AGE-receptor-1 counteracts cellular oxidant stress induced by AGEs via negative regulation of p66shc-dependent FKHR1 phosphorylation

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Submitted 7 August 2007; accepted in final form 15 November 2007

Cai W, He JC, Zhu L, Chen X, Striker GE, Vlassara H. AGE-receptor-1 counteracts cellular oxidant stress induced by AGEs via negative regulation of p66shc-dependent FKHR1 phosphorylation. Am J Physiol Cell Physiol 294: C145–C152, 2008. First published November 21, 2007; doi:10.1152/ajpcell.00350.2007.—Advanced glycation end products (AGEs) promote reactive oxygen species (ROS) formation and oxidant stress (OS) in diabetes and aging-related diseases. AGE-induced OS is suppressed by AGER1, an AGE-receptor that counteracts cellular oxidant stress induced by AGEs (RAGE) and mediates signal output through the receptor for advanced glycation end products (RAGE). RAGE mediates Shc/Ras signal activation, resulting in decreased OS. Akt, FKHR1, and antioxidants; e.g., MnSOD, regulate OS. Serine phosphorylation of p66shc also promotes OS. We examined the effects of two defined AGEs N-carboxymethyl-lysine (CML) and methylglyoxal derivatives (MG) on these cellular pathways and their functional relationship to AGER1 in human embryonic kidney cells (HEK293). Stimulation of HEK293 cells with either AGE compound increased phosphorylation of Akt and FKHR1 by approximately threefold in a redox-dependent manner. The use of p66shc mutants showed that the AGE-induced effects required Ser-36 phosphorylation of p66shc. AGE-induced phosphorylation of FKHR1 led to a 70% downregulation of MnSOD, an effect partially blocked by a phosphatidylinositol 3-kinase inhibitor (LY-294002) and strongly inhibited by an antioxidant (N-acetylcysteine). These pro-oxidant responses were suppressed in AGER1 overexpressing cells and reappeared when AGER1 expression was reduced by small interfering RNA (siRNA). These studies point to a new pathway for the induction of OS by AGEs involving FKHR1 inactivation and MnSOD suppression via Ser-36 phosphorylation of p66shc in human kidney cells. This represents a key mechanism by which AGER1 maintains cellular resistance against OS. Thus the decrease of AGER1 noted in aging and diabetes may further enhance OS and reduce innate antioxidant defenses.

glycoxidation; aging; diabetes; N-carboxymethyl-lysine; methylglyoxal; forkhead transcription factors; manganese superoxide dismutase; receptor for advanced glycation end products

INCREASED OXIDANT STRESS (OS) underlies numerous conditions associated with aging, including diabetes and chronic cardiovascular, kidney, or central nervous system diseases (7, 8, 39). Advanced glycation end products (AGEs) are major contributors to increased OS in these conditions (8, 39). Specific AGE compounds, namely N-carboxymethyl-lysine (CML) and methylglyoxal (MG) derivatives, have been linked to cellular injury due to diabetes and older age (13, 17, 18, 32). Their significance in disease processes is well established, especially since it is recognized that reactive AGEs can readily be derived from the modern diet (9, 17, 18, 32, 39). Thus, whereas it has long been known that AGEs may promote chronic cell injury and elevated OS in disease states, it is now increasingly apparent that they accumulate and induce pathological changes before the onset of recognizable diseases (9, 38).

Intracellular oxidant status is influenced by unopposed or excess reactive oxygen species (ROS) in the cytosol or mitochondria (27). Increased cellular ROS can also arise from AGEs in the absence of elevated glucose; e.g., from myeloperoxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or from lipids (3, 4, 17). ROS may be increased via activation of AGE-sensitive cell surface receptors, such as receptor for advanced glycation end products (RAGE) (5, 33), or nonreceptor-dependent pathways, causing activation of mitogen-activated protein kinase (MAPK)/Ras and nuclear factor (NF)-κB, as well as Akt, forkhead transcription factors (FOXO), p38, or c-JUN pathways, which can promote both inflammatory responses or cellular apoptosis (2, 24).

