Role of the Lysine-Specific Demethylase 1 in the Proinflammatory Phenotype of Vascular Smooth Muscle Cells of Diabetic Mice

Marpadga A. Reddy, Louisa M. Villeneuve, Mei Wang, Linda Lanting, Rama Natarajan

Abstract—Insulin resistance and type 2 diabetes are major risk factors for vascular complications. Vascular smooth muscle cells (VSMCs) derived from db/db mice, an established mouse model of type 2 diabetes, displayed enhanced inflammatory gene expression and proatherogenic responses. We examined the hypothesis that aberrant epigenetic chromatin events may be the underlying mechanism for this persistent dysfunctional behavior and “memory” of the diabetic cells. Chromatin immunoprecipitation assays showed that levels of histone H3 lysine 4 dimethylation (H3K4me2), a key chromatin mark associated with active gene expression, were significantly elevated at the promoters of the inflammatory genes monocyte chemoattractant protein-1 and interleukin-6 in db/db VSMCs relative to db/+ cells. Tumor necrosis factor-α–induced inflammatory gene expression, H3K4me2 levels, and recruitment of RNA polymerase II at the gene promoters were also enhanced in db/db VSMCs, demonstrating the formation of open chromatin poised for transcriptional activation in diabetes. On the other hand, protein levels of lysine-specific demethylase1 (LSD1), which negatively regulates H3K4 methylation and its occupancy at these gene promoters, were significantly reduced in db/db VSMCs. High glucose (25 mmol/L) treatment of human VSMCs also increased inflammatory genes with parallel increases in promoter H3K4me2 levels and reduced LSD1 recruitment. LSD1 gene silencing with small interfering RNAs significantly increased inflammatory gene expression and enhanced VSMC–monocyte binding in nondiabetic VSMCs. In contrast, overexpression of LSD1 in diabetic db/db VSMCs inhibited their enhanced inflammatory gene expression. These results demonstrate novel functional roles for LSD1 and H3K4 methylation in VSMCs and inflammation. Dysregulation of their actions may be a major mechanism for vascular inflammation and metabolic memory associated with diabetic complications. (Circ Res. 2008;103:615-623.)

Key Words: atherosclerosis ■ diabetes ■ histone modifications ■ inflammation vascular smooth muscle cells

Diabetes, insulin resistance, and the metabolic syndrome are associated with significantly increased rates of vascular complications such as atherosclerosis.1–2 Vascular inflammation, dyslipidemia, and enhanced oxidant stress have been shown to be among the major risk factors.3–5 Enhanced expression of proinflammatory cytokines and chemokines is associated with vascular inflammation in type 2 diabetes (T2D), insulin resistance, and atherosclerosis.3,6 Several mechanisms, including hyperglycemia (HG), oxidant stress, increased flux through the polyol and hexosamine pathways, formation of advanced glycation end products, and protein kinase C (PKC) activation2,7,8 have been proposed to be involved in these events. Enhanced inflammatory gene expression and proatherogenic responses in vascular smooth muscle cells (VSMCs) play important roles in the pathogenesis of atherosclerosis and vascular dysfunction.9,10 Diabetogenic agents such as HG and AGEs further enhance these events.5,7 VSMCs cultured under HG (25 mmol/L) conditions to mimic a diabetic state exhibit significantly increased rates of basal and growth factor induced proatherogenic responses including hypertrophy, proliferation, and migration relative to those cultured in normal glucose (NG) (5.5 mmol/L).11–13 Advanced glycation end products (AGEs) and other RAGE (receptor for AGEs) ligands also induce inflammatory genes and migration in VSMCs.14,15

We recently demonstrated that VSMCs derived from db/db mice, a well-established model of insulin resistance, obesity, and type 2 diabetes (T2D), exhibit increased inflammatory gene expression and proatherogenic responses, such as migration and monocyte binding even after culturing for 5 to 8 passages.15,16 This “preadapted state” of VSMCs from diabetic mice may be attributable to “metabolic memory” induced by the prior exposure to sustained hyperglycemia in diabetic animals. Macrophages and endothelial cells isolated from db/db mice also exhibited this preactivated state in short term cultures,17,18 further supporting the role of metabolic...
memory in persistent vascular inflammation. Identifying the molecular mechanisms involved in these events is important to prevent or treat accelerated vascular complications in diabetes.

