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Cell Injury Repair, Aging and Apoptosis

Reduced Oxidant Stress and Extended Lifespan in Mice Exposed to a Low Glycotoxin Diet

Association with Increased AGER1 Expression

Weijing Cai,* John Cijiang He,† Li Zhu,* Xue Chen,* Sylvan Wallenstein,‡ Gary E. Striker,† and Helen Vlassara*

From the Department of Geriatrics, Division of Experimental Diabetes and Aging, the BioMath Department,‡ and the Department of Medicine,† Division of Nephrology, Mount Sinai School of Medicine, New York, New York*

Aging is accompanied by increased oxidative stress (OS) and accumulation of advanced glycation end products (AGEs). AGE formation in food is temperature-regulated, and ingestion of nutrients prepared with excess heat promotes AGE formation, OS, and cardiovascular disease in mice. We hypothesized that sustained exposure to the high levels of pro-oxidant AGEs in normal diets (Reg_{AGE}) contributes to aging via an increased AGE load, which causes AGER1 dysregulation and depletion of anti-oxidant capacity, and that an isocaloric, but AGE-restricted (by 50%) diet (Low_{AGE}), would decrease these abnormalities. C57BL6 male mice with a life-long exposure to a Low_{AGE} diet had higher than baseline levels of tissue AGER1 and glutathione/oxidized glutathione and reduced plasma 8-isoprostanes and tissue RAGE and $p66^{shc}$ levels compared with mice pair-fed the regular (Reg_{AGE}) diet. This was associated with a reduction in systemic AGE accumulation and amelioration of insulin resistance, albuminuria, and glomerulosclerosis. Moreover, lifespan was extended in Low_{AGE} mice, compared with Reg_{AGE} mice. Thus, OS-dependent metabolic and end organ dysfunction of aging may result from life-long exposure to high levels of glycoxidants that exceed AGER1 and anti-oxidant reserve capacity. A reduced AGE diet preserved these innate defenses, resulting in decreased tissue damage and a longer lifespan in mice. ***(Am J Pathol 2007, 170:1893–1902; DOI: 10.2353/ajpath.2007.061281)***

Environmental and genetic factors including elevated oxidant stress (OS), cumulative DNA damage, altered gene expression, telomere shortening, and energy utilization are among the postulated mechanisms of senescence and aging in organisms from yeast to mammals.[1–5] The effects of increased OS are thought to be partly mediated by oxidative changes in proteins, lipids, and nucleic acids, which alter their function.[6] Excess OS can be diminished by manipulating genetic or environmental factors. Genetic models of increased longevity include loss-of-function mutations of the GH/IGF-1 axis and downstream signaling,[7–10] as well as $p66^{Shc}$,[11] the FOXO transcription factors,[12] catalase,[13] and anti-oxidant mimetics.[14] The most widely studied environmental intervention that prevents excess OS and extends lifespan is caloric restriction, which mimics many of the changes observed in the genetic models.[1,5]

Oxidants *in vivo* are multiple, heterogeneous, and include nonenzymatic reaction derivatives of free amine-containing nucleic acids, peptides, or lipids with ambient reducing sugars, termed advanced glycation/lipoxidation products (AGE/ALE or glycotoxins).[15,16] Protein and lipid-derived AGE include N^{ε}-carboxy-methyl-lysine (CML), N^{ε}-carboxyethyl-lysine, methyl-glyoxal-hydroimidazolone, their precursors, and/or derivatives. These are products of normal metabolism in organisms ranging from single cells to mammals and are turned over or neutralized by receptors that promote AGE uptake and degradation, such as AGER1,[17] and are excreted by the kidney.[18] Thus, AGER1 functions to decrease OS. On the other hand, other AGE receptors promote OS after binding, the prime example being RAGE.[19–21] AGE receptors can be up-regulated *in vitro*[22]; however, when AGEs are chronically elevated, ie, in diabetes, AGER1 can be

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Address reprint requests to Helen Vlassara, M.D., Mount Sinai School of Medicine, Box 1640, One Gustave Levy Place, New York, NY 10029. E-mail: helen.vlassara@mssm.edu.

Table 1. Characteristics of Mouse

	Regular	Low
Protein (g)	20.0	20.0
Fat	4.5	4.5
Carbohydrate (g)	54.8	54.8
Total calories (kcal/g)	4.0	4.0
AGE	6.0×10^4	3.0×10^4
Food/day/mouse (g)	5.0	5.0
AGE intake/day (U)	30.0×10	15.5×10^4
Total calories/day (kcal)	20.0	20.1

down-regulated,[23] but OS-promoting receptors, such as RAGE, are enhanced. RAGE promotes the formation of reactive oxygen species, inflammation, stress-responses, and apoptotic events.[19–21] Excess AGEs are present in several diseases common in aging, including diabetes, cardiovascular disease, and chronic kidney disease (CKD).[15,16,18–21,24–26]

Nutrients that are thermally prepared for consumption are a rich source of protein- or fat-derived oxidation derivatives, including AGEs, and a host of toxic compounds in foods, some of which have been implicated in oncogenesis.[27-32] Laboratory rodent food is high in protein, low in fat, supplemented with micronutrients, and routinely heated to ensure safety. The temperatures currently used are sufficiently high to inadvertently cause standard mouse chow to be rich in oxidant AGEs, not unlike levels present in the usual Western diet.[27,30]

Lowering the intake of dietary AGEs or glycotoxins, by restricting the temperature used in nutrient preparation, reduces circulating and tissue levels of AGEs.[33–40] This was associated with a decrease in OS and inflammatory responses in patients with diabetes[37] and CKD,[40] and in animals a Low_{AGE} diet prevents the development of insulin resistance and diabetes,[41,42] cardiovascular disease,[38] and CKD.[43]

Because it was not known whether a decreased intake of AGEs affects normal aging or lifespan in normal animals, we compared the effects of dietary AGE intake on age-related changes such as OS, glucose/insulin metabolism, kidney disease, and lifespan in mice pair-fed a regular diet or an isocaloric diet exposed to lower temperature (Low_{AGE} diet). We found that the Low_{AGE} diet contained lower oxidant AGEs and preserved anti-oxidant reserves, prevented kidney disease, and extended lifespan. These events could be attributable to suppressed OS-regulatory mechanisms and may be related to preservation of the capacity of AGER1 to respond to the increased AGE load that characterizes normal aging.