Certain cell surface receptors, such as RAGE, promote AGE-induced OS and inflammatory responses (5, 33), whereas a second category, which includes AGER1, reduces AGE levels and suppresses OS, RAGE, and inflammation (20, 22). Since the balance between the responses mediated by these two types of receptors may determine cell fate, a clear understanding of the signaling pathways involved becomes critical. AGER1, an integral membrane protein localized in the endoplasmic reticulum (ER) and on plasma membranes, is associated with AGE endocytosis and removal (20, 22). Overexpression of AGER1 in glomerular mesangial cells or human embryonic kidney 293 (HEK293) cells opposes AGE-mediated RAGE, MAPK, and NF-κB-dependent inflammatory responses via inhibition of epidermal growth factor (EGFR) and the EGFR-dependent Tyr phosphorylation of Grb2 and Shc adaptor proteins p52 and p46, which directly transport signals to Ras (10, 20, 22). The third Shc isoform p66shc may be an essential regulator of mitochondrial and cytoplasmic OS and apoptosis, since p66shc mutant mice are resistant to OS and have an extended lifespan (23, 25). p66shc functionally interacts with FKHR1, a homologue of the forkhead family members that tightly regulates the transcription of anti-oxidant genes; e.g., MnSOD and catalase (19, 26). MnSOD in particular is encoded by a nuclear gene, SOD2, which is tightly regulated by ROS via FKHR1; however, this

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anti-oxidant pathway can be blocked by a mechanism regulated by p66shc (19, 23, 26). Oxidants, such as H2O2, ultraviolet light irradiation, or glucose induce phosphorylation of p66shc at CH2-Ser-36 residue, promoting ROS generation and the recruitment and activation of Akt/PKB (19, 23, 26, 34). Activation of Akt/PKB by p66shc leads to phosphorylation of FKHR1, keeping this inactive form in the cytosol (6), and this has been proposed as a mechanism by which sustained OS compromises MnSOD availability and increases the sensitivity of cells to injury and apoptosis. Deletion of p66shc largely blocks FKHR1 phosphorylation and inactivation, leading to enhanced expression of MnSOD and to greater resistance to OS (6, 19, 23, 25, 26, 34). Recent studies reveal an association among AGEs, OS, and p66shc (9, 12, 16, 21). Erk, a kinase previously linked to MAPK and NF-kB activation by AGEs (10, 20, 22, 33), was recently reported as a mediator of p66shc Ser-36 phosphorylation (16), and this interplay may modulate responses to aging and OS.

The present studies were undertaken to explore the effects of well-characterized AGEs on these interactions and to determine whether they are modulated by AGER1. The data suggest that specific AGEs can promote ROS-dependent Ser-36 phosphorylation of p66shc, resulting in inactivation of FKHR1 and suppression of MnSOD in HEK293 cells. This pathway can be blocked by AGER1 overexpression, identifying a novel mechanism by which AGER1 promotes anti-oxidant homeostasis.

MATERIALS AND METHODS

Materials. Phospho-Thr-32 FKHR1 antibody was from Upstate Biotechnology (Lake Placid, NY), anti-Shc antibody from BD Biosciences (Transduction Laboratories), EGFR inhibitor AG 1478 from Calbiochem (La Jolla, CA), MEK inhibitor U0126 and anti-phospho-ERK antibody from Cell Signaling (Beverly, MA), anti-MnSOD antibody from Stressgen Biotechnologies (Victoria, BC, Canada), dihydroethidium from Molecular Probes (Eugene, OR), and phospho-erbB-2 antibody from Calbiochem. The vectors expressing wild-type p66shc (p66WT) or expressing position-36 mutant (serine to alanine) of p66shc (p66SA) were a generous gift from Dr. Doreen Finkel (NHBLI, NIH, Bethesda, MD). Endotoxin-free BSA was used to prepare two chemically distinct AGEs: methylglyoxal (MG)-BSA, containing 22 MG-modified arginine residues per molecule, and CML-BSA, containing 23 CML-modified lysine residues per molecule, characterized by HPLC and GC-MS, as described (10, 11). Endotoxin contaminants were removed by using an endotoxin-binding affinity column (Pierce, Rockford, IL), and final products contained <0.1 U/ml, as tested by a Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD) (10, 11, 20, 22).

