



Research paper

Epigenetic regulation of vascular NADPH oxidase expression and reactive oxygen species production by histone deacetylase-dependent mechanisms in experimental diabetes



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ABSTRACT

Reactive oxygen species (ROS) generated by up-regulated NADPH oxidase (Nox) contribute to structural-functional alterations of the vascular wall in diabetes. Epigenetic mechanisms, such as histone acetylation, emerged as important regulators of gene expression in cardiovascular disorders. Since their role in diabetes is still elusive we hypothesized that histone deacetylase (HDAC)-dependent mechanisms could mediate vascular Nox overexpression in diabetic conditions. Non-diabetic and streptozotocin-induced diabetic C57BL/6J mice were randomized to receive vehicle or suberoylanilide hydroxamic acid (SAHA), a pan-HDAC inhibitor. *In vitro* studies were performed on a human aortic smooth muscle cell (SMC) line. Aortic SMCs typically express Nox1, Nox4, and Nox5 subtypes. HDAC1 and HDAC2 proteins along with Nox1, Nox2, and Nox4 levels were found significantly elevated in the aortas of diabetic mice compared to non-diabetic animals. Treatment of diabetic mice with SAHA mitigated the aortic expression of Nox1, Nox2, and Nox4 subtypes and NADPH-stimulated ROS production. High concentrations of glucose increased HDAC1 and HDAC2 protein levels in cultured SMCs. SAHA significantly reduced the high glucose-induced Nox1/4/5 expression, ROS production, and the formation malondialdehyde-protein adducts in SMCs. Overexpression of HDAC2 up-regulated the Nox1/4/5 gene promoter activities in SMCs. Physical interactions of HDAC1/2 and p300 proteins with Nox1/4/5 promoters were detected at the sites of active transcription. High glucose induced histone H3K27 acetylation enrichment at the promoters of Nox1/4/5 genes in SMCs. The novel data of this study indicate that HDACs mediate vascular Nox up-regulation in diabetes. HDAC inhibition reduces vascular ROS production in experimental diabetes, possibly by a mechanism involving negative regulation of Nox expression.

1. Introduction

Oxidative stress is implicated in numerous diabetes-associated vascular disorders by inducing endothelial cell (EC) dysfunction, alteration of vascular smooth muscle cell (SMC) phenotype, extracellular matrix synthesis or recruitment and activation of immune cells into the blood vessel wall [1].

Among other cellular sources, members of the NADPH oxidase (Nox) family are major contributors to the production of reactive oxygen species (ROS) and the ensuing oxidative stress within the cardiovascular system. Nox1, Nox2, Nox4, and Nox5 (absent in rodents)

are the main Nox subtypes identified in the vascular cells. These enzymes catalyze the one/two electron reduction of molecular oxygen (O_2) using NADPH as electron donor to produce superoxide anion ($O_2^{\cdot-}$)/hydrogen peroxide (H_2O_2). Nox-derived ROS are directly involved in reversible/irreversible oxidation of biological molecules and participate in secondary reactions to generate highly toxic free radicals such as hydroxyl radical ($HO\cdot$) and hydroperoxyl radical ($HOO\cdot$). Although functionally related, Nox subtypes display distinct structural characteristics and activation mechanisms. Nox1 and Nox2 require cytosolic regulatory subunits for their enzymatic activities, Nox4 is constitutively active, whereas Nox5 is Ca^{2+} -dependent. All Nox

Abbreviations: ROS, Reactive oxygen species; NADPH, Nicotinamide adenine dinucleotide phosphate (reduced form); Nox, NADPH oxidase; HAT, Histone acetyltransferase; HDAC, Histone deacetylase; CVD, Cardiovascular disorders; STZ, Streptozotocin; SMCs, Smooth muscle cells; DMSO, Dimethyl sulfoxide; SAHA, Suberoylanilide hydroxamic acid; HRP, Horseradish peroxidase; IFN γ , Interferon gamma; Jak, Janus kinase; STAT, Signal Transducer and Activator of Transcription; AP-1, Activator protein-1; NF-kB, Nuclear factor kappa-light-chain-enhancer of activated B cells; C/EBP, CCAAT/enhancer-binding protein

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subtypes form bound complex with p22phox essential subunit. The enhanced expression of each Nox isoform and the ensuing ROS formation/oxidative stress markers have been directly associated with the severity of structural-functional alterations of the vascular wall in all major cardiovascular disorders (CVD), including diabetes [2,3]. For this reason, in an attempt to counteract the deleterious effects of the oxidative stress in CVD, Nox enzymes have become highly attractive therapeutic targets [4–6].

Recent evidence indicates that the dysregulation of epigenetic mechanisms involving enzymes regulating histone acetylation (e.g., histone acetyltransferases – HAT and histone deacetylases – HDAC) is associated with CVD [7,8]. As a general principle, histone acetylation induces chromatin relaxation and activation of gene expression. HAT and HDAC also act on transcription factors to induce or repress the expression of specific genes.

The HDAC superfamily comprises Zn²⁺-containing (class I, II, and IV) and NAD(+) - dependent (class III) isoenzymes. HDAC1 and HDAC2 belong to class I HDAC, are primarily localized in the nucleus, exhibit high deacetylation activity, and are critically involved in the regulation of chromatin topology and gene expression [9].

Evidence exists that HDACs could play a role in the regulation of Nox expression in endothelial cells and in hypertension conditions [10–12]. Still, the implication of HDACs in mediating diabetic vasculopathies and, in particular, in the regulation of Nox enzymes remains elusive [13–15].

Thus, in this study we aimed at investigating whether in diabetes, HDAC-related mechanisms are involved in the regulation of the expression of vascular Nox. We provide *in vivo* and *in vitro* evidence that HDAC1 and HDAC2 proteins are up-regulated in the aortas of diabetic mice compared to controls and in cultured human aortic SMCs exposed to high glucose concentrations. Employing various pharmacological and molecular interventions we found that HDAC signaling pathways are implicated in the modulation of Nox expression and the ensuing ROS production in experimental diabetes. The data stand for the rationale for further preclinical investigation of HDAC inhibitors in the therapy of diabetes and associated vascular disorders.

2. Materials and methods

2.1. Materials

Standard chemicals, cell culture and molecular biology reagents and kits were obtained from Sigma-Aldrich, Thermo Fisher, and Qiagen. Primary and secondary antibodies were from Santa Cruz Biotechnology and Diagenode. Human HDAC2, HDAC4, HDAC6, and HDAC11 expression vectors were from Dharmacon. AP-1 luciferase *cis*-reporter plasmid was from Stratagene.

