Research Paper

Oxidation of HDAC4 by Nox4-derived H$_2$O$_2$ maintains tube formation by endothelial cells

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ABSTRACT

NADPH oxides produce reactive oxygen species that differ in localization, type and concentration. Within the Nox family only Nox4 produces H$_2$O$_2$ which can directly oxidize cysteine residues. With this post-translational modification, activity, stability, localization and protein-protein interactions of the affected protein is altered. Nox4 controls differentiation, cellular homeostasis and prevents inflammation. Therefore, is likely that epigenetic mechanisms contribute to the effects of Nox4. One group of epigenetic modifiers are class IIa histone deacetylases (HDACs). We hypothesize that Nox4-derived H$_2$O$_2$ oxidizes HDACs and analyzed whether HDACs can be differentially oxidized by Nox4. As an artificial system, we utilized HEK293 cells, overexpressing Nox4 in a tetracycline-inducible manner. HDAC4 was oxidized upon Nox4 overexpression. Additionally, Nox4 overexpression increased HDAC4 phosphorylation on Ser632. H$_2$O$_2$ disrupted HDAC4/Mef2A complex, which de-represses Mef2A. In endothelial cells such as HUVECs and HMECs, overexpression of HDAC4 significantly reduced tube formation. Overexpression of a redox insensitive HDAC4 had no effect on endothelial tube formation. Treatment with H$_2$O$_2$, induction of Nox4 expression by treatment of the cells with TGFβ and co-overexpression of Nox4 not only induced phosphorylation of HDAC4, but also restored the repressive effect of HDAC4 for tube formation, while overexpression of a redox dead mutant of Nox4 did not.

Taken together, Nox4 oxidizes HDAC4, increases its phosphorylation, and eventually ensures proper tube formation by endothelial cells.

1. Introduction

Endothelial cells regulate vascular tone, vascularization processes, influence smooth muscle cell function and mediate immune as well as general inflammatory responses [6,11,31,34]. As such, endothelial dysfunction can contribute to the development of atherosclerosis, hypertension and coronary artery disease [15]. It has become clear that endothelial cells undergo permanent changes in their epigenetic programming, dependent on their metabolic/redox status [23].

Epigenetic programming includes alterations in DNA packing density, which among others is regulated via the acetylation-status of positively charged amino acids, namely lysine and arginine in histone tails. Acetyl transferases, such as p300, neutralize the positive charges by converting the amines into amides, which loosens the histone-DNA binding and eases transcription. In contrast, histone deacetylases remove acetyl groups, which tightens the histone-DNA binding and hinders transcription. In mammals, 18 HDACs sorted into four main classes. Out of those, class II HDACs (class IIa: HDACs 4, 5, 7 and 9 and class IIb: HDACs 6 and 10) appear to be redox-regulated via direct cysteine oxidation [2]. An interesting HDAC in endothelial cells is HDAC4. It maintains VEGFR2 expression as well as autophagy and it enhances angiotensin II-induced inflammation in vascular endothelial cells [5,14]. Besides histones, HDACs have other targets as well. Recently it was shown that fluid shear stress stimulates HDAC5 phosphorylation and its nuclear export in endothelial cells through a calcium/calmodulin-dependent pathway. This results in dissociation of HDAC5 and Mef2, which enhances Mef2 transcriptional activity, leading to the development of an anti-inflammatory phenotype of endothelial cells [38]. Phosphorylated HDAC5 in the nucleus therefore may play a protective role in inflammation. Further, overexpression of HDAC5 in vivo, as found in scleroderma, impaired the expression of key-angiogenic and fibrotic genes [35]. This finding is similar to the
effect of Nox4 knock out, which results in a pro-inflammatory phenotype, in deficits in angiogenesis as well as in enhanced fibrosis [2,12,30].

Metabolic and especially redox regulation have become major fields of research within the last decade. NADPH oxidases are significant sources of reactive oxygen species. The family of NADPH oxidases is composed of seven members. Nox1, 2 and 3 are dependent on cytosolic subunits, the organizer, and activator. All subunits need to form a complex, which additionally contains the small G-protein Rac. The whole complex then allows formation of superoxide (•O₂⁻). Nox5 and DUOX1 and 2 produce •O₂⁻ or H₂O₂ in a Ca²⁺-dependent manner. The only constitutively active NADPH oxidase is Nox4, which produces permanently small amounts of H₂O₂ and thereby facilitates long term processes such as differentiation [9]. Further, the H₂O₂-producing NADPH oxidase Nox4 is important for endothelial health, function and integrity [28]. In fact, Nox4 is expressed at high levels in endothelial cells [30]. Importantly, in endothelial cells as well as in the heart Nox4 elicits positive and protective effects [31,40].

