RAGE-Induced Cytosolic ROS Promote Mitochondrial Superoxide Generation in Diabetes

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ABSTRACT

Damaged mitochondria generate an excess of superoxide, which may mediate tissue injury in diabetes. We hypothesized that in diabetic nephropathy, advanced glycation end-products (AGEs) lead to increases in cytosolic reactive oxygen species (ROS), which facilitate the production of mitochondrial superoxide. In normoglycemic conditions, exposure of primary renal cells to AGEs, transient overexpression of the receptor for AGEs (RAGE) with an adenoviral vector, and infusion of AGEs to healthy rodents each induced renal cytosolic oxidative stress, which led to mitochondrial permeability transition and deficiency of mitochondrial complex I. Because of a lack of glucose-derived NADH, which is the substrate for complex I, these changes did not lead to excess production of mitochondrial superoxide; however, when we performed these experiments in hyperglycemic conditions in vitro or in diabetic rats, we observed significant generation of mitochondrial superoxide at the level of complex I, fueled by a sustained supply of NADH. Pharmacologic inhibition of AGE-RAGE–induced mitochondrial permeability transition in vitro abrogated production of mitochondrial superoxide; we observed a similar effect in vivo after inhibiting cytosolic ROS production with apocynin or lowering AGEs with alagebrium. Furthermore, RAGE deficiency prevented diabetes-induced increases in renal mitochondrial superoxide and renal cortical apoptosis in mice. Taken together, these studies suggest that AGE-RAGE–induced cytosolic ROS production facilitates mitochondrial superoxide production in hyperglycemic environments, providing further evidence of a role for the advanced glycation pathway in the development and progression of diabetic nephropathy.


During oxidative phosphorylation (OXPHOS) under physiologic conditions, there is minimal superoxide leakage, which is immediately scavenged by the antioxidant enzyme manganese superoxide dismutase (MnSOD, SOD2); however, damaged or dysfunctional mitochondria generate excessive superoxide, creating a state of redox imbalance.1 In diabetic complications, a unifying hypothesis has been postulated: That the excessive generation of mitochondrial superoxide by hyperglycemia is the primary initiating event that activates all other pathways of tissue damage.2 There remains debate, however, as to whether oxidative stress is an important early link between hyperglycemia and compli-
cations or is a byproduct of other primary pathogenic mechanisms.

It is thought that excess concentrations of mitochondrial reactive oxygen species (ROS) are mediated by disruption of the activity of OXPHOS enzymes via electron leakage at complex I (NADH:ubiquinone oxidoreductase) or complex III (ubiquinol:cytochrome c oxidoreductase).\(^\text{1,4,5}\) For example, in Friedreich ataxia, a genetically inherited mitochondrial disease, deficiencies of OXPHOS complexes I, II, and III are associated with excessive steady-state generation of mitochondrial superoxide.\(^\text{6}\) Of interest is that progressive renal disease has been reported in patients with mitochondrial respiratory chain abnormalities, with some patients presenting with renal abnormalities as their primary pathology.\(^\text{7,8}\) In addition, a decrease in complex I activity of patients presenting with renal abnormalities has been reported in patients with mitochondrial disease.\(^\text{9}\) PHOS complexes I, II, and III are associated with inherited mitochondrial disease, deficiencies of OXPHOS complexes I, II, and III are associated with excessive steady-state generation of mitochondrial superoxide.\(^\text{6}\) Of interest is that progressive renal disease has been reported in patients with mitochondrial respiratory chain abnormalities, with some patients presenting with renal abnormalities as their primary pathology.\(^\text{7,8}\) In addition, a decrease in complex I activity of patients presenting with renal abnormalities has been reported in patients with mitochondrial disease.\(^\text{9}\)

Advanced glycation end products (AGEs), formed by the nonenzymatic irreversible modification of proteins, are known contributors to the pathogenesis of diabetic renal disease, in particular as effector molecules for the receptor for AGEs (RAGE).\(^\text{13}\) Ligand engagement of RAGE by AGEs also results in the production of cellular ROS.\(^\text{16,17}\)

In this study, we investigated the in vitro and in vivo effects of the AGE-RAGE interaction on mitochondrial function, under both normal- and high-glucose environments. We show that engagement of RAGE by AGEs induces mPT, which subsequently leads to NADH-dependent excessive generation of mitochondrial superoxide radical via complex I deficiency in a high-glucose milieu.

