Online Supplement

Losartan Reverses Permissive Epigenetic Changes in Renal Glomeruli of Diabetic db/db Mice

Marpadga A. Reddy[1], Putta Sumanth[1], Linda Lanting[1], Hang Yuan[1], Mei Wang[1], Daniel Mar[2], Charles E. Alpers[2,] Karol Bomsztyk[2*], Rama Natarajan[1*]

[1]Department of Diabetes, Beckman Research Institute of the City of Hope,

1500 E. Duarte Road, Duarte, CA-91010,

[2]UW Medicine Lake Union, University of Washington, Seattle, WA 98109.

MATERIALS & METHODS

Materials: Antibodies against Histone H3 (ab1791), H3K4me1 (ab8895), H3K4me2 (ab32356), H3K27me3 (ab6002), H3K36me3 (ab9050), H4K20me3 (ab9053), H3K9me2 (ab39289), MCP-1 (ab7202) and PAI-1 (ab28207) were from Abcam (Cambridge, MA). Antibodies against H3K4me3 (17-614), histone H4 (17-10047), H3K9/14Ac (06-599) and H3K9me3 (07-442) were from Millipore (Billerica, MA). RNA polymerase II CTD 4H8 (sc-47701) and RAGE (sc-5563) antibodies was from Santa Cruz Biotech (Santa Cruz, CA). Qiazol, miRNeasy kits, miScript Reverse Transcriptase and SYBR Green PCR reagents, Epigenetic PCR arrays, RT² First Strand Kit and RT² qPCR Master Mixes were from Qiagen, Inc. (Valencia, CA). SYBR green Master Mix was from Applied Biosystems (Foster City, CA).

Animals and Treatments with Losartan: Animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. 10-12 weeks old male type 2 diabetic *db/db* mice (BKS.Cg-m$^{+/+}$lepr^{db}/J) and age matched control littermates *db/+* mice were obtained from Jackson laboratories (Bar Harbor, Maine). Diabetes was confirmed by measuring blood glucose levels (>350 mg/dL in *db/db* vs about 150 mg/dL in db/+ mice) using the Alphatrak glucometer (Abbott Laboratories, Abbott Park, IL). Diabetic db/db mice were then randomly assigned to two groups (28 animals in each group): mice treated without Losartan in drinking water (db/dbH2O) and with Losartan (10 mg/kg/day) in drinking water (db/dbLOS) for 10 weeks. As non-diabetic control, 28 *db/+* mice were treated without Losartan (db/+H2O). Blood pressure was measured at 4 weeks and at the end of the study (10

weeks) using the non-invasive CODA 2 tail cuff acquisition system (Kent Scientific, Torrington, CT). At the end of the study, kidneys from 3-4 mice were pooled and glomeruli were prepared by sieving technique as described before[1]. Glomeruli preparations were used to extract RNA and protein and to prepare samples for chromatin immunoprecipitation (ChIP) assays as described below.

Experiments with mesangial cells: Rat mesangial cells (RMC) were prepared from kidneys of Sprague-Dawley rats and cultured in RPMI medium containing 5.5 mM glucose as described before (3). RMC were grown to 80% confluency, serum depleted for 24 h and treated with normal (5.5 mM) glucose (NG) or high (25 mM) glucose (HG) for 48h. RMC were then pretreated without or with Losartan (1.0 µM) for one hour followed by incubation in NG, HG or HG + 0.1 µM Angiotensin II (HG/A) for an additional 24 h. Treated cells were used to prepare total RNA (for gene expression analysis by RT-qPCR) or fixed with 1% formlaldehyde for Chromatin immunoprecipitation (ChIP) assays with indicated antibodies as described earlier[3]. ChIP-QPCRs were performed as described earlier[3] using promoter specific primers (Table 2).

Urine and serum analysis: Urine and serum analyses were performed as described before[2]. Urine samples (24 h) were collected at the end of the study by placing mice in metabolic cages. Total urine protein (mg) was determined using D_c protein assay kit (Bio-Rad Hercules, CA), and Albumin and creatinine using Albuwell M and Creatinine Companion ELISA (Exocell Inc, Philadelphia, PA) kits. Serum/plasma creatinine was estimated by Olympus AU680 analyzer (RADIL, Columbia, MO).