Materials and Methods

Animals and Diets

C57BL/6 mice ($n = 84$, male, 4 months of age) from the NIA caloric restriction colonies were individually caged and provided free access to water. Mice were assigned to two dietary groups (Table 1): a regular NIH-31 open formula (Harlan Teklad, Madison, WI) ad lib (Reg_{AGE}), and a rodent chow diet (LabDiet 5053; LabDiet, Purina Mills, Richmond, IN), equal in calories, nutrient, and micronutrient content to Reg_{AGE}, but, by limiting exposure to the standard temperature during manufacture, contained less measurable CML-like AGEs (Low_{AGE} diet, ~50% of Reg_{AGE} diet).[38,42,43] Thus, compared with Reg_{AGE}, which is first steam-conditioned and pelleted at 70 to 75°C, for 1 to 2 minutes, and then dried at 55°C for 30 minutes, Low_{AGE} was only exposed to 80°C for 1 minute, during pelleting. Micronutrient content was in excess of established requirements.[38,42,43] Diets were purchased in small amounts (<5 kg) and kept at 4°C. Food consumption was monitored daily, for the first 4 weeks, by weighing the food of each individual mouse to assess exact intake, and weekly thereafter. After establishing the daily intake of Reg_{AGE} mice, the identical amount was given to both Low_{AGE} and Reg_{AGE} mice. Throughout the study, food was completely consumed between feedings. Half of these mice ($n = 20$/dietary group) were used for procedures, ie, blood collection and glucose tolerance testing or sacrifice and tissue retrieval, and the remaining ($n = 22$/diet group) were used for analysis of survival curves.

Body weight was monitored two times per week during the first 3 months and then monthly because none of the groups showed sudden weight loss. At intervals, blood was collected from the tail vein of nonanesthetized mice ($n = 5$/group) and serum was separated and frozen for subsequent analyses. At sacrifice, tissue segments were placed in 2% freshly prepared paraformaldehyde in phosphate-buffered saline or were snap-frozen at −80°C.

All mice were maintained in a specific pathogen-free environment, with the room temperature maintained at 72°F, 50% humidity, and 12:12 light/dark cycles, at the Center for Laboratory Animal Science, Mount Sinai School of Medicine. Sentinel mice in the same room were examined every 3 months and tested sero-negative for pneumonia virus of mice, mouse hepatitis virus, mouse minute virus, lymphocytic choriomeningitis virus, mouse adenovirus, and Sendai virus and tested negative for parasites (eg, pinworm) and other routine pathogens. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare, publication no. NIH 78-23, 1996).

AGE Measurements

Because CML-like AGEs, present in both the normal mouse chow as well as the tissues of animals and humans, and correlate with other AGEs and oxidants, ie, methyl glyoxal or lipid oxidation derivatives such as 8-isoprostanes, it is used as a surrogate marker of other AGEs.[31] AGE concentrations in mouse sera, tissues, urine, and diets were determined by enzyme-linked immunosorbent assay, using monoclonal antibodies reacting with CML-like (4G9; Alteon, Northvale, NJ) or methyl-glyoxal-like epitopes.[31,37,40–42] Based on HPLC/GC-MS, the CML-bovine serum albumin standard contained 23 modified lys/mol, whereas the methyl-glyoxal-bovine serum albumin standard contained 23 modified arginine/mol.[31] CML immunoreactivity (based on 4G9 monoclonal antibody) correlates with that of methyl-glyoxal-derivatives (based on 3D11 monoclonal antibody).[31]

Metabolic Studies

At 4 and 24 months, an intraperitoneal glucose tolerance test (5% dextrose solution; 2 mg/g body weight) was performed in subgroups from each dietary group ($n = 5$), after an overnight fast.[41,42] Blood samples were taken before and at intervals between 5 and 120 minutes after glucose infusion. Blood glucose was determined with an Elite glucometer (Bayer, Mishawaka, IN). Serum insulin levels were measured by enzyme-linked immunosorbent assay (Ultra-Sensitive mouse insulin kit; Alpco Diagnostics, Windham, NH).

Urinary Albumin Excretion Rate

Renal function was evaluated at 4 and 24 months by determining the urinary albumin/creatinine ratio in 24-hour urine samples collected from each group ($n = 5$). Urinary creatinine and albumin were measured using a DCA 2000 microalbumin/creatinine reagent cartridge with a DCA 2000 analyzer (Bayer Corp., Elkhart, IN).

Renal Histopathology

Kidney specimens obtained at 4 and 28 months ($n = 5$), were fixed in 10% buffered formalin and embedded in paraffin. Sections, stained by periodic acid-Schiff (PAS) were used to assess glomerulosclerosis.[43] At least 20 glomeruli per slide were chosen for quantification using IP Lab (BD Biosciences, San Jose, CA) for Macintosh OSX, version 3.9.