**RESULTS**

**Exogenous AGEs Induce RAGE Expression and Generate Cytosolic Hydrogen Peroxide in Normal-Glucose Environments**

Primary rat mesangial cells exposed to 100 μg/ml AGE-modified BSA (AGE-BSA) had increased cellular hydrogen peroxide (H\(_2\)O\(_2\)) production, as compared with BSA vehicle–treated cells alone (Figure 1A). H\(_2\)O\(_2\) production was attenuated by the inhibitor of AGE accumulation alagebrium (ALA) and by the cytosolic NADPH-oxidase inhibitor apocynin (APO; Figure 1A).

Exogenously prepared AGE-BSA and AGE-modified rat serum albumin (AGE-RSA) had approximately 100-fold increases in CML modification (AGE-BSA 67.0 ± 1.2 mmol/mol lysine; AGE-RSA 38.2 ± 3.6 mmol/mol lysine) as compared with the original preparations (BSA 0.5 ± 0.0 mmol/mol lysine and RSA 0.3 ± 0.0 mmol/mol lysine).\(^\text{18,19}\) Importantly, no endotoxin was detected in any of the albumin preparations (as measured by the Limulus Amoebocyte Lysate assay). In addition, no anti-CML antibodies in excess of those seen in RSA-injected rats could be detected in the plasma from AGE-RSA–injected rats by ELISA (data not shown). AGE-RSA injection did not change plasma glucose, glycated hemoglobin, renal function, or systolic BP (Table 1) when compared with RSA-injected rats. Renal cortical RAGE gene (Figure 1B, gene accession no. L33413) and protein expression...
were increased in AGE-RSA–injected rats as compared with RSA (densitometric analysis of RAGE protein expression as determined by Western immunoblotting and corrected to β-actin RSA 202.3 ± 5.7 arbitrary units versus AGE-RSA 220.1 ± 1.3 arbitrary units; n = 3 rats per group; P < 0.05). The cytosol from the renal cortex of AGE-RSA–injected rats also had markedly increased H₂O₂ generation, which was normalized with concomitant ALA therapy (Figure 1C). The RSA vehicle also modestly increased cytosolic H₂O₂ production, although this was significantly lower when compared with AGE-RSA–injected rodents (Figure 1C).

To verify the combined effects of AGE–RAGE interactions on renal H₂O₂ production, we treated RAGE adenoviral vector (Ad-RAGE)–infected cells with AGE–BSA (Ad-RAGE+AGEs) or BSA (Ad-RAGE+BSA). Cytosolic H₂O₂ production was unchanged with Ad-RAGE transfection alone (Figure 1D, Ad-RAGE) or BSA but was significantly increased by more than two-fold with addition of ligand (Ad-RAGE+AGEs), clearly indicating that the AGE–RAGE interaction was required for increased cytosolic H₂O₂ generation in mesangial cells.

AGE-Induced Cytosolic H₂O₂ Liberation Induces mPT and Disrupts Mitochondrial Membrane Potential

Exposure of renal mitochondria to H₂O₂ led to a sequential decrease in absorbance at 540 nm, reflecting induction of mPT (Figure 2A). This decrease in absorbance was inhibited by cyclosporin A (CsA), a classically described agent known to inhibit mPT. In addition, an acute AGE–BSA treatment of mesangial cells infected with the RAGE adenosine virus led to the liberation of cytosolic H₂O₂ (Figure 2B).

