Supporting Information

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SI Materials and Methods

Anti-AGER1, anti-insulin receptor, anti-NF-κB p65, and anti-PY20 antibodies were from Santa Cruz Biotechnology. cDNA of human SIRT1 was a gift of J. C. He (Mount Sinai School of Medicine, New York, NY). Human RAGE siRNA was from Dharmacon RNAi Technologies. Anti-SIRT1, anti-IRS1, anti-IRS2, and anti-phospho-IRS1 (Ser307) were from Millipore. Anti-NAMPT antibody was from BETHYL. Anti-phospho-akt (Ser473), anti-acetylated-lysine, and anti-phospho-JNK (Thr183/Tyr185) antibodies were from Cell Signaling. SIRT1 inhibitor Sirtinol was from CALBIOCHEM. NAD/NADH Assay Kit was from AAT Bioquest. Plasma 8-isoprostanes (Cayman), VCAM-1 (R&D Systems), and adiponectin (Invitrogen) were assessed by commercial kits as per manufacturer's procedures.

Animals and Treatments. C57BL/6 mice (National Institutes on Aging, CR colony), Fo, 8 wk old, were placed on the standard diet (NIH-31 open formula), which had been prepared without heat exposure (low AGE) (1–3). The offspring F1-F3 (n = 18–20/group), after weaning, were assigned to two pair-fed groups, one receiving the low-AGE or the same formula with synthetic MG-BSA added (1 mg/g food), and air-dried, at room temperature (1). The diets (henceforth referred to as MG^- or MG^+ diet, respectively) were identical in nutritional and caloric content but MG^+ diet contained MG derivatives, recently identified as MG-H1 (Fig. S1*A*), and CML to levels equivalent to standard heat-treated chow, and nearly twice those in MG^- diet (Table 1). Data from mice of F3 (n = 12–18/group, 1:1 M/F, age 18 mo), together with age-matched control mice (n = 15, 8:7 M/F), fed NIH-31 open formula (Reg) were used in the present report. All mice were maintained in a pathogen-free environment, (72 °F, 50% humidity, and 12:12-h light: dark cycles) at the Center for Laboratory Animal Science, Mount Sinai School of Medicine. All experimental procedures complied with the guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare, NIH 78–23, 1996).

RNA isolation and reverse transcription–PCR (RT-PCR). Total RNA was isolated and purified from mouse peritoneal microphages, adipocytes, and stromal vascular cells using TRIzol (Sigma) (1, 4). First-strand cDNA synthesis and PCR were performed. The following primers were used: AGER1-F: ACAGCTCTCCAATGGCAAGT; AGER1-R: ATACTGCGTGTGCTGGAGTG, SIRT1-F: TTGTGAAGCTGTTCGTGGAG; SIRT1-R: TGAGGCACTTCATGGGGTAT, TNFα-F: TTCGGCTACCCCAAGTTCAT; TNF-α-R: CGCACGTAGTTCCGCTTTC, IL10-F: GCTCTTACTGACTGGCATGAG; IL10-R: CGCAGCTCTAGGAGCATGTG, CD206-F: TGGGGTGCTGACGAGCCGAA, CD206-R: ACCAGGGAGGCACCCATTCGA, CD11c-F: CCTGAGGGTGGGCTGGAT; CD11c-R: GCCAATTTCCTCCGGACAT, MCP1-F: AGGTCCCTGTCATGCTTCTG; MCP1-R: GCTGCTGGTGATCCTCTTGT, Adiponectin-F: ACAATGGCACACCAGGCCGT; Adiponectin-R: TGCCAGGGGTTCCGGGGAAG.

Isolation of peritoneal macrophages, adipocytes, and stromal vascular cells. Nonelicited peritoneal macrophages were isolated from 18 mo of F3 MG^- and MG^+ mice. Cells were plated in 12-well tissue culture plates in RPMI supplemented with 10% FBS. Nonadherent cells were removed after 2 h and total RNA was isolated from the adherent macrophages (5, 6).

Mouse white adipose tissue from inguinal fat pads was rinsed in saline and cut into small pieces, and digested with collagenase (Sigma-Aldrich, 2 mg/mL, at 37 °C for 45 min) and characterized as described (6–8). Cells were filtered through a nylon mesh and fractionated by centrifugation (× 1,000 rpm). Floating cells (adipocytes) and pellet (nonadipocytes or stromal vascular cells) were washed by PBS buffer three times and used in RNA extraction.