Determination of Glutathione (GSH), Oxidized Glutathione (GSSG), and F2-Isoprostanes (8-Isoprostane)

At 4 and 24 months, five animals from each of the diet groups were anesthetized. Whole blood was collected via cardiac puncture and plasma was separated by centrifugation. Levels of GSH and GSSG in whole blood were analyzed colorimetrically (Oxis Research, Portland, OR) using the manufacturer's recommendations and quantified by an enzyme-linked immunosorbent assay reader (412 nm).[31,41] 8-Isoprostane (8-epi-$PGF_{2\alpha}$) levels were determined in fresh plasma samples, using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).[41]

Western Blot Analysis: AGER1, RAGE, p66^{shc}

Equal amounts of tissue (kidney cortex, spleen) protein extracts (50 μg) were separated on 10% or 8% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked in TTBS (Tris-buffered saline with 0.1% Tween 20) containing 5% powdered milk for 1 hour. Primary antibody incubations were performed in TTBS with 5% powdered milk overnight at 4°C. After washing, the membranes were incubated with the appropriate secondary peroxidase-conjugated antibody for 1 hour in TTBS. Immunoreactive proteins were visualized using the enhanced chemiluminescence system from Roche (Indianapolis, IN). For reprobing, blots were stripped with a buffer containing 50 mmol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, and 0.1 mol/L β-mercaptoethanol before probing with a second primary antibody. Primary antibodies were as follows: anti-AGER1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) (0.2 μg/ml), anti-RAGE was from Affinity BioReagents (Golden, CO) (1 μg/ml), and anti-p66^{Shc} was from BD Biosciences, Transduction Laboratories (San Jose, CA) (0.25 μg/ml).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis: Transforming Growth Factor (TGF)-β1, Collagen IV

Total RNA was extracted from mouse kidney tissue using TRI Reagent (Sigma, St. Louis, MO) and RT-PCR was performed using a kit from Boehringer-Mannheim (Indianapolis, IN). The following primers were used[43]: TGF-β1 sense: 5′-ATACAGGGCTTTCGATCCAGC-3′; antisense: 5′-GTCCAGGCTCCAAATATAGG-3′. A1 IV collagen sense: 5′-TAGGTGTCAGCAATT AGG-3′: antisense: 5′-TCACTTCAAGCATAGTGGTCCG-3′. PCR products were separated on 1% agarose gel. β-Actin was also performed for each sample as a control of interassay variance. All experiments were repeated at least three times.

Statistics

Mouse survival curves were calculated by the Kaplan-Meier method, and statistical differences between these curves were evaluated by the log rank test. Per time point analyses of weight or serum AGEs were performed by one-way analysis of variance followed by Bonferroni's multiple comparisons test. Data are shown as mean ± SEM. $P < 0.05$ was defined as significant.

Results

Food Intake and Body Weight

The caloric and nutrient composition of the diets was identical, but the AGE levels were twofold higher in the

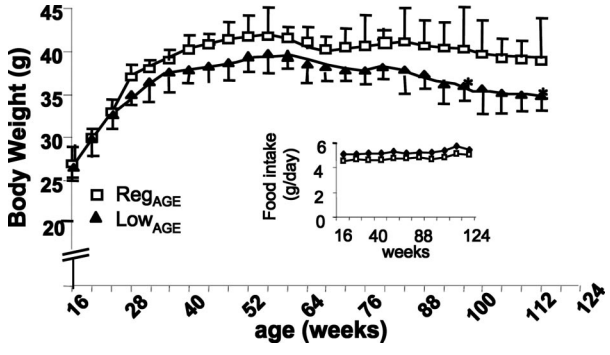

Figure 1. Body weight changes. Mice were exposed to Reg_{AGE} or Low_{AGE} diets as per Table 1 ($n = 22$/group). Food intake (g/day) is shown between 4 and 24 months of age (**inset**). Data are shown in M ± SEM. After 56 weeks: Low_{AGE} versus Reg_{AGE}, $^*P < 0.001$.

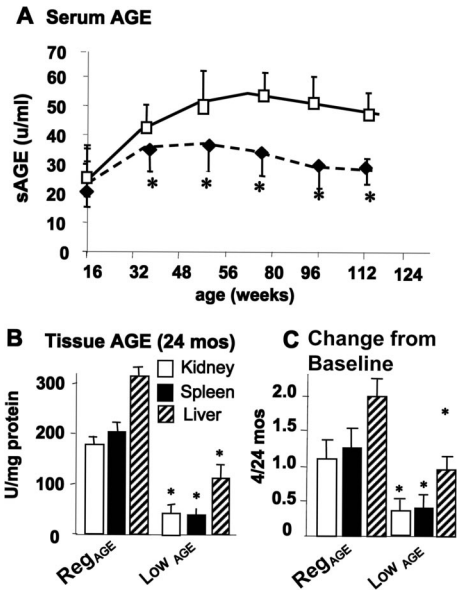

Figure 2. Serum and tissue AGE levels. **A:** Serum AGE concentrations in mice pair-fed Reg_{AGE} (**open symbols**) or Low_{AGE} diets (**filled symbols**). Low_{AGE} versus Reg_{AGE}, $^*P < 0.05$. **B:** Kidney and spleen AGE were assessed at 4 and 24 months of age ($n = 8$/group). Low_{AGE} versus Reg_{AGE}, $^*P < 0.05$. **C:** Kidney and spleen AGE levels, shown as x-fold above baseline (4 months) ($n = 5$/group). Data are shown as M ± SEM. Low_{AGE} versus Reg_{AGE}, $^*P < 0.05$.

Reg_{AGE} than in the Low_{AGE} diet (Table 1). Food intake by either Reg_{AGE} or Low_{AGE} mice stabilized at 5.0 ± 0.27 g/day and remained unchanged throughout the study (Figure 1, inset). The body weight did not differ between the groups, until ~96 weeks of age, when a modest decline in total weight was noted in Low_{AGE} mice ($P < 0.01$), despite identical food consumption (Figure 1).

