Redox Biology 15 (2018) 12–21

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

The NADPH organizers NoxO1 and p47phox are both mediators of diabetes-induced vascular dysfunction in mice

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ARTICLE INFO

Keywords:
NADPH oxidase
NoxO1
Nox1
p47phox
Superoxide
Reactive oxygen species

ABSTRACT

Aim: NADPH oxidases are important sources of reactive oxygen species (ROS). Several Nox homologues are present together in the vascular system but whether they exhibit crosstalk at the activity level is unknown. To address this, vessel function of knockout mice for the cytosolic Nox organizer proteins p47phox, NoxO1 and a p47phox-NoxO1-double knockout were studied under normal condition and during streptozotocin-induced diabetes.

Results: In the mouse aorta, mRNA expression for NoxO1 was predominant in smooth muscle and endothelial cells, whereas p47phox was markedly expressed in adventitial cells comprising leukocytes and tissue resident macrophages. Knockout of either NoxO1 or p47phox resulted in lower basal blood pressure. Deletion of any of the two subunits also prevented diabetes-induced vascular dysfunction. mRNA expression analysis by MACE (Massive Analysis of cDNA ends) identified substantial gene expression differences between the mouse lines and in response to diabetes. Deletion of p47phox induced inflammatory activation with increased markers of myeloid cells and cytokine and chemokine induction. In contrast, deletion of NoxO1 resulted in an attenuated interferon gamma signature and reduced expression of genes related to antigen presentation. This aspect was also reflected by a reduced number of circulating lymphocytes in NoxO1-/- mice.

Innovation and conclusion: ROS production stimulated by NoxO1 and p47phox limit endothelium-dependent relaxation and maintain blood pressure in mice. However, NoxO1 and p47phox cannot substitute each other despite their similar effect on vascular function. Deletion of NoxO1 induced an anti-inflammatory phenotype, whereas p47phox deletion rather elicited a hyper-inflammatory response.

1. Introduction

NADPH oxidases of the Nox family are important sources of reactive oxygen species (ROS). In the vascular system, Nox1, Nox2, Nox4 and Nox5 are expressed. Nox1 and Nox2 have been associated with endothelial dysfunction during disease conditions such as diabetes [1,2],

Abbreviations: CBA, Cytometric Bead Assay; eNOS, endothelial Nitric Oxide Synthase; Gpx3, Glutathione peroxidase 3; IFNγ, Interferon gamma; MACE, Massive Analysis of cDNA ends; Nox, NADPH oxidase; PMA, Phorbol Myristate Acetate; ROS, Reactive Oxygen Species; SMCs, Smooth Muscle Cells; STZ, Streptozotocin

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https://doi.org/10.1016/j.redox.2017.11.014
Received 16 August 2017; Received in revised form 1 November 2017; Accepted 16 November 2017
Available online 22 November 2017

hypertension [3–5] and atherosclerosis [6,7]. This is a consequence of the fact that both enzymes produce nitric oxide-inactivating superoxide anions ($\bullet O_2^-$). Moreover, Nox1 and Nox2 are induced or activated by forces leading to cardiovascular disease [8]. Nox1 and Nox2 differ from other vascular Nox enzymes in their dependency on cytosolic proteins required for their activation. In the case of Nox2 the organizing protein p47phox tethers the activating protein p67phox to the catalytic subunits which leads to activation. A similar situation exists for Nox1, for which NoxO1 serves as the organizer and NoxA1 as the activator. Protein interactions of p47phox require its serine phosphorylation and displacement of an autoinhibitory region, a mechanism missing in NoxO1. Therefore, in overexpression systems, Nox1-NoxO1-NoxA1 complexes have high basal activity, whereas Nox2-p47phox-p67phox becomes active only after phosphorylation [9]. Overexpression has also demonstrated a degree of exchangeability between the components of the different complexes. The resulting activities, however, were always substantially lower than for the prototypic complexes [10,11].

To infer the possibility of such mixed complexes occurring within the cell, the relevant proteins have to be present in the same compartment and same cell type. The expression of p47phox predominates in myeloid cells [12], but expression in smooth muscle cells (SMCs) has been reported, albeit at lower level [13–15]. NoxO1 is highly expressed in colon and testis [16]. Its expression and function in the vascular system has been only recently shown and a quantitative analysis is still missing. NoxO1-/- mice produce lower basal vascular $\bullet O_2^-$ and show an increased angiogenesis by promoting tip cell formation [17]. Mice transfected with NoxO1 siRNA exhibit attenuated eNOS uncoupling in diabetes [1]. In cultured endothelial cells, NoxO1 expression can be induced by oscillatory flow, leading to eNOS uncoupling [18]. Such findings illustrate a paucity of information concerning cell-specific expression as well as supporting the view that components of the Nox1 and Nox2 systems are expressed and functionally relevant in the vascular system. Considering that Nox1 and Nox2 generate the same product and that the enzymes are similar to some extent, crosstalk between the Nox enzymes could be inferred. This aspect was studied here using p47phox-/-, NoxO1-/- and p47phox-NoxO1-double knockout mice under basal condition and streptozotocin-induced diabetes. Moreover, with the present study we provide a first phenotypic characterization of NoxO1-/- mice in a vascular disease context.