To assess AGE–induced mPT more directly, we treated primary rat mesangial cells with AGE–BSA or BSA for 30 min and cells loaded with calcine–AM with or without cobalt chloride. Mesangial cells that were exposed to AGE–BSA had a significant decrease in calcine fluorescence, indicative of mPT, when compared with cells treated with BSA (Figure 2C). The AGE–mediated decrease in calcine fluorescence was prevented by co-incubation with CsA, confirming the process of mPT. In addition, pretreatment with PEG-catalase (PEG–CAT), a cell-permeable form of catalase, prevented the AGE–BSA–induced mPT (Figure 2C), implicating intracellular H₂O₂ in the process.

To explore further AGE–RAGE–induced mitochondrial dysfunction, we used flow cytometric analysis of cells loaded with the potentiometric dye JC-1 to assess changes in mitochondrial membrane potential (ΔΨm). Primary rat mesangial cells were exposed to the uncoupler CCCP as a positive control, which demonstrated a significant loss in red fluorescence in the third (10³) and fourth (10⁴) log, resulting in a leftward shift (Figure 2D, top right), compared with the control empty vector (Figure 2D, top left). This finding is consistent with a decrease in ΔΨm (i.e., depolarization of the inner mitochondrial membrane). Mesangial cells overexpressing RAGE via infection with the human full-length Ad-RAGE had a modest but NS loss in red fluorescence compared with the empty vector (Figure 2D, middle left). After addition of the AGE ligand, this leftward shift became more pronounced (Figure 2D, middle right). This was not seen with BSA treatment (Figure 2D, bottom left). In addition, pretreatment with PEG–CAT prevented the AGE–BSA–induced shift (Figure 2D, bottom right). Data from four independent experiments are collated in Figure 2E. Taken together, these data clearly demonstrate AGE–RAGE–induced disruption of ΔΨm and enhanced susceptibility to mPT.

AGE-RAGE Interaction Disrupts the Mitochondrial Respiratory Chain in Normoglycemia, but Hyperglycemia Is Essential for Superoxide Overproduction

To evaluate further whether AGE–induced cytosolic oxidative stress and subsequent mPT could be associated with or could influence changes in the activity of the respiratory chain, we investigated the enzyme complexes I through IV. Initially we identified in an advanced model of diabetes (streptozotocin [STZ]–induced diabetes in Sprague–Dawley rats with no insulin treatment) that complex I activity is selectively decreased by 50% in renal glomeruli compared with control rats (50 ± 10% of control; n = 6 rats per group; P < 0.001), whereas the remaining OXPHOS complexes were unchanged (complex II 110 ± 8, complexes II+III 105 ± 6, complex III 145 ± 30, and complex IV 125 ± 30% of control; n = 6 rats per group). In addition, another previous study would have shown this pattern of electron transport complexes in long-term diabetes if it had corrected for the marker of mitochondrial activity, citrate synthase, which is elevated in renal mitochondria from diabetic rats (control 76.9 ± 9.1 vs. diabetic 114.5 ± 4.0 mmol/min per mg; n = 5 rats per group; P < 0.01). On the basis of these results, we focused on complex I alone as a target of dysfunction in our rodent models.

Table 1. Rat biochemical and metabolic parameters: AGE injection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>AGE-RSA</th>
<th>AGE-RSA+ALA</th>
<th>RSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>7.4 ± 0.2</td>
<td>7.4 ± 0.2</td>
<td>7.6 ± 0.2</td>
<td>7.9 ± 0.3</td>
</tr>
<tr>
<td>GHB (%)</td>
<td>4.1 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>4.1 ± 0.5</td>
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<tr>
<td>Kidney wt/body wt (×10⁻³)</td>
<td>5.4 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>GFR (ml/min)</td>
<td>4.3 ± 0.1</td>
<td>4.4 ± 0.2</td>
<td>4.1 ± 0.2</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>118 ± 2</td>
<td>118 ± 2</td>
<td>123 ± 3b</td>
<td>121 ± 2</td>
</tr>
<tr>
<td>AER (mg/24 h)</td>
<td>1.2 ± 0.8</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