Histology: Kidneys were fixed in 10% buffered formalin to prepare paraffin embedded sections, and glomerular hypertrophy and ECM deposition were evaluated using Periodic Acid Schiff (PAS) staining and indicated antibodies as described previously[1, 2]. Slides were visualized under Olympus BX51 microscope (40X objective) and images were collected using Pixera 600 digital camera and InStudio software (Pixera Corp). Glomerular surface area and mesangial expansion were determined using Image-Pro Plus 5.1 (Media Cybernetics, Rockville, MD). Images for PAI-1, RAGE and MCP-1 staining were collected using Olympus DP-72 camera (40X objective) and color intensity was quantified using smart segmentation in Image-pro-premier software (Media Cybernetics, Rockville, MD). Results were expressed as fold over db/+H2O or % of db/+H2O.

Total RNA extraction and Quantitative Reverse transcriptase-Polymerase Chain Reaction (QRT-PCR): Glomeruli were lysed in Qiazol and total RNA was prepared using miRNAeasy columns using on column DNAse I digestion according to manufacturer specifications (Qiagen, Valencia, CA). The cDNA was prepared with 0.5- 1.0 µg of RNA using miScript Reverse Transcriptase Kit in a 20 µl reaction volume at 37 ^{0}C for 60 min, the reaction terminated at 95 ^{0}C for 5 min and cDNAs were diluted (5 fold). Gene expression was determined by Realtime quantitative PCR (QPCR) using miScript SYBR Green PCR Kits (Qiagen, Valencia, CA) or SYBR green master mix (Applied Biosystems) using gene specific primers (Table 2) in a final volume of 20 µl on 7500 Realtime PCR systems (Applied Biosytems). QPCR data was analyzed by the $2^{-\Delta\Delta Ct}$ method[3] and results normalized with internal control genes were expressed as fold over db/+H2O or % of db/+H2O mice.

Epigenetic PCR arrays: Mouse Epigenetic Chromatin Modification Enzymes PCR Arrays (PAMM-085a) from Qiagen (Valencia, CA) contain primers for 84 genes involved in epigenetic mechanisms including DNA methylation and histone PTMs, and 6 housekeeping genes for normalization, in a 96 well format suitable for Realtime PCR analysis. RNA (1 µg) from glomeruli was used to prepare cDNAs using RT² First Strand Kit, and realtime PCR in array plates was performed using SYBR green based RT² qPCR Master Mixes on 7500 Realtime PCR systems. We performed QPCR array analysis with two glomerular preparations from each group. Each glomerular preparation consists of pooled glomeruli from 3 mice, thus representing data from 6 mice per group. Data analysis was performed by $2^{-\Delta\Delta Ct}$ method using manufacturer provided templates in Microsoft Excel software and results were expressed as fold over db/+H2O samples. Results obtained represent Mean±SEM of two QPCR arrays per group.

Matrix ChIP procedure [4-6].

Chromatin preparation.

Glomeruli samples were cross-linked with formaldehyde final concentration 1.42% for 15 minutes at room temperature and then formaldehyde was quenched with 125 mM glycine for 5 minutes at room temperature as previously described [6]. Cross-linked samples were lysed using IP buffer [150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 50 mM Tris–HCl (pH 7.5)] containing 10 µg/µl leupeptin, 0.5 mM PMSF, 30 mM p-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, and 10 mM β-glycerophosphate [5, 7]. Chromatin was sheared using Diagenode Bioruptor (175 µl IP buffer, 30 rounds 30 seconds ON/30 seconds OFF, high power, 4 ^oC). The suspension was cleared by centrifugation at 12,000 g (10 minutes at 4 ^oC), and stored at -80 ^oC.

Microplate-based chromatin immunoprecipitation.

Step1. Protein A-coated microplate preparation. UV-irradiated 96-well plates were coated with protein A by incubating overnight at room temperature with 20-100 μl of 2 μg/μl protein A in PBS per well.

Step2. Blocking well walls. Prior to use, plates were washed twice with 200 μl of PBS and well walls were blocked with 200 μl of blocking buffer for 60 minutes at room temperature. The wells were blocked with with IP buffer/5% BSA containing 100 μg/ml sheared salmon sperm DNA (ChIP blocking buffer). In all steps, vacuum aspirator wand was used to clear wells of buffers.

Step3. Attachment of ChIP antibodies to well walls. ChIP wells were cleared of blocking buffer and then ChIP antibodies diluted in ChIP blocking buffer were incubated in wells for 60 minutes at room temperature. Wells were cleared and then appropriate amount of sheared chromatin diluted in ChIP blocking buffer was added to wells.

