Proinflammatory Stimuli Engage Brahma Related Gene 1 and Brahma in Endothelial Injury

Fei Fang, Dewei Chen,* Liming Yu, Xin Dai,* Yuyu Yang, Wenfang Tian, Xian Cheng, Huihui Xu, Xinyu Weng, Mingming Fang, Jiliang Zhou, Yuqi Gao, Qi Chen, Yong Xu

Rationale: Endothelial dysfunction inflicted by inflammation is found in a host of cardiovascular pathologies. One hallmark event in this process is the aggregation and adhesion of leukocyte to the vessel wall mediated by the upregulation of adhesion molecules (CAM) in endothelial cells at the transcriptional level. The epigenetic modulator(s) of CAM transactivation and its underlying pathophysiological relevance remain poorly defined.

Objective: Our goal was to determine the involvement of Brahma related gene 1 (Brg1) and Brahma (Brm) in CAM transactivation and its relevance in the pathogenesis of atherosclerosis.

Methods and Results: In the present study, we report that proinflammatory stimuli augmented the expression of Brg1 and Brm in vitro in cultured endothelial cells and in vivo in arteries isolated from rodents. Overexpression of Brg1 and Brm promoted while knockdown of Brg1 and Brm abrogated transactivation of adhesion molecules and leukocyte adhesion induced by inflammatory signals. Brg1 and Brm interacted with and were recruited to the CAM promoters by nuclear factor κB/p65. Conversely, depletion of Brg1 and Brm disrupted the kinetics of p65 binding on CAM promoters and crippled CAM activation. Silencing of Brg1 and Brm also altered key epigenetic changes associated with CAM transactivation. Of intrigue, 17β-estradiol antagonized both the expression and activity of Brg1/Brm. Most importantly, endothelial-targeted elimination of Brg1/Brm conferred atheroprotective effects to Apoe−/− mice on a Western diet.

Conclusions: Our data suggest that Brg1 and Brm integrate various proinflammatory cues into CAM transactivation and endothelial malfunction and, as such, may serve as potential therapeutic targets in treating inflammation-related cardiovascular diseases. (Circ Res. 2013;113:986-996.)

Key Words: atherosclerosis ■ Brg1 ■ endothelial dysfunction ■ epigenetics ■ inflammation ■ transcription

Endothelial dysfunction is a prominent feature and in many cases serves as a direct cause for cardiovascular disease (CVD), due largely to the fact that vascular endothelium provides a physical and functional sanctuary to the wholesomeness of the cardiovascular system.1,2 One of the early events shared among different CVDs is active recruitment of leukocytes to the vascular wall.3 Circulating leukocytes normally are unable to interact with the vascular endothelium due to insufficient surface contact. Under pathological circumstances, induction of adhesion molecules (CAM) primarily at the transcriptional level in endothelial cells allows the leukocytes to adhere and aggregate at focal points and perpetuates the maladaptive response.4 Mounting evidence has correlated inflammation with impaired endothelial functionality and ensuing cardiovascular mishaps.5 A host of inflammatory factors act either singularly or synergistically to amend the vascular endothelial transcriptome as a means of advancing their pathogenic agenda. Specifically, increased CAM expression by proinflammatory stimuli has been observed in a number of cardiovascular pathologies, which include atherosclerosis, hypoxia-induced pulmonary hypertension, and acute lung injury.6–8 Nuclear factor κB (NF-κB) has emerged as a central coordinator key to the transactivation of CAM genes in response to inflammatory cues.9 The cofactors involved in this process that fine-tune NF-κB–dependent CAM activation and their pathophysiological relevance in CVD, however, are not well understood.

Cardiovascular episodes are intimately associated with epigenetic alterations on promoters that ultimately lead to the
Proinflammatory Stimuli Activate the Expression of Brg1 and Brm in Endothelial Cells

To assess the relevance of Brg1/Brm in the activation of adhesion molecules in endothelial cells, we first measured the expression of Brg1 and Brm in response to proinflammatory stimuli. Treatment of HUVEC (EAhy926), human aortic artery endothelial cell (HAEC), human umbilical vein endothelial cell (HUVEC), human embryonic kidney cell (HEK293), and the Brg1/Brm-negative adenoma cell (SW-13) were maintained according to the vendors’ recommendations. Promoter activity was measured by transfection reporter assays. Expression of mRNA and protein was measured by real-time quantitative PCR and Western blotting, respectively. Interaction between endothelial cells and leukocytes was assessed by leukocyte adhesion assay in vitro and immunostaining in vivo. Knockdown of endogenous proteins was mediated by either small interfering RNA (siRNA) or short hairpin RNA carried by lentiviral particles. Protein binding to DNA was assayed by DNA affinity pulldown assay and chromatin immunoprecipitation (ChIP) assay. ApoE– mice were fed a high-fat diet for 6 to 8 weeks to induce atherosclerosis. All animal experiments have been performed following guidelines by the intramural Committee on Ethical Conduct of Animal Studies.

