as adiponectin and PPARγ (Fig. 1B) when compared with those from adipocytes at passage number 5 (low passage (LP)). The impaired adipogenesis of HP preadipocytes was correlated with
as RAGE mRNA (Fig. 2D). However, glycated BSA showed no effect on C/EBPβ in differentiated HP preadipocytes (Fig. 2C).

We next examined whether pre-exposure of senescent HP preadipocytes to glycated BSA is necessary for restoring the impaired adipogenic potential of senescent preadipocytes. As depicted in Fig. 3A, a 48-h pretreatment (i.e. day −2 to day 0) of HP preadipocytes with MG-BSA or GA-BSA resulted in an increase of lipid droplet accumulation after 8 days of differentiation. We further confirmed the pro-adipogenic function of glycated BSA in replicative senescent MEF cells and cells in the SV fraction of adipose tissues isolated from mice of different ages, such as 12 weeks old and 1 year old. Although LP MEF cells (i.e. passage 1–2) were normally differentiated with accumulated lipid droplets regardless of the treatments, HP MEF cells (i.e. passage 7–10) showed impaired adipogenesis as judged by ORO staining, and this impaired adipogenic ability was restored when the HP MEF cells were pretreated with MG-BSA or GA-BSA for 48 h prior to induction of adipogenesis (Fig. 3B). Furthermore, 1-year-old primary inguinal SV cells pretreated with MG-BSA or GA-BSA for 48 h resulted in accumulation of lipid droplets after 8 days of adipogenesis, whereas glycated BSA showed no effect on adipogenesis in primary inguinal SV cells isolated from 12-week-old mice as judged by ORO staining (Fig. 3C) and gene expression analysis adipocyte markers such as PPARγ, adiponectin, and FAS (Fig. 3D). Collectively, these results supported the hypothesis that AGES restore the adipogenic potential of aged preadipocytes both in vitro and ex vivo.

RAGE Is Required for the Pro-adipogenic Effect of Glycated BSA in Senescent Preadipocytes—Our findings of the glycated BSA-restored adipogenic potential of senescent preadipocytes with an elevated level of RAGE led us to hypothesize that RAGE mediates AGE-induced adipogenesis in senescent preadipocytes. To test this, we first confirmed the effect of glycated BSA on the protein levels of RAGE in HP preadipocytes. We found that senescent HP preadipocytes treated with MG-BSA or GA-BSA for 48 h displayed an ~2- or 3-fold increase in RAGE level, respectively (Fig. 4A, left panel). Immunofluorescence of glycated BSA-treated HP preadipocytes further confirmed induction of RAGE by glycated BSA detected predominantly in the cytoplasm and nucleus (Fig. 4A, right panel). We next investigated whether the increase in RAGE by glycated BSA is required for AGE-induced adipogenesis in HP preadipocytes. To ascertain this, we utilized neutralizing antibody against RAGE. We found that glycated BSA-induced adipogenesis of senescent HP preadipocytes was blunted by neutralizing the antibody against RAGE (Fig. 4B). Moreover, a lentiviral knockdown of RAGE mRNA in senescent HP preadipocytes as confirmed by PCR (Fig. 4C, left panel) and immunoblot (Fig. 4C, right panel) analyses abrogated glycated BSA-induced adipogenesis in senescent HP 3T3-L1 cells (Fig. 4D) with reduced levels of PPARγ and FAS after 8 days of differentiation (Fig. 4E).

These findings suggested that RAGE is required for AGE-induced restoration of the adipogenic potential of senescent preadipocytes.