2.2. Animal model and treatment protocol

At 8 weeks of age, male C57BL/6J mice (The Jackson Laboratories) were rendered diabetic by injecting 55 mg/kg streptozotocin (STZ), intraperitoneally (i.p.) for 5 consecutive days [16]. One week after the last STZ injection, the glucose level was measured in the blood collected by tail puncture and only the animals with glycemia > 300 mg/dL remained in the study as diabetics. Citrate buffer-injected C57BL/6J mice were employed as controls. The animals divided into 3 experimental groups received (i.p.) vehicle (DMSO) or 15 mg/kg suberoylanilide hydroxamic acid (SAHA), a pan-HDAC inhibitor, every other day for 4 weeks. The experimental groups were: (i) control (non-diabetic) animals + vehicle (n = 12/group), (ii) diabetic mice + vehicle (n = 12/group), and (iii) diabetic mice + SAHA (n = 12/group). The dose and the procedure of SAHA administration were established in accordance with previous *in vitro* and *in vivo* studies [17]. The study was performed in agreement with the guidelines of EU Directive 2010/63/EU and the experimental protocols were approved by the Ethical

Committee at the Institute of Cellular Biology and Pathology “Nicolae Simionescu”.

2.3. Cell culture

Considering the prevalence of SMCs in the structure of the aortic wall, the *in vitro* studies were done on previously established and characterized human aortic SMC line [18]. SMCs were grown to confluency ($\approx 2 \times 10^6$ cells) in tissue culture plates (\emptyset 60 mm) in presence of 10% fetal bovine serum (FBS) and then made quiescent by culturing for 24 h culture in FBS-free Dulbecco's modified Eagle's Medium (DMEM, 5.5 mM glucose). The cells were further exposed (24 h) to DMEM containing normal (5.5 mM) or high (11–25 mM) glucose concentrations in the absence or presence of SAHA (1–10 μ M).

2.4. Real-time polymerase chain reaction analysis

Total RNA was extracted from the aortas of mice and cultured SMCs subjected to the above experimental conditions and reverse-transcribed into cDNA as previously described [19]. The Nox1, Nox4, Nox5, and p22phox mRNA expression levels were determined by amplification of cDNA employing SYBR® Green I and quantified by using the comparative C_T method [20]. The β -Actin mRNA expression was used for internal normalization. The sequences of oligonucleotide primers used in quantitative real-time PCR reactions are shown in Table 1 (Supplemental file).

2.5. Western blot analysis

Aortic tissue homogenization, cell lysate preparation and Western blot assays were performed as previously indicated [19]. Equal quantities of protein (aortic tissue: 30 μ g, cells: 50 μ g) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto nitrocellulose membranes (Biorad), blocked in TBS Blotto A reagent (sc-2333), and then exposed to the primary antibodies against HDAC1 (rabbit polyclonal, sc-7872, 1:200), HDAC2 (rabbit polyclonal, sc-7899, 1:200), Nox1 (rabbit polyclonal, sc-25545, 1:200), Nox2 (goat polyclonal, sc-5827 and mouse monoclonal, sc-130543, 1:200), Nox4 (rabbit polyclonal, sc-30141, 1:200), Nox5 (rabbit polyclonal, sc-67006, 1:200), malondialdehyde (goat polyclonal, sc-130087, 1:100) or β -Actin (mouse monoclonal, sc-47778, 1:500). Anti-rabbit IgG-HRP (sc-2370, 1:2000) and anti-mouse IgG-HRP (sc-2031, 1:2000) secondary antibodies were applied. The protein bands were digitally detected (ImageQuant LAS4000, Fujifilm, Japan) and quantified (TotalLab™). The protein expression level of β -Actin was used for internal normalization.

2.6. Measurement of ROS production

NADPH-stimulated ROS production was determined in the aortic segments and SMC homogenates as previously described [21,22]. The reaction was done in 50 mM phosphate buffer (pH 7.0) containing protease inhibitor cocktail, 5 μ M lucigenin, 1 mM CaCl₂ (for SMC homogenate), and 100 μ M NADPH. The reaction was initiated by the addition of aortic segments (\approx 500 μ g of vessel dry weight) or cell homogenate (\approx 100 μ g of protein) and the light emission was recorded in a luminometer (Berthold). After subtracting the blank chemiluminescence signal, the ROS production was calculated from the ratio of mean light units (MLU) to vessel dry weight (aorta) or total protein level (SMCs) and expressed as MLU/ μ g or MLU/ μ g protein, respectively.

The formation of ROS in the intact SMCs was evaluated employing carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) probe [23]. Briefly, SMCs were grown to confluence in tissue culture plates (\emptyset 60 mm) and incubated with 5 μ M DCF-DA for 1 h at 37 °C in serum-free culture medium. After loading with DCF-DA, the cells were washed,

detached, resuspended in HEPES-buffered saline solution (pH 7.4), and distributed at $\approx 5 \times 10^5$ cells/well into a 96-well microplate reader (Tecan). The DCF fluorescence emission was detected at 535 nm with an excitatory wavelength of 485 nm. The intracellular ROS production was calculated from the ratio of relative fluorescence units (RFU) to protein concentration and expressed as RFU/ μ g protein.

2.7. Transient transfection and luciferase reporter gene assay

Twenty-four hours before transfection, SMCs were seeded at 1×10^5 cells/well ($\approx 80\%$ confluence) into 12-well tissue culture plates. Transient transfection was performed as in [22] using Superfect™ reagent according to the manufacturer's protocol (Qiagen). Previously characterized pGL3-based luciferase reporter gene constructs [24,25] carrying the proximal promoter regions (≈ 1000 bp) of human Nox1, Nox4, and Nox5 genes were used. The optimized plasmid concentrations were as follows: 0.9 μ g/ml of Nox promoter-luciferase construct/pAP-1-luciferase cis-reporter plasmid [enhancer element configuration: AP-1 (7 \times); enhancer element sequence: (TGACTAA)₇], 0.1 μ g/ml pSV- β -galactosidase expression vector (Promega), and 0.3 μ g/ml of empty vector, HDAC2 (MHS1010–202697141, clone ID 4475960), HDAC4 (MHS6278–202759515, clone ID 6067363), HDAC6 (MHS6278–202755468, clone ID 2984860) or HDAC11 (MHS6278–202756917, clone ID 3906049) expression vectors. Promoter activity was calculated from the ratio of firefly luciferase to β -galactosidase levels (Beta-Glo™ assay system, Promega).