Methods

An expanded Methods section can be found in the Online Data Supplement. Briefly, human umbilical vein endothelial cell (HUVEC; EAhy926), human aortic artery endothelial cell (HAEC), human embryonic kidney cell (HEK293), and the Brg1/Brm-negative adenoma cell (SW-13) were maintained according to the vendors’ recommendations. Promoter activity was measured by transfection reporter assays. Expression of mRNA and protein was measured by real-time quantitative PCR and Western blotting, respectively. Interaction between endothelial cells and leukocytes was assessed by leukocyte adhesion assay in vitro and immunostaining in vivo. Knockdown of endogenous proteins was mediated by either small interfering RNA (siRNA) or short hairpin RNA carried by lentiviral particles. Protein binding to DNA was assayed by DNA affinity pulldown assay and chromatin immunoprecipitation (ChIP) assay. ApoE– mice were fed a high-fat diet for 6 to 8 weeks to induce atherosclerosis. All animal experiments have been performed following guidelines by the intramural Committee on Ethical Conduct of Animal Studies.

Results

Proinflammatory Stimuli Activate the Expression of Brg1 and Brm in Endothelial Cells

To assess the relevance of Brg1/Brm in the activation of adhesion molecules in endothelial cells, we first measured the expression of Brg1 and Brm in response to proinflammatory stimuli. Treatment of HUVEC (EAhy926) with tumor necrosis factor α (TNF-α), oxLDL, or LPS all led to significant induction of Brg1 and Brm levels (Figure 1A and 1C; Online Figure 1A and IB). Similarly, inflammatory treatments induced Brg1/Brm expression in HAECs and HPECs (Figure 1B and 1C; Online Figure IA and IB). In contrast, levels of BAF47 and BAF155, another 2 components of the mammalian SWI/SNF complex, were not significantly altered (Figure 1A and 1B; Online Figure IA). Interestingly, 17β-estradiol (E2), known to curb the inflammatory response in the vascular endothelium, tampered the upregulation of Brg1/Brm expression (Online Figure IC). More importantly, Brg1 and Brm levels were elevated in arteries in vivo in rodents challenged with inflammatory stress (Figure 1D and 1E; Online Figure ID). Collectively, these data illustrate that expression levels of Brg1 and Brm might be correlated with intracellular inflammatory state.

Brg1 and Brm Are Involved in the Transcriptional Activation of Adhesion Molecules by Proinflammatory Stimuli

Having observed that the expression of Brg1 and Brm was upregulated by proinflammatory stimuli in endothelial cells, we next evaluated the role of Brg1 and Brm in inflammation-induced CAM expression. By themselves, neither Brg1 nor Brm impacted promoter activities of adhesion molecules; they, however, potently augmented promoter activities of adhesion molecules in the presence of an individual proinflammatory signal (Online Figure IIA). The catalytic moiety of Brg1/Brm was clearly required for their function because enzyme-deficient forms of Brg1 and Brm failed to activate TNF-α–dependent transcription of CAM genes (Figure 2A). Coexpression of Brg1 and Brm resulted in additional activation of CAM promoters (Online Figure IIB). Similar results were obtained in HUVECs treated with oxLDL or LPS (Figure 2B), suggesting that Brg1/Brm may be a common link in inflammation-induced transactivation of CAMs. In further support of this notion, overexpression of Brg1 and Brm in HUVECs enhanced activation of endogenous CAM expression in the presence of proinflammatory stimuli both at the mRNA level (Figure 2C; Online Figure IIC) and the protein level (Figure 2D; Online Figure IID). To further confirm the correlation between Brg1/Brm and CAM transactivation, we used SW-13 cells in which both Brg1 and Brm are lacking. Ectopic expression of Brg1 and Brm significantly enhanced CAM induction by TNF-α (Online Figure IIE–G).

In contrast, knockdown of Brg1/Brm by either siRNA (Figure 3A; Online Figure IIIA for validation) or short hairpin RNA (Online Figure IIIB) attenuated promoter activities of all 3 CAM genes. In keeping with reduced CAM transcription, depletion of Brg1/Brm abrogated mRNA and protein levels of CAMs in HUVECs (Figure 3B and 3C; Online Figure IIIIC) and HAECs (Figure 3D and 3E; Online Figure IIID) treated with TNF-α. Of note, codepletion of Brg1 and Brm did not achieve additional suppression, indicating that proteins other than Brg1/Brm may become a rate-limiting factor when Brg1/Brm levels are sufficiently low. As a result, adhesion of leukocytes was severely crippled in the absence of Brg1/Brm (Figure 3F). Consistently, knockdown of Brg1/Brm also abolished activation of CAM message levels by oxLDL (Online Figure IIIE and IIIF) or LPS (Online Figure III G and IIIH). Taken together, these data outline a scenario wherein Brg1 and Brm are intimately involved in the transactivation of CAMs by proinflammatory stimuli.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Brg1</td>
<td>Brahma related gene 1</td>
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<tr>
<td>Brm</td>
<td>Brahma</td>
</tr>
<tr>
<td>HAEC</td>
<td>human aortic artery endothelial cell</td>
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<tr>
<td>HPEC</td>
<td>human pulmonary artery endothelial cell</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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Figure 1. Proinflammatory stimuli activate the expression of Brahma related gene 1 (Brg1) and Brahma (Brm) in endothelial cells. 

