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BRG1 regulates NOX gene transcription in endothelial cells and contributes to cardiac ischemia-reperfusion injury

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ABSTRACT

Excessive accumulation of reactive oxygen species (ROS) is considered a major culprit for a host of cardiovascular diseases. In vascular endothelial cells, ROS production is mediated by NAPDH oxidases (NOX). In the present study we investigated the role of the chromatin remodeling protein BRG1 in NOX trans-activation as well as its implication in cardiac ischemia-reperfusion injury. We report that in response to hypoxia-reoxygenation (HR) BRG1 was recruited to the NOX promoter regions in both immortalized endothelial cells and primary microvascular endothelial cells. BRG1 knockdown attenuated the induction of NOX genes by HR stimulation. Suppression of NOX trans-activation by BRG1 silencing was paralleled by the loss of active histone modifications (acetylation of histones H3 and H4) and the re-appearance of repressive histone modification (dimethylation of histone H3K9) surrounding the NOX promoter. Of interest, the H3K9 demethylase KDM3A bound to the NOX promoters with kinetics similar to BRG1 and interacted with BRG1 to activate NOX transcription. KDM3A depletion ameliorated NOX induction and ROS production in endothelial cells exposed to HR. Finally, mice with endothelial-specific deletion of BRG1 were protected from cardiac ischemia-reperfusion injury. In conclusion, our data suggest that BRG1 may link epigenetic activation of NOX transcription in endothelial cells to cardiac ischemia reperfusion injury.

1. Introduction

Reactive oxygen species (ROS) serves as a double-edged sword in the regulation of pathophysiological events. On the one hand, ROS plays an essential role in hematopoiesis guiding the differentiation of progenitor cells into mature red blood cells [1]. ROS also stimulates phagocytosis and enhances bactericidal activities of macrophages thereby boosting the innate immunity and host survival [2]. On the other hand, ROS may potentially disrupt the structural and functional integrity of major macromolecules (e.g., proteins) and thus cause irrevocable damages to the organism [3]. Accumulation of ROS, when produced in magnitude beyond the scavenging capacity of the body, is considered a major culprit for a host of human diseases [4,5].

ROS production in mammalian cells is catalyzed by a myriad of specialized enzyme families, of which the NADPH oxidases (NOX) family of proteins are the most extensively studied. At least twenty different members of the NOX family, including both catalytic and regulatory subunits, have been identified and characterized [6]. Different NOX family members can be distinguished by their tissue distribution, subcellular localization, and/or the specific ROS variety they produce. NOX1, for instance, is preferentially expressed in epithelial cells whereas NOX2 is almost exclusively expressed in phagocytes [7,8]. Induced expression of NOX proteins, however, can be detected in a much wider range of cell types. The mechanisms underlying the activation of different NOX genes, primarily at the transcriptional level, by disease-related stimuli are yet to be elucidated.

Brahma related gene 1 (BRG1) is the catalytic subunit of the mammalian SWI/SNF chromatin remodeling complex that plays pivotal roles in transcriptional regulation. Earlier investigations have found that germline deletion of BRG1 in mice causes developmental arrest

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[9]. A close examination of BRG1-null embryos reveals severe defects in capillary formation indicating that BRG1 is essential for vasculogenesis. Interestingly, both endothelial-specific and endocardial-specific deletion of BRG1 lead to embryonic lethality affirming the role of BRG1 as a crucial gatekeeper in determining endothelial lineage specification [10]. What remains unclear is how BRG1 regulates endothelial function (or dysfunction for that matter) after organogenesis is concluded. We have previously shown that endothelial-restricted, short hairpin RNA (shRNA) mediated depletion of BRG1 in adult mice protects against pressure overload induced cardiac hypertrophy and fibrosis [11]. Mechanistically, BRG1 forms a complex with the histone H3K4 trimethyltransferase complex to activate the transcription of endothelin, which in turn stimulates cardiomyocyte hypertrophy. Here we report that BRG1 regulates NOX trans-activation in endothelial cells and contributes to cardiac ischemia reperfusion injury. Therefore, our data links a specific BRG1-mediated transcriptional event in endothelial cells to the pathogenesis of cardiac ischemia-reperfusion injury.