aData are means ± SEM; n = 6 to 10 per group. AER, albumin excretion rate; GHB, glycated hemoglobin.
bP < 0.01 versus control.
In vivo, long-term injection of AGE-RSA (16 wk) induced a 50% suppression of complex I activity in renal glomeruli as compared with both sham-injected rats (control) and rats injected with RSA (Figure 3A). Of note, modest inhibition of complex I activity was also seen in rodents injected long-term with RSA alone. Generation of mitochondrial superoxide was unchanged by long-term AGE-RSA injection in the absence of hyperglycemia (Figure 3B).

Consistent with previous studies, diabetic rats had significantly elevated plasma glucose and glycated hemoglobin and an increase in kidney-to-body weight ratio compared with control rats (Table 2). By 16 wk of diabetes, significant elevations in systolic BP and albumin excretion rate were evident, which were further increased by 32 wk of diabetes (Table 2). Induction of RAGE gene expression was seen at both 16 and 32 wk of diabetes (see Supplemental Appendix; Figure 2). As observed in the AGE injection model, there was increased cytosolic H$_2$O$_2$ at weeks 16 and 32 of diabetes (see Supplemental Appendix; Figure 3).

Renal glomerular complex I activity was unchanged after 16 wk of diabetes; however, by 32 wk, there was a significant decline in glomerular complex I activity of 30% compared with control (Figure 3C). In contrast to results for AGE-injected rodents, by 32 wk of disease, complex I deficiency in diabetic glomeruli was accompanied by a significant overproduction of mitochondrial superoxide (Figure 3D). Strikingly, renal mitochondria from diabetic rats treated with the cytosolic NADPH oxidase inhibitor, APO, or ALA, the inhibitor of AGE accumulation had less superoxide production with levels similar to those seen in healthy rats (Figure 3D). Furthermore, ALA restored glomerular complex I activity to control levels (Figure 3C). The superoxide overproduction was observed in the context of decreased activity of MnSOD in renal mitochondria from diabetic rats (see Supplemental Appendix; Figure 4).
production. Bars represent means ± SEM, n = 6 to 10 rats per group. *P < 0.001 versus control; †P < 0.001 versus 16-wk control; ‡P < 0.05 versus 32-wk control; §P < 0.05 versus AGE-RSA; #P < 0.05 versus control; ¥P < 0.05 versus 32-wk diabetes.

Table 2. Rat biochemical and metabolic parameters: STZ-induced diabetes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16 Wk</td>
<td>32 Wk</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Diabetes</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>7.4 ± 0.2</td>
<td>34.5 ± 0.5b</td>
</tr>
<tr>
<td>GHB (%)</td>
<td>4.1 ± 0.2</td>
<td>17.3 ± 1.0b</td>
</tr>
<tr>
<td>Kidney wt:body wt (×10⁻³)</td>
<td>5.1 ± 0.1</td>
<td>10.9 ± 0.6b</td>
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<tr>
<td>GFR (ml/min)</td>
<td>4.3 ± 0.1</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>106 ± 2</td>
<td>140 ± 4e</td>
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<tr>
<td>AER (mg/24 h)</td>
<td>1.2 ± 0.8</td>
<td>29.9 ± 7.5b</td>
</tr>
</tbody>
</table>

AG-E RAGE Interaction and High Glucose Are Critical for Superoxide Overproduction, Facilitated by Induction of mPT

Mesangial cells overexpressing RAGE and exposed to AGEs (Ad-RAGE+AGE) had decreased complex I activity in both normal- and high-glucose environments (Figure 4A). There was, however, no increase in mitochondrial superoxide production in Ad-RAGE–infected cells exposed to normal glucose and AGEs (Figure 4B). Although no excess superoxide generation was evident in Ad-RAGE–infected cells incubated in high glucose in the absence of AGE-BSA (Figure 4B), there was a three-fold increase upon exposure to both high glucose and AGEs (Figure 4B). Similarly, increased cytochrome C release from the mitochondria was evident only in Ad-RAGE–infected cells incubated with both high glucose and AGEs (Figure 4C). In addition, caspase 3 was activated in Ad-RAGE–infected mesangial cells exposed to AGEs and high glucose, and this increase in apoptosis was inhibited by the mPT inhibitor adenosine S’ diphosphate (ADP; see Supplemental Appendix; Figure 5).