Western analysis. After incubation with MG-BSA or BSA at 37 °C for the indicated time periods, cell proteins were separated on 8% SDS/PAGE gels and transferred onto nitrocellulose membranes, which were probed with the appropriate primary and secondary antibodies, and visualized by enhanced chemiluminescence (Roche) (2).

Immunoprecipitation. Cell lysates (300 μg of protein) were incubated with the appropriate antibody at 4 °C, overnight, and further incubated with A/G PLUS-agarose immunoprecipitation reagent beads (60 μL) (Santa Cruz Biotechnology), at room temperature, for 2 h. Bound immune complexes were washed five times in the RIPA lysis buffer and used for immunoblotting, after SDS/PAGE and transferred to a nitrocellulose membrane (2, 9).

NAD/NADH and intracellular H_2O_2 (ROS) determination. NAD and NADH were measured using an Amplite Fluorimetric NAD/NADH Assay Kit (AT Bioquest), as per manufacturer's instructions (10). After stimulation with MG-BSA (60 μg/mL for 72 h) in the presence or absence of anti-oxidants *N*-acetylcysteine (NAC; 5 mM) and apocynin (300 μM), cells were lysed. NAD+, NADH, and [NAD/NADH] ratio measures were based on an enzymatic cycling reaction, using a fluorescent plate reader (excitation: 560 nm and emission: 590 nm). Intracellular ROS were measured as hydrogen peroxide by using 2′,7′-dichlorofluorescein diacetate (DCFDA) (Molecular Probes) as a probe (11). Cells were treated with or without 60 μg/mL MG-BSA for 4 h. After incubation with 5 μM DCFDA for 45 min, the DCF was measured (at excitation 485 nm and emission 530 nm).

Glucose tolerance. An IGTT was performed at 6-mo intervals after age 12 mo. The 5% dextrose solution was injected after overnight fast (2 mg/g body weight, i.p., n = 6) and plasma glucose (Elite glucometer, Bayer) or insulin (by ELISA, Ultra-Sensitive mouse insulin kit, Alpco Diagnostics) levels were measured in tail-blood samples for 120 min (2, 3, 9).

Glucose uptake. Adipocytes were isolated from 18-mo-old mouse WAT, after mild digestion by collagenase (Sigma-Aldrich), 2 mg/mL, at 37 °C for 45 min (8). Cells were filtered through a nylon mesh and fractionated by centrifugation ((× 1,000 rpm). Adipocytes were incubated with or without insulin (100 nM, 20 min at 37 °C) followed by the addition of 0.5 uCi 2-deoxy-[^{3}H] glucose (2DOG) for 3 min. The incubation was terminated with cytochalasin B (Sigma), cells were separated by centrifugation through silicone oil, and cell-associated radioactivity was determined. Nonspecific transport was determined under the same conditions except that cytochalasin B was added to the medium before the addition of cells and nonspecific values were subtracted. In a similar way, 2DOG uptake was measured in skeletal muscle isolated from mouse hind limbs, and preincubated with or without insulin (100 nM) for 20 min (12). To examine insulin action in vivo, regular insulin (5 units/kg, Novo Nordisk) was injected i.p. and 30 min later, liver, skeletal muscle, and inguinal fat were removed, weighed and frozen in liquid nitrogen for analysis (7, 8, 13).

Cell culture and transfection. The 3T3-L1 preadipocytes (ATCC) were maintained in DMEM (10% bovine calf serum). Differentiation medium consisting of DMEM supplement with 10% BCS, and a mixture containing 500 μM isobutyl-methyl-xanthine, 4 μg/mL insulin, and 1 μM dexamethasone was added to pre-adipocytes two days after reaching confluence (day 0). On day 7,

cells were washed with PBS and incubated in DMEM plus 1% BCS for 4 h, followed by MG-BSA or BSA (60 μg/mL) in serum-free medium. For preparation of cell lysates for insulin receptor signaling studies, MG-BSA–treated differentiated 3T3-L1 adipocytes were stimulated with or without insulin (100 nM, at 37 °C for 10 min) before they were harvested. The 3T3-L1 adipocyte transient transfection with AGER1, shAGER1 or Sirt1 was performed by using lower concentration of Lipofectamine 2000 (Invitrogen). The ratio of Lipofectamine 2000 to plasmid DNA was 0.6 (volume:mass) (14).