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as in [25] using reagents and protocols from Santa Cruz Biotechnology. The primary antibodies (2 μ g/ChIP) used for immunoprecipitation were as follows: HDAC1 (rabbit polyclonal, sc-7872), HDAC2 (rabbit polyclonal, sc-7899), p300 (rabbit polyclonal, sc-584), PolII/POLR2A (mouse monoclonal, C15200004), H3K27ac (rabbit polyclonal, C15410174), H3K9ac (rabbit polyclonal, C15410004), H3pan (mouse monoclonal, C15200011), and rabbit IgG (rabbit polyclonal, C15410206).

2.9. Statistical analysis

Data obtained from at least three independent experiments were expressed as mean \pm standard deviation. Statistical analysis was done by *t*-test and one-way analysis of variance followed by Tukey's post hoc test; $P < 0.05$ was considered as statistically significant.

3. Results

3.1. In vivo set-up of experimental diabetes

To investigate the long-term effects of diabetic conditions on vascular HDAC and the possible mechanistic links between HDAC, Nox expression and ROS production we employed STZ-induced diabetic C57BL/6J mice following the standard dosage and procedures as indicated above. The mice exhibiting hyperglycemia (glycemia > 300 mg/dl) were randomized in experimental groups that received either SAHA for 4 weeks or the vehicle in the same conditions. As expected at the end of the study, all diabetic animals had lower body weights and elevated blood glucose levels as compared to non-diabetic (control) animals. SAHA treatment did not affect significantly either the body weights or the blood glucose levels (Fig. 1).

3.2. HDAC1 and HDAC2 proteins are up-regulated in the aortas of diabetic mice

Previous studies showed that different HDAC subtypes are up-regulated in experimental models of heart failure, hypertension, and

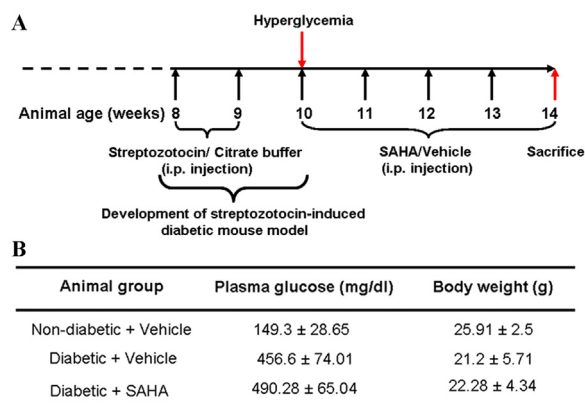


Fig. 1. The experimental design and characterization of the diabetic animal model. (A) Diagram showing the setup and the time periods of administration of HDAC pharmacological inhibitor (SAHA) or its vehicle (DMSO) to the diabetic C57BL/6J mice. (B) At the end of the experiment (14 weeks) plasma glucose level increased significantly in diabetic mice regardless of the SAHA or vehicle administration whereas the body weight of diabetic mice decreased as compared to non-diabetic (control) mice. Values represent the mean \pm SD ($n = 12$ per group).

aortic aneurisms [7]. Thus, we questioned whether diabetic conditions (i.e. hyperglycemia) have an impact on these proteins. To this purpose, HDAC1 and HDAC2 expression levels were assessed by Western blot assays using protein extracts derived from aortas isolated from diabetic and non-diabetic mice. The results showed that after 4 weeks of hyperglycemia the protein level of HDAC1 was significantly augmented (~ 1.7 fold) and that of HDAC2 was significantly increased (~ 3.5 fold) in the aortas of diabetic mice as compared to non-diabetic control animals (Fig. 2).

3.3. Pharmacological inhibition of HDAC down-regulates Nox expression and NADPH-stimulated ROS formation in the aortas of diabetic mice

To determine whether the up-regulated HDAC affects the expression of vascular Nox and ROS production, the diabetic animals were treated for 4 weeks with SAHA, a clinically approved HDAC inhibitor. At the end of the treatment the animals were sacrificed, the aortas were dissected out and further processed for RNA isolation and purification, protein extraction, and ROS measurement. In the aortas of diabetic mice compared to controls, the gene and protein expression levels of Nox isoforms were found significantly elevated. The induction of Nox1, Nox2, and Nox4 expression was blunted in SAHA-treated animals indicating the involvement of HDAC signaling in the regulation of these Nox subtypes under diabetic conditions (Fig. 3).

To investigate whether the HDAC-dependent pathways play a role in mediating NADPH-stimulated ROS production in the aortas of mice, we employed the lucigenin-enhanced chemiluminescence assay, a method that partially reflects the overall activity of Nox enzymes [26]. Consequently, the involvement of other enzymatic sources contributing to lucigenin signal should be taken into account. We found that NADPH-stimulated ROS production was significantly higher in aortic segments derived from diabetic mice as compared to non-diabetic animals (12700.56 ± 2196.01 MLU/ μ g vs. 4131.22 ± 1109.24 MLU/ μ g). Long-term administration (4 weeks) of SAHA to diabetic mice led to a significant decrease in ROS production (8514.28 ± 1180.64 MLU/ μ g) as compared to vehicle-treated diabetic mice (12700.56 ± 2196.01 MLU/ μ g) (Fig. 4).

3.4. High glucose concentrations up-regulate HDAC1 and HDAC2 protein expression in cultured human aortic SMCs

To validate the results obtained on the animal model, the in vivo experiments were complemented by in vitro studies on cultured human