2. Results

2.1. NoxO1 and p47phox can activate an overexpression system of Nox1 and NoxA1

To confirm the function of NoxO1 and p47phox in a complex containing Nox1/NoxA1, different combinations of the proteins were overexpressed in HEK293 cells (Fig. 1). The combination of Nox1/NoxA1/NoxO1 produced 230 fold more $\bullet O_2^-$, as measured with L-012 chemiluminescence in intact cells than GFP transfected controls in non-stimulated conditions. PMA (phorbol myristate acetate), which was used to activate the complex, further increased the signal by approx. 30%. The ROS formation of the combination of p47phox with Nox1/NoxA1 had a very different profile: under basal conditions, *i.e.* in the absence of PMA, only a negligible signal was generated. Upon PMA stimulation, however, this combination yielded a 700% increase in the L-012 signal. These results are in agreement with previous publications [16,19] and confirm the preference of Nox1 for NoxO1 and the constitutive activity of this complex.

2.2. NoxO1 is mainly expressed in vascular cells whereas p47phox is mainly present in adventitial myeloid cells

Most of the expression data for p47phox in the vascular system were generated by RT-PCR. This is because this technique is highly sensitive and specific for the target gene. RT-PCR, however, is not the method of choice to identify cell specific expression differences within a tissue. Given the lack of cell-specific expression data, it is plausible that the p47phox mRNA detected in the vascular system is a reflection of a few contaminating myeloid cells [20]. To address this aspect, *in situ* hybridization by RNAscope® was employed to cell-specifically visualize the mRNA of NoxO1 and p47phox in the aortic wall. By this technique, NoxO1 signal in the adventitia was only 55% of that of the media. In contrast, the average abundance of p47phox in the adventitia was 300% of that in the media, showing a predominant localization of p47phox in adventitial cells whereas NoxO1 was mainly detected in the media (Fig. 2A & C). In the adventitial layer, p47phox but not NoxO1 co-localized with Adgre1 (Adhesion G Protein-Coupled Receptor E1 also called Egf-Like Module Containing, Mucin-Like, Hormone Receptor-Like Sequence (F4/80)), a marker for macrophages. Although RNAscope is limited in sensitivity, the data suggest that high levels of NoxO1 and p47phox are unlikely to reside together in the vascular tissue. Considering that the vascular adventitia is rich in inflammatory cells containing p47phox, the majority of the signal for this activator likely reflects its expression in myeloid cells.

2.3. NoxO1 and p47phox contribute to basal blood pressure and vascular tonus

To investigate a contribution of NoxO1 and p47phox to the vascular homeostasis the resting blood pressure was measured in mice and vascular function of the mesenteric artery was determined *ex vivo* in a wire myograph system. Knockout mice of p47phox or NoxO1 individually or combined exhibited a reduced systolic blood pressure as compared to WT animals. In p47phox-/- and NoxO1/p47phox-double knockout mice, but not in NoxO1-/- mice, was the diastolic pressure

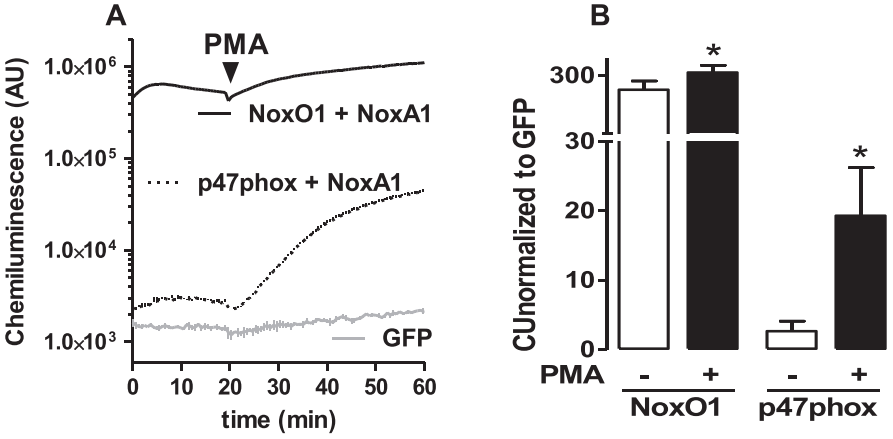

Fig. 1. Canonical (Nox1/NoxA1/NoxO1) and hybrid (Nox1/NoxA1/p47phox) activation of Nox1 in overexpression system using HEK cells. A: HEK cells were transfected with Nox1 and its activating subunits as indicated. ROS were measured by chemiluminescence with L012 (200 μmol/L). Stimuli-dependent activation of Nox1 was triggered by PMA (phorbol myristate acetate, 100nmol/L). B: quantification of the signal upon normalization to GFP control. n = 5, *p < 0.05 without PMA *vs.* with PMA.