Nox4 plays a pivotal role in angiogenesis via the maintenance of Hif-1 and consequently VEGFR2 expression [36]. Nox4 knock-out results in a pro-inflammatory phenotype, in deficits as well as in enhanced fibrosis [2,12,30]. In vivo, HDAC4 contributes to the manifestation of cardiac hypertrophy after transverse aortic constriction (TAC) surgery [1,19], while Nox4 overexpression protects from TAC-induced hypertrophy [40]. We hypothesize that HDAC4 and Nox4 may have opposite roles and interact. Accordingly, we thought to investigate this potential interaction of Nox4 and class II HDACs.

2. Results

2.1. Endothelial HDAC4 is susceptible to H₂O₂-dependent oxidation and specifically oxidized by Nox4

HDAC expression may vary between certain endothelial cell lines or origins [35,41]. We analyzed for the expression of members of class IIa HDACs in endothelial cells (HUVECs and HMECs) as well as in the model system of HEK293 cells (Supplemental Fig. 1A-D). In all three cell lines, the most abundant HDAC was HDAC7, followed by HDAC4 and very little HDAC5, whereas HDAC9 was nearly at the detection limit.

The NADPH oxidase Nox4 is an essential, direct, and constitutively active source of H₂O₂ in endothelial cells [30]. In order to analyze whether Nox4 is involved in oxidation of HDACs, an inducible Nox4 overexpression system in HEK293 cells was utilized (Nox4-tet-on cells). Tetracycline-dependent Nox4 overexpression was confirmed by qPCR and Western blot, while expression of other NADPH oxidases, such as Nox5 remained unaffected by tetracycline (Supplemental Fig. 2A+B). Tetracycline treatment and subsequent upregulation of Nox4 resulted in an increased catalase-sensitive H₂O₂ production, which was inhibited by the flavoprotein inhibitor DPI [17] (Supplemental Fig. 2C). In this work, DPI-treated cells served as a control, to show that the redox change in tetracycline-treated cells is likely due to a flavoenzyme-mediated ROS production and not due to some other effects tetracycline may have on the cells. Although we are aware of the many side effects DPI may have, it still is the most potent and reliable inhibitor of NADPH oxidases, with a fail-safe inhibition of ROS formation in the utilized system of Nox4 overexpression upon tetracycline treatment in HEK293 cells. Using this artificial system, we found that HDAC7 was not prone to Nox4-mediated oxidations (Supplemental Fig. 2D). In contrast, HDAC5 was oxidized (Supplemental Fig. 2E). In the DPI control, however, HDAC5 oxidation was highly variable and not significantly reduced. We conclude that other events related to tetracycline, rather than Nox4 derived ROS, elicit HDAC5 oxidation. In contrast, HDAC4 oxidation was enhanced when Nox4 was induced by tetracycline in Nox4-tet-on cells. DPI reduced Nox4-dependent HDAC4 oxidation (Fig. 1A). Neither mRNA nor protein expression of HDAC4 was altered by Nox4 overexpression (Fig. 1B&C). Further experiments were conducted to prove that the effects seen so far are specifically mediated by Nox4-derived

![Fig. 1. Oxidation of HDAC4 upon Nox4 overexpression and treatment with H2O2.](image)

(A) BIAM switch redox assay (n = 5) (One-way ANOVA with Tukey post-hoc test, *p < 0.05). (B) relative mRNA expression analyzed by qPCR (n > 4) and (C) relative protein expression of HDAC4 analyzed by Western blot and normalized to β-actin (n = 3) in Nox4 tet-on cells with or without tetracycline (1 μg/ml, 24 h) and DPI (3 μM, 3 h). (D) BIAM switch redox assay (n > 3) (One-way ANOVA with Dunnett post-hoc test, *p < 0.05) and (E) relative protein expression of HDAC4 in HUVECs with H2O2 (10/100/1000 μM, 30 min or 6 h) (n = 4). Representative Western blots are depicted. All bar graphs show means ± SEM.
H₂O₂ and do not represent a general oxidation of HDACs by ROS. Nox5 overexpressing HEK293 cells (Supplemental Fig. 3) were used to model the effect of plasma membrane bound NADPH oxidases and subsequent •O₂⁻ production [33]. HEK293-Nox5 cells constitutively overexpress Nox5, which is inactive in normal cell culture conditions but can be activated by phorbol myristate acetate (PMA) treatment. Challenging HEK293-Nox5 with PMA induced superoxide production, while Nox4-induction in the Nox4-tet-on cells did not have such an effect (Supplemental Fig. 3B). Accordingly, in HEK293-Nox5 cells treated with PMA Prx3 was oxidized (Supplemental Fig. 3C), which serves as positive control for cellular effects of Nox5-derived superoxide, as it has been shown to be oxidized by Nox5 before [17]. Although Nox5-derived •O₂⁻ technically is sufficient to oxidize target proteins, such as Prx3 in HEK-Nox5 cells, neither HDAC4 nor HDAC5 was oxidized in PMA-treated HEK-Nox5 cells (Supplemental Fig. 3D). Taken together, the data suggest HDAC4 to be the most likely redox target in endothelial cells. Indeed, HDAC4 can be oxidized by H₂O₂ in HUVECs and HMECs as shown by a modified BIAM (biotinylated iodoacetamide)-based redox-immunoprecipitation [17]. With the aid of this redox-IP, we were able to detect selectively reversible thiol oxidations. According to the concept of over-oxidation and irreversible modification by high concentrations of H₂O₂, BIAM labeling of HDAC4 was increased at 100 μM H₂O₂ and decreased when cells were treated with 1 mM H₂O₂ (Fig. 1D and Supplemental Fig. 4A). HDAC4 protein abundance in HUVECs and HMECs is not affected by H₂O₂ (Fig. 1E and Supplemental Fig. 4B).