A–C, Human umbilical vein endothelial cells (HUVECs; A) and human aortic artery endothelial cells (HAECs; B) were treated with tumor necrosis factor α (10 ng/mL) for 3 hours or oxidized low-density lipoprotein (oxLDL) (100 μg/mL) for 24 hours. mRNA (A, B) and protein (C) levels of Brg1 and Brm were probed with quantitative PCR and Western blotting, respectively. Protein quantifications were performed with Image Pro based on 3 independent experiments.

D, E, ApoE−/− mice were fed a Western (high-fat diet, HFD) or a control (ad libitum [AL]) diet for 8 weeks. Aortic arteries were isolated and Brg1/Brm expression was probed by quantitative PCR (D) and Western blotting (E). Data are shown as mean±SD of triplicates and are representative of 1 experiment out of 3 performed.
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Brm1 Promotes Endothelial Inflammation

Brg1 and Brm Interact With and Are Recruited to the Promoters by p65

All 3 promoter constructs used in this study (Figure 2A) harbor a conserved NF-κB site, alluding to the possibility that Brg1 and Brm may interact with and be recruited to the CAM promoters by NF-κB. Indeed, communoprecipitations confirmed that Brg1/Brm formed a complex with p65 (Online Figure IVA and IVB). Inflammatory stimuli augmented occupancies of Brg1 and Brm to regions where NF-κB/p65 binds on all 3 CAM promoters as evidenced by ChIP assays (Figure 4A; Online Figure IVC). In contrast, there was less binding of Brg1/Brm on the intronic regions of CAM genes, and the binding was not responsive to inflammatory signals (Figure 4A; data not shown). Importantly, ex vivo ChIP assay revealed that there was more binding of Brg1 and Brm on the intercellular adhesion molecule 1 (ICAM-1) promoter in arteries isolated from mice that developed atherosclerosis (Figure 4B). Of note, consistent with its ability to curtail

Figure 2. Brahma related gene 1 (Brg1) and Brahma (Brm) enhance transcriptional activation of adhesion molecules by proinflammatory stimuli. A, Promoter luciferase fusion constructs for intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin genes were transfected into human umbilical vein endothelial cells (HUVECs) with either wild-type (WT) or enzyme-deficient (ED) Brg1 or Brm as indicated followed by treatment with tumor necrosis factor α (TNF-α). Luciferase activities were expressed as normalized relative luciferase unit (NRLU). B, ICAM-1 promoter construct was transfected into HUVECs with either WT or ED Brg1 or Brm followed by treatment with oxidized low-density lipoprotein or lipopolysaccharide (LPS) as indicated. Luciferase activities were expressed as NRLU. C, D, Brg1 or Brm expression constructs were transfected into HUVECs followed by treatment with TNF-α. CAM levels were assessed by quantitative PCR (C) and Western blotting (D). Data are shown as mean±SD of triplicates and are representative of 1 experiment out of 3 performed.
the expression of Brg1/Brm, 17β-estradiol also disrupted the binding of Brg1 and Brm to the CAM promoters (Online Figure IVD).

To further probe the requirement of p65 for the recruitment of Brg1/Brm, DNA affinity pulldown assays were performed with nuclear protein extracted from HUVECs and HAECs. TNF-α treatment led to an increase in the binding of Brg1/Brm to a biotin-labeled ICAM-1 probe, which was abolished by cold oligos containing the p65 consensus sequence (Online Figure IVE). Similar observations were made in 293 cells transfected with exogenous Brg1 and Brm (Online Figure IVF). Finally, ablation of p65 by siRNA significantly hampered the recruitment of Brg1/Brm to the CAM promoter as demonstrated by ChIP assays (Figure 4C) and blunted the enhancement of CAM transactivation (Online Figure IVG and IVH).

We next tackled the question whether Brg1 and Brm could affect the binding of p65 on CAM promoters in response to inflammatory signals. Ectopic expression of Brg1 or Brm in Brg1/Brm-negative SW-13 cells significantly enhanced the occupancy of p65 on the CAM promoters as demonstrated by both ChIP analysis (Figure 5A) and DNA affinity pulldown assay (Online Figure VA and VB), indicating that Brg1 and Brm may facilitate the binding of p65. Meanwhile, treatment of endothelial cells with TNF-α led to a strong and sustained recruitment of p65 on all 3 CAM promoters up to 9 hours post-exposure. Knockdown of Brg1 or Brm markedly accelerated the clearance of p65 from the CAM promoters in ChIP assays.
Brg1 and Brm Coordinate Key Epigenetic Alterations in Response to Proinflammatory Stimuli

Because Brg1 and Brm constitute a key component of the epigenetic machinery, we examined how depletion of Brg1 and Brm would impact the chromatin structure of the CAM genes. Specific siRNA for Brg1 or Brm significantly reduced their association with the CAM chromatin in response to TNF-α (Online Figure VIA). Consequently, active histone markers including acetylated histone H3 and methylated histone H3 lysine 4 were erased (Figure 6A–C). This was accompanied by impaired recruitment of the basic transcriptional machinery as evidenced by inefficient binding of RNA polymerase II. Curiously, levels of the histone variant H2A.Z that is commonly associated with active transcription was only modestly affected. In addition, elimination of Brg1 and Brm also abrogated active histone modifications and disrupted polymerase II binding on the CAM chromatin when endothelial cells were treated with oxLDL (Online Figure VIIB) or LPS (Online Figure VID). In all, these data strongly suggest that Brg1 and Brm contribute to CAM transactivation by maintaining a transcriptionally friendly chromatin environment for the access of RNA polymerase.