Recently, epigenetic mechanisms involving covalent modifications of nucleosomal histones have emerged as another level of gene regulation.23 Epigenetic histone marks are posttranslational modifications of exposed amino-terminal tails of nucleosomal histones H2, H3, and H4 in chromatin. These include acetylation, phosphorylation, sumoylation, methylation, and ubiquitination.22,23 Such histone modifications in chromatin act sequentially or in concert with others to form a “histone code” that is read by chromo- or bromodomain-containing regulatory proteins to initiate downstream biological responses, including transcriptional activation or repression.22-24 Methylation of histone H3 at lysine 4 (H3K4) is usually associated with active gene expression. H3K4 can be monomethylated (me1), dimethylated (me2), or trimethylated (me3) on ε-amino side chains of Lys residues by specific histone H3K4 methyltransferases such as SET7/9 or MLL.21 The recent discovery of lysine-specific demethylase 1 (LSD1), which specifically removes histone H3K4 monomethylation (H3K4me1) and -me2 demonstrated the dynamic nature of H3K methylation.24 LSD1, also known as p110b, BHC110, or NPAO, belongs to a family of amine oxidases and catalyzes lysine demethylation in a flavin adenine dinucleotide–dependent manner.24,25 It has 3 domains, an amino-terminal SWIRM domain, a central Tower domain, and a carboxyl-terminal amine oxidase–like domain. The SWIRM domain enables LSD1 association with chromatin, whereas the Tower domain interacts with other nuclear factors such as Co-REST, and the amine oxidase–like domain contains subdomains for the binding of substrate and cofactor FAD.25-26 LSD1 is associated with several other factors in the nucleus which regulate its activity including Co-REST, histone deacetylase (HDAC)1, HDAC2, and BHC80.24 Interaction with Co-REST is essential for the demethylase activity of LSD1 on nucleosomal histones. Co-REST knockdown by small interfering (si)RNAs reduces LSD1 protein levels, suggesting that it may also play a role in LSD1 protein stability.27,28 LSD1 expression is regulated in human diseases such as prostrate cancer.25 However, nothing is known about its role in the vessel wall in diabetes or in VSMC inflammatory gene expression.

Because H3K4 dimethylation (H3K4me2) is an epigenetic mark of gene activation and its removal by LSD1 can lead to gene repression, we examined the hypothesis that aberrant H3K4 methylation through dysregulation of LSD1 functions by the diabetic milieu may be a key mechanism involved in sustained inflammatory gene expression associated with the preactivated state of diabetic db/db VSMCs. Our novel results show that LSD1 levels are decreased in db/db VSMCs, and this is associated with reduced LSD1 occupancy and increased H3K4me2 levels at the promoters of inflammatory genes, along with parallel enhanced responses to inflammatory stimuli. Furthermore, results obtained with gene silencing or overexpression of LSD1 could demonstrate its functional role in VSMC inflammatory gene expression and monocyte–VSMC binding, suggesting that LSD1 and H3K4 methylation play key roles in vascular inflammation.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Mouse VSMCs (MVSMCs) were isolated from thoracic aortas of 9- to 11-week-old male 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. Blood glucose levels of db/db mice were >450 mg/dl versus <140 mg/dl in db/+ mice. Human VSMCs (HVSMS) were from Cascade Biologics (Portland, Ore). Gene expression was determined by real-time reverse transcription–quantitative PCR (RT-QPCR) and normalized to internal control β-actin. Chromatin immunoprecipitation (ChIP) assays with indicated antibodies were performed with lysates from formaldehyde fixed cells. ChIP-enriched DNA samples were analyzed by QPCR using primers that amplify indicated gene promoter regions (supplemental Figure 1 and Table 1 in the online data supplement). Data were analyzed by the 2-ΔΔCt method and normalized with input samples. VSMCs were transfected using Nucleofection kits from Amaxa (Gaithersburg, Md). Monocyte–VSMC binding assays were performed by incubating fluorescently labeled monocytes with VSMCs for 60 minutes and washed, and the fluorescence of bound monocytes was measured. All results are expressed as the means±SEM. Statistical significance (set at 0.05) was tested by ANOVA or Student’s tests using GraphPad Prism software.

