

RESEARCH DESIGN AND METHODS

Materials. Methylglyoxal, glyoxal, acetol, NADPH, glutathione, mammalian protease inhibitor, DTT, EDTA, EGTA, GSH, DL-glyceraldehyde were purchased from Sigma Chemical Co (St. Louis, MO, USA). Anti-argpyrimidine, anti-CML, and anti-deoxyglucosone derived imidazolone antibodies were purchased from Cosmo Bio Ltd. Anti-glyoxalase I and Anti-HSP27 antibodies were obtained from Novus Biologicals and Abcam Inc. respectively. Methylglyoxal was distilled and the concentration was determined by GC-MS analysis as mentioned below. Sorbinil and tolrestat were gifts from Pfizer and American Home Products, respectively. Bromobenzylglutathione cyclopentyl diester (BBGC) was synthesized as mentioned (1). All the other reagents were of the highest purity available.

Cloning, expression, and purification of AKRs. Expression constructs for the AKR genes were generated as described previously (2). The proteins were purified by column chromatography on a Ni-affinity column. The catalytic activities of His-Tag proteins were similar to those of the non His-Tag proteins and deletion of the His-Tag by thrombin did not affect enzyme activities. Purity of each enzyme was established by SDS/PAGE and the catalytic activity was measured with their standard substrates.

Enzyme activity measurements of AKRs. After purification, the enzymes were reduced by incubating with 0.1 M DTT at 37°C in 0.1 M Tris-HCl (pH 8.0). Excess DTT was removed by gel filtration on a Sephadex column (PD 10 column) pre-equilibrated with N₂-saturated 0.1 M potassium phosphate (pH 7.0) containing 1mM EDTA. Catalytic activity was measured at room temperature in a 1 ml mixture containing 0.1 M potassium phosphate buffer (pH 7.0) with 0.15 mM NADPH and indicated substrate concentrations (2;3). For tissue activity measurements, frozen mouse hearts were thawed and homogenized in 3 volumes (30% v/v) of buffer (10 mM HEPES; pH 7.0, 1mM EGTA, 1mM EDTA, 1mM DTT and protease inhibitor 1:100). The homogenate was centrifuged at 13000×g for 20 min and the supernatant was passed through a PD-10 column pre-equilibrated with N₂-saturated buffer (potassium phosphate pH 6.0 containing 1 mM DTT). Enzyme activity was measured at 25°C in a 1 ml mixture containing 0.1 ml tissue homogenate (100-700 µg protein), 0.1 M potassium phosphate (pH 6.0) containing 0.4 M ammonium sulfate, 0.15 mM NADPH and substrate (10mM DL glyceraldehyde/1 mM glyoxal/1 mM methylglyoxal/1 mM 3-deoxyglucosone). Initial velocity was monitored by the rate of oxidation of NADPH at 340 nm for 5 min. The control cuvette contained all the components of the reaction mixture except the substrate.

Measurement of glyoxalase I activity. For the measurement of glyoxalase I activity, cytosolic fractions of the heart homogenates were prepared as described above and passed through a PD10-column equilibrated with phosphate buffer (pH 7.0). Glyoxalase I activity was measured as described by Di Ilio et al (4). Samples were incubated with 1mM glyoxal + 1mM GSH or 1mM methylglyoxal + 1mM GSH for 3 min at room temperature. Formation of S-glycolylglutathione and S-D-lactoylglutathione were monitored at 240 nm.

Computer simulation experiments. Computer simulations were performed based on experimental data in order to assess the relative contribution of AR and glyoxalase I pathways for the metabolism of glyoxal and methylglyoxal. The model assumes that the concentration of AGE precursors is maintained at a steady-state level due to the equilibrium between synthesis and

elimination. Hence, the relative contribution of each pathway is proportional to its velocity. The velocities of AR and glyoxalase I were calculated using Michaelis-Menten equation using the kinetic constants in *Supplemental Table 1*. The contribution of AR was calculated as $100 * V_{AKR} / (V_{AR} + V_{Gly})$, and that of glyoxalase I as $100 * V_{Gly} / (V_{AR} + V_{Gly})$.