Cell culture. HEK293 cells (ATCC CRL-1573) were maintained in MEM (supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS) at 37°C in 5% CO2. Before treatment, cells were washed in PBS 1× and incubated with serum-free media for 12 h. Established stable HEK293 cell lines overexpressing human AGE-R1 (AGER1) were also used (10, 22).

Western blot analysis. Cells were exposed to ligands at 37°C, rinsed (2× in ice-cold PBS), and lysed in 500 µl lysis buffer (New England Biolabs). After brief sonication, equal amounts of cell protein were separated on 10% or 8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes, which were treated with the appropriate primary and secondary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence system (Roche). For reprobing, blots were stripped with buffer (50 mM of Tris·HCl, pH 6.8, 2% SDS, and 0.1 M β-mercaptoethanol), before the addition of a new primary antibody (10).

Transient transfection. Quiescent cells were transiently transfected with wild-type p66shc (p66WT), vector alone (p66v), or Ser-36 mutant p66shc (p66SA), using lipofectamine plus reagent (GIBCO-BRL), and 48 h later cells were exposed to CML-BSA, MG-BSA, or BSA before harvesting for Western blot analysis (10, 22, 26). Intracellular ROS. Intracellular superoxide anion levels were detected using dihydroethidium (HE) (35). Briefly, cells were stimulated at the indicated AGE concentrations (100 µg/ml CML-BSA, or 40 µg/ml MG-BSA, 100 µg/ml BSA) at 37°C for up to 4 h, washed with PBS, and incubated with HE (10 µM) for another 45 min, and fluorescence was measured in the cellular extract with a fluorometer (extinction and emission wavelengths were 501 and 521 nm, respectively).

AGER1 siRNA. Quiescent HEK293 cells were transfected with AGER1 small interfering RNA (siRNA) using Lipofectin reagent (GIBCO-BRL), as previously described (10). After 48 h cells were treated with CML-BSA for another 24 h, and the lysates were analyzed for MnSOD expression by Western blot analysis.

RESULTS

AGEs induce Ser-36 phosphorylation of p66shc and FKHR1 phosphorylation, both events are suppressed in HEK293 cells overexpressing AGER1. We previously observed that AGEs promote rapid Tyr phosphorylation of p46 and p52shc, but not of p66shc in mesangial and HEK293 cells, both of which express p66shc (10). HEK293 cells stimulated with CML-BSA (100 µg/ml) showed rapid serine phosphorylation of p66shc, which peaked by 5–15 min (Fig. 1A). Similarly treated cells overexpressing AGER1 showed no changes in phospho-p66shc levels from baseline values (Fig. 1A). The MEK-Erk cascade is involved in p66shc Ser-36 phosphorylation, with (28) or without EGFR transactivation (16), and AGEs promote Erk signaling through the transactivation of EGFR in HEK293 cells, a response that is blocked in cells overexpressing AGER1 (10). To determine whether AGE-induced serine phosphorylation of p66shc was a downstream target of the EGFR-MEK-Erk pathway, HEK293 cells were stimulated with AGEs or EGF in the presence or absence of the EGFR kinase inhibitor AG1478 and the MEK inhibitor U0126 (Fig. 1, B and C). Since AGEs are heterogeneous, CML-BSA (13, 17, 32, 38) and a structurally distinct, but equally common AGE, MG-BSA (13, 17, 18, 39) were studied. Stimulation with these two types of AGE-modified BSA or EGF failed to promote Ser phosphorylation of p66shc in the presence of EGFR or MEK activation inhibitors (Fig. 1, B and C). These data suggest that AGEs promote p66shc Ser phosphorylation via the EGFR-MAPK-Erk pathway (16). Also, AGER1 inhibition of this pathway occurred most likely via an interaction with EGFR (10). Furthermore, AGEs promoted p66shc protein expression, persisting for at least 24 h, an effect not evident in AGER1 cells (Fig. 1D).