Results

Histone H3K4me2 Levels Are Increased at Monocyte Chemoattractant Protein-1 and Interleukin-6 Promoters in VSMCs From Diabetic Mice

Our recent studies demonstrated that MVSMCs derived from diabetic db/db mice displayed enhanced monocyte chemoattractant protein (MCP-1) and interleukin (IL)-6 gene expression and proatherogenic responses relative to those derived from db/+ mice, even after culture in vitro for several weeks.16 Because chromatin histone H3K4me2 is associated with actively transcribed genes, we examined the hypothesis that increased MCP-1 and IL-6 gene expression may be attributable to elevated levels of H3K4me2 on these gene promoters in db/db MVSMCs. We first compared the mRNA levels of MCP-1 and IL-6 genes in db/+ and db/db MVSMCs by RT-QPCR. Results showed that MCP-1 and IL-6 mRNA levels were significantly increased in db/db MVSMCs relative to db/+ mice (Figure 1A), further confirming our previous findings.16 Then, cell lysates from formaldehyde fixed cells were subjected to ChIP assays using histone H3K4me2 antibodies. ChIP-enriched DNA samples were analyzed by QPCR using primers spanning nuclear factor (NF)-κB sites (supplemental Figure 1 and Table 1) of IL-6 promoter (−60) and MCP-1 promoter (−487), as well as MCP-1 enhancer (−2474 and −2342). H3K4me2 levels were significantly increased at the promoters of IL-6 (Figure 1B) and MCP-1 (Figure 1C), as well as MCP-1 enhancer (Figure 1D) in MVSMCs from db/db mice relative to db/+.

In contrast, expression of the housekeeping gene cyclophilin A (CypA) (Figure 1A) and its promoter H3K4me2 levels (Figure 1E) showed no significant changes. Immunoblotting of cell lysates did not show any changes in global H3K4me2 or H3K4me3 levels in db/db MVSMCs (supplemental Figure
Tumor Necrosis Factor-α–Induced Gene Expression and H3K4me2 Levels Are Enhanced in Diabetic MVSMCs

We next examined whether the formation of open chromatin leads to enhanced responses to proinflammatory stimuli in diabetic db/db MVSMCs. MVSMCs were stimulated with tumor necrosis factor (TNF-α) (10 ng/mL), a proinflammatory cytokine increased in diabetic conditions. RT-QPCR results showed that TNF-α–induced gene expression of MCP-1 and IL-6 was significantly enhanced in db/db MVSMCs relative to db/+ cells (Figure 2A). Furthermore, ChIP assays showed that TNF-α–induced H3K4me2 was significantly increased on the IL-6 promoter (Figure 2B), MCP-1 promoter (Figure 2C), and MCP-1 enhancer (Figure 2D) in db/db MVSMCs compared to db/+.

LSD1 Protein Levels Are Reduced in db/db MVSMCs

Next, we examined whether the increased promoter H3K4me2 in db/db MVSMCs could be attributable to decreased levels of LSD1, an H3K4me2 demethylase. Immuno-
noblotting of cell lysates with LSD1-specific antibodies showed that the protein levels of LSD1 were significantly reduced in db/db MVSMCs compared to db/+/H11001 (Figure 3A, top panel, and bar graph in 3B), whereas β-actin levels were not altered (Figure 3A, bottom panel). We also examined the protein levels of a cofactor, Co-REST, that is required not only for demethylation activity of LSD1 on nucleosomal histones but also for LSD1 protein stability.28 As shown in Figure 3A (middle panel), levels of Co-REST protein were similar in db/db and db/+/H11001 cells. These results demonstrate that LSD1 protein levels are reduced in diabetic conditions and may contribute to the enhanced H3K4me2 at the promoters of inflammatory genes in db/db MVSMCs.