Synthesis and GC/MS analysis of $^{13}C_3$ -methylglyoxal, $^{13}C_3$ -acetol. $^{13}C_3$ -methylglyoxal was synthesized by mixing $^{13}C_3$ -acetone (4.1 mmoles) with selenium dioxide (4.7 mmol) in 15 ml water. The reaction mixture was refluxed for 3 h under nitrogen and $^{13}C_3$ -methylglyoxal was separated by distillation. $^{13}C_3$ NMR spectrum of the distillate was recorded in D_2O at 500 MHz and showed predominant signals for hydrated methylglyoxal at δ 22 - 24 Hz (CH₃), 90 - 106 Hz (HCOH) and 220 Hz (CO). The NMR spectrum of $^{13}C_3$ -methylglyoxal is in agreement with Nemet et al (5). $^{13}C_3$ -acetol was synthesized by incubating 100 μ M of $^{13}C_3$ -methylglyoxal with 100 μ g recombinant AKR1B1 and 150 μ M NADPH in 0.1 M potassium phosphate buffer pH 7.0 at 37°C. The reaction was monitored by following the decrease in absorbance at 340 nm. The enzyme was removed by ultrafiltration. The concentrations of $^{13}C_3$ -methylglyoxal and $^{13}C_3$ -acetol were determined by GC/MS analysis using commercially available methylglyoxal and acetol as external standards. α -oxoaldehydes were derivatized with aqueous PFBHA solution. The reaction was left for 30 min at room temperature. The derivatives were then extracted with 2 ml hexane. Six drops of concentrated sulfuric acid were added to each vial to remove excess PFBHA. The organic fraction was separated by centrifugation at 3000 \times g for 2 min, collected, and evaporated under a stream of nitrogen gas. The dried samples were derivatized with 4 μ l of BSTFA in 20 μ l acetonitrile for 30 min at 65°C. The mixture was cooled to room temperature and 1 μ l aliquots were used for analysis. The GC/MS analysis was performed using a HP5890/HP5973 GC/MS system (Hewlett Packard; Palo Alto, CA, USA). Under these conditions the retention time for the derivatized adducts of methylglyoxal and acetol were 13.1, and 7.5 min, respectively. Data from GC-MS analyses of methylglyoxal and acetol are shown in Fig. 2 A.

High glucose-induced expression of AGEs in endothelial cells. Human umbilical vein endothelial cells (Clonetics, Walkersville, MD, USA) were cultured in endothelial basal medium (EBM, Clonetics) supplemented with human endothelial growth factor (hEGF), hydrocortisone, gentamicin/amphotericin B (GA), bovine brain extract (BBE) and 2% fetal bovine serum (FBS, EGM SingleQuots[®], Clonetics/Lonza, Walkersville, MD, USA) as suggested by the supplier under standard cell culture conditions (37 °C, 5% CO₂). To examine the formation of AGEs, HUVEC were maintained in 0.4% fetal bovine serum in presence of 5.5 or 30 mM glucose as described by Shinohara et al (6) or with 30 mM glucose +50 μ M sorbinil for 7 days. The medium was changed every 12 h. At the end of the protocol, the cells were lysed in ice-cold PBS containing the protease inhibitor cocktail, centrifuged at 12000 \times g for 20 min and the supernatant was used for Western blotting probed with anti-argpyrimidine and anti-CML antibodies. Peptides for MS analysis were obtained from protein spots of interest that were excised from silver-stained gels and digested with trypsin using previously published procedures (7). Trypsin-generated peptides were applied by a thin-film spotting procedure for mass-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis on an α -cyanohydroxycinnamic acid matrix (7). Mass spectral data were obtained with a TOF-Spec 2E instrument (Micromass) and a 337-nm laser in the reflectron mode. Data were obtained by averaging 10 spectra, each of which was the composite of 10 laser firings. Mass axis was

calibrated by using peaks from tryptic auto-hydrolysis. Peptide masses obtained by MALDI-TOF/MS analyses were used to search the National Center for Biotechnology Information (NCBI) database via Mascot search engine (www.matrixscience.com) to identify the intact proteins.

Generation of glyoxal-modified BSA. CML-BSA was prepared as described by Ikeda et al (8). Briefly, BSA (50 mg/ml; 45 mM lysine equivalent) was incubated with glyoxylic acid (45 mM) and NaBH₃CN (150 mM) in 0.2 M phosphate, pH 7.4 for 24 h at 37 °C. Excess glyoxylic acid and NaBH₃CN was removed by dialysis against PBS.