These data suggest HDAC4 to be specifically oxidized by Nox4-derived H₂O₂, which has no impact on the expression of HDAC4. We continued to analyze the consequences of Nox4-mediated HDAC4 oxidation.

2.2. Mef2A physically interacts with HDAC4, which is counteracted by H₂O₂ and Nox4 activity

One major component of HDAC4-directed regulation of cellular processes is the repression of Mef2 transcription factors. Out of the four Mef2 isoforms (Mef2A-D), mainly Mef2A, C and D interact with HDAC4, which alters their physiological functions [22,37]. In contrast, any physiological consequences of Mef2Bs interaction with HDAC4 are uncertain [10]. Mef2A is the most abundant isoform in HEK293 cells, HMECs and HUVECs (Fig. 2A). Overexpression of HDAC4 in HEK293 cells impaired the activity of Mef2A as analyzed with the aid of a luciferase reporter gene assay (Fig. 2B). Importantly, HDAC4 physically interacts with Mef2A and H₂O₂ treatment disrupts this interaction (Fig. 2C). We therefore thought to analyze the effect of Nox4-derived H₂O₂ on Mef2A activity. We overexpressed a redox dead mutant of Nox4 (RD-Nox4; which does not produce H₂O₂), or wild type Nox4. If overexpressed together with HDAC4 and Mef2A, Nox4 was able to increase Mef2A activity, while RD-Nox4 was not (Fig. 2D). We conclude that Nox4-derived H₂O₂ oxidizes HDAC4, which disrupts the HDAC4/-Mef2A complex and allows an increased Mef2A activity. Indeed, binding of HDAC4 to Mef2 in the nucleus inhibits Mef2s function [22]. Enhanced nuclear export of HDAC4 upon oxidation [19] may interfere with the repressive effect of the deacetylase on Mef2 and subsequently activate this transcription factor family. Indeed, when we analyzed for a correlation of Nox4 and Mef2A target genes in databases of human samples of endothelial cells, we found several of them to correlate with Nox4 in their expression (Supplemental Fig. 4C).

2.3. HDAC4 phosphorylation is enhanced by H₂O₂ and Nox4 activity

The inhibitory activity of class IIa HDACs on Mef2 isoforms is
overcome by their phosphorylation on conserved serine residues [18]. Phosphorylation leads to the disruption of Me2-HDAC complexes [21]. In the artificial system of tetracycline-inducible Nox4 expression in HEK-Nox4-tet-on cells, HDAC4 phosphorylation was increased upon Nox4 induction which was absent upon prevention of Nox4 activity by treatment of the cells with DPI (Fig. 3A). In endothelial cells treatment with increasing concentrations of \( \text{H}_2\text{O}_2 \) increased HDAC4-phosphorylation in a dose dependent manner (Fig. 3D). We also thought to analyze for the endogenous effect of Nox4 expression in endothelial cells. Endothelial cells endogenously express Nox4 and transforming growth factor (TGF) β is known to increase Nox4 expression in endothelial cells [25]. Therefore, we utilized TGFβ as an inducer of endogenous Nox4 expression. Indeed, TGFβ potently and robustly increased the expression of Nox4 as well as \( \text{H}_2\text{O}_2 \) production in HMECs (Fig. 3B). Further, TGFβ treatment increased the abundance of phosphorylated HDAC4 and prevention of Nox4 dependent ROS formation by DPI reduced the phosphorylation of HDAC4 back to the basal level (Fig. 3D).

Our data suggest that Nox4 overexpression results in oxidation of HDAC4. This may ease HDAC4 phosphorylation at Ser632 and consequently, via disruption of the HDAC4/Mef2A complex, enhances Mef2A activity.