AGE-activated RAGE Binds to p53 and Inhibits p53 Expression in Senescent Preadipocytes—Given that a tumor suppressor p53 has been shown to modulate adipose tissue function and senescence (40), we attempted to examine whether p53 modulates AGE-RAGE-induced adipogenesis in senescent preadipocytes. First, we examined the effect of glycated BSA on the protein levels of p53 and p21, a downstream target of p53, in HP preadipocytes. As expected, senescent HP preadipocytes treated with glycated BSA showed an increase in RAGE (Fig. 5A). This was accompanied by reduced levels of p53 and phosphorylated p53 (Fig. 5A), suggesting a suppressive role of glycated BSA in p53 expression in senescent preadipocytes. We next investigated whether RAGE expression is required for glycated BSA-suppressed protein levels of p53 and p21 in senescent preadipocytes. As shown in Fig. 5A, a lentiviral knockdown of RAGE in senescent preadipocytes reversed glycated BSA-suppressed total and phosphorylated forms of p53 and p21. Consistent with this, glycated BSA also suppressed mRNA levels of p53 and p21 in control senescent preadipocytes, whereas this was reversed in MG- or GA-BSA-treated RAGE knockdown senescent preadipocytes (Fig. 5B). Apparently, glycated BSA-suppressed p21 expression was through altered DNA binding ability of p53 to the promoter region of p21 in MG- or GA-BSA-treated control senescent preadipocytes as judged by ChIP assay (Fig. 5C). However, RAGE knockdown resulted in increased DNA binding ability of p53 to the p21 promoter in both BSA- and glycated BSA-treated senescent preadipocytes (Fig. 5D). Because our finding in Fig. 4A demonstrated the presence of RAGE in the cytosolic space of senescent preadipocytes,
we further sought the direct binding between RAGE and p53, and the effect of glycated BSA on the RAGE-p53 binding. Our immunoprecipitation assay revealed a physical interaction between RAGE and p53 in senescent preadipocytes, and this was not affected by the treatment with glycated BSA (Fig. 5D).

Consistent with our finding of AGE-impaired p53 function in senescent preadipocytes, differentiating senescent preadipocytes pretreated with different concentrations of pifithrin-α/H9251, a p53 inhibitor, for 24 h followed by a 48-h treatment with glycated BSA prior to the initiation of adipogenesis resulted in a dose-dependent increase in adipogenesis as judged by ORO staining (Fig. 5E). Overall, our results indicate that AGE-RAGE-induced adipogenesis in senescent preadipocytes is through AGE-dependent impairment of p53 expression and function in senescent preadipocytes. Interestingly, we also demonstrated a direct interaction between RAGE and p53 in senescent preadipocytes, but its role in adipogenesis and the function of senescent preadipocytes still remain elusive.

**DISCUSSION**

Senescent preadipocytes are known to exhibit reduced replicative and adipogenic capacity, increased pro-inflammatory responses, and susceptibility to lipotoxicity during aging (3). Much of our current understanding of the biological function of AGEs has been largely limited to their actions in diabetic and cardiovascular complications during aging through AGE-RAGE-induced oxidative stress and inflammation (41, 42). The AGE-RAGE pathway has been shown to be associated with aging of humans and animals (14, 43), as well as the cellular differentiation program of various cell types (44, 45). Moreover, RAGE has shown to participate in high fat diet-induced complications such as cardiac inflammation (15), atherosclerosis (46), renal implication (17) with potential adiposity. However, the role of the AGE-RAGE axis in adipose aging remains unknown.

Here, we report a novel function of AGEs in restoring impaired adipogenic potential of senescent preadipocytes through RAGE-dependent modulation of p53 function via RAGE-dependent suppression of p53 and possibly by a physical interaction between RAGE and p53 as depicted in Fig. 6. Our findings thus infer that senescent preadipocytes are likely to resume adipogenesis when these cells are exposed to diet-driven exogenous and endogenously generated glycated proteins through altered p53 expression and function, thereby impacting adipose development during aging.