FACS analysis. Localization of AGEs in HUVEC was determined by flow cytometry. To determine the concentration of extracellular AGEs, HUVECs grown under high and normal glucose conditions were harvested with trypsin, washed and 10⁶ cells were incubated with the anti-argyprimidine antibody for 30 min on ice. After incubation the cells were pelleted, resuspended in PBS containing a phycoerythrin conjugated anti-rabbit IgG and incubated for an additional 30 min. After washing, the fluorescence properties of these cells were analyzed on an LSR II flow cytometer (Becton-Dickinson). Cells stained with an isotype control antibody were used to detect background staining and the mean fluorescence intensity of these samples was subtracted from the mean fluorescence intensity of anti-AGE-stained cells. To determine the intracellular AGEs, the cells were first fixed by incubation with 2% paraformaldehyde for 30 min at room temperature. They were then washed with PBS and permeabilized with 0.3% saponin for 20 min at room temperature. After washing, the cells were stained and analyzed as above except 0.1% saponin was included during all incubations and washes.

Metabolic studies. For metabolic studies, cells were incubated with 1 mM methylglyoxal for 24 h with and without sorbinil (50 µM) or tolrestat (20 µM). After treatment, the cells were lysed in phosphate-buffered saline (PBS: 0.01 M) containing protease inhibitors followed by three cycles of freezing, thawing and sonication at 4°C. The samples were centrifuged at 12000×g for 20 min. The supernatant was analyzed for acetol formation by GC-MS as described above.

Generation of AR and FR-1 Transgenic Mice. The AR transgene was constructed by inserting the 0.95-kb rat AR cDNA next to the mouse α-MyHC promoter in the multiple cloning site of the Myo vector. The integrity of the final transgene construction (α-MyHC) was verified by restriction mapping and nucleotide sequence. Before the microinjection, the transgene construct was freed from all the bacterial sequences 5' to the digestion with Not-1, producing a 8.0-kb linear DNA fragment. The linearized α-MyHC-AR fragment was injected into the mouse eggs (C57BL/6). The injected eggs were implanted in foster mothers. DNA from tail biopsies of F₀ mice was genotyped by PCR. The primers used to detect AR were α-MyHC (5'-ccccataagagtttgagtgcac) and AR (5'-ctatcgacatgggtatcgcc). The PCR protocol was as follows: 94 °C for 2 min, 32 cycles of 94 °C for 0.5 min, 58 °C for 0.5 min, 72 °C for 0.75 min. Positive offspring, identified by PCR screening after pronuclear injection, were used to establish founders. Similar strategy was used to generate cardio-specific FR-1 transgenic mice. AR-null mice were obtained from Dr. Stephen Chung, University of Hong Kong.

Perfusion of isolated mouse hearts. Following anesthesia (sodium pentobarbital 60 mg/kg of body weight and heparin (10 U/g), the thorax of mice was opened and the hearts were removed

and immediately placed in ice-cold modified Krebs-Henseliet (KH) buffer. The composition of the KH buffer was (in mM): NaCl, 118; KCl, 4.7; MgCl₂, 1.25; CaCl₂, 3.0; KH₂PO₄, 1.25; EDTA, 0.5; NaHCO₃, 25; and glucose 10, pH 7.4. The buffer was heated to 37 °C continuously gassed (95% O₂ and 5% CO₂). Hearts were continuously perfused at a constant pressure of 80 mm Hg. Coronary flow rate was measured periodically during the entire perfusion period. After a stabilization period of 30 min, the perfusate was switched to KH buffer containing 20 μM methylglyoxal. Perfusion was continued for another 30 min and the perfusate was collected for analysis.

Induction of diabetes. Diabetes was induced in 6 weeks old male WT (n=7) and AR-null (n=7) mice by repeated low dose streptozotocin (STZ; 65mg/kg/day for 6 consecutive days, i.p) treatment as described by Park et al.(9). Control mice were treated with vehicle only (0.05 mM sodium citrate, pH 4.5). Blood glucose was measured one week after the last STZ injection in blood collected from the tail vein of non-fasted mice. All the STZ-injected mice had blood glucose > 400 mg/dl (*Supplemental table 2*). Mice were maintained on normal chow for 12 weeks and then euthanized. At euthanization blood was withdrawn by heart puncture in 10 mM EDTA and hearts were isolated. All animal protocols were approved by the Institutional Animal Care Committee at the University of Louisville.