Serum and Tissue AGE

Differences in the dietary content of CML-like AGEs were reflected in the serum and tissue AGE of each cohort. Serum AGE (sAGE) levels in the Low_{AGE} group did not change throughout the study, whereas after 4 months of age sAGE levels increased in the Reg_{AGE} group and remained higher throughout the study ($P < 0.05$) (Figure 2A). The difference in AGE levels between the spleen, kidney cortex, or liver tissues from the two groups of mice at 24 months of age were even more pronounced than in the serum (more than twofold, $P < 0.05$) (Figure 2, B and C). Similar differences were noted in other tissues (data not shown).

Glucose and Insulin Metabolism

There were no significant differences in fasting blood glucose between the two cohorts, at 4 or 24 months of age (Table 2). Although all mice had elevated plasma insulin at 2 years, Reg_{AGE} mice had an ~3.5-fold higher fasting plasma insulin greater than baseline, 4 months ($P < 0.05$), and approximately twofold higher than 24-month Low_{AGE} mice ($P < 0.01$) (Table 2). Low_{AGE} (24 months) mice, however, had a less than 50% increase in fasting insulin from their baseline ($P < 0.01$) (Table 2). Furthermore, both glucose and insulin responses to intraperitoneal glucose tolerance test, as well as the glucose/insulin ratio (GIR) were markedly impaired in 24-month-old Reg_{AGE} mice (Figure 3, A–E). By comparison, 24-month Low_{AGE} mice had nearly normal responses to glucose challenge, ie, insulin returned to baseline levels within 30 minutes (Figure 3, B and D), and the GIR remained within the normal range (twofold more than Reg_{AGE} mice) (Figure 3E).

Glomerular Morphology and the Urinary Albumin Excretion Rate

At 24 months, the Reg_{AGE} group exhibited more severe glomerulosclerosis (Figure 4, A and B), manifest by an increased fractional mesangial volume per glomerulus (periodic acid-Schiff-positive area ~58%) (Figure 4C), which was associated with an inflammatory infiltrate. Low_{AGE} mice, had lower fractional mesangial volume (periodic acid-Schiff-positive area ~38%, $P < 0.001$) and fewer inflammatory cells in both the glomeruli and interstitium (Figure 4, A and B). The glomerulosclerosis in the Reg_{AGE} group was associated with increased levels of TGF-β1 and collagen-IV mRNA relative to Low_{AGE} glomeruli ($P < 0.05$) (Figure 4, D and E). The Reg_{AGE} group also had interstitial inflammatory cell infiltrates and fibrosis, which was associated with tubular atrophy and thickening of the tubular basement membranes (Figure 4A). These histological changes were associated with changes in the albumin excretion rate in both groups of 24-month-old mice. However, compared with Reg_{AGE} mice, the Low_{AGE} group had a significantly lower albumin excretion rate ($P < 0.05$) (Figure 4F), consistent with the lower levels of serum and kidney AGEs in this group (Figure 2, A–C).

Table 2. Fasting Plasma Glucose and Insulin Levels in C57BL6 Mice at 4 and 24 Months of Age

	4 months		24 months	
Groups	Glucose (mmol/L)	Insulin (nmol/L)	Glucose (mmol/L)	Insulin (nmol/L)
Reg_{AGE}	6.4 ± 0.5	0.16 ± 0.06	6.6 ± 1.3	0.56 ± 0.2*
Low_{AGE}	5.9 ± 1.3	0.17 ± 0.08	5.8 ± 0.8	0.29 ± 0.04*§

*$P < 0.01$ versus 4 months.
§$P < 0.05$ versus age-matched Reg_{AGE} group.

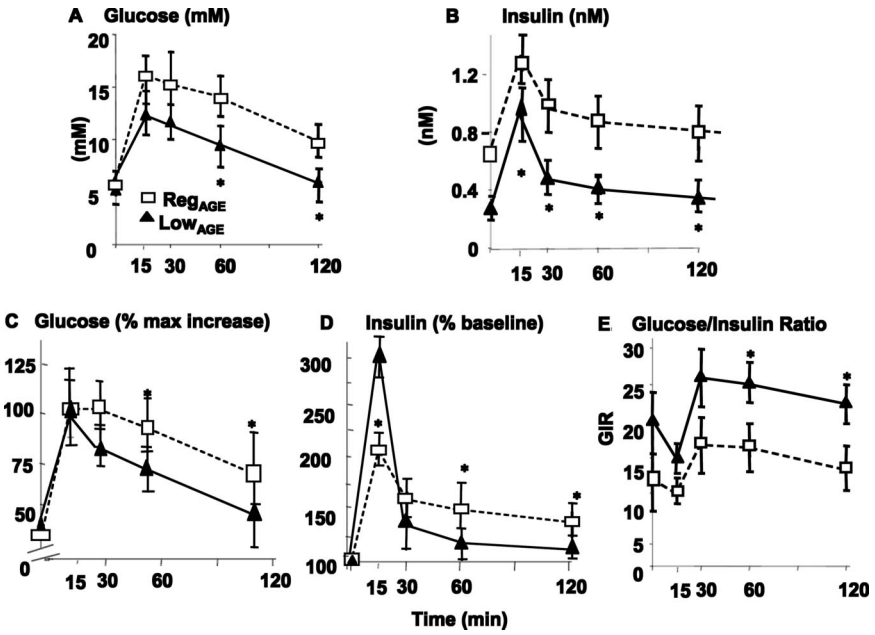

Figure 3. Changes in glucose and insulin response to intraperitoneal glucose tolerance test. Glucose response (**A**, **B**), insulin response (**C**, **D**), and glucose/insulin ratio (GIR) (**E**) in Reg$_{AGE}$ or Low$_{AGE}$ C57BL6 mice. At 4 and 24 months, after an overnight fast, an intraperitoneal glucose tolerance test (5% dextrose solution; 2 mg/g body wt) was performed in subgroups of mice ($n = 6$) from each diet. Blood samples were taken before and at intervals (15 to 120 minutes) after glucose infusion. **A:** Differences in glucose levels between Low$_{AGE}$ and Reg$_{AGE}$; **B:** in percentage from baseline, $^*P < 0.05$ at 60 to 120 minutes; **C:** in plasma insulin levels; and **D:** in percentage from baseline, $^*P < 0.01$. **E:** Glucose-to-insulin (GIR) between Low$_{AGE}$ versus Reg$_{AGE}$ at 60 to 120 minutes, $^*P < 0.01$.