**Occupancy of LSD1 Is Decreased on Promoters of Inflammatory Genes in db/db MVSMCs**

We next examined whether LSD1 occupancy was also altered at the inflammatory gene promoters in diabetic cells. ChIP assays with LSD1 antibody showed that occupancies of LSD1 at the promoters of IL-6 (Figure 3C) and MCP-1 (Figure 3D) genes were significantly decreased in db/db MVSMCs relative to db/+/H11001 MVSMCs. TNF-α treatment (30 minutes) could also reduce LSD1 recruitment to the inflammatory gene promoters in both db/db and db/+/H11001 MVSMCs. However, these decreases at MCP-1 promoter, MCP-1 enhancer, and IL-6 promoter were significantly more pronounced in db/db MVSMCs relative to db/+/H11001 (Figure 3F and 3G). In contrast, similar changes were not seen at the CypA promoter (Figure 3G). These results demonstrate for the first time that enhanced gene expression under diabetic conditions in VSMCs could be attributable to reduced expression as well as reduced recruitment of LSD1, a negative regulator of H3K4me2.

**HG Increases Expression of Inflammatory Genes and H3K4me2 Levels**

HG is a major risk factor for the development of vascular complications and can promote VSMC dysfunction.2,4,7 Therefore, we examined whether HG can regulate inflammatory genes and alter histone lysine methylation using HVSMCs grown in NG (5.5 mmol/L), HG (25 mmol/L), or the osmotic control mannitol (5.5 mmol/L glucose plus 20 mmol/L mannitol) for 2 weeks. RT-QPCR data revealed that HG significantly increased mRNA levels of IL-6 (Figure 4A) and MCP-1 (Figure 4B) relative to NG or mannitol. Furthermore, ChIP assays showed that HG significantly increased H3K4me2 on both MCP-1 and IL-6 gene promoters compared to NG (Figure 4C). Conversely, LSD1 occupancy was significantly reduced at both these promoters (Figure 4D). These results demonstrate that HG can increase promoter H3K4me2 by decreasing LSD1 recruitment.

**LSD1 Negatively Regulates Inflammatory Gene Expression in VSMCs**

Next, we investigated the functional role of LSD1 in inflammatory gene expression. HVSMCs were transfected with siRNA oligonucleotides targeting LSD1 (siLSD1) or control oligonucleotides (siNeg). Cell lysates were collected 72 hours...
posttransfection and analyzed by immunoblotting with LSD1 antibody. As shown in Figure 5A, LSD1 protein levels were greatly reduced in HVSMCs transfected with siLSD1 compared to siNeg. Next, HVSMCs were transfected with enhanced green fluorescent protein (EGFP) expression vector, siLSD1, or siNeg and 72 hours posttransfection stimulated with TNF-α (10 ng/mL, one hour) to examine the effect of LSD1 gene silencing on inflammatory gene expression. LSD1 mRNA levels were reduced by 50% in HVSMCs transfected with siLSD1 compared to EGFP or siNeg, confirming LSD1 gene silencing (Figure 5B). Furthermore, TNF-α-induced MCP-1 and IL-6 expression was significantly greater in HVSMCs transfected with siLSD1 relative to siNeg or EGFP plasmid (Figure 5C). These results demonstrate that LSD1 negatively regulates MCP-1 and IL-6 in VSMCs and further support a role for LSD1 in modulating VSMC inflammatory responses.

**LSD1 Plays a Key Role in the Enhanced Inflammatory Gene Expression in Diabetes**

Having demonstrated the role of LSD1 in inflammatory gene expression, we next hypothesized that LSD1 gene silencing in control db/db MVSMCs will mimic the diabetic state and increase inflammatory genes similar to levels present in db/db MVSMCs. Conversely, overexpression of LSD1 in db/db MVSMCs should reverse their diabetic phenotype by reduc-

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**Figure 4.** HG effect on inflammatory gene expression, H3K4me2, and LSD1 recruitment. A and B, IL-6 and MCP-1 expression in HVSMCs treated with normal glucose (NG), high glucose (HG), and mannitol (MN). Gene expression was analyzed by RT-QPCR and normalized to β-actin, and results were expressed as fold change over NG (means±SEM). "P<0.05, "P<0.01 vs NG (n=3). C, HVSMCs were subjected to ChIP assays using H3K4me2 antibody, and ChIP-enriched DNA samples were analyzed by QPCR using human IL-6 and MCP-1 promoter primers. Results were normalized to input and expressed as fold change over NG (means±SEM). "P<0.03, "P<0.01 vs NG (n=3). D, HVSMC samples were subjected to ChIP assays using LSD1 antibody, and ChIP samples were analyzed by QPCR using human IL-6 and MCP-1 promoter primers. Results were normalized to input and expressed as percentage of NG. *P<0.04, "P<0.004 vs NG (n=3).