The specificity of AGEs for Ser phosphorylation of p66shc and its direct downstream events was further explored using HEK293 cells transiently transfected with either a Ser-36 mutant of p66shc (p66SA), wild-type p66shc (p66WT), or (p66V) vectors (23, 26). A p66shc protein with slightly reduced gel mobility was present in cells transfected with either the dominant negative p66shc (p66SA) or the wild-type (p66WT) vector but not in cells transfected with an empty vector (p66v) (Fig. 2A). CML-BSA or MG-BSA treatment for 10–15 min induced a threefold increase in FKHR1 Thr-32 phosphorylation in p66WT cells compared with p66v cells but not in AGE-stimulated cells transfected with mutant p66SA, indicating that
these events were largely due to p66\textsuperscript{Shc} Ser-36 phosphorylation (Fig. 2, B and C). Furthermore, AGE-induced FKHR1 phosphorylation was blocked in cells overexpressing AGER1 co-transfected with either p66\textsuperscript{WT} or p66\textsuperscript{V} (Fig. 2, D and E). Thus AGER1 inhibited AGE-induced p66\textsuperscript{Shc} Ser-36 phosphorylation, a key step leading to phosphorylation of FKHR1. Akt is upstream from FKHR1 (6, 34) and AGE stimulation induced Akt phosphorylation in a time-dependent manner from 5 to 20 min (Fig. 3A). This time course mirrored the AGE-stimulated phosphorylation of FKHR1, which also peaked between 5 and 20 min and decreased at 30 min (Fig. 3B). An antioxidant (N-acetylcysteine, NAC), added to determine whether FKHR1 phosphorylation was due to AGE-induced ROS, blocked the response induced by either CML-BSA or MG-BSA (Fig. 3C). Whereas FKHR1 phosphorylation by AGEs was dose dependent in HEK293 cells transfected with an empty vector, AGER1 cells were unresponsive to AGE levels, up to 200 \textmu g/ml (Fig. 3D).
AGE-induced FKHRL1 phosphorylation is associated with reduced expression of MnSOD in HEK293 cells but not in AGER1 overexpressing cells. We tested the effects of CML-BSA, MG-BSA, or native BSA on MnSOD levels, since FKHRL1 regulates its expression (19, 25). Both AGEs led to significant suppression of MnSOD expression within 4 h (CML by 70% and MG by 50%, respectively) (Fig. 4, A and B), which was sustained for 24 h by CML (Fig. 4B). MnSOD suppression was nearly completely prevented by 5 mM NAC and partially (~50%) by a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY-294002). Thus the suppression of MnSOD levels by CML-BSA and MG-BSA involves ROS and the PI3K pathway.

Fig. 3. AGE-induced reactive oxygen species (ROS) production mediates FKHRL1 phosphorylation via Akt in HEK293 cells and is blocked in AGER1 cells. A: Akt phosphorylation following treatment of cells with CML-BSA (100 μg/ml) for 0–30 min (as in Fig. 1). Blots were probed with antibodies to phospho-Akt and total-Akt. FKHRL1 phosphorylation is increased in cells after exposure to CML-BSA (100 μg/ml) for 0–30 min (B) and is inhibited by NAC (5 mM, for 15 min) (C). Blots were probed with antibodies to phospho-Thr-32 FKHRL1 or total FKHRL1. D: FKHRL1 phosphorylation was blocked in AGER1 cells, but not in HEK293 cells, after exposure to CML-BSA (0–200 μg/ml) for 15 min. Representative blots (top and middle) and densitometric data from 3 independent experiments (bottom) are shown as the ratio (means ± SD) to unstimulated controls (*P < 0.01, **P < 0.001) or to stimulated HEK293 cells (#P < 0.01).