MG-BSA preparation. Low-endotoxin BSA (Sigma) was incubated in sodium phosphate buffer with pure MG (1 mmol/L) (provided by Y. Al-Abed, Manhasset, NY) for 3 d, dialyzed against ammonium bicarbonate buffer (pH 8, 4 °C), then lyophilized to dryness and stored at −80 °C. Based on HPLC, the thus-derived MG-BSA (MG-AGE) contained 19.2 MG-Arg/mol BSA. Based on ELISA using anti-MG mab (3D11), it contained 9.2 nmol MG/mg, and by an anti-CML mab (4G9), 48 CML U/mg (15). Control or unmodified BSA contained 0.01 nmol MG/mg and 1.3 CML U/mg. All reagents were prepared under endotoxin-free conditions, and for cell assays were passed through a polymyxin column (Detoxi-gel; Pierce) testing negative by Limulus assay (E-Toxate, Sigma; <0.2 ng endotoxin/mL).

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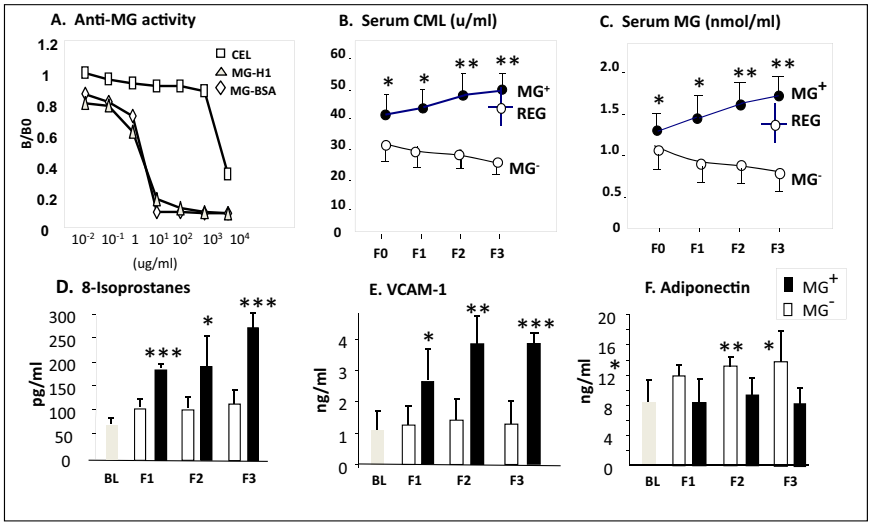

Fig. S1. (*A*) Anti-MG monoclonal antibody (3D11) is highly reactive with the MG-derivative hydroimidazolone (MG-H1). Competitive ELISA was performed against antigen MG-BSA (10ng), with serial dilutions of competitor antigens MG-derived hydroimidazolone (MG-H1), carboxy-ethyllysine (CEL), MG-BSA and with 3D11 antiserum added sequentially. Absorbance (at 405 nm) was read on a micro-ELISA plate reader. 3D11 mab, which has been shown to be immune-reactive with MG- and AGE-BSA (at ~0.5 μg/mL) and AGE-LDL (at~100 μg/mL), but not with CML-BSA, native-LDL, oxidized-LDL, BSA, ovalbumin, glucose, or lysine alone (5), is here shown to be reactive with MG-H1, but not with CEL. (*B*) Chronic exposure of four successive generations of C57BL6 mice to MG^+ or MG^- diet leads to progressive changes in serum CML, (*C*) serum MG derivative, MG-H1, (*D*) plasma 8-isoprostanes, (*E*) plasma VCAM-1, and (*F*) plasma adiponectin, (n = 12–15/group, at least 6–8 mice/generation). Blood levels in 4 mo mice fed standard (Reg) diet (n = 8) were used as baseline. Data in *B–F* are shown as means ± SEM $*P < 0.05$, $**P < 0.01$, $***P < 0.01$ indicate values vs. those in MG^- mice of the respective generation.

