ONLINE SUPPLEMENT

ROLE OF THE LYSINE SPECIFIC DEMETHYLASE 1 IN THE PRO-INFLAMMATORY PHENOTYPE OF VASCULAR SMOOTH MUSCLE CELLS OF DIABETIC MICE

Marpadga A. Reddy [1], Louisa M. Villeneuve[1, 2], Mei Wang[1], Linda Lanting[1], and Rama Natarajan[1, 2*]

From [1]Department of Diabetes, [2]Graduate School of Biological Sciences, Beckman Research Institute of City of Hope, Duarte, CA 91010

MATERIALS AND METHODS

Materials: Antibodies specific for H3K4me2 H3K4me3 and LSD1 were purchased from from Abcam, Inc (Cambridge, MA). RNA Pol II and p65 (NF-kB) antibodies were from Santa Cruz Biotech (Santa Cruz, CA). LSD1 expression vector was purchased from OriGene Technologies, Inc (Rockville, MD), RNAesay kits from Qiagen (Valencia, CA), Gene Amp RNA PCR kits and SYBR green Realtime PCR kits from Applied Biosystems, Inc (Foster City, CA). Complete protease inhibitors were from Roche Applied Science (Indianapolis, IN). Nucleofection kit was from Amaxa, Inc (Gaithersberg, MD), SmBM medium was from Cascade Biologics (Portland, OR), DMEM-F/12 was from Invitrogen, Inc (San Diego, CA), and Chemiluminescence kit was from Pierce, Inc (Rockford, IL). Realtime PCR primers for β-Actin gene, LSD1 and control siRNA oligonuceotides were from Ambion, Inc (Austin, TX). Fluorescent label 2',7'-Bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) was obtained from A. G. Scientific, Inc. (San Diego, CA). Protein A and Protein A G agarose beads were from Upstate, Inc (Billerica, MA), Luciferase assay kits were from Promega, Inc. (Madison, WI). All other reagents and biochemical supplies were from Sigma-Aldrich, Inc (St. Louis, MO) unless indicated otherwise.

Cell Culture: All animal studies were performed according to an institutionally approved protocol. Mouse VSMCs (MVSMC) were isolated from 9-10 weeks old *db/db* diabetic mice (BKS.Cg-m+/+leprdb/J, stock no. 000642; The Jackson Laboratory, Bar Harbor, ME) and control heterozygote non diabetic *db/+* mice as described earlier (1). Cells were cultured in DMEM/F-12 medium supplemented with fetal bovine serum (10%), Streptomycin (100 ug/ml), Penicillin (100 U/ml) and Plasmocin (50 ug/ml) and used between passages 5 and 8 in order to obtain sufficient numbers of cells and also maintain the diabetic phenotype. Human vascular

smooth muscle cells (HVSMC) were purchased from Cascade Biologics (Portland, OR). HVSMC were cultured in SmBM medium according to manufacturer's instructions and used between passages 4-8. When stimulated with TNF-α, MVSMC were first serum depleted for 48 hours in DMEM/F12 supplemented with 0.2% BSA, while HVSMC were serum depleted for 24 hours in SmBM medium supplemented with 0.5% FBS.

RNA isolation and RT-QPCR: Total RNA was extracted using RNAeasy columns and 1.0 ug of total RNA used to synthesize cDNA with Gene Amp RNA PCR kit with random hexamers in a final volume of 20 ul as described by the manufacturer. Gene expression was then analyzed by Quantitative Realtime PCR (QPCR) using SYBR green reagent with gene specific primers (listed in Table 1) and β-actin gene primers (internal control) on GeneAmp 7300 (Applied biosystems, Foster City, CA) in a 20 ul reaction volume. In each reaction, dissociation Standard graphs were generated with Applied Biosystems software using serial dilutions of cDNA to determine the amplification efficiency (E = 10^(-1/slope). Relative levels of gene expression were calculated in Microsoft Excel, after normalization with internal control β-actin gene using formula E_{gene}^($Ct_{control}$-Ct_{sample})/E_{actin}^($Ct_{control}$-Ct_{sample}), where E is the amplification efficiency of test gene (Egene) or internal control β-actin gene and Ct is the threshold value (2). Results were expressed as fold over control or % of control as indicated.

Western blotting: Preparation of cell lysates and immunoblotting with indicated antibodies were performed as previously described (20). Blots were developed using Chemiluminescence method and scanned with GS-800 densitometer to determine the intensity of protein bands using Quantity One software (Bio-Rad, Inc, Hercules, CA).

Transfection of VSMC: Both HVSMC and MVSMC were transfected with indicated siRNA oligonucleotides or plasmids using Nucleofection equipment (Amaxa, Inc., Gaithersberg MD) as

described earlier (3). Transfected cells were allowed to recover for 48 hours and serum depleted for 24 hours prior to stimulation for gene expression or monocyte-VSMC binding.