Step4. Capture of chromatin to well walls. Microplate(s) were covered with a sealing film and capture of chromatin complexes to well walls was done by floating the microplates in ultrasonic water bath (Branson 3510) for 60 minutes at 4 ^{o}C.

Step5. Well washes. Wells were washed three times with ice-cold IP buffer followed by three washes with ice-cold TE buffer (pH 7.0).

Step6. Elution of PCR-ready DNA. After washes, appropriate amount of elution buffer (25 mM Tris, pH 10, 1 mM EDTA, and 0.2 μg/μl proteinase K containing 1% IP buffer) was added to each well. Input DNA was prepared by adding the same amount of chromatin to wells containing elution buffer. The plates were then sealed with PCR sealing film. PCR-ready DNA was eluted/purified in a 96-well PCR cycler applying one cycle of heating (55 ^{o}C for 15 minutes)

followed by one cycle to reverse cross-linking and to inactivate proteinase K (95 ^{o}C for 15 minutes). All PCR reactions were run in quadruplicates with primers designed using the Primer3 software (Table 2).

Statistical Analysis: Statistical analysis was performed by t-tests when comparing two groups and by ANOVA with multiple comparison tests to compare more than two groups using Prism software (Graphpad, San Diego, CA). $p<0.05$ was considered statistically significant. For Matrix ChIP assays, data were acquired, analyzed and graphed using in-house generated PCRCrunch and GraphGrid software. The software includes a tool that automatically adjusts the computed pairwise p-value for the significance t-test between each pair of group mean values and the results are indicated in the grid below the graphs [8]. Each vertical line and its attached horizontal component is associated with the bar above it. The Bonferroni corrected p-value of the t-test between any two groups is indicated by the size of the solid circle at the intersection of their respective lines (Fig S1). Data represents Mean±SEM.

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Fig.S1. Matrix ChIP data presentation[8]. A. GraphGrid analysis. Solid yellow circles positioned at line intersections designate comparison between a given pair of means. The blue bar above the circle is one of the means. The second mean is found by tracing left along horizontal line from the circle all the way to farthest left angle corner where the vertical line designates the other blue bar for the paired comparison. The graph illustrates bar (means + SEM). Three different paired statistical comparisons are done as shown with the numbered circles. B. Statistical analysis is done using Bonferroni correction. Statistical differences between two means (p value) are shown by the size of the solid circle. : $p<0.05$ by small circle, $p<0.01$ by large circle, and no circle indicating the differences are not statistically significant.

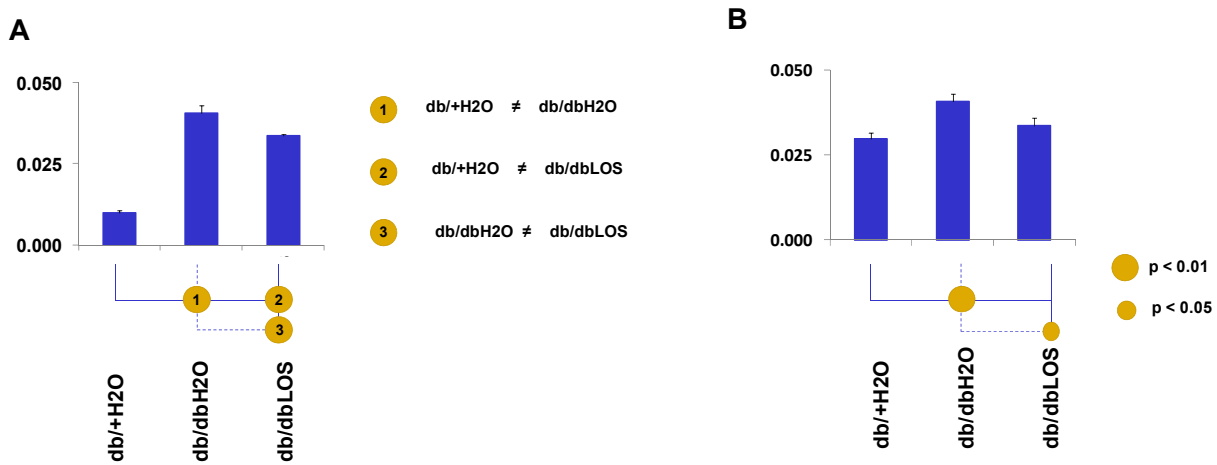

Fig. S2. Expression of epigenetic enzymes using QPCR Arrays. Expression of indicated genes was analyzed in QPCR arrays and results were shown as fold over db/+H2O. QPCR array analysis was performed with two glomerular preparations from each group. Each glomerular preparation consists of pooled glomeruli from 3 mice, thus representing data from 6 mice per group. Results shown represent Means of data from two QPCR arrays per group.