Brg1 and Brm Deficiency Averts Detrimental Effects Exerted by Proinflammatory Stimuli In Vivo

Finally, we probed the in vivo relevance of Brg1- and Brm-mediated CAM transactivation in atherosclerosis. To this end, we used an endothelial-specific targeting system to knock down Brg1/Brm in vivo. Indeed, infection of in vitro–cultured endothelial cells but not embryonic kidney cells resulted in suppression of Brg1/Brm expression (Online Figure VIIA). Moreover, immunostaining also revealed marked downregulation of Brg1/Brm expression in the vascular endothelium (Online Figure VIIB), but not in circulating leukocytes (Online Figure VIIC). With endothelial-specific silencing of Brg1/Brm, there was a significant reduction in atherosclerotic lesion in mice (Figure 7A and 7B). Concomitantly, expression of ICAM-1, VCAM-1, and E-selectin was normalized in the aortic arteries of atherosclerotic mice (Figure 7C). Consequently, adhesion of CD3+ lymphocytes, CD45+ activated leukocytes, and F4/80+ macrophages were reduced (Figure 7D). In aggregate, these data clearly support the notion that silencing of Brg1 and Brm stalled development of atherosclerosis by normalizing endothelial function in vivo.

Discussion

Inflammation-triggered, endothelium-dependent recruitment of leukocyte to the vessel wall and consequently the establishment of a proinflammatory milieu within the vasculature
represents a hallmark event in the pathogenesis of a range of CVDs. Key to this process is the transactivation of adhesion molecules mediated by NF-κB in response to inflammatory stimuli. Here we report that epigenetic factors Brg1 and Brm contribute to inflammation-induced CAM transactivation and are critically involved in the pathogenesis of atherosclerosis.

By shaping up the chromatin structure, Brg1 and Brm play a versatile role in transcriptional regulation. Our data indicate that Brg1 and Brm represent an integral part of the proinflammatory program initiated by divergent injurious signals. Brg1 and Brm seem to be crucial in orchestrating key histone modifications indicative of an active transcription state that correlates with CAM upregulation (Figure 6; Online Figure VIB). It has been well documented that Brg1 and Brm are required for the recruitment and stabilization of several enzymes that catalyze the active histone marks, including MLL1 (H3K4 methylation), CBP (histone H3 and H4 acetylation), and p300 (histone H3 and H4 acetylation). These modifications combined likely set up the stage for the recruitment of the basic transcriptional machinery. In fact, 2 recent articles highlight the importance of Brg1/Brm in stress-induced gene expression. Naito et al provide evidence that ischemic stress harnesses
Brg1 to induce the expression in TNF-α and macrophage chemotactic protein-1 in renal epithelial cells. Meanwhile, He and Luo pointed to the indispensability of Brg1 in human papilloma virus 18 E6/E7 gene expression in HeLa cells. Of particular interest, although Brg1 is responsible for RNA polymerase II recruitment to the TNF-α and MCP-1 promoters in response to ischemia without altering histone status, acetylation of histones H3 and H4 is clearly dependent on Brg1 for E6/E7 transactivation, which is more in line with our data presented here. Relying on extracellular and intracellular cues, Brg1 and Brm can be engaged in both active and repressive chromatin modification by switch binding partners. Paradoxically, Fish et al. have reported that Brg1 helps restore endothelial nitric oxide synthase expression during hypoxia/reoxygenation cycle by preventing the eviction of acetylated histones H3 and H4 on the endothelial nitric oxide synthase promoter in endothelial cells, indicating that Brg1 is beneficial in maintaining the vascular tone under stress conditions. These discrepancies reflect the gene-, cell-, and signal-specific nature of transcriptional regulation mediated by Brg1/Brm and need to be clarified by further research.