Chromatin Immunoprecipitation (ChIP) assays: ChIP assays were performed and ChIP enriched DNA analyzed by Realtime QPCR as described earlier (4). Briefly, cells were fixed with 1% formaldehyde at 37^0C for 10 min, washed with phosphate buffered saline containing protease inhibitors and lysed in Tris, pH 8.1 containing 1% SDS, 1 mM PMSF and complete protease inhibitor cocktail. Cell lysates were sonicated to fragment chromatin to 500 bp size, diluted in ChIP dilution buffer to reduce SDS concentration to 0.1% and immunoprecipitated overnight at 4^0C with indicated antibodies. Next day, immune complexes were collected on Protein A-Agarose beads (except for p65 Ab, which were collected with Protein G-Agarose beads) and beads were washed to remove non-specific binding. DNA was eluted from the beads, crosslinks reversed, and DNA was extracted. ChIP enriched DNA samples and input DNA samples were analyzed by QPCR with SYBR reagent in 7300 Realtime PCR machine (Applied Biosystems) using primers specific for IL-6 or MCP-1 promoters or MCP-1 enhancer near NF-κB sites (Prime locations are shown in Fig. S1 and sequences are listed in Table 1). QPCR data was analyzed using the $2^{\wedge-\Delta\Delta Ct}$ method as described earlier and normalized with input samples (4). Results were expressed as fold over control or % of control.

Monocyte-VSMC binding: Binding of THP-1 monocytes to HVSMC was performed as described earlier (5, 6) with some modifications. HVSMC were plated in 24 wells, serum depleted and either left alone or stimulated with TNF-α (10 ng/ml) for 6 hours. Monolayers were washed with serum depletion medium to remove TNF-α and incubated with THP-1 cells that were pre-labeled with fluorescent BCECF/AM (50,000 cells/well) for one hour at 37 ^{0}C in the tissue culture incubator. Then the monolayers were gently washed four times with serum depletion

medium to remove unbound monocytes. Bound monocytes were lysed with 100 mM Tris, pH 8.0 containing 1% Triton X-100. Fluorescence in each well was determined using a fluorescent plate reader and results expressed as fluorescence units.

Fig. S1. Maps of IL-6 and MCP-1 promoters: Arrows indicate location of ChIP primers used in the study. Map not drawn to scale.

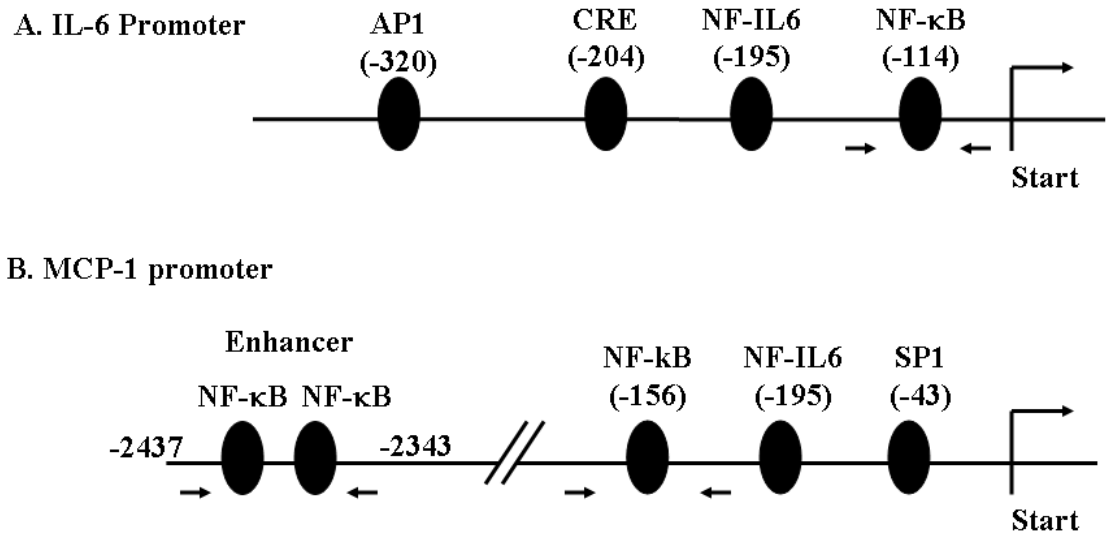

Fig. S2. No significant differences in H3K4me2 and H3K4me3 levels in db/db and db/+ MVSMC: Cell lysates from db/+ and db/db MVSMC were immunoblotted with indicated antibodies.