GSH/GSSG Ratio and Plasma 8-Isoprostane Levels

Comparison of GSH and GSSG levels in fresh blood cells from each group revealed a significantly higher GSH/GSSG ratio in Low$_{AGE}$ mice, compared with Reg$_{AGE}$ mice at 24 months (Figure 5A) ($P < 0.001$). At 24 months, the intracellular GSH/GSSG ratio in Reg$_{AGE}$ was reduced ~25% below the 4-month baseline level (Figure 5A, inset). In contrast, in 24-month Low$_{AGE}$ mice, the GSH/

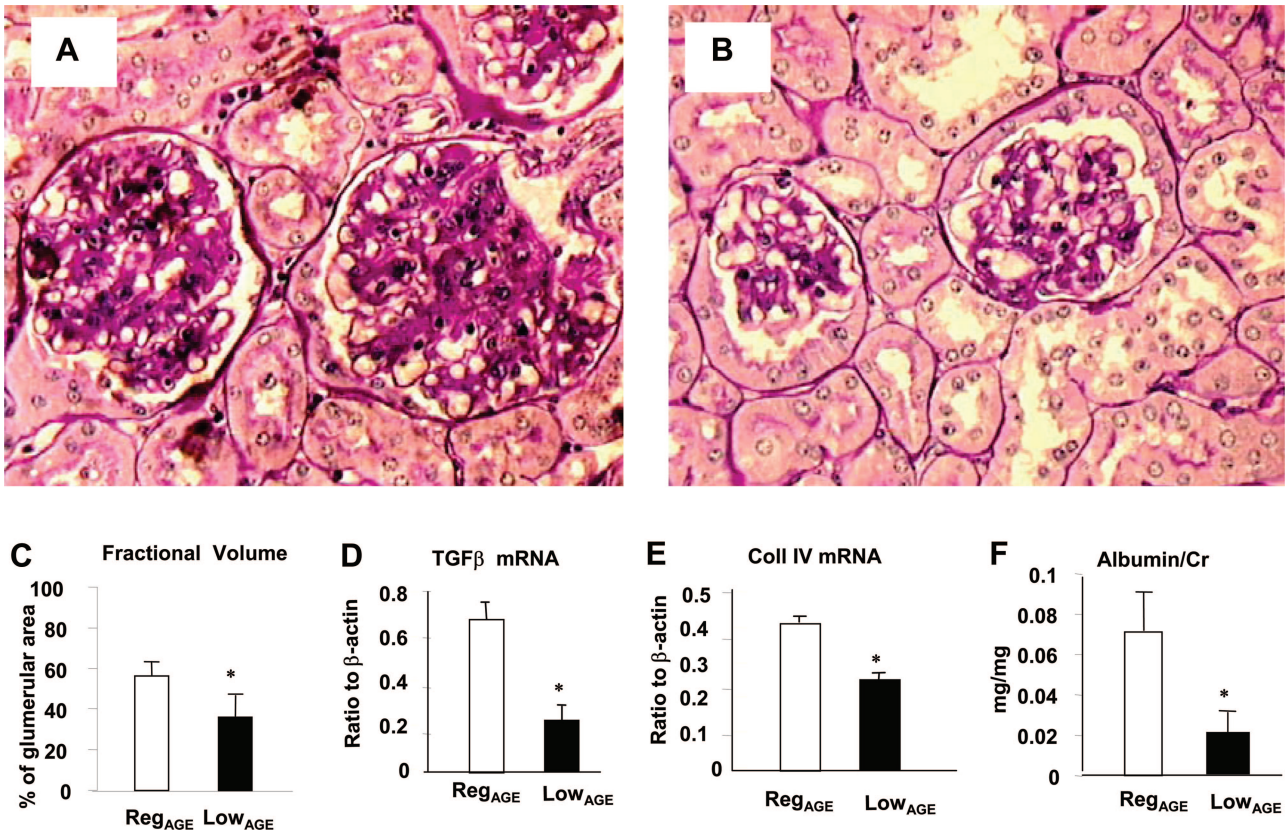

Figure 4. Changes in glomeruli and renal function. Morphology of renal cortex from Reg$_{AGE}$ (**A**) and Low$_{AGE}$ mice (**B**), $n = 6$/group (periodic acid-Schiff). **C:** Fractional mesangial volume ($^*P < 0.05$). **D:** TGF-β ($P < 0.05$) and **E:** collagen type IV mRNA levels in Reg$_{AGE}$ versus Low$_{AGE}$ ($^*P < 0.05$). **F:** Albumin/creatinine ratio ($^*P < 0.05$). Data are shown as M ± SEM of triplicate values. Original magnifications, ×200.