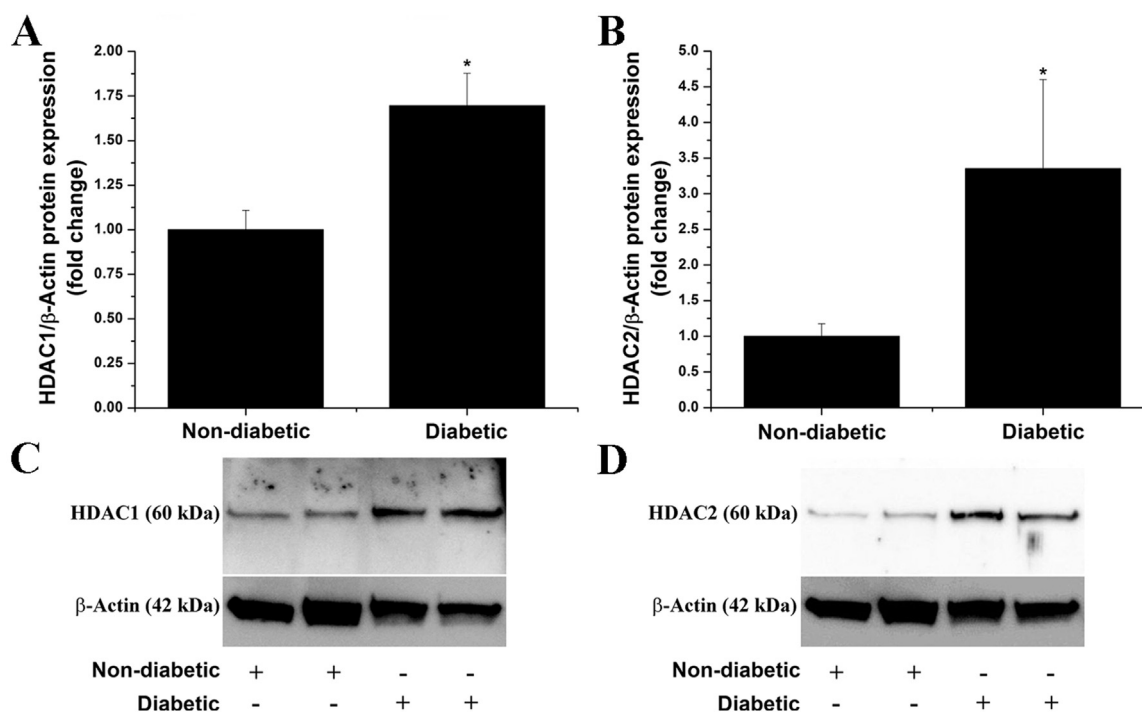


Fig. 2. Analysis of HDAC1 and HDAC2 protein expression in the aortas of non-diabetic and diabetic mice. (A, B) As revealed by Western blot analyses the relative expression of HDAC1 and HDAC2 proteins increased significantly in aortas of diabetic versus non-diabetic animals. (C, D) Representative immunoblots showing the induction of HDAC1 and HDAC2 proteins in the aortas of mice under diabetic conditions. $n = 4$, $*P < 0.05$. P -values were taken in relation to non-diabetic animals.

aortic SMCs. To mimic hyperglycemia-induced pathological changes of the vascular wall in diabetes, we searched for the effect of high glucose on HDAC1 and HDAC2 protein expression in cultured SMCs. Confluent quiescent SMCs were exposed for 24 h to increasing concentrations of glucose (5.5–25 mM) and then subjected to Western blot analysis (as described in the methods section). The results showed that exposure of SMCs to rising glucose levels resulted in a steady glucose concentration-dependent induction of HDAC1 (≈ 2.5 -fold at 25 mM glucose) and HDAC2 (≈ 2 -fold at 25 mM glucose) proteins (Fig. 5). We may safely hypothesize that high concentration of glucose alone, as an independent risk factor, induces epigenetic alterations in the vasculature, at least in part, by up-regulating enzymes of the HDAC superfamily.

3.5. Implication of HDAC-dependent pathways in mediating high glucose-induced Nox expression, ROS production, and protein carbonylation in human aortic SMCs *in vitro*

To test and validate our *in vivo* findings regarding the regulation of Nox expression and ROS production by HDAC in diabetic conditions, quiescent human aortic SMCs in culture were exposed for 24 h to normal (5.5 mM) or high (25 mM) concentrations of glucose in the absence or presence of SAHA (1–10 μ M). The gene and protein expression of Nox1, Nox4, and Nox5 subtypes were determined by real-time PCR and Western blot, respectively. The results showed that Nox isoforms were significantly up-regulated (Nox1: mRNA ≈ 2.9 -fold and protein ≈ 1.5 -fold; Nox4: mRNA ≈ 2.8 -fold and protein ≈ 1.75 -fold; Nox5: mRNA ≈ 1.9 -fold and protein ≈ 1.4 -fold) in SMCs exposed in culture to high glucose (25 mM) conditions when compared with cells exposed to normal glucose (5.5 mM). These results complement and confirm our previous work regarding the effect of high glucose on Nox expression in SMCs [22]. Interestingly, the high glucose-augmented gene and protein expression of the Nox subtypes were reduced to control levels (5.5 mM glucose condition) in high glucose-exposed cells treated with SAHA (Fig. 6). Notably, the mRNA expression level of the p22phox essential subunit was significantly increased (≈ 1.9 -fold) by high glucose concentration and was diminished following SAHA

treatment (Fig. 1 in Supplemental file). Collectively, these data demonstrate a similar pattern on HDAC-mediated, high glucose-induced up-regulation of Nox subtypes in cultured SMCs.

To assess whether HDACs are implicated in the production of ROS in SMCs, two different methods were used, namely NADPH-stimulated lucigenin-enhanced chemiluminescence assay (using cell homogenates) and DCF-based fluorescence method (employing intact cells). We found that high glucose concentration induced excess formation of ROS in SMCs in either cell homogenates (≈ 2.25 -fold) or living cells (≈ 1.3 -fold). Moreover, in both detection systems, SAHA reduced the high glucose-augmented production of ROS by a concentration mediated mechanism (Fig. 7A and B).

To further validate the ROS measurements in SMCs, the levels of malondialdehyde (MDA)-modified proteins were determined for an indirect estimation of MDA formation, an important biomarker of oxidative stress that results from oxidative decomposition of polyunsaturated fatty acids. We determined that exposure of cultured SMCs to high glucose concentration (25 mM) induced a marked accumulation (≈ 2 -fold) of MDA-protein adducts when compared with cells exposed to normal glucose (5.5 mM). Interestingly, SAHA treatment caused a concentration-dependent inhibition of MDA-protein adducts formation (Fig. 7C and D).