2.4. HDAC4 mediated inhibition of angiogenesis is rescued by \( \text{H}_2\text{O}_2 \) and Nox4 activity

As an indicator of the physiological relevance of the data in this manuscript, we sought to investigate the effect of HDAC4 on angiogenesis. Active Mef2A, as well as phosphorylation of HDAC4, has been shown to enhance angiogenesis [16,27]. We first analyzed the effect of HDAC4 overexpression and we were able to verify that HDAC4 is a negative regulator of angiogenesis in HUVECs and HMECs (Fig. 4A and B). Treatment of HDAC4 overexpressing cells with \( \text{H}_2\text{O}_2 \) or TGFβ restored tube formation capacity (Fig. 4C and Supplemental Fig. 5A). Overexpression of Nox4 along with HDAC4 returned tube formation back to the basal level, while overexpressing a redox dead mutant of Nox4 (RD-Nox4) did not rescue the anti-angiogenic effect of HDAC4 overexpression (Supplemental Fig. 5B).

Oxidation is a prerequisite for HDAC4 to exit the nucleus, while phosphorylation ensures its accumulation in the cytosol [39]. We analyzed the effect of a redox-insensitive mutant of HDAC4 (C667/669A; RI-HDAC4). Upon overexpression in HEK293 cells, RI-HDAC4 accumulated substantially more in the nucleus than WT-HDAC4 does (Fig. 5A). Redox assays performed in Nox4-tet-on cells transfected with the RI-HDAC4 mutant did not provide reasonable evidence for a Nox4-mediated oxidation of RI-HDAC4 (Fig. 5B). Further, RI-Nox4 did not induce RI-HDAC4s phosphorylation (Fig. 5C). Instead, RI-HDAC4 is already phosphorylated at a higher level than WT-HDAC4, for an unclear reason. Eventually, overexpression of RI-HDAC4 in HMECs did not reduce the angiogenic potential of these cells (Fig. 5D).

We conclude that Nox4-mediated oxidation of HDAC4 is a prerequisite for HDAC4 to leave the nucleus, but also for the formation of the HDAC4/Mef2A complex. We conclude that HDAC4, oxidized by Nox4, has less capacity to inhibit Mef2A, which ensures endothelial tube formation.
3. Discussion

HDAC4 contributes to the manifestation of cardiac hypertrophy after TAC surgery [1,19], while Nox4 overexpression protects from TAC-induced hypertrophy [40]. Accordingly, we hypothesized that Nox4 may counteract the effects of HDAC4. Based on the finding of a high Nox4 expression in endothelial cells, we analyzed a potential connection of Nox4 and the histone deacetylase HDAC4 as well as its physiological relevance in endothelial cells. We provide evidence that Nox4-derived H$_2$O$_2$ oxidizes HDAC4 and thereby eases the exit of HDAC4 from the nucleus. Most prominently Nox4 is localized in the endoplasmic reticulum and the perinuclear membrane system [13]. As HDAC4 shuttles between cytosol and nucleus, it is possible that HDAC4 passes an area similar to a micro domain with high H$_2$O$_2$ concentration made by Nox4, a redox cloud [29]. In cardiac myocytes, oxidation of HDAC4 can be reversed by the reductase Trx1 and reduction of Cys667 and 669 in HDAC4 inhibits its nuclear export independently of its phosphorylation status [1]. Here we verify that oxidation is a prerequisite for HDAC4 to stay in the nucleus and show that Nox4 overexpression forces phosphorylation of HDAC4. Potentially both mechanisms regulate subcellular localization of HDAC4.

A mechanism how phosphorylation prevents nuclear accumulation of HDAC4 would be its binding to 14-3-3. Two models are discussed for this effect: Binding of 14-3-3 proteins could unmask a nuclear localization site (NLS) in HDAC4 and 5 [7] or expose a nuclear export site (NES) in HDAC5 [20]. Although it is uncertain, whether binding of 14-3-3 promotes nuclear export or prevents nuclear import, it is established that 14-3-3 binding controls the subcellular localization of class IIa HDACs. The Sadoshima lab published elegant studies showing that oxidation of HDAC4, facilitates nuclear export more than phosphorylation does. They found that phosphorylation is a follow up of oxidation. Accordingly, some conformational change may enable a better association of HDAC4 with its kinases. This however needs to be addressed in a separate study. Different to the work of Matsushima et al., 2013 performed in cardiac myocytes [19], we provide evidence that Nox4