One of the intriguing issues regarding regulation of human pathobiology by Brg1 and Brm is their functional redundancy, that is, how well one can compensate for the loss of the other both in embryogenesis and adult life. Brg1 and Brm clearly assume opposing roles in osteoblast differentiation. On the other hand, although Brm null mice are viable and slightly overweight due to increased cell proliferation, whole-body or tissue-specific Brg1 deletion causes lethality in mice, arguing for the irreplaceable role of Brg1 and as such lending support to the notion that Brm is nonessential in organogenesis especially in the cardiovascular system. There has been, however, few definitive reports dissecting the role of Brg1 and Brm in postembryonic life. In hepatocyte, Brm, but not Brg1, is required for hypoxia-induced transactivation of the erythropoietin gene and for the activation of Cyp7a1 gene in cholesterol metabolism. Hang et al demonstrated, by using a tetracycline-inducible system that switches off Brg1 expression in the heart in adult mice, that Brg1 depletion greatly improves, but not completely abolishes, pressure overload–induced cardiac hypertrophy, leaving open the question whether Brm could be responsible for the residual cardiomyocyte oversize observed in these mice. In the present study, we have observed that Brg1 and Brm play differential roles in regulating CAM transcription in response to different stimuli. For instance, knock down of Brg1 does not seem to abrogate completely VCAM promoter activity/mRNA induced by TNF to basal levels (Figure 3A and 3B); on the other hand, Brg1 siRNA dramatically downregulates VCAM mRNA levels induced by LPS to below basal levels (Online Figure IIIG). This clearly reflects the complicated interplay between Brg1/Brm, specific gene/chromatin structure, and the upstream signal. Several key factors may contribute to the target specificity and hence biological relevance for Brg1 and Brm. Structurally, Brg1 differs from Brm in that Brg1 possesses a unique N-terminal domain that confers to Brg1 the ability to interact with and be recruited by zinc finger–type transcription factors such as Kruppel like factor and GATA. Alternatively, tissue-specific expression/distribution of Brg1 and Brm may also play a role in their dedicated functionality. Compared with Brg1 that is preferentially expressed in epithelial cells, Brm expression is more enriched in smooth muscle and endothelial cells in healthy humans. It is noteworthy that under pathological conditions, the expression patterns of Brg1 and Brm could be significantly altered (Figure 1; Online Figure I).
demonstrate here that there were subtle differences in CAM transactivation by Brg1 and Brm. Overall, however, there was little difference in the dependency of leukocyte adhesion on Brg1 or Brm in cultured endothelial cells (Figures 3F; data not shown), consistent with previous findings that Brg1 and Brm are equally important in maintaining the contractile phenotype of smooth muscle cells in vitro. Recently, it has been demonstrated that smooth muscle cell–specific knockout of Brg1 on a Brm-null background led to increased neonatal death and an aggravated phenotype in surviving mice, strongly supporting a Brg1-independent role for Brm in the vasculature in vivo. Because we targeted both Brg1 and Brm in our animal models, it remains to be determined whether they contribute differentially to inflammation-inflicted endothelial injury in vivo. High-throughput ChIP-seq profiling of Brg1/Brm binding in endothelial cells under different stress cues (LPS versus TNF-α) will enable a thorough analysis of the differential contribution of Brg1/Brm to gene expression.

Figure 7. Brahma related gene 1 (Brg1) and Brahma (Brm) depletion alleviates atherosclerotic lesion in Apoe<sup>−/−</sup> mice. Apoe<sup>−/−</sup> mice were injected with endothelial-specific lentivirus targeting Brg1/Brm (Endo-siBrg1/Brm) or scrambled sequence (SCR) and fed a Western (high-fat diet) diet for 8 weeks to induce atherosclerosis as described under Methods; n=12 to 14 mice for each group. A, Representative images of thoracic aorta isolated from mice injected with SCR or siBrg1/Brm and stained with oil red O. B, Representative images of aortic sinuses stained with oil red O. C, Expression levels of CAM genes in aortic arteries were assessed by quantitative PCR (n=8–10 mice for each group) and immunohistochemistry (n=5 mice for each group; representative images are shown); scale bar, 20 μm. D, Representative images of immunofluorescence staining of aortic arteries with indicated antibodies; scale bar, 20 μm. Arrows show immune cells adhered to the endothelium. Quantifications were performed using Image Pro and data are expressed as relative adhesion. ICAM-1 indicates intercellular adhesion molecule 1; and VCAM-1, vascular cell adhesion molecule 1.
Our data also revealed some subtle differences between Brg1 and Brm in terms of CAM transactivation by TNF-α. For instance, Brm induced VCAM-1 and E-selectin promoter activities better than Brg1 in the presence of TNF-α (Figure 2A). As shown in Figure 1C and 1E, endogenous Brm levels seem to be lower than endogenous Brg1 levels, possibly rendering the cells more sensitive to the introduction of exogenous Brm than to Brg1 in terms of promoter activity. On the other hand, it is noteworthy that we used partial deletions of the CAM promoter constructs (ICAM-1, −393/+42; VCAM-1, −519/+20; and E-selectin, −188/+25) with the NF-κB-binding motif included, which may not give the whole picture wherein Brg1/Brm regulates endogenous gene transcription. Indeed, endogenous VCAM-1 and E-selectin mRNA (Figure 2C) and protein (Figure 2D) levels respond to Brg1 overexpression and Brm overexpression equally well. The only way to compare the postembryonic functionalities of Brg1 and Brm in the endothelium would be to generate an inducible (eg, dox or estrogen) Cre line similar to the one used by Hang et al.13

To our best knowledge, the results as summarized in this article represent heretofore the first direct evidence that links Brg1/Brm-dependent CAM transactivation in endothelial cells to the pathogenesis of atherosclerosis (Figure 7) in mice. Brg1 and Brm have been shown to exert cell-specific effects in the cardiovascular system.11,13,32 Therefore, nonselective depletion of Brg1/Brm in vivo might have engendered equivocal data interpretation. For instance, atherosclerosis is widely believed to be associated with inflammation-induced endothelial injury to the pathogenesis of atherosclerosis by coordinating epigenetic regulation of NF-κB-dependent CAM transactivation. In light of the observation that Brg1 inactivation in the heart protects against pathological cardiac hypertrophy in mice,13 our findings reinforce the notion that (inadvertent) reactivation of certain genes (eg, Brg1) required for embryonic development may pose great health risk in adult life. Therefore, targeting Brg1/Brm may offer new hope in the prevention or intervention of cardiovascular disorders.