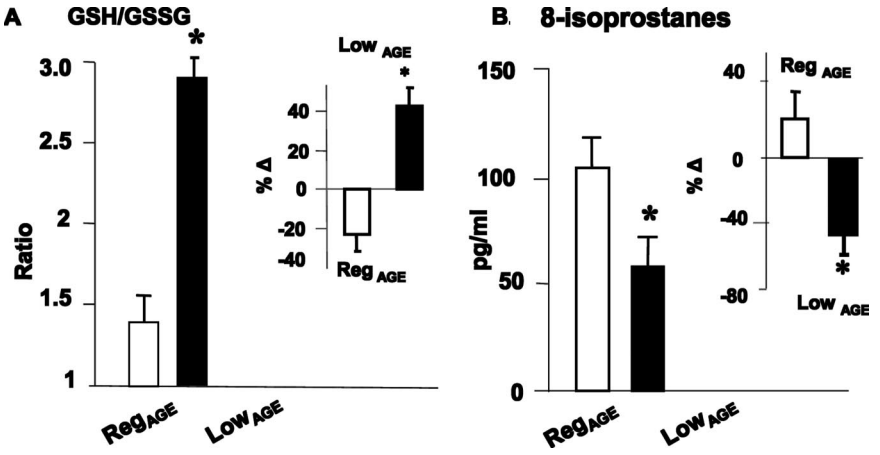

Figure 5. Changes in OS indicators. **A:** GSH/GSSG ratio. Levels of GSH and GSSG were measured in whole blood at 4 (baseline) and 24 weeks of age (n = 6/group). Reg_{AGE} versus Low_{AGE} mice. $^{**}P < 0.001$. The relative change from baseline is shown in the **inset**. **B:** Plasma levels of isoprostane 8-epi-$PGF_{2\alpha}$ are shown as M ± SEM pg/ml (n = 6/group). Reg_{AGE} versus Low_{AGE}, $^{**}P < 0.01$. The relative change from baseline is shown in the **inset**.

GSSG ratio was elevated by ~40% above that at 4 months (baseline) (Figure 5A and inset). Furthermore, at 24 months the levels of endogenous lipid peroxidation products, plasma 8-isoprostanes were higher in Reg_{AGE} mice, compared with Low_{AGE} ($P < 0.01$) (Figure 5B). Of note, in the 24-month Low_{AGE} group, 8-isoprostanes were ~45% lower than at the 4-month baseline ($P < 0.02$), whereas in Reg_{AGE} mice they were ~20% higher than at baseline (Figure 5B, inset).

AGER1, RAGE, and p66^{shc}

The tissue expression of AGE receptors was assessed in spleen, kidney, and liver from young (4 months) and old (24 months) mice (Figure 6). AGER1 protein levels in old Low_{AGE} mouse spleen tissue was increased by threefold, compared with age-matched and 4-month Reg_{AGE} mice ($P < 0.01$ versus 4-month Reg_{AGE}; $P < 0.01$ versus 24-month Reg_{AGE}) (Figure 6A). In fact, AGE-R1 expression in 24-month Reg_{AGE} mouse tissues was as low as at 4 months. By comparison, RAGE expression in these mice was 3.5-fold higher than at 4 months of age ($P < 0.01$) (Figure 6B). However, RAGE levels in old Low_{AGE} mice were significantly lower than in old Reg_{AGE} ($P < 0.01$) and did not different from those at 4 months (Figure 6B). Similar differences were noted in kidney and liver tissues (Figure 6, C and D).

Protein levels of p66^{shc}, an adaptor molecule implicated in oxidant injury and lifespan,[13,14] were also examined in kidney, spleen, and liver tissues of young and aging mice exposed to the two diets (Figure 7, A and B). The expression of p66^{shc} in old Low_{AGE} mice was significantly lower than in old Reg_{AGE} or the 4-month baseline (~55 to 60%; $P < 0.01$, respectively). This was in direct contrast to the 24-month Reg_{AGE} mice, which had an

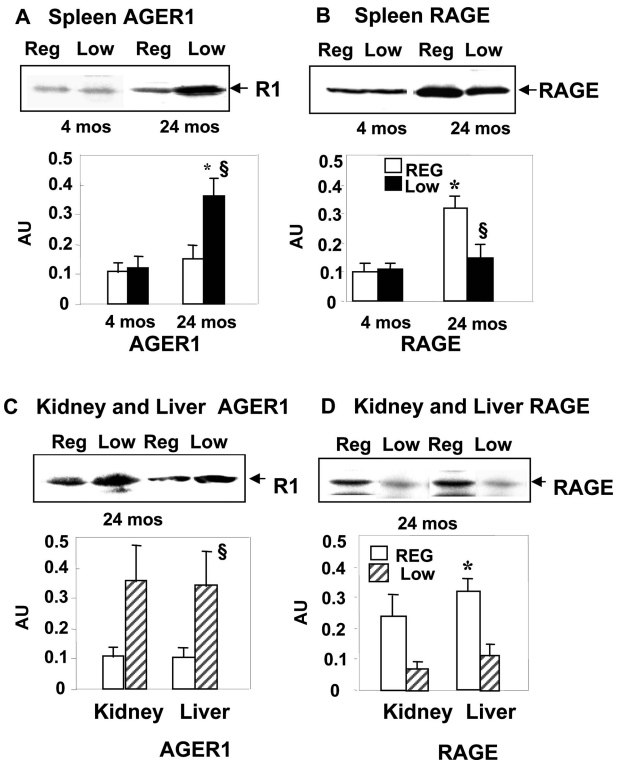

Figure 6. Levels of AGER1 and RAGE. AGER1 (**A**) and RAGE (**B**) protein levels were assessed in spleen tissues from Reg_{AGE} and Low_{AGE} mice (n = 6/group) at 4 and 24 months. AGER1 expression (**C**) in kidney and liver and RAGE expression (**D**) in kidney and liver of the same mouse groups were also assessed at 24 months by Western blotting and densitometric analysis. Data are shown as M ± SEM of three independent experiments ($^{*}P < 0.01$ versus 4-month Reg_{AGE}; $^{\S}P < 0.01$ versus 24-month Reg_{AGE}).