**Figure 5.** LSD1 siRNA increases inflammatory gene expression in HVSMCs. A, HVSMCs were transfected with LSD1 siRNA (siLSD1) or control (siNeg) oligonucleotides, and 3 days after transfection, cell lysates were analyzed by immunoblotting using the indicated antibodies. B, HVSMCs were transfected with pEGFP vector plasmid, siLSD1 or siNeg. Three days after transfection, total RNA was analyzed by RT-PCR, LSD1 levels were normalized to 18S RNA and expressed as percentage of EGFP (means±SEM). *P<0.05 vs pEGFP (n=3). C, HVSMCs were transfected with pEGFP plasmid vector, siNeg, or siLSD1 and serum-depleted for 48 hours posttransfection. On the following day (72 hours posttransfection), cells were stimulated with TNF-α (10 ng/mL) for 1 hour. Total RNA was used to determine IL-6 or MCP-1 expression by RT-QPCR. Results were expressed as fold change over respective control (means±SEM). *P<0.01, "P<0.002 vs EGFP (n=3).
Role of LSD1 in Monocyte–VSMC Binding

Evidence shows that VSMC–monocyte binding plays a key role in the development of atherosclerosis by promoting subendothelial monocyte retention, survival, and differentiation. 29 We previously demonstrated that VSMC–monocyte interactions are enhanced in diabetic MVSMCs, which may contribute to accelerated vascular complications. 16 Because this can be mediated by proinflammatory chemokines such as MCP-1, we next examined whether LSD1 can play a role in these heterotypic cellular interactions. HVSMCs were transfected with siLSD1 or control siNeg and 72 hours later, were serum-depleted and treated with or without TNF-α for 6 hours. Then VSMC–monocyte binding assays were performed as described in the expanded Materials and Methods section (online data supplement) using fluorescently labeled human monocyte THP-1 cells. As shown in Figure 8, TNF-α significantly increased monocyte binding to HVSMCs transfected with siNeg. However, transfection of HVSMCs with siLSD1 significantly enhanced both basal and TNF-α–induced monocyte binding compared to siNeg-transfected cells. These results demonstrate that, by negatively regulating or repressing key chemokines, LSD1 can play a key functional role in preventing VSMC–monocyte binding.

Discussion

In this report, we have demonstrated that the aberrant H3K4 dimethylation due to functional dysregulation of the H3K4 demethylase LSD1 function could be key underlying mech-

![Image](Image)

Figure 6. LSD1 knockdown enhances inflammatory gene expression in nondiabetic db/+ MVSMCs. A, Western blotting of db/+ MVSMC cell lysates transfected with siNeg or siLSD1 with indicated antibodies. B through E, db/+ MVSMCs were transfected with siNeg or siLSD1, and expression of MCP-1 (B and C) and IL-6 (D and E) was determined in total RNA isolated from basal or TNF-α–stimulated cells (10 ng/mL for 1 hour) (means±SEM). *P<0.02, **P<0.01 vs siNeg (n=3).

Figure 7. Inhibition of enhanced inflammatory gene expression by LSD1 overexpression in diabetic MVSMCs. A, The cells were transfected with plasmid expression vectors for EGFP (pEGFP) or LSD1 (pLSD1), and 48 hours later, cell lysates were immunoblotted with indicated antibodies. B, MVSMCs were transfected with pEGFP or pLSD1 and 24 hours later serum-depleted for another day. Then TNF-α–induced (10 ng/mL, 1 hour) MCP-1 and IL-6 gene expression was determined by RT-QPCR.