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Fig. 4. Tube formation assay with HDAC4 overexpression. (A) HUVECs were transfected with GFP or HDAC4. After 24 h cells were seeded on matrigel. (B + C) HMECs were transfected with GFP or HDAC4. After 24 h cells were treated with TGFβ (10 ng/ml) or H2O2 (30 μM) for 24 h and seeded on matrigel. Representative images after 4 h of tube formation are shown. Numbers of nodes were counted and total tube length was measured with an image angiogenesis analyzer (Carpentier, 2012) in ImageJ and summarized in the statistics (n > 4). All bar graphs show means ± SEM (One-sample t-test for GFP vs. HDAC4, One-way ANOVA with Sidak post-hoc test for TGFβ/H2O2 treatment *p ≤ 0.05).
overexpression in HEK293 as well as treatment of HMECs with H2O2 or TGFβ induces phosphorylation of HDAC4. We can only speculate, why this is so. The phosphatase PP2A dephosphorylates multiple serines including the 14-3-3 binding sites of HDAC4, which promotes HDAC4 nuclear import [24]. In fibrosarcoma cells the absence of Nox4 allows an accumulation of PP2A in the nucleus [11]. These findings open room for the following speculation concerning the situation in endothelial cells: In the absence of Nox4 PP2A dephosphorylates HDAC4, which accumulates in the nucleus, where it prevents angiogenesis. Indeed, one consequence of a Nox4 knock out is a reduced angiogenesis [12,36] and here we show that overexpression of HDAC4 itself prevents angiogenesis. Additionally in another work, utilizing HEK-tet-on cells for Nox4 overexpression, we identified the kinase MARK2 as a target of Nox4-derived redox modification [17]. In HDAC7, MARK2 phosphorylates Ser155 as a prerequisite for the phosphorylation of the nearby 14-3-3 binding site in a signal-independent manner [4]. The authors of that study propose a signal-independent multisite hierarchical phosphorylation by a variety of kinases. This may also include Ser632, which was analyzed in this work. Although it is just speculation, oxidation of MARK2 could serve as an activator stimulus and facilitate HDAC4 phosphorylation. Alternatively, oxidized HDAC4 may interact easier with kinases of the CaMKII family.

A major function of endothelial cells is to perform angiogenesis. TGFβ can promote angiogenesis through multiple factors, including increased endothelial Nox4 expression and TGFβ-mediated tube formation of HUVECs which is abolished when Nox4 is inhibited [25]. TGFβ treatment elevates Nox4 expression in lung fibroblasts and leads to nuclear export of HDAC4 enabling myofibroblast differentiation [8]. Those findings are in line with the results of this study and therefore likely also to apply in endothelial cells. Our results indicate that a TGFβ-induced Nox4 expression is likely to rescue the anti-angiogenic effect of HDAC4 overexpression in endothelial cells. In summary, here we show that overexpression of HDAC4 is abrogated by TGFβ treatment, likely based on a subsequent Nox4 expression.

4. Innovation

Overexpression of HDAC4 inhibits endothelial tube formation. Nox4-derived H2O2 specifically oxidizes HDAC4 and forces its phosphorylation. As a consequence Nox4 derived H2O2 can prevent the anti-angiogenic effect of HDAC4 overexpression in endothelial cells.

5. Materials and methods

5.1. Cell culture

Human embryonic kidney (HEK) 293 cells were purchased from
ATCC (Manassas, USA) and used as an artificial model system. HEK293 cells overexpressing Nox4 in a tetracycline-inducible manner (Nox4-tet-on) were kindly provided by K.H. Krause, University of Geneva, Switzerland [32] as well as stably Nox5 overexpressing HEK293 cells (HEK293-Nox5) [26]. All HEK cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), high glucose, GlutaMAX (Gibco, Carlsbad, USA), supplemented with 10% fetal calf serum (FCS, #S0115, Biochrom, Berlin, Germany), 1% MEM Non-essential Amino Acid Solution (Biochrom, Berlin, Germany), 1% MEM Non-essential Amino Acid Solution (Biochrom, Berlin, Germany), and gentamicin (50 μg/ml, Gibco). Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (#CC-2519, Lot No. 371074, 369146, 314447, 192485, 186864, 177172, Walkersville, USA) and PeloBiotec (#PB-CH-190-813, Lot No. QC-18P13F11, Planegg, Germany). HUVECs were cultured in dishes coated with gelatin in endothelial growth medium (EGM). EGM was composed of endothelial basal medium (EBM) supplemented with human recombinant epidermal growth factor (EGF), EndoCGS-Heparin (PeloBiotec), 8% FCS, penicillin (50 U/ml) and streptomycin (50 μg/ml) (#15140-122, Gibco). Human microvascular endothelial cells (HMECs) were purchased from Centers for Disease and Prevention (Eggert:32967; RRID:Addgene_32967).

5.2. Transfection

5.2.1. Lipofection of HEK293 cells and HMECs

HEK293 cells were transiently transfected with Lipofectamine 2000 reagent (#11668019, Thermo Fisher Scientific, Carlsbad, USA). For 6 cm dish 50 μl transfection medium (HEK293 medium without antibiotics) including 1.5 μg DNA were mixed with 35 μl transfection medium including 3.5 μl Lipofectamine 2000. 35 μl transfection medium with the respective DNA amount were mixed with 17.5 μl transfection medium including 1.75 μl Lipofectamine 2000 per well in a 12-well plate. During 10 min incubation of the transfection mixture at room temperature, HEK293 cells were washed and the growth medium was replaced with 1.5 ml (6 cm dish) or 0.5 ml (12-well plate) transfection medium. The transfection mixture was then added dropwise and after 4 h the medium was changed to HEK293 growth medium.