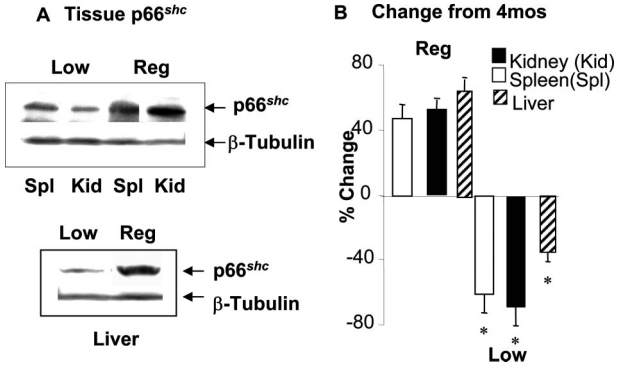

Figure 7. Levels of p66^{shc} protein in kidney, spleen, and liver tissues (n = 6/group). Western blots (**A**) and densitometry data (**B**) also indicate relative change from 4 months of age (baseline). Data shown are M ± SEM of three independent experiments (n = 6/group, $^{*}P < 0.01$).

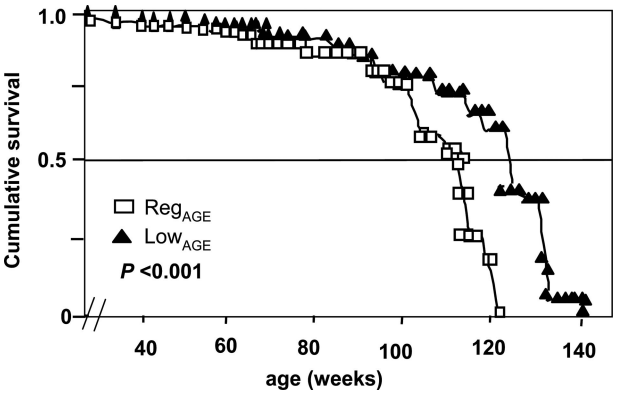

Figure 8. Survival. Kaplan-Meier survival curves in Reg_{AGE} mice (**open squares**) and Low_{AGE} mice (**filled triangles**) ($n = 22$/group). Lifespan of the Low_{AGE} group was significantly longer than in Reg_{AGE} ($P < 0.001$). Differences between the curves were estimated by the log rank test.

increase in $p66^{shc}$ (~40 to 50%) greater than the 4-month baseline ($P < 0.01$) (Figure 7, A and B).

Survival

Median and maximal survival (at 10th percentile) of each group are shown in Figure 8 and Table 3, along with all lifespan percentiles. Lifespan in Low_{AGE} mice was longer than in Reg_{AGE} (median, 15%; maximal, 6%, respectively; $P < 0.001$). At the median survival for RegAGE, 75% of Low_{AGE} mice were alive, whereas at the maximal survival level for Reg_{AGE}, 40% of Low_{AGE} mice were alive.

Discussion

The mice exposed to a low-glycoxidant (Reg_{AGE}) diet had reduced levels of OS, less severe age-related metabolic and kidney changes, and a longer lifespan, relative to mice exposed to an isocaloric diet of standard AGE content (Low_{AGE}). The findings suggest that avoidance of certain thermally induced oxidants in the standard diet, such as oxidant AGEs, may be beneficial. The expression and function of AGER1, a receptor responsible for AGE removal and degradation of AGEs, was enhanced in several key organs of Low_{AGE} mice, but not of Reg_{AGE} mice. Preservation of AGER1 function may have involved the negative regulation of the pro-oxidant RAGE, and lifespan-related $p66^{sch}$ proteins. In combination with a reduced oxidant AGE intake, such a novel pathway may have contributed to the prevention in the decline of innate defense mechanisms and the appearance of organ failure in these aging mice.

The C57BL6 male mice given the Reg_{AGE} diet appeared normal throughout the study. Their weight reached a plateau in adulthood and remained constant, whereas the mean body weight of the Low_{AGE} mice declined slightly at the end of the study. Because a significant reduction in caloric intake (~40%) is required for the lifespan extension observed in this study,[1] the late and modest differences in body weight of these pair-fed mice could not be attributed to a significantly different energy intake, pointing to other factor(s), including the levels of oxidants. Age-related weight gain or retention is associated with increased intra-abdominal fat and insulin resistance, despite the concurrent loss of muscle mass attributable to both a decrease in physical activity and the effects of aging.[44] Therefore, the impaired glucose and insulin responses in 24-month Reg_{AGE} mice could be attributable to increased fat stores. The 24-month Low_{AGE} mice had a pattern of glucose and insulin utilization similar to that of 4-month mice, consistent with an absence of age-related excess fat. A dissociation between energy intake and body weight was previously observed in mice pair-fed fed high-fat diets that varied only in AGE content and in normal adults.[41,44,45] The Reg_{AGE}, high-fat mice weighed more and had larger abdominal fat stores, containing higher levels of AGE-modified lipids and were less insulin sensitive than mice fed a Low_{AGE}, high-fat diet. Consistent with that study, the current differences in weight could not be explained by differences in food intake because the mice were pair fed. The modest differences in body weight may also be related to a difference in the rate of turnover of AGE fat in the Reg_{AGE} diet group, compared with the Low_{AGE} mice. Because AGEs stimulate the production of proinflammatory cytokines, including tumor necrosis factor-α,[46] which may promote insulin resistance,[47,48] the greater tissue AGE burden in Reg_{AGE} mice might have contributed to the age-related impaired glucose, insulin, and fat utilization. The attenuation of these metabolic events in aged Low_{AGE} mice in the current study was consistent with the lower AGE intake and tissue AGE deposits.