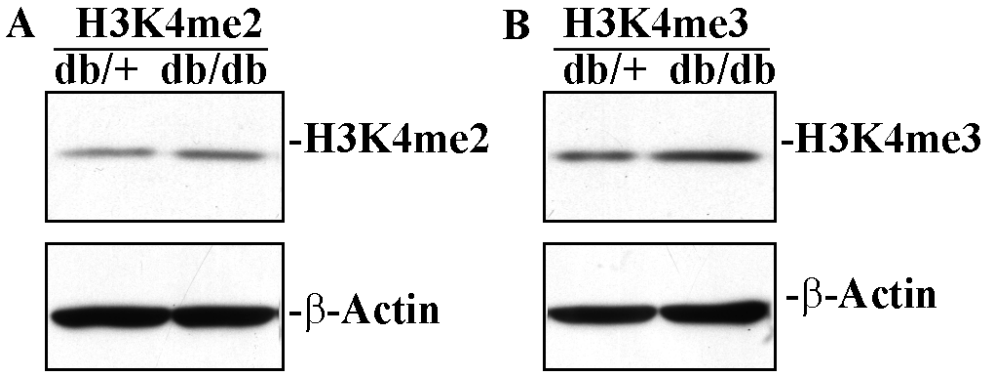

Fig. S3. Recruitment of p65 (NF-κB) at the IL-6 and MCP-1 promoters in MVSMC from db/+ and db/db mice: Cell lysates from MVSMC were subjected to ChIP assay using p65 antibody. ChIP DNA samples were analyzed by QPCR using primers derived from IL-6 and MCP-1 promoters and MCP-1 enhancer spanning NF-κB binding sites.

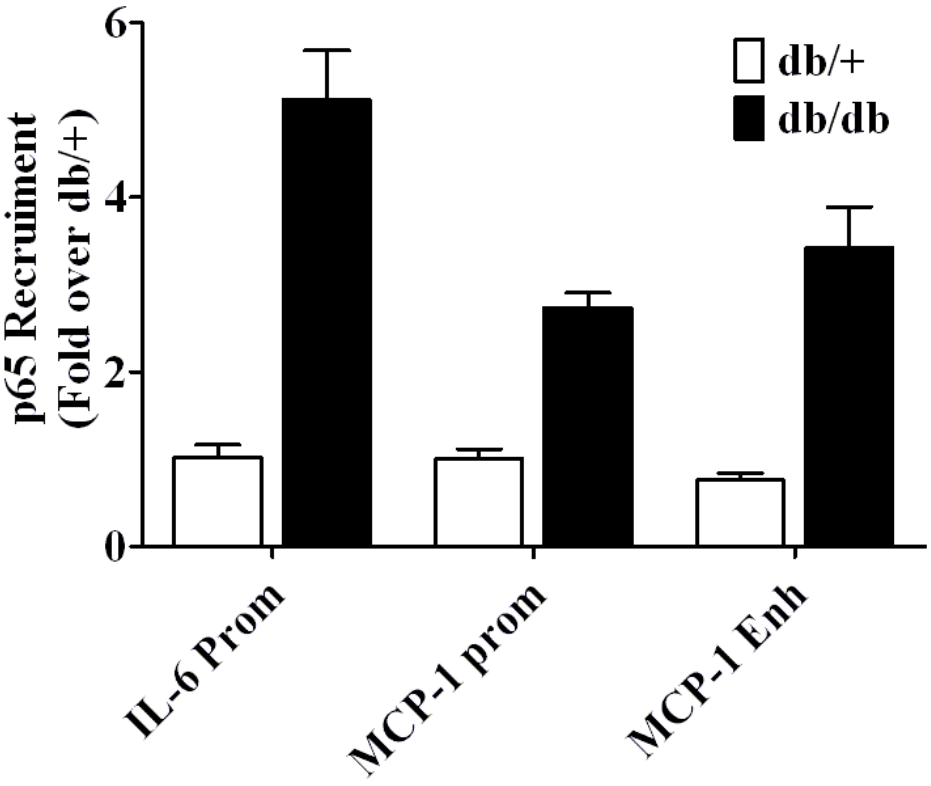

Table 1. Primer sequences:

Primer*	Forward primer	Reverse primer	Annealing Temp.
cDNA Primers			
mIL-6	ACAAAGCCAGAGTCCTTCAGAG	ACCACAGTGAGGAATGTCCAC	56
mMCP-1	AGGTCCCTGTCATGCTTCTGG	CAGCACTTCTTTGGGACACCTGCTG	61
mCypA**	ATGGTCAACCCCACCGTGT	TTCTTGCTGTCTTTGGAACTTTGTC	58
hIL-6	TCCTGCAGAAAAAGGCAAAG	GCCCAGTGGACAGGTTTCT	60
hMCP-1	GTGAGGAACAAGCCAGAGCTG	TGCGCAGAATGAGATGAGTTG	58
ChIP Primers			
mIL-6pro	CGTTTATGATTCTTTCGATGCTAAACG	GTGGGCTCCAGAGCAGAATGAG	60
mMCP-1pro	ACCAAATTCCAACCCACAGTTTC	TGCTCTGAGGCAGCCTTTTATT	58
mMCP-1enh	ATTTCCACGCTCTTATCCTACTCTG	TCACCATTGCAAAGTGAATTGG	58
mCypApro2	CGCGGCAATGGGAAGATACGCG	AGAGTCCGAGGGCAGTTAGGG	58
mCypApro3	GCAGGTAGGTCCTTGAGCTTGTC	CGCTAGAAGACCCTTCACCATAGcG	58
hIL-6pro	CTTCGTGCATGACTTCAGCTTT	CGTCCTTTAGCATCGCAAGAC	58

*m = mouse; h = human.

**Solinas G *et al.* (2007) JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. *Cell Metabolism* 6:386-397.

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