3.6. HDAC subtypes differentially regulate Nox1, Nox4, and Nox5 transcription

Based on the fact that SAHA is a pan-HDAC inhibitor, we next questioned whether representative HDAC isoforms belonging to each class of the Zn^{2+} -containing HDAC superfamily may influence the transcription of the Nox subtypes. Thus, to uncover the possible HDAC-gene promoter mechanistic links we performed co-transfection experiments using Nox1, Nox4, and Nox5 gene promoter-luciferase constructs and wild-type expression vectors for class I (HDAC2), class IIa (HDAC4), class IIb (HDAC6), and class IV (HDAC11) HDAC. The results showed that transient overexpression of HDAC2 led to a significant up-regulation of luciferase level directed by Nox1 (≈ 1.4 -fold), Nox4 (\approx

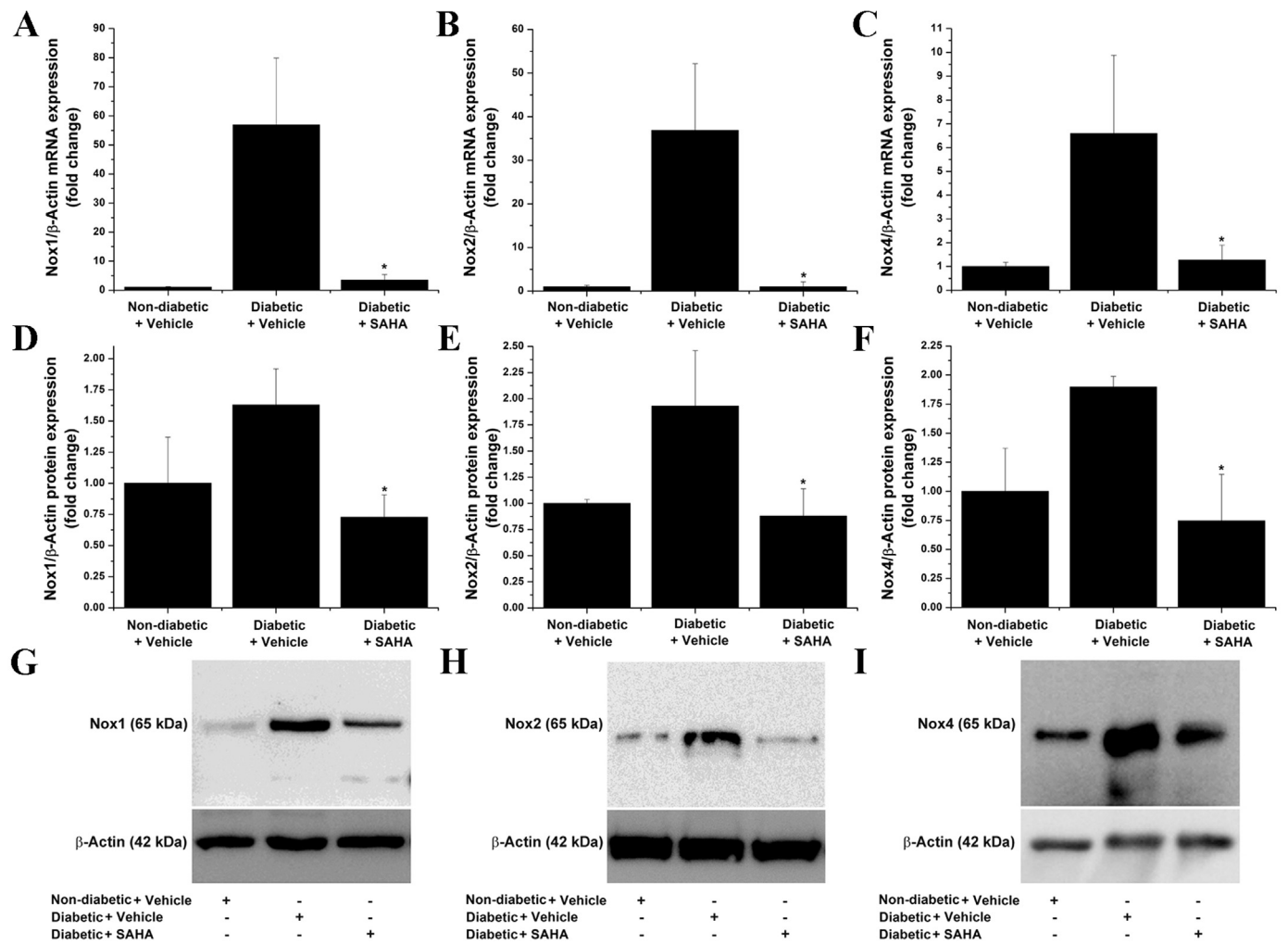


Fig. 3. Pharmacological inhibition of HDAC down-regulates the expression of Nox subtypes in the aortas of diabetic mice. (A-C) Relative mRNA expression of Nox1, Nox2, and Nox4 isoforms assessed by quantitative real-time PCR in the aortas of non-diabetic, diabetic, and SAHA-treated diabetic mice. (D-F) Western blot analysis of Nox1, Nox2, and Nox4 protein levels in each experimental condition. (G-I) Representative immunoblots depicting the modulation of Nox proteins expression. n = 5, *P < 0.05. Note that the up-regulation of Nox1, Nox2, and Nox4 mRNA and protein levels in the aortas of diabetic mice is blunted following SAHA treatment. P-values were taken in relation to vehicle-treated diabetic mice condition.

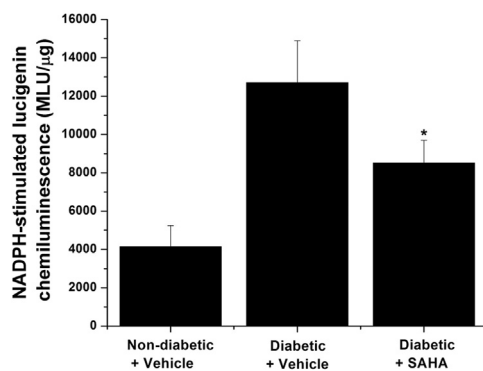


Fig. 4. Pharmacological inhibition of HDAC mitigates NADPH-dependent ROS formation in the aortas of diabetic mice. Lucigenin enhanced chemiluminescence assay was employed to measure NADPH-stimulated ROS production in aortic samples derived from non-diabetic animals and diabetic mice treated with vehicle or SAHA for 4 weeks. n = 5, *P < 0.05. P-values were taken in relation to diabetic state.

1.8-fold), and Nox5 (\approx 1.75-fold) proximal promoter regions. Likewise, significant increases in Nox1 (\approx 1.5-fold) and Nox5 (\approx 1.5-fold) promoter activities were detected in HDAC6-overexpressing cells. In contrast, transient overexpression of HDAC4 or HDAC11 displayed

opposite effects, acting as negative regulators of Nox4 and/or Nox5 promoters (Fig. 8). These results indicate that HDAC subtypes differentially regulate the transcriptional activation of the Nox isoforms via direct or indirect mechanisms.