Western blotting for the quantification of AGEs in the plasma and hearts of diabetic mice. For identification and measurement of AGEs, hearts from WT and AR-null mice were homogenized in PBS containing the protease inhibitor cocktail. Tissue homogenates were centrifuged at 12000×g for 20 min and supernatants (30-40 μg protein) were loaded on non-reducing gels. For the analysis of circulating AGEs, plasma (10-150 μg protein) from diabetic and non-diabetic mice was separated by SDS-PAGE and AGEs were identified by Western analysis using anti-argpyrimidine and anti-CML monoclonal antibodies, diluted 1:2000 in 0.5 % (v/v) blocking solution in TBS at room temperature.

Generation of AR-null/apoE-null mice. For the generation of AR-null/apoE-null mice, AR-null mice (on C57 BL/6 background) were bred with apoE-null mice. The resulting heterozygote for both genes were interbred with each other to generate homozygote AR-null/apoE-null mice. To identify apoE genotype, following set of primers were used: F: 5'-GCCTAGCCGAGGGAGAGCCG-3'; R(WT): 5'-TGTGACTTGGGAGCTCTGCAGC-3' and R(null): 5'-GCCGCCCGACTGCATCT-3'. For genotyping AR, following 3 primers were used (10): F(WT): 5'-GGGCTATACGGAGAACTGTGT-3'; F(null): 5'-ATCAGCAGCCTCTGTTCCAC-3' and R: 5'-TGACCTTCTCTAGAGGCTCTT-3'. Diabetes was induced in these mice by STZ injection as described above.

Atherosclerotic lesion analyses. For the morphometric analysis, innominate arteries were isolated under the dissecting microscope and fixed in formalin. Serial sections of 5 μm-thickness were cut throughout the innominate artery and every 10th section was stained with hematoxylin and eosin. Digital images were acquired and percent lesion area was calculated using the Metamorph 4.5 software.

Immunohistochemical analysis. Immunohistochemical analyses was performed on formalin-fixed sections of hearts and innominate arteries as described before (11;12). Briefly, sections

(5 μm) were de-paraffinized, rehydrated, and endogenous peroxidase activity was blocked with 3% H_2O_2 . Sections were incubated with anti-argpyrimidine (1:200 dilution) and anti-CML (1:1500 dilution) and anti-deoxyglucosone imidazolone (1:25 dilution) antibodies for 18 h at 4 $^\circ\text{C}$ and Biocare Medical MM-HRP-Polymer kit was used for detection. Digital images were acquired at $\times 40$ magnification from 3 sections from each mouse. Digital images were acquired using Spot advanced camera and analyzed using the Metamorph 4.5 software (Universal Imaging Corp) as described (11;12). For each stain, the threshold was predetermined, and held constant for all sections analyzed from each protocol. The samples were analyzed by one blinded observer.

Statistical analysis. Data are expressed as mean \pm SEM. Student's t test was used for the data analysis when the data were restricted to two groups. For multiple group analysis one way Anova with Holm Sidak corrections was used. Statistical significance was accepted at $P < 0.05$ level.