Fig. 4. AGEs suppress MnSOD protein expression in HEK293 cells. A: cells were exposed to CML-BSA (100 μg/ml), MG-BSA (40 μg/ml), or BSA (100 μg/ml) for up to 4 h. B: blots were probed with antibodies to MnSOD and β-tubulin. Representative blots (top) and densitometric data of 3 independent experiments are expressed as the percentage of controls (CL). **P < 0.01 vs. 1 h. B: cells, pretreated with LY-294002 (25 μM and 50 nM) or N-acetylcysteine (NAC, 1 mM and 5 mM), or buffer for 1 h were exposed to CML-BSA (100 μg/ml) for 24 h. Blots were probed with antibodies to MnSOD and β-tubulin. Representative blot (top) and densitometric data from 3 independent experiments are shown as fold increase above control (bottom). *P < 0.01 vs. CML without inhibitors.
The dose-dependent inhibitory effect of CML-BSA on MnSOD seen in HEK293-V cells was significantly attenuated in AGER1 cells (Fig. 5, A and B). This finding was AGER1 specific, since reducing AGER1 levels by siRNA transfection resulted in reappearance of CML-induced MnSOD inhibition. Therefore, the AGER1 actions include the regulation of MnSOD levels.

**AGER1 blocks the RAGE-induced reduction in the expression of MnSOD by AGEs.** Baseline levels of AGER1 production in HEK293 cells were reduced by transduction with AGER1 siRNA and increased by AGER1 (Fig. 6A). Both CML and MG reduced MnSOD levels (Fig. 6B). The levels of MnSOD were further decreased in cells transduced with RAGE. However, the levels of MnSOD remained unchanged in cells cotransfected with both AGER1 and RAGE. Thus CML and MG reduce MnSOD levels, and the levels are further decreased by the overexpression of RAGE. However, AGER1 blocks the RAGE-induced reduction of MnSOD expression. This is the first time that this action of AGER1 has been shown.

**AGER1 overexpression suppresses the AGE-mediated cellular ROS generation associated with p66^{shc} activation.** CML-BSA or MG-BSA stimulation resulted in significant increases in superoxide levels in p66^{V} cells (~60% greater than control) and in p66^{WT} HEK293 cells (~175% greater than control) (Fig. 7A). In addition, the AGE-mediated ROS production was absent in AGER1 cells transiently cotransfected with p66^{V} and significantly attenuated in AGER1 cells transiently cotransfected with p66^{WT} (Fig. 7B). Superoxide production in AGER1 cells by either AGE stimulant was significantly lower in p66^{SA} than in p66^{WT} transfectants, whether or not they coexpressed AGER1 (Fig. 7, A and B). Thus Ser-36 phosphorylation of p66^{shc} is important for AGE-promoted ROS production and FKHRL1 phosphorylation. Since p66^{shc} phosphorylation was blocked in cells overexpressing AGER1, the expression levels of AGER1 may be key in its inhibition of ROS after AGE stimulation.

**Fig. 5. AGER1 inhibits AGE-induced MnSOD suppression.** AGER1, HEK293, and HEK293 cells transfected with pooled AGER1 small interfering RNA (siRNA) were exposed to CML-BSA (0–100 μg/ml) for 24 h. Blots were probed with antibodies to MnSOD and β-tubulin antibodies. A representative blot (A) and densitometry data (B) from 3 independent experiments comparing 0 to 50 or 100 μg/ml CML-BSA are shown. *P < 0.005; **P < 0.01 vs. 0; #P < 0.05 vs. HEK 293 cells.