To investigate the possible transcription factors interfering in transducing the effects of HDAC on Nox1, Nox4, and Nox5 transcription, we performed transfection assays using a control plasmid carrying the luciferase gene reporter under the control of AP-1 transcription factor, an important regulator of Nox expression and also a known target of HDAC. We found that HDAC2 overexpression resulted in a significant activation of AP-1 transcription factor (\approx 1.8-fold). Conversely, a significant reduction of AP-1 transcriptional activity was detected in HDAC4-overexpressing cells. No significant variations were detected in AP-1 activity in response to HDAC6 or HDAC11 overexpression (Fig. 9).

3.7. HDAC1 and HDAC2 are constitutively located within the proximal promoter regions of the Nox1, Nox4, and Nox5 genes

To investigate the physical interaction of HDAC1 and HDAC2 proteins with the proximal promoter regions corresponding to Nox1–5 subtypes, cultured SMCs were subjected to ChIP assays. Previously characterized primer sets flanking STAT, NF- κ B, and C/EBP transcription factor binding sites located in the proximal promoter regions of

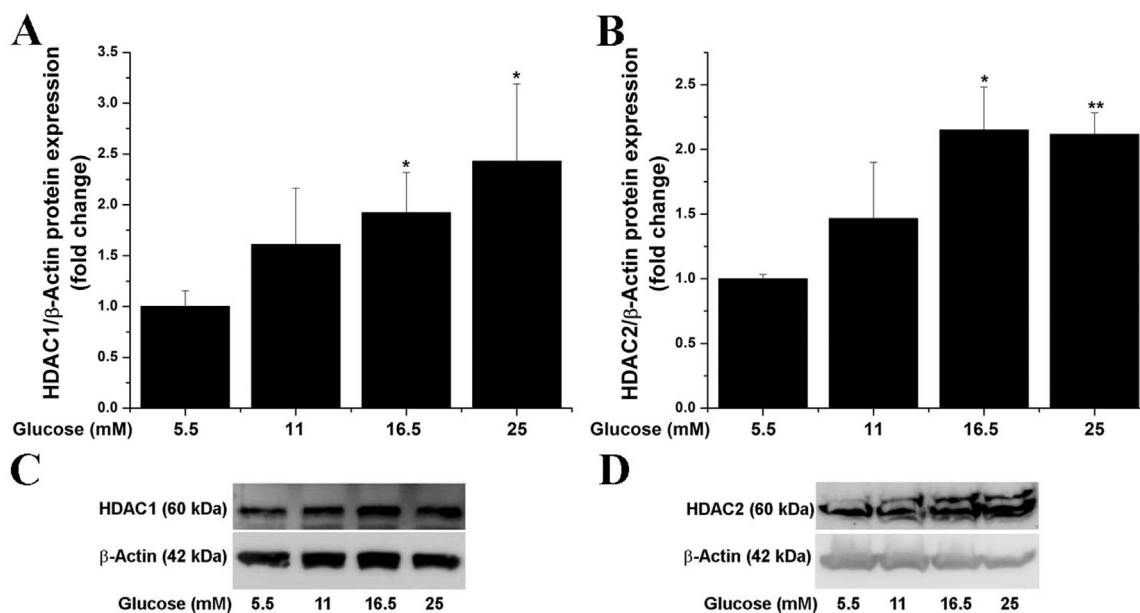


Fig. 5. In human aortic SMCs, glucose induces an increase in HDAC1 and HDAC2 protein expression in a concentration-dependent manner. (A, B) Induction of HDAC1 and HDAC2 protein levels quantified by Western blot analyses in cells exposed to 5.5 – 25 mM glucose. (C, D) Representative immunoblots depicting the increase in HDAC1 and HDAC2 proteins in response to rising glucose concentrations. $n = 4-5$, * $P < 0.05$, ** $P < 0.01$. P -values were taken in relation to SMCs exposed to 5.5 mM glucose.

Nox1, Nox4, and Nox5 genes were used [24,25,27,28]. The results showed that HDAC1 and HDAC2 proteins form bound complexes with Nox1–5 promoters in SMCs exposed to normal concentration of glucose. Notably, HDAC1 and HDAC2 colocalized with p300 (HAT) protein in the area of active transcription sites within Nox1–5 promoter regions as judged by the occurrence of POLR2A (the largest subunit of RNA polymerase II) or epigenetic marks of active gene expression (e.g., H3K27ac, H3K9ac) (Fig. 10A). No PCR amplification products were obtained when primary antibodies were replaced with rabbit IgG or were omitted (“no antibody” negative control) (Fig. 10B). The positive or negative interactions among Nox1/4/5 gene promoters and HDAC/p300/POLR2A/H3K27ac/H3K9ac are schematically depicted in Fig. 10C. Collectively, these findings demonstrate that both HAT and HDAC are involved in the regulation of the basic promoter activity of each Nox subtype.

To assess the level of histone acetylation within Nox1–5 gene promoter regions in response to high concentration of glucose, cultured SMCs were exposed to 5.5 mM or 25 mM glucose for 24 h and subjected to chromatin processing and analysis. Primers that tested positive for H3K27ac (primer sets 2 for Nox1/4/5) were used in ChIP real-time PCR assays. As shown in Fig. 10D, high glucose induced H3K27ac enrichment at the Nox1 (≈ 9 -fold), Nox4 (≈ 2 -fold), and Nox5 (≈ 2 -fold) gene promoters in SMCs.

4. Discussion

Emerging evidence indicates that HDAC-dependent epigenetic mechanisms play an important role in mediating cell proliferation, migration, and apoptosis, oxidative stress and inflammatory reactions in several experimental models of CVD [29,30]. Thus far, the impact of diabetic conditions on vascular HDAC system and its potential implication in the regulation of Nox enzymes have not been investigated.

Based on the current knowledge, we hypothesized that up-regulation of Nox and the ensuing ROS production as reported in numerous clinical and experimental studies on diabetes-associated CVD [21] may be directly or indirectly affected by alterations in histone acetylation system. To address this issue and to find out whether, in response to diabetic insults, induction of HDAC-dependent pathways is mechanistically involved in the up-regulation of Nox and ROS overproduction,

we designed *in vivo* and *in vitro* experiments employing diabetic mice and cultured human aortic SMCs.