REFERENCES

1. Thornalley,PJ, Ladan,MJ, Ridgway,SJ, Kang,Y: Antitumor activity of S-(p-bromobenzyl)glutathione diesters in vitro: a structure-activity study. *J Med Chem* 39:3409-3411, 1996
2. Spite,M, Baba,SP, Ahmed,Y, Barski,OA, Nijhawan,K, Petrash,JM, Bhatnagar,A, Srivastava,S: Substrate specificity and catalytic efficiency of aldo-keto reductases with phospholipid aldehydes. *Biochem J* 405:95-105, 2007
3. Srivastava,S, Watowich,SJ, Petrash,JM, Srivastava,SK, Bhatnagar,A: Structural and kinetic determinants of aldehyde reduction by aldose reductase. *Biochemistry* 38:42-54, 1999
4. Di Ilio,C, Angelucci,S, Pennelli,A, Zezza,A, Tenaglia,R, Sacchetta,P: Glyoxalase activities in tumor and non-tumor human urogenital tissues. *Cancer Lett* 96:189-193, 1995
5. Nemet,I, Vikic-Topic,D, Varga-Defterdarovic,L: Spectroscopic studies of methylglyoxal in water and dimethylsulfoxide. *Bioorg Chem* 32:560-570, 2004
6. Shinohara,M, Thornalley,PJ, Giardino,I, Beisswenger,P, Thorpe,SR, Onorato,J, Brownlee,M: Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. *J Clin Invest* 101:1142-1147, 1998
7. Jensen,ON, Larsen,MR, Roepstorff,P: Mass spectrometric identification and microcharacterization of proteins from electrophoretic gels: strategies and applications. *Proteins Suppl* 2:74-89, 1998
8. Ikeda,K, Higashi,T, Sano,H, Jinnouchi,Y, Yoshida,M, Araki,T, Ueda,S, Horiuchi,S: N (epsilon)-(carboxymethyl)lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. *Biochemistry* 35:8075-8083, 1996
9. Park,L, Raman,KG, Lee,KJ, Lu,Y, Ferran,LJ, Jr., Chow,WS, Stern,D, Schmidt,AM: Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med* 4:1025-1031, 1998
10. Yang,JY, Tam,WY, Tam,S, Guo,H, Wu,X, Li,G, Chau,JF, Klein,JD, Chung,SK, Sands,JM, Chung,SS: Genetic restoration of aldose reductase to the collecting tubules restores maturation of the urine concentrating mechanism. *Am J Physiol Renal Physiol* 291:F186-F195, 2006

11. Srivastava,S, Ramana,KV, Tammali,R, Srivastava,SK, Bhatnagar,A: Contribution of aldose reductase to diabetic hyperproliferation of vascular smooth muscle cells. *Diabetes* 55:901-910, 2006
12. Srivastava,S, Chandrasekar,B, Gu,Y, Luo,J, Hamid,T, Hill,BG, Prabhu,SD: Downregulation of CuZn-superoxide dismutase contributes to beta-adrenergic receptor-mediated oxidative stress in the heart. *Cardiovasc Res* 74:445-455, 2007

Supplemental Table 1: Steady state kinetic parameters for the metabolism of AGE precursors by AR and glyoxalase I.

Substrates	AR		Glyoxalase I	
	K _m (μ M)	V _{max} (nmol/min/mg protein)	K _m (μ M)	V _{max} (nmol/min/mg protein)
Methylglyoxal	35	8.0	315	105
Glyoxal	333	6.0	19721	65

AR and glyoxalase I activities were determined as described under *Research Design and Methods*.

Supplemental Table 2: Parameters measured in non-diabetic and diabetic mice.

Group	Mice	Treatment	Starting body weight (gm)	Final body weight (gm)	Blood Glucose (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
I	C57BL/6	Vehicle	22.6 \pm 0.4	29.8 \pm 0.5	231 \pm 7	89 \pm 4	36 \pm 2
II	C57BL/6	STZ	20.8 \pm 0.2	21.2 \pm 0.5*	566 \pm 30*	84 \pm 5	38 \pm 5
III	AR-null	Vehicle	22.7 \pm 0.4	28 \pm 0.5	249 \pm 11	84 \pm 4	39 \pm 3
IV	AR-null	STZ	19.4 \pm 0.9	20.5 \pm 1*	744 \pm 55*	131 \pm 7*	36 \pm 8
V	apoE-null	Vehicle	19.8 \pm 0.4	29.0 \pm 0.7	189 \pm 5	457 \pm 19	124 \pm 10
VI	apoE-null	STZ	19.8 \pm 0.4	23.1 \pm 0.6*	552 \pm 22*	1141 \pm 85*	101 \pm 9
VII	AR/apoE-null	Vehicle	20.4 \pm 0.6	31.0 \pm 0.8	180 \pm 5	614 \pm 19	128 \pm 9
VIII	AR/apoE-null	STZ	19.4 \pm 0.7	19.5 \pm 0.7*	540 \pm 30*	1360 \pm 128*	109 \pm 10

Diabetes was induced by streptozotocin (STZ). Mice were euthanized after 12 weeks of diabetes. Values are mean \pm SEM. *P<0.01 Groups II vs I, IV vs III, VI vs V and VIII vs VII.