To confirm that AGE-RAGE interactions caused excess mitochondrial superoxide generation via mPT in high-glucose environments, we incubated Ad-RAGE–infected primary mesangial cells with various mPT inhibitors in the presence and absence of AGE-BSA. Indeed, significant inhibition of AGE-RAGE–induced mitochondrial superoxide generation was observed with the addition of two disparate inhibitors of mPT, CsA and ADP (Figure 4D).

Mitochondrial superoxide generation was also elevated in renal cortices from wild-type (WT) mice after 24 wk of diabetes when compared with control WT mice (Figure 4E). Conversely, no increase in the production of mitochondrial superoxide was seen in renal cortices from diabetic RAGE−/− mice. This lack of mitochondrial ROS production in diabetic RAGE−/− mice occurred in the context of reduced albuminuria in these mice when compared with WT diabetic mice (WT control 155 ± 41, WT diabetic 410 ± 75 µg/24 h [P < 0.05 WT control versus WT diabetic]; RAGE−/− control 142 ± 51; RAGE−/− diabetic 158 ± 50 µg/24 h [P < 0.05 WT diabetic versus RAGE−/− diabetic]). Furthermore, analysis of apo-
tic cells in renal cortices demonstrated a diabetes-specific increase in WT mice when compared with control WT mice (Figure 4F). Diabetes, however, was not associated with an increase in apoptosis in renal cortices from diabetic RAGE/H11002/H11002 mice. In addition to this, cytosolic ROS production was blocked in diabetic RAGE/H11002/H11002 mice compared with WT diabetic mice (Supplemental Figure 6).

**OXPHOS Substrate Availability Is Crucial for Superoxide-Induced Renal Dysfunction in Diabetes**

Finally, we examined the substrate availability of the pyridine nucleotide NADH, which is essential for complex I function in vivo. Mesangial cells infected with Ad-RAGE in high-glucose environments had significant increases in mitochondrial NADH content that were not seen in Ad-RAGE cells incubated in normal-glucose conditions (Figure 5A). Indeed, in normal glucose, Ad-RAGE–infected cells had a depletion of NADH content within the mitochondria when compared with the empty vector alone. In addition, in rat renal cortical mitochondria, NADH content was depleted 50% after long-term AGE injection in healthy normoglycemic rodents when compared with control and RSA-treated rats (Figure 5B). This depletion was not via an inhibition of glycolysis, because cytosolic lactate concentration was similar between groups (data not shown). In contrast, there was no depletion of NADH content in renal mitochondria from high-glucose conditions when compared with normal glucose (data not shown). In normal glucose, Ad-RAGE–infected cells had a depletion of NADH content within the mitochondria when compared with the empty vector alone. In addition, in rat renal cortical mitochondria, NADH content was depleted 50% after long-term AGE injection in healthy normoglycemic rodents when compared with control and RSA-treated rats (Figure 5B). This depletion was not via an inhibition of glycolysis, because cytosolic lactate concentration was similar between groups (data not shown). In contrast, there was no depletion of NADH content in renal mitochondria from high-glucose conditions when compared with normal glucose (data not shown).