**Fig. 6. AGER1 blocks the RAGE-induced reduction in the expression of MnSOD by AGEs.** A: cells were transduced with vector (V), small interfering AGER1 (siAGER1), or RAGE and harvested at 48 h. Blots were probed with antibodies to AGER1, RAGE, and β-tubulin. Representative blots are shown. B: cells were transduced with vector, RAGE, or AGER1+RAGE and harvested at 48 h. These cells were then exposed to BSA (100 μg/ml) (C), MG-BSA (40 μg/ml) (MG), or CML-BSA (100 μg/ml) (CML) for 4 h. Blots were probed with antibodies to MnSOD and β-tubulin. Representative blots are shown.

**DISCUSSION**

We found that AGEs promote p66^{shc} Ser-36 phosphorylation and FKHRL1 transcription factor inactivation in a ROS-dependent manner. AGE-induced ROS mediate early changes in the p66^{shc} signaling pathway and also causes prolonged suppression of MnSOD. These data implicate p66^{shc} in AGE-induced oxidant damage and cell injury via EGFR, MEKK, and Erk. Since cells that over express AGER1 are resistant to both AGE-induced oxidant damage and cell injury, the data also implicate AGER1 in the control of inflammatory signals, via MAPK-NF-κB and also in the downregulation of transcriptional stress-response systems, via p66^{shc}. Thus AGER1 may represent an important factor in cellular defenses against AGE or oxidant injury.

ROS, such as H_{2}O_{2}, O_{2}^{\cdot-}, and hydroxyl radicals, generated from AGEs have been implicated in aging and related disorders; i.e., diabetes, cardiovascular and kidney disease, and Alzheimer’s disease (9, 13, 17, 18, 33, 39). We recently found that two Shc isoforms (p52^{shc} and p46^{shc}) but not p66^{shc} undergo Tyr phosphorylation in response to AGEs via EGFR (10). These data link AGEs to MAPK activation and proinflammatory responses via tyrosine kinase receptors. Others found that p66^{shc} plays a crucial role in the regulation of oxidative stress responses (23, 26). We now show that AGEs also lead to p66^{shc} Ser-36 phosphorylation in HEK293 cells overexpressing wild-type p66^{shc} but not in cells transfected with a dominant-negative Ser-36 mutant p66^{shc} p66^{SA}. Since FKHRL1 phosphorylation, induced by either CML-BSA or MG-BSA, was blocked in cells overexpressing p66^{SA}, p66^{shc} may functionally interact with FKHRL1 under conditions of high levels of at least two common in vivo AGE prototypes CML and MG (8, 9, 11, 13, 17, 18, 32, 38, 39). The Ser-36 phosphorylation of p66^{shc} is thought to serve in the generation
levels of p66Shc protein, associated with reduced AGEs, lipid exposed to a diet with a reduced oxidant burden had lower lifespan in mice (23, 25). We have found that aging mice

of ROS for the recruitment and activation of Akt/PKB and the phosphorylation and inactivation of FKHR1 (6, 26, 36). The fact that the phosphorylation of FKHR1 induced by AGEs was abolished by the addition of NAC suggests that this AGE-mediated event is ROS dependent. Although AGEs induced significant amounts of superoxide anions in cells overexpressing p66WT, this was blocked in p66SA cells, suggesting that, although certain early redox-dependent AGE responses are important, p66Shc Ser-36 phosphorylation is critical for their downstream effects in HEK293 cells. Furthermore, this response was blocked in cells overexpressing AGER1, showing that AGER1 interferes with the phosphorylation of p66Shc and the generation of ROS due to AGE stimulation.

p66Shc protein levels correlate with oxidant injury and the lifespan in mice (23, 25). We have found that aging mice exposed to a diet with a reduced oxidant burden had lower levels of p66Shc protein, associated with reduced AGEs, lipid peroxides (8-isoprostanes), and RAGE, as well as a marked reduction of kidney disease (9). This low OS state was linked to preservation of normal AGER1 levels, suggesting that this receptor could be functionally linked to low OS and p66Shc protein (9). Herein, AGE stimulation of HEK293 cells resulted in increased p66Shc protein expression between 30 min and 24 h. Thus AGEs may promote sustained increase of cytosolic and mitochondrial p66Shc levels (23, 25, 28), and this might account for the elevated levels of p66Shc protein seen in high-AGE states such as diabetes and aging (9, 30, 31, 39).