A number of intriguing questions have been brought forth by our findings. First, mechanisms by which RAGE, a membrane-bound protein, is found in the cytosol and interacts with p53 in senescent preadipocytes should be determined. Although most of the predicted and experimentally tested proteolytic cleavage sites in RAGE are found in the extracellular domain to generate soluble forms of RAGE, it has been reported to also be detected in the intracellular space in various cell types such as muscle satellite cells, astrocytes, and amyloid-β-stimulated murine cortical neuron (47–49). Increasing evidence points to the role of proteolytic cleavage in regulating the function of RAGE isoforms in both the extracellular and intracellular space (50). Interestingly, aging-dependent truncation of RAGE and its detection in the cytosol of human muscle satellite cells has been reported (47). Moreover, generation of the truncated form of RAGE in aged human muscle satellite cells appears to be
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On the one hand, p53 has been known to inhibit adipogenesis and to be involved in maintaining the proper function of the adipose tissue (56) and 3T3-L1 cells (57), possibly through suppression of the key adipogenic transcriptional factor such as PPARγ (58). On the other hand, activated p53 is known to be involved in the development of early aging in adipose tissue with reduced adipose tissue deposition and adipose inflammation in animals (23, 60, 61). p53 appears to modulate adipose aging because inhibition of p53 function in adipose tissue has been reported to suppress senescence-like phenotypes with improved insulin sensitivity (61). Collectively, these studies indicate that future study should be directed to understanding the role of RAGE in p53-dependent cross-talk between autophagy and apoptosis in adipose aging. It should also be noted that RAGE knockdown in senescent preadipocytes resulted in a slight increase in p53 protein (Fig. 5A) but a decrease in p53 mRNA (Fig. 5B) in the absence of glycated BSA treatment. Although more studies are needed to address this discrepancy, this result suggests a possibility of the potential role of RAGE in regulating p53 protein stability.

Nevertheless, our findings define a novel pro-adipogenic function of AGEs in senescent preadipocytes. Although our study is largely based on *in vitro* and *ex vivo* studies, we would predict that a chronic dietary intake of AGEs would positively contribute to adipose development during aging. Serum AGE levels in humans appear to be influenced by diet-driven AGE intake. For example, administration of single AGE-rich diet resulted in an increase in serum AGE levels from ~10 to 80 units/ml in normal subjects and to ~120 units/ml in diabetic subjects after several h of ingestion, where 1 unit is defined to be equivalent to ~10 μg of CML-BSA (62). Accordingly, serum AGEs levels found in this study would be in the range of 0.8–1.2 mg/ml glycated BSA. In addition, Sandu et al. (14) reported that normal mice fed a high fat diet containing 995.4 units/mg AGEs for 6 months resulted in elevation of AGE levels in the serum and adipose tissue at 124 ± 7 units/ml and 400 units/mg (i.e. 1.24 mg of CML-BSA/ml and ~4 mg of CML-BSA/mg), respectively. Supporting this notion, a recent study demonstrated that mice fed a standard chow diet supplemented with 1 mg of MG-BSA/g of diet for 18 months showed elevated AGE levels in the serum as well as in adipose tissue to 0.43 mg of CML-BSA/ml and 190 nmol of MG/g of tissue, respectively (63). Collectively, these studies suggest that the AGE concentration we used in our study is likely to be achievable in the sera of humans and animals after a chronic oral administration of an AGE-rich diet.

Recent studies showed that chronic AGE-rich standard diet feeding studies in animals showed an increase in body weight with no report on the change in adipose mass with elevated insulin resistance (14, 64, 65). Conversely, a long term of feeding study of mice with AGE-restricted standard diet resulted in reduced body weight gain with enhanced insulin sensitivity and life span at the end of the study (66). Moreover, Monden et al. (59) and Cai et al. (63) recently demonstrated a causative role of dietary AGEs and RAGE expression in adiposity, and its related insulin resistance and cellular anti-oxidant system. Cai et al. (63) further showed that oral administration of an AGE-rich diet promotes insulin resistance in mice by severe deficiency of...
an anti-AGE advanced glycation receptor 1 (AGER1) and SIRT1 in peripheral tissues with impaired glucose uptake, altered insulin signaling pathway, and inflammatory activation in adipose tissue (63). Although these elegant studies clearly indicated a novel pathogenic role of AGEs and an altered RAGE level in obesity and type 2 diabetes, the direct role of the AGE-RAGE axis in aging-dependent impairment of adipogenic potential in aged preadipocytes and its contribution to aging-related metabolic and inflammatory dysfunction are unknown. To our knowledge, our study is the first report that AGEs are able to restore the senescence-impaired adipogenic potential of aged preadipocytes. These findings implicate that AGE-induced adipogenesis in senescent preadipocytes is likely to contribute to exacerbating aging-related adiposity.

In conclusion, our finding of the pro-adipogenic function of AGE-RAGE axis in senescent preadipocytes through a mechanism by which RAGE suppresses p53 function and expression may provide new insights into the aging-dependent adiposity by glycated proteins derived from the diet and/or chronically dysregulated metabolic conditions such as hyperglycemia.

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