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Disclosures
None.

References
Brg1 and Brm are activated by proinflammatory stimuli in endothelial cells in vitro and in vivo. Brg1 and Brm promote CAM transactivation by influencing nuclear factor κB (NF-κB)/p65 kinetics and chromatin structure. 

Endothelial-specific targeting of Brg1/Brm attenuates atherogenesis in mice.

Prolinflammatory stimuli–induced endothelial injury underlies the pathogenesis of several cardiovascular diseases. Transcriptional activation of adhesion molecules facilitates the interaction between the endothelium and circulating leukocytes contributing to the amplification and perpetuation of chronic inflammation. Here we report that several proinflammatory stimuli elevate the levels of Brg1 and Brm, core components of the mammalian chromatin remodeling complex, in cultured endothelial cells in vitro and in mice. Overexpression of Brg1/Brm enhances, while depletion of Brg1/Brm attenuates, CAM transactivation. Brg1 and Brm interact with NF-κB/p65 and fine tune the binding of p65 to target promoters. Brg1/Brm helps establish a transcription-friendly chromatin structure for the recruitment of the basic transcription machinery. Endothelial-specific targeting of Brg1/Brm in mice decreases atherogenesis. Thus, pharmaceutical inhibition of Brg1/Brm in the vascular endothelium represents a novel strategy for CVD treatment.
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Methods

Cell culture and treatment

Human umbilical vein endothelial cells (HUVEC/EAh926, ATCC), human monocyte/macrophage cells (THP-1, ATCC), human adrenal carcinoma cells (SW13, ATCC), and human embryonic kidney cells (HEK293, Invitrogen) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone). Human primary aortic endothelial cells (HAEC, Cambrex/Lonza) and human primary pulmonary endothelial cells (HPEC, Cambrex/Lonza) were maintained in EGM-2 media with supplements supplied by the vendor; experiments were performed in primary cells between 3rd and 6th passages. Three separate batches of cells were used in this study.

Plasmids, Transient Transfection, and Luciferase Assay

Flag-tagged Brg1 (WT and ED) and HA-tagged Brm (WT and ED) constructs were kindly provided by Dr. Imbalzano and Dr. Muchardt, respectively. Flag-tagged Brm, V5-tagged p65 and promoter luciferase fusion constructs for ICAM-1, VCAM-1, and E-selectin genes have described previously.3-8 Small interfering RNA (siRNA) sequences for human Brg1 were as follows: #1, AACATGCACCAGATGCACAAG; #2, GCCCATGGAGTCCATGCAT; #3, GTAGCTCAGGAGTCGATAG. siRNA sequences for human Brm were as follows: #1, AAGTCCTGGACCTCCAAGTGT; #2, GGAGGTGCTAAGACACTTATG; #3, CATTCAAGCCACTGTTA. Transient transfections were performed with Lipofectamine 2000 (Invitrogen). Luciferase activities were assayed 24-48 hours after transfection using a luciferase reporter assay system (Promega). Experiments were routinely performed in triplicate wells and repeated at least three times.

Mice

All protocols were approved by the intramural Committee on Ethic Conduct of Animal Studies. 8-week old, male Apoe-/- mice were fed on a high-fat diet (HFD) for 8 weeks to induce atherosclerosis. As a control, age and sex-matched Apoe-/- mice were fed on a normal chow diet for the same period of time. siRNA targeting both Brg1 and Brm (GCUGGAGAAGCAGCAGAAG) were cloned into an endothelium-specific expression vector (Tie2p/eas) and packaged using an endothelium-specific envelope (2.2) as previously described.9 At week 1 and week 3, these mice were injected with lentivirus via tail vein. Atherosclerotic lesions were gauged by en face analysis of the whole aorta and by cross-sectional analysis of the proximal aorta essentially described previously.10

Protein Extraction, Immunoprecipitation, and Western Blot

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor tablet (Roche). Nuclear proteins were prepared with the NE-PER Kit (Pierce) following manufacturer’s recommendation. Specific antibodies or pre-immune IgGs (P.I.I.) were added to and incubated with cell lysates overnight before being absorbed by Protein A/G-plus Agarose beads (Santa Cruz). Precipitated immune complex was released by boiling with 1X SDS electrophoresis sample buffer. Alternatively, FLAG-conjugated beads (M2, Sigma) were added to and incubated with lysates overnight. Precipitated immune complex was eluted with 3X FLAG peptide (Sigma). Western blot analyses were performed with anti-FLAG, anti-V5, anti-β-actin (Sigma), anti-VCAM-1 (Abgent), anti-ICAM-1, anti-E-selectin, anti-Brg1, anti-Brm, and anti-p65 (Santa Cruz) antibodies. Experiments were repeated at least three times.