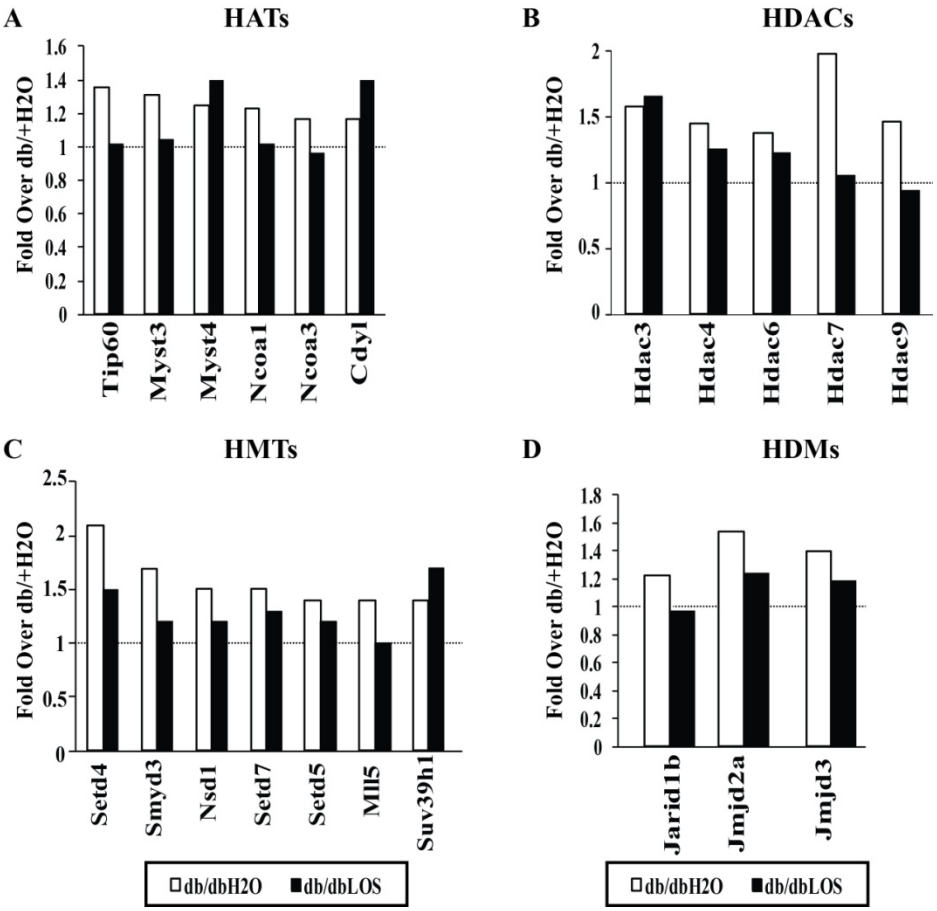

Table S1. QPCR Array data of more epigenetic enzymes. QPCR Array data of genes not shown in Fig. S2. QPCR array analysis of indicated genes was performed with two glomerular preparations from each group and results were expressed as fold over db/+H2O. Results shown represent Means of two QPCR arrays per group.

	db/dbH2O	db/dbLOS
Histone Acetyl Transferases		
Ciita	0.92	0.66
Myst1	1.18	0.91
Myst2	1.2	1.23
Histone deacetylases		
Hdac5	1.22	0.9
Hdac6	1.38	1.23
Hdac10	1.35	1.14
Histone Methyl Transferases		
Ash1l	1.3	1.3
Smyd1	1.3	1.4
Dot1l	1.3	0.9
Setd3	1.2	1.4
Mll3	1.2	1.1
Ehmt1	1.2	1
Setd1a	1.2	1.3
Setd6	1.2	1.1
Setdb1	1.2	1
Setd1b	1.2	1
Suv420h1	1.2	1
Arginine Methyl Transferases		
Prmt1	1.21	1.18
Prmt3	1.31	1.21
Carm1	1.25	0.96
Prmt6	1.12	1.38
Prmt7	1.28	1.25
Kinases		
Aurka	1.29	1.2
Aurkb	1.4	0.99
Aurkc	1.07	0.52
Nek6	1.8	2.07
DNA Methyl Transferases		
Dnmt1	1.61	1.56
Dnmt3a	1.31	0.77
Dnmt3b	1.95	1.26