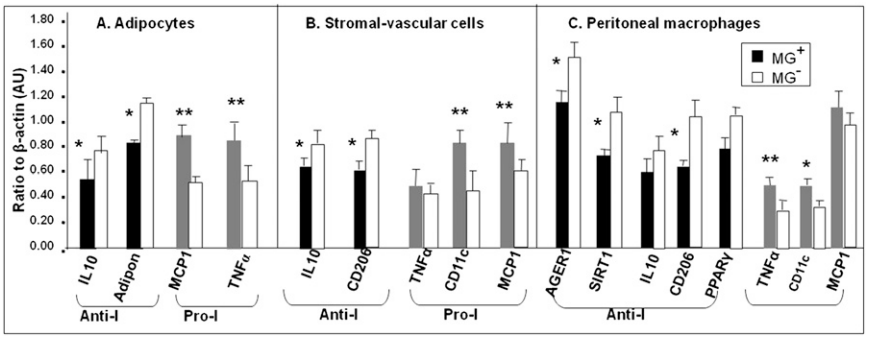

Fig. S2. WAT cells and peritoneal macrophages from MG$^+$ mice share a proinflammatory phenotype. Primary WAT adipocytes and stromal vascular cells, as well as nonelicited peritoneal macrophages were isolated from MG$^+$ F3 (filled bars) or MG$^-$ F3 mice (open bars) (n = 5–7 per group) and mRNA levels of proinflammatory (Pro-I) genes, MCP-1, TNF-α, CD11c, or anti-inflammatory (Anti-I) genes, adiponectin (in adipocytes), IL10 and CD206, as well as AGER1, SIRT1, and PPAR-γ (in macrophages) were measured by RT-PCR. Data (means ± SEM) indicate the ratio of each PCR product to β-actin. *$P < 0.05$, **$P < 0.01$ vs. MG$^-$ mice.

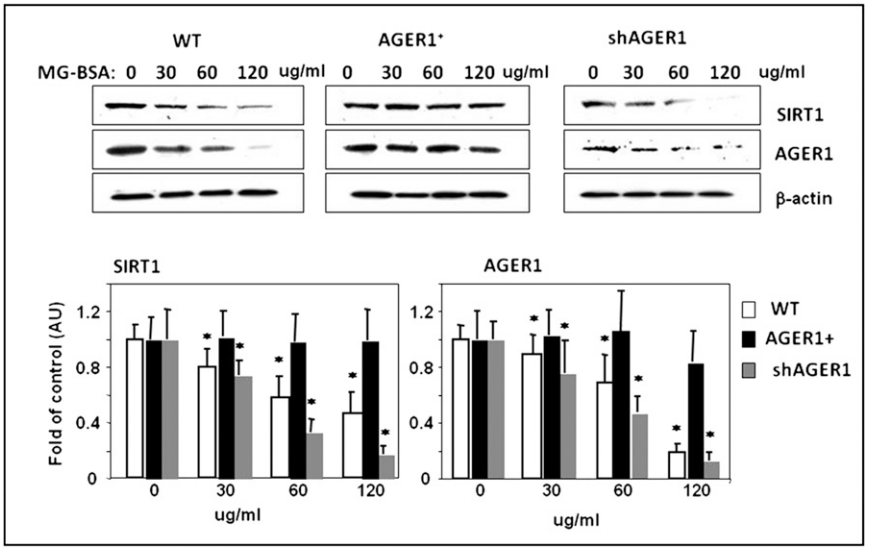

Fig. S3. SIRT1 expression in MG-stimulated differentiated 3T3-L1 adipocytes is AGER1 dependent. WT, transduced (AGER1$^+$), or silenced for AGER1 (shAGER1) 3T3-L1 cells were preincubated with different concentrations of MG-BSA for 72 h, and cell lysates were subjected to Western blotting with anti-AGER1 or anti-SIRT1 antibodies. Representative gel images and densitometry data (means ± SD, n = 3) show the ratio of each protein to β-actin. *$P < 0.05$ vs. nonstimulated control cells.