The novel findings of this study are: (i) HDAC1 and HDAC2 are up-regulated in the aortas of diabetic mice as compared to non-diabetic animals; (ii) pharmacological inhibition of HDAC reduces the gene and protein expression of Nox isoenzymes and the NADPH-stimulated ROS overproduction in the aortas of diabetic mice; (iii) *in vitro*, high glucose concentrations induce a steady up-regulation of HDAC1 and HDAC2 proteins in human aortic SMCs; (iv) pharmacological inhibition of HDAC down-regulates the expression of Nox subtypes, ROS production, and the formation of MDA-protein adducts in high glucose-exposed SMCs; (v) transient overexpression of HDAC2 induces activation of Nox1–5 transcription and AP-1; (vi) HDAC1, HDAC2, and p300 proteins physically interact with the proximal promoters of Nox1–5 genes at the sites of active transcription in cultured SMCs; (vii) histone acetylation is enriched at the promoters of Nox genes in high glucose-exposed human aortic SMCs.

The beneficial effects of various HDAC inhibitors were reported in a number of experimental models of cardiovascular and metabolic diseases. *In vitro* and *in vivo* studies on neointima formation following vascular injury, hypertension, pulmonary arterial hypertension (PAH), and heart failure demonstrated that HDAC inhibitors, at relatively low concentrations, negatively interfere with ROS formation, inflammation, fibrosis, and pathological remodeling of the vascular wall and ischemic myocardium [7,31–33]. In experimental diabetes, HDAC inhibitors promote pancreatic β -cell development, proliferation, differentiation and function, and mitigate advanced diabetic microvascular complications [34,35]. However, the precise implication of HDAC-dependent pathways in these pathologies and the rationale of using HDAC inhibitors as potential therapeutic agents remain elusive.

HDAC inhibitors were initially designed as antiepileptic drugs and are currently considered for alternative treatment of depression and several neurodegenerative disorders including Alzheimer and Parkinson diseases. In the last decade, numerous HDAC inhibitors have been developed as novel cytostatic agents for cancer therapy (clinical trials phase I to III). Most HDAC isoforms are up-regulated in cancer cells, a condition that triggers histone hypoacetylation-induced chromatin condensation and subsequent transcriptional repression of key genes that control cell proliferation and growth arrest, differentiation

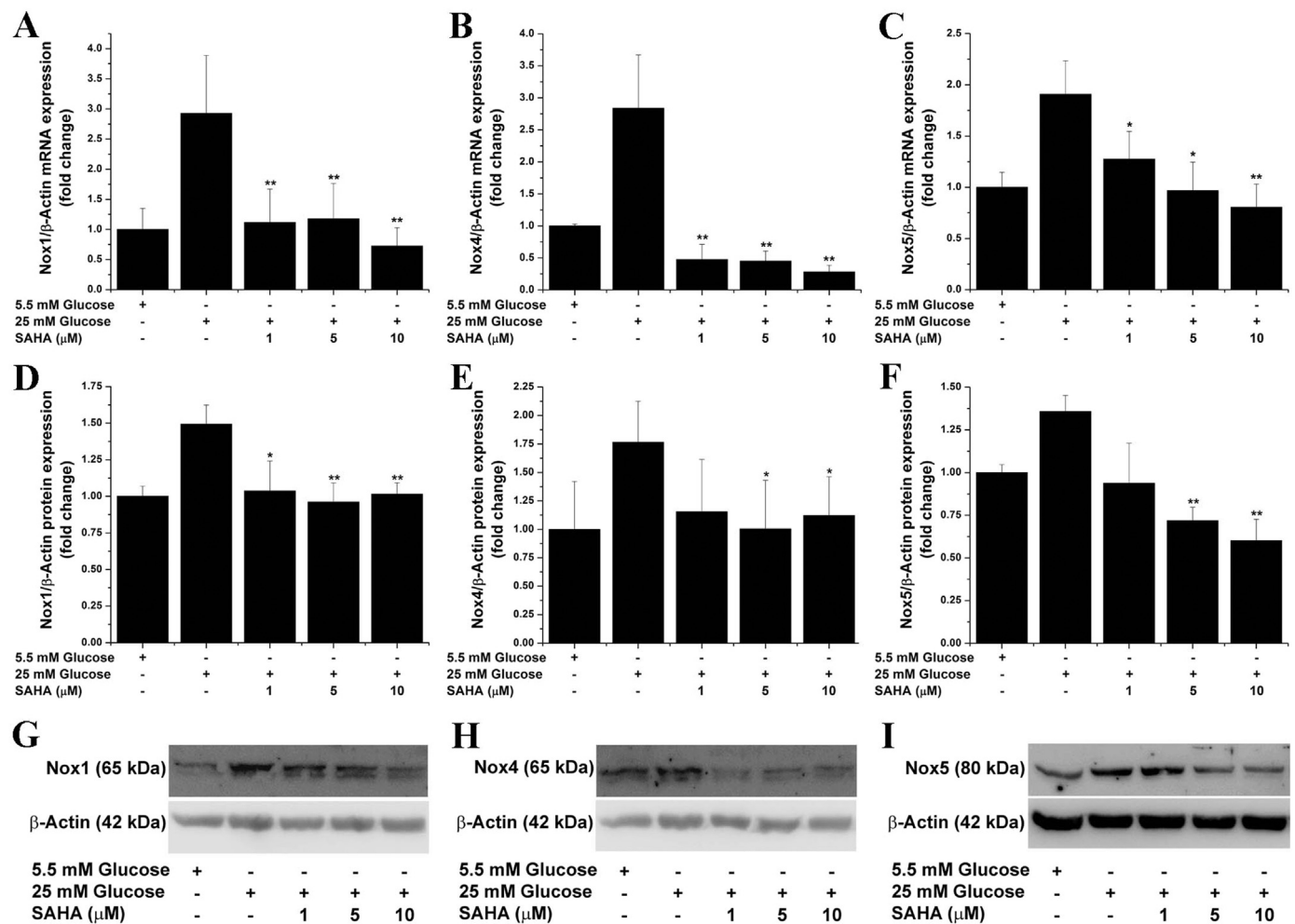


Fig. 6. HDAC-mediated, high glucose-induced up-regulation of Nox subtypes in cultured human aortic SMCs. (A–C) Concentration-dependent effects of SAHA on Nox1, Nox4, and Nox5 mRNA and (D–F) protein expression in high glucose-treated SMCs. Quiescent confluent cells were exposed (24 h) to normal (5.5 mM) or high (25 mM) glucose concentrations in the absence/presence of vehicle or SAHA (1–10 μ M). Quantification of Nox gene and protein levels was done by real-time PCR/Western blot using β -Actin as internal control. (G–I) Representative immunoblots depicting the modulation of Nox proteins in response to SAHA in high glucose-exposed cells. $n = 5–6$, * $P < 0.05$, ** $P < 0.01$. P -values were taken in relation to the cells exposed to 25 mM glucose.

and apoptosis. Thus, in an attempt to restore the balance between histone acetylation/deacetylation in different maladies, HDAC inhibition could represent an important therapeutic strategy. In addition, HDAC-mediated lysine-deacetylation of non-histone proteins, including transcription factors, has emerged as important signaling pathways in cell physiology and pathology. This particular aspect could explain several histone modifications - independent beneficial effects of some HDAC inhibitors [36].