TNF-α–induced gene expression was expressed as percentage of db/+ MVSMCs (means±SEM). *P<0.05, **P<0.001, #P<0.01 vs db/+ transfected with pEGFP (n=3).
Diabetes Interventions and Complications (EDIC) Trial in memory phenomenon was observed in the Epidemiology of TNF-α-induced MCP-1 and IL-6 gene expression were enhanced in db/db VSMCs and correlated with increased H3K4me2 levels at these gene promoters. Interestingly, these responses persisted for a few passages in culture suggesting that db/db VSMCs exhibit metabolic memory. The metabolic memory phenomenon was observed in the Epidemiology of Diabetes Interventions and Complications (EDIC) Trial in which patients with type 1 diabetes who were previously on standard therapy continued to develop microvascular complications in spite of subsequent intensive therapy. It was also demonstrated in vitro in cultured endothelial cells and ARPE-19 retinal cells. However, the nuclear mechanisms were not identified. Histone lysine modifications are considered relatively more stable than other histone modifications such as acetylation and phosphorylation. Thus, the enhanced H3K4me2 levels in VSMCs of diabetic mice may confer short-term memory to the gene transcription machinery and propagation of this could be a novel mechanism for metabolic memory, leading to persistent dysfunctional behavior of cultured db/db VSMCs relative to db/+ cells.

Elevated levels of H3K4me2 on MCP-1 and IL-6 promoters could result from either increased recruitment of the corresponding histone methyltransferases or reduced recruitment of demethylases. Our present studies demonstrated that both protein levels and occupancies of the H3K4me2 demethylase LSD1 at the MCP-1 and IL-6 promoters were significantly reduced in db/db VSMCs, suggesting that the dysfunction of this demethylase could play a key role in the sustained proinflammatory phenotype of db/db VSMCs. This is supported by our observations of increased inflammatory gene expression after LSD1 gene silencing in nondiabetic VSMCs and its inhibition by overexpression of LSD1 protein in db/db MVSMCs. These results also demonstrate a negative regulatory role of LSD1 in MCP-1 and IL-6 expression in VSMCs. It is possible that the expression or promoter occupancies of related histone H3K4 methyltransferases such as SET7/9 or MLL are also altered under diabetic conditions. H3K4me2 may also cooperate with other chromatin marks such as H3K9/14 acetylation and arginine (H3R17) or H3K9 methylation because evidence shows that concerted action of factors regulating these modifications would determine transcription status. Altered balance of such interactions under diabetic conditions may promote formation of open chromatin poised for transcriptional activation. We have previously shown that the arginine methyltransferase CARM1 and the histone acetyltransferase CBP/p300 play important roles in the regulation of inflammatory genes in monocytes in diabetic conditions may promote formation of open chromatin accessible to transcription factors and coactivators resulting in enhanced transcription.

MCP-1 and IL-6 genes are regulated by NF-κB as well as CREB transcription factors. Our previous work suggested that NF-κB and CREB activities are enhanced under diabetic conditions including in db/db VSMCs. Additional studies are needed to determine whether LSD1 interacts with these transcription factors and whether this affects LSD1 recruitment and H3K4 methylation. Our present studies show that the protein levels of LSD1 were also reduced in db/db MVSMCs, indicating another level of LSD1 regulation under diabetic conditions. This was not attributable to the loss of Co-REST, a cofactor required for nucleosomal activity as well as of LSD1 protein stability. LSD1 has been identified as a nuclear phosphoprotein in HeLa cells, and, interestingly, the amino acid sequence 150 to 182 surrounding the phospho-serine revealed a potential PEST domain. PEST domains are present in proteins that are targeted for proteosomal degradation, such as like IkBa. Additional studies are needed to identify the roles of phosphorylation and the PEST domain in modulating LSD1 protein stability in diabetes. Our results indicate that both decreased LSD1 protein levels and promoter recruitment play key roles in enhanced inflammatory gene expression in MVSMCs.
Increased levels of inflammatory cytokines and chemokines can enhance atherogenic responses such as monocyte–VSMC binding. Our findings that LSD1 negatively regulates chemokine expression and monocyte–VSMC binding, coupled with reduced LSD1 levels in diabetic cells, further indicate that the dysregulation of LSD1 function may be among the mechanisms mediating the enhanced proatherogenic responses of diabetic VSMCs. They also demonstrate a new role for a histone lysine demethylase in VSMCs.

Hyperglycemia is a major risk factor associated with vascular complications in diabetes. Our studies with HVSsMs demonstrated that HG treatment alone could lead to an increase in inflammatory gene expression, along with enhanced H3K4me2 and reduced LSD1 occupancy at the promoters of these inflammatory genes. Thus, HG-mediated signaling could be the initial trigger for aberrant histone methylation and metabolic memory in diabetic VSMCs. Studies are in progress to identify the key HG-triggered signaling pathways that may regulate histone lysine methylation under diabetic conditions.