Fig. S3. Expression of Histone Methyl Transferases and Histone Demethylases using RT-QPCR . Expression of indicated genes was analyzed by RT-QPCR and results were expressed as fold over db/+H2O. RT-QPCRs were performed as described in Methods section. Data shown represents Mean±SEM of 5 samples per group. Several of the genes tested showed a trend towards increased expression in db/dbH2O mice vs db/+H2O, which were inhibited in db/dbLOS mice. However, these changes between the three groups were not statistically significant for the indicated genes.

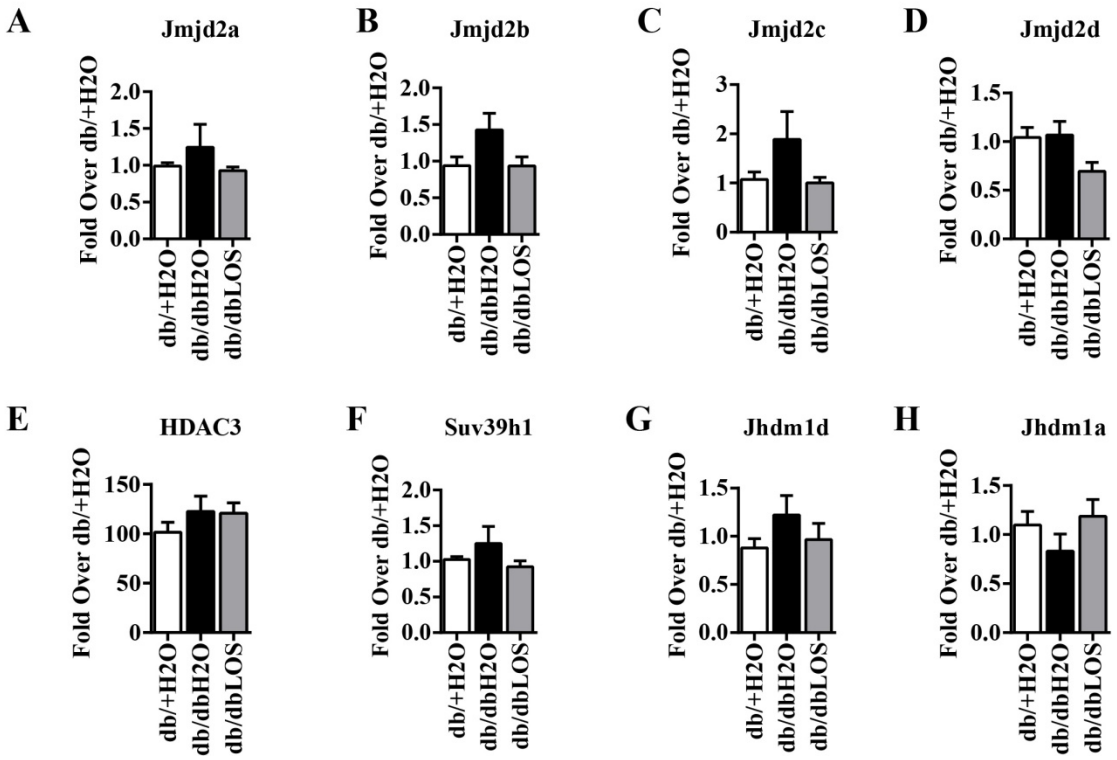

Table S2. Effect of Losartan on gene expression in rat mesangial cells cultured under diabetic conditions.

Rat mesangial cells were treated with NG (5.5 mM), HG (25 mM) for 48 h and pretreated with 1.0 μM Losartan (LOS) for one h followed by treatment with NG, HG or HG + 0.1 μM Angiotensin II (HG/A) for an additional 24 h. Total RNA was used to analyze expression of indicated genes was by RT-QPCR, normalized with internal control Cyclophilin A and results were expressed as % of NG. Data represents Mean±SEM (n=2).

	HG	HG+LOS	HG/A	HG/A+LOS
RAGE	364±172	180±72	575±17	120±32
PAI-1	200±84	93±35	347±65	101±31
MCP-1	151±62	81±5	280±57	74±24