**Figure 4.** The AGE-RAGE interaction and high glucose amplify renal mitochondrial superoxide generation, facilitated by induction of mPT. (A through C) Representative of primary rat mesangial cells cultured in normal glucose (5.5 mM) or high glucose (25 mM) and infected with the human FL Ad-RAGE or the control empty vector for 72 h. At 24 h after infection, AGE-BSA (100 μg/ml) was added to one group (Ad-RAGE+AGES). (A) Mitochondrial complex I activity expressed relative to citrate synthase activity. (B) Mitochondrial NADH-driven superoxide generation was measured by lucigenin-enhanced chemiluminescence. (C) Cytochrome C release from mitochondria determined by ELISA. Inhibitors of mPT, CsA (10 μM) and ADP (100 μM), were also given to some groups 24 h after infection in the presence of 100 μg/ml AGE-BSA. (D) Mitochondrial NADH-driven superoxide generation from cells treated with high glucose (25 mM) after inhibition of mPT. Bars represent means ± SEM, n = 3 independent cell culture experiments. (E and F) Representative of renal cortical samples from control (□) and diabetic (■) WT (C57BL/6J) and RAGE−/− mice at week 24 after the induction of STZ diabetes (n = 10 mice per group). (E) NADH-driven superoxide production. (F) Terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling in renal cortices expressed per 1000 cells counted. *P < 0.001 versus control empty vector high glucose; **P < 0.05 versus control empty vector normal glucose; †P < 0.01 versus Ad-RAGE in high glucose; ‡P < 0.05 versus Ad-RAGE+AGES in normal glucose; §P < 0.05 versus wild type control 24W; #P < 0.001 versus Ad-RAGE+AGES; ¥P < 0.05 versus WT diabetic 24 wk.
diabetic rodents at week 16 or 32 compared with nondiabetic rats (Figure 5C), consistent with the presence of sufficient substrate levels to drive complex I and thereby generate superoxide radicals.
cells isolated from STZ-induced diabetic rats,29 and AGE-RAGE interactions were postulated to contribute to podocyte apoptosis.30 Furthermore, other groups demonstrated protection against the induction of diabetic nephropathy by inhibition of cellular apoptosis,31,32 which is consistent with the improved renal function in the context of less renal apoptosis seen in RAGE−/− mice from this study.

Studies have suggested that patients with genetic mutations leading to a dysfunctional mitochondrial respiratory chain can present with renal disease as their primary pathology.7,8 One such disorder, Friedreich ataxia, has specific electron leakage at complexes I and III leading to excessive generation of superoxide.9 In our study, we showed that within diabetic glomeruli, there seems to be a specific defect in complex I rather than other complexes of the respiratory chain. In other contexts, opening of the mPT pore induces electron leakage, leading to increased mitochondrial ROS production at the level of complex I.11 In that study, mitochondrial ROS production was observed only in the presence of a continued supply of the complex I substrate NADH to the respiratory chain, because mPT led to depletion of NADH through diffusion of pyridine nucleotides out of the mitochondria. Hence, these investigators postulated that mPT induces a decline in the activity of complex I, which dramatically increases ROS production as long as electrons are provided to complex I.11 In addition, a recent study elegantly demonstrated that transient opening of the mPT pore stimulated spontaneous bursts of superoxide generation by the mitochondrial respiratory chain.33 Indeed, our own studies in RAGE overexpressing cells exposed to AGE-BSA demonstrated an abrogation of excess mitochondrial superoxide production in the presence of inhibitors of mPT. Furthermore, although a decline in complex I activity could be seen in various settings of normoglycemia in this series of studies, there was no increase in superoxide generation as a result of the depletion of mitochondrial NADH. Indeed, our AGE-injected nondiabetic rats had little evidence of progressive nephropathy at the time point studied. In our diabetic rats, however, the mitochondrial NADH pool seemed sufficient to supply reducing equivalents to complex I, thereby sustaining mitochondrial superoxide production. Furthermore, this mitochondrial superoxide overproduction was exacerbated by decreased activity of mitochondrial MnSOD.