DNA affinity pull-down assay

Nuclear proteins (~200µg) were incubated with biotin-labeled ICAM-1 DNA probe (5’-TAGTTGGAATTCCGGAGC-3’) at room temperature for 1 hour in 1X binding buffer (20mM HEPES pH7.9, 0.1mM EDTA, 4% glycerol, 2mM DTT) supplemented with BSA (50µg per reaction), poly-dIdC, and sonicated salmon sperm DNA (100 µg per reaction). DNA-protein complexes formed were then captured by incubating with the streptavidin beads (Promega) for 1 hour at 4 ºC on a shaking platform. Ternary complex (biotin-labeled DNA-protein-streptavidin) was washed three time with 1X binding
buffer supplemented with 0.01% Triton X and 100mM KCl for 10 minutes each wash. The bound proteins were eluted with 1X SDS electrophoresis sample buffer by incubating at 90°C for 10 minutes and analyzed by SDS-PAGE gel electrophoresis followed by Western. Experiments were repeated at least three times.

**RNA Isolation and Real-time PCR**

RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen). Real-time PCR reactions were performed on an ABI Prism 7500 system. Primers and Taqman probes used for real-time reactions to detect ICAM-1, VCAM-1, E-selectin, Brg1, and Brm were purchased from Applied Biosystems. Experiments were repeated at least three times.

**Chromatin Immunoprecipitation (ChIP)**

Chromatin was cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet. DNA was fragmented into ~500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-Brg1, anti-Brm, anti-p65, anti-RNA Pol II (Santa Cruz), anti-acetyl histone H3, anti-acetyl histone H4, anti-dimethyl H3K4, anti-trimethyl H3K4 (Millipore), anti-histone H2A.Z (Abcam) or pre-immune IgG. Precipitated genomic DNA was amplified by real-time PCR with primers listed in supplemental Table I. Experiments were repeated at least three times.

**Leukocyte Adhesion Assay**

THP-1 cells were stained with a fluorescent dye (2′,7′-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein tetrakis (acetoxymethyl) ester) (Sigma) for 30 min at 37°C. After several washes with PBS, THP-1 cells were co-incubated for 30 min with endothelial cells. Unbound leukocytes were removed by washing and the number of adhered cells was visualized by fluorescence microscopy and analyzed with Image-Pro Plus (Media Cybernetics).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described. Briefly, parallel sections were blocked with 10% normal goat serum for 1 hour at room temperature and then incubated with anti-ICAM-1 (Abgent, 1:50), anti-VCAM-1 (Abgent, 1:50), or anti-E-selectin (R&D, 1:50). Staining was visualized by incubation with an appropriate biotinylated 2° antibody and developed with a streptavidin-horseradish peroxidase kit (Pierce) for 20min. Sections were counterstained with hematoxylin. Pictures were taken using an Olympus IX-70 microscope. For each primary antibody, we stained at least 3 slides from each individual mouse. For each experimental group, 5 mice were included.

**Immunofluorescence staining**

The plastic-embedded sections were incubated with primary antibodies, anti-CD31 (BD Biosciences), anti-Brg1 (Santa Cruz), anti-Brm (Santa Cruz), anti-CD3 (BD Biosciences), anti-CD45 (BD Biosciences), and anti-F4/80 (BD Biosciences), followed by incubation with donkey secondary antibodies (Jackson ImmunoResearch). The nuclei were counterstained with DAPI (Sigma).

**Statistical analysis**

Data are presented as mean±SD. For experiments concerning multiple groups, one-way ANOVA with post-hoc Scheffe analyses were performed to evaluate the differences. The differences between two (control and experimental) groups were determined by two-sided, unpaired Student’s t-test. p values smaller than .05 are considered significant. For in vivo experiments, all p values are spelled out. For in vitro experiments, p values between .05 and .01 are spelled out. Otherwise, * designates p values smaller than .01.
References