HMECs were transiently transfected with Lipofectamine 3000 and P3000TM Enhancer Reagent (#L3000-015, Thermo Fisher Scientific). For 3.5 cm dish 50 μl EBM were mixed with 5 μl Lipofectamine 3000 and another 125 μl EBM with 5 μl P3000TM Enhancer Reagent and 2 μg DNA. After 5 min incubation at room temperature both components were mixed and incubated for another 20 min at room temperature. The mixture was added dropwise to the dishes filled with HMECs in 1 ml EBM. After 4 h media was changed to HMEC-EGM.

5.2.2. Electroporation of HUVECs

HUVECs were transfected by electroporation using the Neon Transfection System (Thermo Fisher Scientific) according to the manufacturers’ protocol. Cells were then cultured in EBM with 5% FCS. Medium was changed to EGM after 6 h.

5.2.3. Overexpression plasmids

The following plasmids were used: GFP (#pEGFP C1, Clontech, Mountain View, USA), flag-N1- and flag-R1-HDAC4-myc (gift from Johannes Backs, Heidelberg, Germany), Me2A-flag (#32970, Addgene, Cambridge, USA), Nox4 and an inactive Nox4 mutant (redox dead Nox4, RD-Nox4) in which Thr595 is mutated to lysine. 3XMe2-luc was a gift from Ron Prywes (Addgene plasmid #32967; http://n2t.net/addr; RRID:Addgene_32967).

5.3. H2O2 measurement with Amplex Red

Nox4-tet-on cells or HMECs were seeded on 12-well plates. Cells were washed with HEPES-Tyrode (HT) buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 0.5 mM MgCl2, 5 mM glucose, 0.36 mM Na2HPO4). 300 μl of pre-warmed assay buffer (50 μl Amplex Red (#A12222, Invitrogen), 2 U/ml horseradish peroxidase (#P-6782, Sigma-Aldrich) in HT buffer) were added to the cells. Nox4-Tet-on cells were incubated for 3 min and HMECs for 30 min in the incubator. 200 μl were transferred to a 96-well plate and fluorescence was measured (Exc./Em. 540/585 nm). To calculate the catalase-sensitive portion fluorescence PEGL-catalase (500 U/ml, #C4963, Sigma-Aldrich) was added to the assay buffer 30 min before starting the assay.

5.4. Superoxide measurement with cytochrome c

Nox4-tet-on and HEK293-Nox5 cells were seeded on 12-well plates. Cells were washed with PBS and resuspended in 1 ml HT buffer. Cells were counted and adjusted 4 × 10^6 cells/ml. 50 μl cell suspension were transferred to a 96-well plate and 200 μl assay buffer (20 μM cytochrome c (#PC7752, Sigma-Aldrich) and 3000 U catalase (#C1345, Sigma-Aldrich)) were added. After incubation for 45 min at 37 °C, absorbance at 550 nm was measured. To calculate SOD-sensitive superoxide production every sample was subsequently measured including 500 U SOD (#S7571, Sigma-Aldrich) in the assay buffer.

5.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Isolation of mRNA was performed with the aid of an RNA-Mini-Kit (Bio&Sell, Feucht, Germany) according to the manufacturer’s protocol. Genomic DNA was digested with DNase I (#453628001, Roche, Basel, Switzerland). For cDNA synthesis, Random hexamer primers (Promega, Madison, USA) together with Superscript III Reverse Transcriptase (Invitrogen, Darmstadt, Germany) were used. qRT-PCR was performed with the aid of an AriaMx qPCR cycler (Agilent Technology, Santa Clara, USA). Primers are listed in Table 1. cDNA was detected using qT® SYBR® Green in a Supermix (BioRad, Hercules, USA). cDNA levels were calculated using the ΔΔct method and gene expression was normalized to β-actin.

5.6. Western blot

Cells were washed with PBS, harvested by scratching them off the plate in RIPA lysis buffer containing Tris-HCl (50 mM), NaCl (150 mM), deoxycholate (1%), Triton X-100 (1%), sodium dodecyl sulfate (SDS) (0.1%), phenylmethylsulfonyl fluoride (PMSF, 1 mM), orthovanadate (OV, 2 mM), okadaic acid (OA, 10 mM) and protease inhibitor mix (PIM).