Recent studies have linked the dysregulation of histone lysine modifications in the pathogenesis of diseases such as cancer. LSD1 mRNA and protein levels were significantly upregulated in high-risk tumors and were suggested to be biomarkers for aggressive prostate cancer. Our studies demonstrating aberrant histone K4 methylation and dysfunction of LSD1 in diabetic cells further highlight the critical roles of epigenetic modifications in human diseases and the need to develop novel therapies based on these chromatin events.

Acknowledgments

We are grateful to Dr Li Meng for her assistance and to all those who generously provided plasmid expression vectors and reagents.

Sources of Funding

These studies were supported by grants from the NIH (National Heart, Lung, and Blood Institute and National Institute of Diabetes and Digestive and Kidney Diseases) (to R.N.), a predoctoral fellowship grant from the Myrtle Carr Foundation (to L.M.V.), and a Junior Faculty Award from the American Diabetes Association (to M.A.R.).

Disclosures

None.

References


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Circ Res. 2008;103:615-623; originally published online August 7, 2008;
doi: 10.1161/CIRCRESAHA.108.175190

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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ROLE OF THE LYSINE SPECIFIC DEMETHYLASE 1 IN THE PRO-INFLAMMATORY PHENOTYPE OF VASCULAR SMOOTH MUSCLE CELLS OF DIABETIC MICE

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MATERIALS AND METHODS

Materials: Antibodies specific for H3K4me2, H3K4me3 and LSD1 were purchased from Abcam, Inc (Cambridge, MA). RNA Pol II and p65 (NF-κB) 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 oligonucleotides were from Ambion, Inc (Austin, TX). Fluorescent label 2',7'-Bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxyethyl ester (BCECF/AM) was obtained from A. G. Scientific, Inc. (San Diego, CA). Protein A and Protein 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 9-11 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). Blood glucose levels were >450 mg/dl in db/db mice compared to <140 mg/dl in db/+ mice. 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

\[
E_{\text{gene}}^{(C_{\text{control}}-C_{\text{sample}})/E_{\beta-\text{actin}}^{(C_{\text{control}}-C_{\text{sample}})}},
\]

where \( E \) is the amplification efficiency of test gene (\( E_{\text{gene}} \)) or internal control β-actin gene and \( C_t \) 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 (3). 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°C 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°C 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 (Primer locations are shown in Online Fig. 1 and sequences are listed in Online Table 1). QPCR data was analyzed using the \(2^{-\Delta\Delta C_t}\) 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 °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.
**Online Figure I.** Maps of IL-6 and MCP-1 promoters: Arrows indicate location of ChIP primers used in the study. Map is not drawn to scale.

A. IL-6 Promoter

- AP1 (-320)
- CRE (-204)
- NF-IL6 (-195)
- NF-κB (-114)

B. MCP-1 promoter

- Enhancer
  - NF-κB NF-κB
  - NF-κB (-156)
  - NF-IL6 (-195)
  - SP1 (-43)
Online Figure II. 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.
Online Figure III. 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.
Online Table 1. Primer sequences:

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing Temp.</th>
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<tbody>
<tr>
<td><strong>cDNA Primers</strong></td>
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<tr>
<td>mIL-6</td>
<td>ACAAAGCCAGAGTCTCTCAGAG</td>
<td>ACCACAGTGAGGAATGTCCAC</td>
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<tr>
<td>mMCP-1</td>
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<tr>
<td>mCypA**</td>
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<tr>
<td>hIL-6</td>
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<tr>
<td>hMCP-1</td>
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<td>58</td>
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<tr>
<td><strong>ChIP Primers</strong></td>
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<tr>
<td>mIL-6pro</td>
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<td>mMCP-1enh</td>
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<td>CGTCTTTTAGCATCGCAAGAC</td>
<td>58</td>
</tr>
</tbody>
</table>

*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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kinase in S100B-induced activation of the receptor for advanced glycation end products

and p300 with NF-kappaB and CREB in angiotensin II-induced IL-6 expression in

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