2. Methods

2.1. Cell culture

Immortalized human endothelial cells (EAhy926, ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone). Human primary cardiac microvascular endothelial cells were purchased from Lonza and maintained as previously described [12]; three different batches of primary cells were used in this study. For hypoxia-reoxygenation, cells were exposed to hypoxia (95% N_2 and 5% CO_2) for 1 h followed by 3 h, 12 h, or 24 h of re-oxygenation (95% O_2 and 5% CO_2).

2.2. Animals

Endothelial conditional BRG1 knockout (ecKO) mice were derived by crossbreeding the Smarca4-Flox strain (Jackson Laboratories) and the Cdh5-ERT2-Cre strain [13]. Protocols for animal experiments were reviewed and approved by the intramural Committee on Ethical Conduct of Animal Studies and adhered to the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Myocardial ischemia/reperfusion was performed as previously described [14]. Briefly, the mice were anaesthetized with a mixture of ketamine (120 mg/kg) and xylazine (6 mg/kg). Following left thoracotomy, the left anterior descending coronary artery was ligated with a 6–0 silk ligature over a 1 mm polyethylene tube (PE-10) for 30 min before reperfusion. The control mice were sham operated wherein the ligature around the LAD was not tied. The mice were sacrificed 24 h after reperfusion. Post-MI heart functions were evaluated by echocardiography (GE Vivid 7 equipped with a 14-MHz phase array linear transducer, S12, allowing a 150 maximal sweep rate).

2.3. DHE staining

DHE staining was performed essentially as previously described [15]. Frozen heart sections or endothelial cells were stained with DHE (10 μM, Sigma) at 37 °C for 30 min. Fluorescence was visualized by cofocal microscopy (LSM 710, Zeiss). Quantifications were performed with Image Pro.

2.4. Protein extraction and western blot

Tissue and cell lysates were obtained as previously described [16]. Western blot analyses were performed with anti-BRG1 (Abcam), anti-NOX2 (Proteintech), anti-NOX4 (Proteintech), anti-KDM3A (Bethyl Laboratories), and anti-β-actin (Sigma) antibodies. All experiments were repeated three times.

2.5. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed essentially as described before [17]. Briefly, 100 μg formaldehyde cross-linked nuclear proteins were precipitated with anti-BRG1 (Abcam), anti-trimethyl H3K4 (Millipore), anti-dimethyl H3K9 (Millipore), anti-trimethyl H3K27 (Millipore), and anti-KDM3A (Bethyl Laboratories). Precipitated genomic DNA was amplified by real-time PCR. Primer sequences are available upon request. All experiments were repeated three times.

2.6. 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 were purchased from Applied Biosystems. Ct values for genes of interest were normalized to 18 s rRNA and expressed as relative mRNA expression levels. All experiments were repeated three times.

2.7. 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.

3. Results

3.1. BRG1 regulates HR-induced NOX trans-activation in endothelial cells

To determine whether BRG1 contributes to the induction of NOX enzymes by hypoxia-reoxygenation (HR) in endothelial cells, the following experiments were performed. NOX2 and NOX4, but not NOX1, were up-regulated in immortalized vascular endothelial cells (EAhy926) exposed to one cycle of HR; depletion of BRG1, however, significantly attenuated the induction of NOX2 and NOX4 expression at both mRNA (Fig. 1A) and protein (Fig. 1B) levels. Similar experiments performed in human primary cardiac microvascular endothelial cells demonstrated that BRG1 silencing abolished the up-regulation of NOX2 and NOX4 expression by HR (Fig. 1C, D). Next, DHE staining was performed to verify whether regulation of NOX expression by BRG1 correlated with intracellular ROS levels. Indeed, ROS levels were elevated in endothelial following HR but were effectively suppressed by BRG1 knockdown (Fig. 1E). Finally, we performed chromatin immunoprecipitation (ChIP) assay to ascertain whether BRG1 could directly bind to the NOX promoters. As shown in Fig. 1F, Brg1 was detected on the NOX2 and NOX4 promoters, but not on the GAPDH promoter, following HR stimulation with the occupancy peaking at 12 h after reoxygenation. In addition, we were able to detect a stronger binding of BRG1 to the NOX promoters, but not the GAPDH promoter, in the reperfused hearts compared to the sham hearts in mice subjected to ischemia-reperfusion injury (Fig. 1G). We therefore conclude that BRG1 plays a role in HR-induced ROS accumulation in endothelial cells likely by directly activating the transcription of NOX genes.