If phosphorylation was investigated, cells were just washed quickly and shocked frozen in liquid nitrogen before lysis. The lysate was centrifuged (17,000 g, 10 min, 4 °C) and the supernatant was collected. Protein concentration was determined using Bradford assay. Samples were boiled in Lammli buffer under reducing conditions, separated by SDS-PAGE and transferred to a nitrocellulose membrane by Western blotting. Fluorescence-based detection of secondary antibodies was performed using the Odyssey CLx imaging system (Licon, Bad Homburg, Germany). Primary antibodies: HDAC4 (HEK293 cells: #sc-46672, Santa Cruz Biotechnology, Mountain View, USA), flag-WT- and flag-RI-HDAC4-myc (gift from T. Schader et al., Redox Biology 36 (2020) 101669). Table 1

| Gene | Forward primer (5′→3′) | Reverse primer (5′→3′) |
|------|------------------------|------------------------|
| β-actin | AAAAGACCTGGTACGGCCAAAC | GTCACTACCTTGTCGGTGTAT |
| HDAC4 | CAAAGAGAGCTGGTACCTTCCACACAG | GCCCTACCGAGGAGAACGGAGG |
| HDAC5 | TTCAAGGCTGGTTCGCTGGCCTG | AGGCTCTGTCGAGTGGGTCCT |
| HDAC7 | TCCTCTGGTAAGGGTACGCTGAC | ACCAGCTGCTCCAGGGGCTACTC |
| HDAC9 | TCAGGACATGGAAGGACGCTG | TGCAAGGTGCCTACGCATTTCC |
| Me2A | TACATACTAAGGACCGAGGTT | TTGGACATGATGCGGCTT |
| Me2C | TACGAGGATTAGTTGACAGG | GGGTAGCAAGGAGGACGTG |
| Me2D | TCCTCTGGTAAGGGTACGCTGAC | ACCAGCTGCTCCAGGGGCTACTC |
| Nox4 | TCGGGACAAATAGGGCAAGTG | CCATCGGAGTTCCAGCATG |
| Nox5 | GTCCTGGTTACCTTGCTGACTAA | GAGGGCGGCTTGATTTAG |

Table 1

qRT-PCR primers (human).
Santa Cruz, Heidelberg, Germany; HUVECs and HMECs: #ab12172, Abcam, Cambridge, UK; phospho HDAC4 (#ab39408, Abcam), HDAC5 (#sc-133106, Santa Cruz), HDAC7 (#sc-74563, Santa Cruz), GFP (#632381, Clontech), peroxisiredoxin (Prx) 3 (#LF-PA0030, Abfrontier/ Biomol, Hamburg, Germany), Me2A (#ab32866, Abcam), phospho Me2A (#ab36046, Abcam), Myc tag (#ab32, Abcam), Flag tag (#F3165, Sigma–Aldrich), Nox4 (#NB110-58851, Novus Biologicals, Wiesbaden, Germany), Topoisomerase I (#sc-3542, Santa Cruz) and β-actin (#A1978, Sigma–Aldrich).

5.7. Redox–immunoprecipitation/BIAM switch assay

Alkylation of thiol groups was performed in living cells before harvest. N-ethylmaleimide (NEM, 50 mM, #4259, Sigma–Aldrich) was added to the medium and left for 5 min at room temperature. Cells were then washed with PBS containing 100 mM NEM and 500 μl trichloroacetic acid (TCA, 20%) were added. Cells were scrapped off, transferred into a reaction tube, and incubated on ice for 15 min. Samples were centrifuged (13000 g, 30 min, 4°C) and washed with 10% and then 5% TCA. The pellet was resuspended in denaturation buffer (pH 8.5, 100 mM Tris-HCl, 8 M Urea, 5 mM EDTA, 0.5% SDS) with NEM (25 mM) and incubated for 1 h at 37°C. After addition of 800 μl acetone, proteins were precipitated over night at −20°C. Samples were centrifuged (13000 g, 30 min, 4°C) and washed twice with acetone. Then pellets were resuspended in denaturation buffer with 5 mM DTT for 5 min at 37°C to reduce oxidized thiols, which were subsequently labeled by adding DB with EZ-Link Iodoacetyl-LC-Biotin (BIAM, 2 mg/ml, #21333, Thermo Fisher Scientific) for 1 h at 37°C and 850 rpm. Proteins were precipitated by adding 800 μl acetone and stored overnight at −20°C. Samples were centrifuged (13000 g, 30 min, 4°C) and washed twice with acetone. The pellet was resuspended with lysis buffer composed of Tris-HCl (50 mM), EDTA (5 mM), Triton X-100 (1%), SDS (1%), PMSF (1 mM), OV (2 mM), OA (10 mM) and PIM. After incubation for 1 h at 37°C the protein amount was determined by Lowry assay. 500 μg protein were adjusted to 1 μg/μl with lysis buffer. Labeled proteins were pulled down with 100 μl Pierce™ Streptavidin agarose beads (#20353, Thermo Fisher Scientific) slurry overnight at 4°C. Beads were washed five times with lysis buffer and boiled with 1.5x Lämml buffer. Samples were analyzed on Western blot.