A previous study found that mitochondria from the renal cortex of rats with diabetes exhibited a diminution of OXPHOS via decreased complex III activity and increased superoxide formation.7 In that particular study, however, the measurement of OXPHOS enzymes was not normalized to citrate synthase activity, a procedure considered critical to correct for changes in total mitochondrial number between kidneys from control and diabetic rats; therefore, in that study, one cannot exclude that the decreased OXPHOS reported is in fact due to changes in other subunits of the respiratory chain. Nevertheless, the observation in that study of increased renal mitochondrial superoxide production in STZ-induced diabetes is consistent with the work described in this study. This is further supported by our studies, which included evaluation of the role of RAGE per se. Specifically, the diabetes-induced increases in renal mitochondrial superoxide generation were observed in WT mice but not in kidneys from diabetic RAGE−/− mice.

In conclusion, our data indicate that chronic hyperglycemia, leading to enhanced NADH-derived electron delivery to complex I, is required in conjunction with AGE-RAGE-induced cytosolic H$_2$O$_2$ activation of mPT to generate excessive mitochondrial superoxide (Figure 6). This indicates not only does the cytosol act in concert with the mitochondria to generate cellular ROS but also that cytosolic ROS generation may cause or amplify some of the specific mitochondrial defects observed in diabetes. These defects include complex I deficiency, enhanced susceptibility to mPT, and ultimately overproduction of superoxide, which is considered to play a central role in inducing diabetic end-organ injury. The observations presented here suggest that pharmacologic inhibition of cytosolic ROS production may be an appropriate strategy to reduce excessive mitochondrial superoxide production in diabetic complications. In addition, it is postulated that therapies that target complex I may restore mitochondrial integrity and potentially confer a degree of renoprotection in diabetes.

**CONCISE METHODS**

An expanded version of the Concise Methods section is available in the Supplemental Appendix.

**Figure 6.** Schematic representation of the interplay between AGE and RAGE and high glucose in promoting mitochondrial superoxide production in the diabetic kidney. AGEs binding to RAGE induce cytosolic H$_2$O$_2$ production. Cytosolic H$_2$O$_2$ facilitates induction of mPT, promoting a deficiency in complex I of the mitochondrial respiratory chain. Hyperglycemia provides increased mitochondrial NADH availability for OXPHOS, which, when coupled with a deficient complex I activity, amplifies mitochondrial superoxide generation. Both the AGE-RAGE interaction and hyperglycemia synergistically coordinate overproduction of mitochondrial superoxide and promote diabetic kidney disease.
In Vivo Rodent Studies
All animal experiments were performed in accordance with the Alfred Medical Research and Education Precinct Animal Ethics committee.

STZ-Induced Diabetes.
Experimental diabetes was induced in male Sprague–Dawley rats or WT (C57BL/6; Jackson Laboratories, Bar Harbor, ME) and RAGE-deficient mice (RAGE−/−)35,36 with STZ as described previously37 with control rodents followed concurrently. Diabetic and control animals were randomized (n = 10 per group) and followed for 16 or 32 wk for rats or 24 wk for mice. A subset of diabetic rats (n = 10) received either the NADPH oxidase inhibitor APO (15 mg/kg per d, oral gavage) or the AGE inhibitor ALA chloride [4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride, a gift from Synvista, Ramsey, NJ; 10 mg/kg per d, oral gavage] from weeks 16 to 32. Two units of Ultralente insulin (Novo Nordisk, Bagsværd, Denmark) were administered daily to diabetic rats to prevent ketoacidosis and improve survival.

Exogenous AGE Administration.
Endogenous-free AGE-RSA was prepared as described previously.19 Exogenous AGE-RSA was administered daily to healthy 8-wk-old Sprague-Dawley rats (n = 10 per group); AGE-RSA (20 mg/kg per d, intraperitoneally), RSA (20 mg/kg per d, intraperitoneally), or saline (controls) for 16 wk. An additional AGE-RSA subgroup (n = 10) was randomized to receive treatment with the AGE inhibitor ALA chloride (10 mg/kg per d, oral gavage) for the duration of the study. Sixteen weeks was chosen on the basis of previous studies that documented the time course of renal disease in this rat model.38