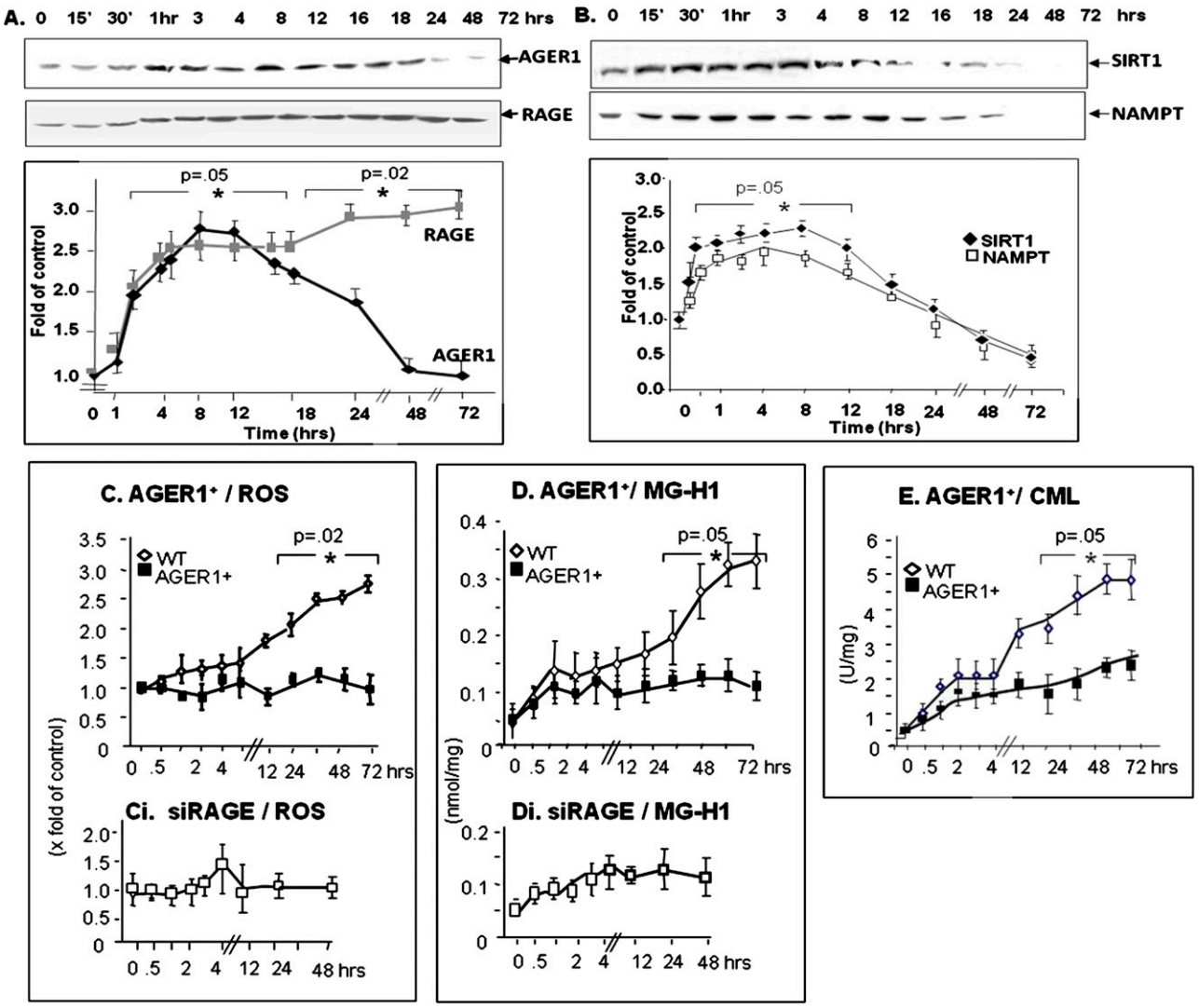

Fig. S4. Time-dependent changes in MG-stimulated differentiated 3T3-L1 cells: (*A*) AGER1, RAGE, (*B*) SIRT1 and NAMTP protein levels, (*C*) intracellular ROS, (*D*) MG-H1, and (*E*) CML. Cells were exposed to MG-BSA (60 μg/mL) for up to 72 h. Western analysis was performed by the indicated primary antibodies. Representative continuous gel images and densitometric analysis data are shown as fold (means ± SEM) above nonstimulated, control cells ($n = 3$). (*C–E*) AGER1 transduction inhibits long-term MG-induced intracellular ROS and AGE accumulation. WT (open symbols) or AGER1-transduced (AGER1$^+$, closed symbols) 3T3-L1 cells exposed to MG-BSA (60 μg/mL) for 72 h. In addition to ROS, measured by dichlorofluorescein DCF (*C*), intracellular MG-H1 (*D*), and CML (*E*) were assessed by ELISAs. Data (means ± SEM, $n = 3$) are shown as fold of nontransduced control cells (for ROS), U/mg (for CML) or nmol/mg (for MG-H1). Additional 3T3-L1 cells transduced with human RAGE siRNA were stimulated with MG for 48 h and ROS (*Ci*) or MG-H1 (*Di*) were measured as above. *$P < 0.05$ vs. control in each group.