Online Figure I: Pro-inflammatory stimuli activate the expression of Brg1 and Brm in endothelial cells. (A, B) Human umbilical vein endothelial cells and human primary pulmonary endothelial cells were treated with LPS (1μg/ml) for 12 hours. mRNA (A) and protein (B) levels of Brg1 and Brm were probed with qPCR and Western. Protein quantifications were performed with Image Pro based on three independent experiments. (C) HUVECs were pre-treated with estradiol (E2, 10^-6M) prior to exposure to TNF-α or oxLDL. mRNA levels of Brg1 and Brm were measured by qPCR. (D) Age and sex matched C57/BL mice were challenged with LPS (25mg/kg) via intraperitoneal injection or PBS of equal volume and sacrificed 8 hours afterwards. Pulmonary arteries were isolated and Brg1/Brm mRNA levels were probed by qPCR. N=4 for each group.
Online Figure II: Brg1 and Brm enhance transcriptional activation of adhesion molecules by pro-inflammatory stimuli. (A) The ICAM-1 promoter luciferase plasmid was transfected into HUVECs with increasing amount of Brg1 or Brm followed by treatment with oxLDL (50μg/ml), LPS (1μg/ml), or TNF-α (10ng/ml). Luciferase activities were expressed as NRU. (B) Promoter luciferase plasmids were transfected into HUVECs with Brg1 and Brm expression constructs either separately or together as indicated followed by treatment with TNF-α for 3 hours. Luciferase activities were expressed as NRU. (C, D) HUVECs were transfected with Brg1 and Brm expression constructs either separately or together as indicated followed by treatment with TNF-α for 3 hours. mRNA (C) and protein (D) levels of CAM molecules were probed by qPCR. (E) Promoter luciferase plasmids were transfected into SW-13 cells with Brg1 and Brm expression constructs as indicated followed by treatment with TNF-α for 3 hours. Luciferase activities were expressed as NRU. (F, G) SW-13 cells were transfected with FLAG-Brg1, FLAG-Brm, or an empty vector (EV) followed by treatment with TNF-α (10ng/ml) for 3 hours. mRNA (F) and protein (G) levels were measured by qPCR and Western.
Online Figure III: Brg1 and Brm enhance transcriptional activation of adhesion molecules by pro-inflammatory stimuli. (A) HUVECs were transfected with siBrg1 (#1, #2, #3), siBrm (#1, #2, #3), or SCR. mRNA and protein levels of Brg1 and Brm were measured by qPCR and Western. (B) Promoter luciferase fusion constructs for ICAM-1, VCAM-1 and E-selectin genes were transfected into HUVECs with either control shRNA vector (shC) or shRNA plasmid targeting Brg1 (shBrg1) and Brm (shBrm) followed by treatment with TNF-α. Luciferase activities were expressed as NRU. (C, D) HUVECs (C) or HAECs (D) were transfected with indicated siRNAs followed by treatment with TNF-α. mRNA levels of CAM molecules were assessed by qPCR. (E, F) HUVECs (E) or HAECs (F) were transfected with indicated siRNAs followed by treatment with oxLDL. mRNA levels of CAM molecules were assessed by qPCR. (G, H) HUVECs (G) or HPECs (H) were transfected with indicated siRNAs followed by treatment with LPS. mRNA levels of CAM molecules were assessed by qPCR.
Online Figure IV: Brg1 and Brm interact with and are recruited to the promoters by p65. (A) 293 cells were transfected with FLAG-Brg1, FLAG-Brm, and/or V5-p65 as indicated. Immunoprecipitation assays were performed with indicated antibodies as described under Methods. (B) HUVECs were treated with TNF-α for 3 hours. Nuclear protein was used for immunoprecipitation with indicated antibodies. (C) HUVECs were treated oxLDL and ChIP assays were performed with anti-Brg1, anti-Brm, or IgG. Precipitated DNA was amplified by primers spanning the promoter region of indicated CAM gene. (D) HUVECs were pre-treated with estradiol (E2, 10^-6M) prior to exposure to TNF-α or oxLDL. ChIP assays were performed with anti-Brg1 or anti-Brm. (E) HUVECs and HAECs were treated with or without TNF-α for 3 hours. DNA affinity pull-down experiments were performed with a biotin-labeled ICAM-1 probe as described under Methods. (F) 293 cells were transfected with FLAG-Brg1, FLAG-Brm, and/or V5-p65 as indicated. DNA affinity pull-down assays were performed as described under Methods. (G, H) SW-13 cells were transfected with indicated plasmids or siRNAs followed by treatment with TNF-α. Expression of CAM genes was evaluated by qPCR (F) and Western (G).
Online Figure V: Brg1 and Brm stabilize p65 binding to the CAM promoters. (A) SW-13 cells were transfected with FLAG-Brg1 or FLAG-Brm followed by treatment with TNF-α. DNA affinity pull-down experiments were performed with a biotin-labeled ICAM-1 probe as described under Methods. (B) SW-13 cells were transfected with FLAG-Brg1, FLAG-Brm, sip65, or SCR followed by treatment with TNF-α. DNA affinity pull-down experiments were performed with a biotin-labeled ICAM-1 probe as described under Methods. (C, D) HUVECs were transfected with siBrg1, siBrm, or SCR followed by treatment with oxLDL (C) or LPS (D). ChIP assays were performed with anti-p65. Data were expressed as relative enrichment of p65 binding.
Online Figure VI: Brg1 and Brm integrates key epigenetic events taking place on the CAM promoter in response to inflammation. (A-C) HUVECs were transfected with siBrg1, siBrm, or SCR followed by treatment with TNF-α (A), oxLDL (B, C), or LPS (D). ChIP assays were performed with indicated antibodies.
**A**

Relative mRNA levels

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**B**

Relative Brg1 staining in CD31+ cells

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**C**

Relative mRNA levels

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Online Figure VII: Endo-siBrg1/Brm specifically targets Brg1 and Brm in the vascular endothelial cells. (A) Human aortic endothelial cells (HAEC), pulmonary arterial endothelial cells (HPEC), and embryonic kidney cells (HEK293) were infected with endothelial-specific virus targeting Brg1/Brm (Endo-siBrg1/Brm) or scrambled sequences (SCR). 72 hours after infection, mRNA levels of Brg1 and Brm were measured by qPCR. (B) Apoe<sup>-/-</sup> mice were injected with indicated lentivirus and induced to develop atherosclerotic lesions as described under Methods. Immunostaining was performed with anti-Brg1 or anti-Brm (Red) and anti-CD31 (green) followed by counterstaining with DAPI. Arrows, typical endothelial cells with Brg1/Brm expression. Scale bar, 20μm (C) CD3<sup>+</sup> and LyG6<sup>+</sup> cells were isolated from mouse peripheral blood by FACS and pooled together for qPCR analysis.
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