Construction and Generation of Recombinant Adenovirus Vectors.
A recombinant adenovirus encoding human RAGE was constructed and generated. Full-length human RAGE cDNA (gift of Prof. H. Yamamoto39) was inserted into the shuttle plasmid pAdTrack-CMV containing a GFP reporter and was then subcloned into the adenoviral backbone plasmid AdEasy-1 (Ad-RAGE). Human embryonic kidney 293 cells were then infected to package the virus using lipofectamine 2000 reagent (Invitrogen, Melbourne, Australia). The pAdTrack-CMV shuttle vector without the gene of interest was used as a control (empty vector). RAGE expression was verified using Western immunoblotting (see Supplemental Appendix, Figure 1).

Adenovirus–Mediated RAGE Gene Transfer In Vitro.
Primary mesangial cells were isolated from rat kidney as described previously.40 Mesangial cells seeded into 75-cm² tissue culture flasks were grown for 3 d to 60% confluence in 5.5 mM D-glucose in DMEM containing 10% FCS; then the medium was replaced with DMEM containing either 5.5 or 25.0 mM D-glucose and the purified Ad-RAGE vector or empty vector (2.85 × 10⁹ optical particle units). After 24 h, 100 µg/ml AGE-BSA or 100 µg/ml BSA was added, and cells were cultured for an additional 2 d. In some studies, inhibitors of mitochondrial pore transition, ADP (100 µM) and CsA (10 µM), or the antioxidant PEG-catalase (500 U/ml), were concomitantly administered with AGE-BSA. Transfection efficiency was 80 to 90% on the basis of GFP fluorescence.

Mitochondrial Superoxide Production.
Superoxide production in renal cortex was determined by lucigenin-enhanced chemiluminescence as described previously.34 Superoxide production in freshly isolated mitochondria from primary mesangial cells was measured as described previously.1

H₂O₂ Production.
Please refer to the expanded version of the Methods section of the Supplemental Appendix.

Superoxide Dismutase Activity.
Please refer to the expanded version of the Methods section of the Supplemental Appendix.

Mitochondrial Membrane Potential.
Mitochondrial membrane potential was assessed using the MitoProbe JC-1 assay kit (Molecular Probes, Eugene, Oregon) by flow cytometry, according to the manufacturer’s instructions.

OXPHOS Complex Activity.
Isolation of renal glomeruli was performed as described previously.41 Respiratory chain complexes and citrate synthase were assayed in glomerular supernatants and in mesangial cell mitochondrial preparations as described previously for other tissues.42 For accounting for variations in mitochondrial number between samples, complex activity was expressed relative to citrate synthase, which is much more resistant to oxidative inactivation than the respiratory chain complexes.43

Apoptosis.
Please refer to the expanded version of the Methods section of the Supplemental Appendix.

Mitochondrial Permeability Transition.
Please refer to the expanded version of the Methods section of the Supplemental Appendix.

Mitochondrial NADH Content.
Mitochondria (50-µl suspension) from renal cortex or from primary rat mesangial cells were alkaline treated (50 µl of 1 M NaOH) at 90°C for 5 min to destroy the oxidized NAD⁺ isomer. Samples were quenched on ice and neutralized, and NADH content was measured using a modification of the method originally described by Nisselbaum and Green.44

Real-Time Reverse Transcription–PCR.
Please refer to the expanded version of the Methods section of the Supplemental Appendix.

Statistical Analysis
All statistical computations were performed using GraphPad Prism 4.0a for Mac OS X (GraphPad Software, San Diego, CA). Values of experimental groups are given as mean, with bars showing the SEM, unless otherwise stated. One-way ANOVA with Tukey posttest analysis or two-way ANOVA with Bonferroni posttest analysis was used to
determine statistical significance. When appropriate, two-tailed t test were performed. P < 0.05 was considered to be statistically significant.

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DISCLOSURES

None.

REFERENCES