5.8. Co-immunoprecipitation (Co-IP)

HEK293 cells were seeded on 6 cm dishes and transfected with 1 μg Me2A and 0.5 μg WT- or RI-HDAC4 plasmids, respectively. Before harvesting cells were washed with PBS. Cells were scraped in lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1% Nonidet P40) including OV, OA, PIM and PMSF. The lysate was sonicated three times for 10 s and 30 Hz. After that samples were centrifuged (15,000 g, 10 min, 4°C) and the supernatant was collected in a new tube. Protein concentration was determined by Bradford assay. 1000 μg of protein were adjusted to a volume of 800 μl. Samples were pre-cleared for 30 min at 4°C with 40 μl protein G beads (# 11004D, Thermo Fisher Scientific) which were washed before five times with lysis buffer. Pre-cleared lysates were separated from the beads and 4 μg of Me2A-, myc tag- or IgG-antibody were added and incubated overnight at 4°C. 40 μl protein G beads per sample were washed five times with lysis buffer and used for IP for 4 h at 4°C. Beads were washed five times with lysis buffer and boiled for 5 min at 95°C with 1.5x Lämml buffer under reducing conditions. Samples were analyzed on Western blot.

5.9. Nuclear extraction

Cells were scratched off the plate in Hank’s buffer and centrifuged (17,000 g, 1 min, 4°C). The pellet was resuspended in nuclear extraction buffer containing HEPES (10 mM), KCl (10 mM), EDTA (0.1 mM), EGTA (0.1 mM), PMSF (1 mM) and PIM. After incubation on ice for 15 min, Nonidet P40 (1.5%) was added, samples were centrifuged (17,000 g, 1 min, 4°C) and the cytosolic protein lysate (supernatant) was transferred into a new tube. The lysate and the pellet, containing nuclear proteins, were boiled in Lämml buffer and used for Western blot.

5.10. Mef2 luciferase reporter assay

Promegax luciferase reporter assay was used to measure luciferase activity (#E1501, Promega, Madison, USA). Cells were seeded on 12-well plates and transfected for 48 h. 1 μg 3xMe2-luc and 0.25 μg of the other plasmids were used for transfection. Cells were washed with PBS and scraped with 120 μl lysis buffer (1x passive lysis buffer (Promega) with PIM). Lysates were stored overnight at −80°C. Samples were centrifuged (15,000 g, 1 min, 4°C) and supernatant was transferred to a new tube. 20 μl were mixed with 100 μl luciferase assay reagent in a 96-well plate followed by measurement of luciferase activity.

5.11. Tube formation assay

Tube formation assays were performed in μ-Slide Angiogenesis coverslips (#81507, Ibidi, Planegg, Germany). Matrigel was thawed on ice over night. 10 μl of matrigel (#356231, Corning, Corning, USA) were added per well and allowed to polymerize for 30 min at 37°C. HUVECs and HMECs were transfected and after 24 h cells were starved in EBM+0.1% BSA. Cells were, trypsinized and counted. 5000 HUVECs or 3000 HMECs were seeded onto the matrigel in 50 μl EBM with 1% FCS. After 4 h incubation in a cell culture incubator at 37°C, 30 μl medium were replaced by 30 μl PBS with 4% FFA in order to fix the cells. Tube formation was analyzed by counting nodes and measuring total tube length using ImageJ [3].

5.12. Immunofluorescence

Immunofluorescence was performed in μ-Slide 8 Well chambers (#80826, Ibidi). 25,000 HMECs per well were seeded in EBM+0.1% BSA for the indicated periods. Then, cells were fixed in 2% PFA and washed twice with PBS. To remove PFA PBS with 4% FBS was added for 10 min and cells were washed twice with PBS afterwards. 0.05% Triton X-100 in PBS was used to permeabilize the cells for 10 min. PBS+3% BSA was added for 30 min to reduce unspecific binding. Phospho HDAC4 (#ab39408, Abcam) was diluted in PBS+3% BSA (1:100) and incubated over night at 4°C. Cells were washed three times with PBS+0.3% Tween and once with PBS. Secondary antibody AlexaFluor488 donkey anti-rabbit (#A21206, Thermo Fisher Scientific) 1:500 in PBS+3% BSA was added for 30 min at room temperature. Cells were stained with DAPI for 10 min and washed three times with PBS+0.3% Tween and once with PBS. Images were taken with a confocal microscope LSM 510 Meta and analyzed with ImageJ.

5.13. Statistics

Statistical analysis was performed with GraphPad Prism 8. Means with standard error of the mean (SEM) are shown. The number of biological replicates is defined by “n”. Differences were considered as significant, if p < 0.05.

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Declaration of competing interest

The authors declare that they have no conflicts to disclose.

Abbreviations

| Abbreviation       | Definition                                      |
|--------------------|-------------------------------------------------|
| Nox                | NADPH oxidase                                   |
| HDAC               | Histone deacetylase                             |
| TGFβ               | Transforming growth factor beta                 |
| HUVEC              | Human umbilical vein endothelial cells          |
| HEK293             | Human embryonic kidney                         |

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101669.

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