The significant differences in kidney disease severity between 24-month Reg_{AGE} and Low_{AGE} mice provide further support for the hypothesis that the high levels of oxidant AGEs in the normal diet promote tissue injury. The CKD of aging, like cardiovascular disease and the metabolic syndrome, is associated with increased OS.[49] CKD is also a major contributor to cardiovascular disease, often preceding vascular decline. Several strains of mice manifest severe CKD with aging, including C57BL6 mice.[50,51] We previously found that the kidneys of 24-month Reg_{AGE} C57BL6 mice had increased glomerulo-

Table 3. Lifespan Percentiles

	Median						
	Max	90%	75%	50%	25%	Mean ± SE	Log rank
Reg_{AGE}	121	~120	117	108	89	102.7 ± 4.2	6.9
Low_{AGE}	137	~129	127	121	104	112.9 ± 4.7	−11.2

Low_{AGE} versus Reg_{AGE}: $P < 0.001$.

sclerosis and albuminuria, as well as stable phenotypic changes in isolated mesangial cells, and that these changes were transferred by bone marrow transplantation to naïve young recipients.[51] The current study shows that reduced AGE intake by a Low_{AGE} diet prevented the CKD of aging. The effects of a high or low AGE intake on tissue injury are not restricted to aging because similar effects have been observed in studies of diabetes-related kidney or vascular disease and wound healing.[38,52]

The age-related decrease of GSH:GSSG ratio in the 24-month Reg_{AGE} mice, in addition to reflecting an excess in total intracellular reactive oxygen species production, may provide a direct link between increased OS and organ decline, metabolic changes, and lifespan during aging. This possibility was supported by the increase in endogenous lipid peroxidation products, ie, 8-isoprostanes, as well as in pro-oxidant AGEs. These changes were not observed in Low_{AGE} mice, which maintained a strong anti-oxidant balance throughout life. These data suggest that depletion of antioxidant stores during aging of experimental mice, and possibly humans, may result from chronic overexposure to oxidants derived from the normal Western diet. This hypothesis is supported by a recent study in normal, ambulatory patients.[45]

Cellular responses to AGEs depend on at least two general types of receptors, which have opposing actions. RAGE exemplifies the class that promotes OS.[22,23,53] AGER1 is a member of a group that mediates removal and detoxification of AGEs, resulting in suppression of OS.[17,54,55] In this study, AGER1 expression in tissues from 24-month Low_{AGE} mice was more than threefold higher than at initiation of the study (4 months). This was accompanied by a marked increase in anti-oxidant reserves and lower levels of tissue AGEs, RAGE, and other native oxidants, ie, 8-isoprostanes. We have previously shown that AGEs can up-regulate cellular AGE uptake[22] and that this is at least partly AGER1 mediated.[17] The increased expression of AGER1 in the Low_{AGE} mice suggests that AGER1 can respond to fluctuations in AGE load *in vivo*, if this load does not exceed a certain threshold, which remains to be defined. The current study also demonstrates that if the external AGE burden is moderated, ie, with a low AGE diet, AGER1 levels remain responsive to AGE increases, ie, because of aging, leading to effective control of tissue AGE as well as of OS. In contrast, under conditions of chronically excessive exogenous AGE, AGER1 levels, like other anti-oxidant defenses, fail to respond, indicating that the capacity of the body to handle oxidants was exceeded. Sustained exposure to high AGE levels, and other oxidants, may lead to a down-regulation of other receptor systems, eg, scavenger receptor B, or CD36,[56,57] further contributing to high OS during aging. Of interest, increased tissue AGER1 expression in old Low_{AGE} mice, matched by a lower RAGE, serve as additional *in vivo* evidence that AGER1 may contribute to the regulation of RAGE *in vivo*.[17,54]

Of interest, in the context of the current study, were the changes observed in the expression of $p66^{shc}$, an OS-regulatory adaptor protein[11,12] that has been negatively linked to longevity.[11] Tissue $p66^{shc}$ levels were increased in 24-month Reg_{AGE} (40 to 50%) compared with the values at 4 months of age. In contrast, $p66^{shc}$ levels were significantly decreased in 24-month Low_{AGE} mice (~60% less than at 4 months). This decrease in $p66^{shc}$ involved multiple tissues and was commensurate with the reduced OS levels in 24-month Low_{AGE} mice, as well as with the reduction in the severity of kidney disease in this group. A similar protection was reported against renal/vascular tissue injury in $p66^{shc}$ mutant mice.[58,59] The inverse correlation between low $p66^{shc}$ and increased AGER1 levels in Low_{AGE} mice may be partly explained on the basis of an AGER1-mediated inhibition of the EGFR signaling pathway,[54] because the activation of this pathway involves $p66^{shc}$.[60] The fact that AGER1 may act to prevent the induction of $p66^{Shc}$ could provide a new mechanistic link between AGER1 stimulation and $p66^{shc}$ suppression. Although further studies are required to fully explain these events, such an interaction may have contributed to extended lifespan in Low_{AGE} mice. The increased AGER1 expression, combined with reduced AGE, OS, RAGE, and $p66^{Shc}$ levels in Low_{AGE} mice does not prove a causal relationship, but it suggests that homeostatic mechanisms might be able to better compensate in older age, if exogenous oxidants did not exceed the body's inherent capacity to take up, degrade, and excrete these compounds. Interestingly, soluble RAGE, which binds circulating AGEs, was recently found to be elevated in the healthy elderly.[61]

In summary, the current study suggests that age-related changes in OS, glucose and insulin metabolism, renal injury, and lifespan in normal mice are events that can be modulated by the degree of exposure to external (dietary) oxidants. Although restricting AGE intake can be immediately tested in humans as a single intervention,[37,40] future studies may also examine the combined effects of low AGE diets with other dietary and therapeutic interventions.

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