Enhanced ROS levels in diabetes and aging are associated with sustained mitochondrial ROS overproduction or cytosolic NADPH oxidase activation (3, 8, 17, 24, 27), both of which are linked to p66Shc (16, 28, 29). The Akt/PKB and FKHR1 system is important in the pro-oxidant stress responses of p66Shc (6, 14, 19, 26, 28, 30). Specific activation of FKHR1, a transcriptional regulator of key antioxidant enzymes, i.e., MnSOD, protects against cellular oxidants (19, 25). Herein, exposure to AGEs led to a time- and dose-dependent phosphorylation of Akt and FKHR1, responses that were suppressed by AGER1 overexpression. CML-BSA and MG-BSA markedly enhanced FKHR1 phosphorylation in cells overexpressing wild-type p66Shc, consistent with FKHR1 inactivation (6, 34). On the other hand, stable expression of the dominant negative interfering p66SA restored normal FKHR1 activity and rendered these mutant cells resistant to AGE-induced OS. These data suggest that in the setting of high AGEs, p66Shc serine phosphorylation can contribute to excess OS via FKHR1 inactivation. This may be of particular importance for cells and tissues where FKHR1 defends against ROS-induced DNA damage by regulating the expression of proteins involved in the repair process (1). The fact that NAC blocked AGE-mediated FKHR1 phosphorylation further supports the view that AGE-inducible redox changes are early events.

Mitochondrial MnSOD normalizes mitochondrial ROS and also prevents OS-dependent NF-B activation (1, 6). In the current study we showed that AGEs can induce a dose-dependent decrease in MnSOD protein expression, which appeared by 4 h and persisted for up to 24 h. The effects of AGEs on Akt and FKHR1 phosphorylation and suppression of MnSOD expression were ameliorated by NAC, as well as by a PI3K inhibitor (LY-294002), indicating that AGE redox effects were also propagated via Akt and FKHR1 phosphorylation. Whereas the cause of MnSOD suppression was not fully determined, the mechanisms may include FKHR1 inactivation due to p66Shc Ser-36 phosphorylation. These AGE-induced changes were blocked by AGER1 overexpression. This effect was AGER1 specific, based on data showing complete reversal by AGER1 siRNA. Furthermore, the AGE-induced reduction of MnSOD mediated by RAGE was blocked in cells cotransduced with both RAGE and AGER1. This suggests that AGER1 blocks the ROS induced by RAGE and extends our previous finding that AGER1 blocks ROS formation by a RAGE ligand (S-100) (10). The possible effect of AGER1 on other AGE receptors was not investigated and this could also be informative. In addition, the effects of AGER1 on ROS generated in the mitochondria and/or endoplasmic reticulum remain to be elucidated.

This study points to a new pathway for the induction of OS by two defined AGEs in human kidney cells, which involves downregulation of FKHR1 activity and MnSOD expression...
via serine phosphorylation of p66\textsuperscript{shc}. Since this pathway is blocked by AGER1, the data also identifies a new pathway by which AGER1 defends against OS. Thus the observed decrease of AGER1 found in aging and diabetes (9, 15) may underlie the increased OS and reduced innate antioxidant defenses in these conditions. These data, together with earlier findings, point to a series of phosphorylation events that are linked to AGE-induced stress-responses and that are counteracted by AGER1. Whereas the present studies considered HEK cells, the data also identifies a new pathway by which AGER1 regulates stress responses via p66\textsuperscript{shc}. AGER1 may thus represent a therapeutic target.

ACKNOWLEDGMENTS

We thank Ina Katz for providing invaluable editorial assistance.

GRANTS

This work was supported by National Institutes of Health Grants AG-009453 (to H. Vlassara) and AG-023188 (to H. Vlassara).

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