The rationale of using HDAC inhibitors in cardiovascular disorders is based on the expression patterns of HDAC isoforms in humans and in various experimental models of disease. An augmented level of HDAC1, 2, 4, and 7 was detected in human samples obtained from patients undergoing abdominal aortic aneurysm repair and in the aortas of angiotensin II-infused apolipoprotein-E-deficient mice [37]. Increased HDAC1 and HDAC5 protein levels were found in the lung specimens derived from patients experiencing idiopathic PAH and in lungs and right ventricles of hypoxia-exposed rats [33]. Likewise, elevated HDAC3, 4, and 5 protein levels correlated with increased expression of Nox 2 and Nox4 isoforms have been detected in the pulmonary arteries in a rat model of PAH [12]. Our data extend these observations on vascular HDAC expression pattern in experimental diabetes. We found that in the aortas of diabetic mice (compared with control animals) the HDAC1 and HDAC2 protein are up-regulated concurrently with the increased expression of Nox1, Nox2, and Nox4 isoforms and NADPH-

stimulated ROS production. To further investigate the mechanistic link between these proteins, the *in vivo* experiments were complemented by *in vitro* studies on cultured human aortic SMCs. We found that exposure of these cells to increasing concentrations of glucose led to a steady up-regulation of HDAC1 and HDAC2 proteins, indicating that high glucose could trigger the induction of HDAC isoforms in diabetes. Interestingly, it was reported that mitogenic (serum) stimulation of cultured rat aortic SMCs results in a time-dependent induction of HDAC1, 2, and 3 proteins [31]. In line with this evidence, we have recently shown that high glucose promotes human aortic SMCs proliferation in a concentration-dependent manner. This is particularly important since high glucose-induced vascular SMCs growth, proliferation, and migration is one of the main pathological manifestations of diabetes-associated vascular disorders [22].

Others and we have shown that various protein kinases, nuclear receptors, pro-inflammatory and mitogenic transcription factors are coordinately involved in the up-regulation of Nox and ROS production [24,25,27,28]. Thus, targeting of up-stream regulators of Nox (i.e., HDACs), may represent an appropriate strategy to mitigate the up-regulated Nox expression and the ROS overproduction in diabetes.

Previous studies demonstrated that HDAC inhibition down-regulates the dexamethasone-induced Nox1 expression in A7r5 cells [38] and Nox4 in cultured human ECs [10,11]. Recently, it was demonstrated that HDAC inhibitors down-regulate the expression of Nox2 and

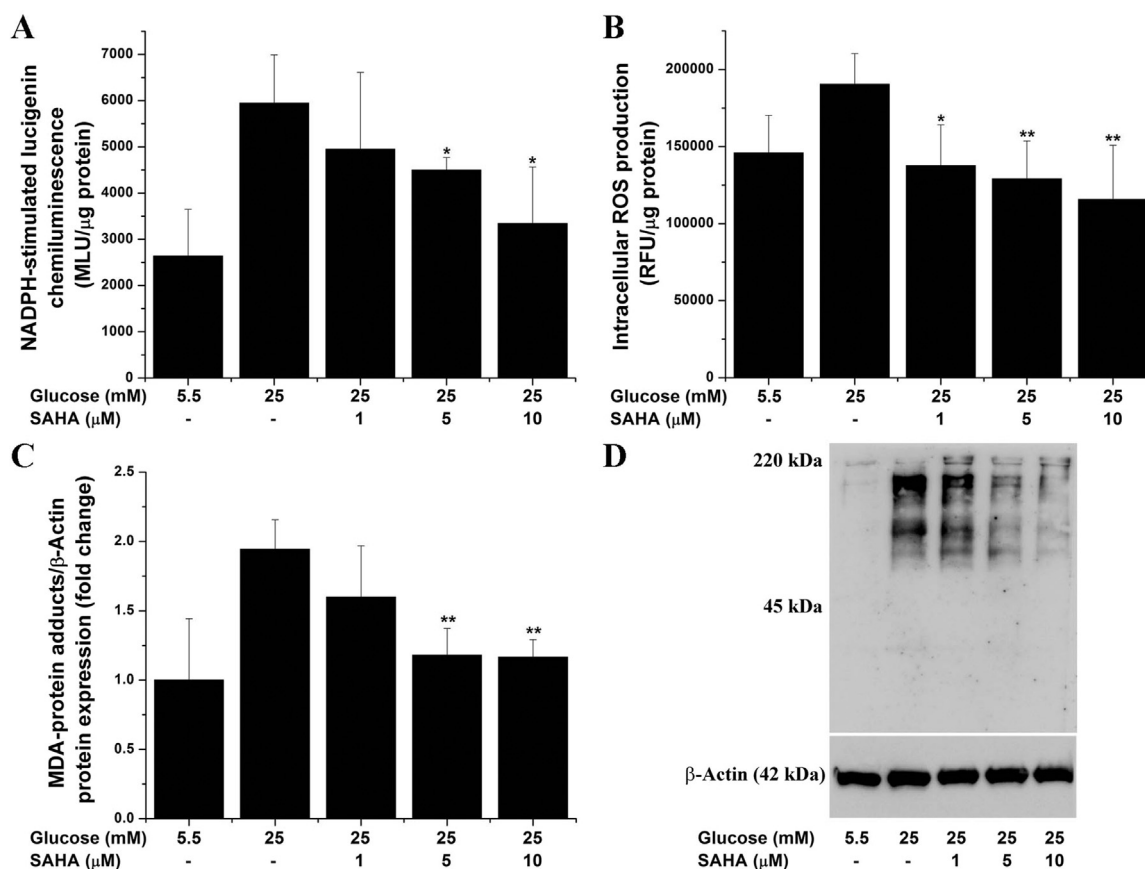


Fig. 7. Pharmacological inhibition of HDAC decreases the high glucose-induced ROS production and the formation of MDA-protein adducts in human aortic SMCs. (A) NADPH-stimulated ROS formation assessed by lucigenin enhanced chemiluminescence. (B) Intracellular formation of ROS measured by DCF fluorescence assay. (C) Western blot analysis of MDA-protein adducts formation. (D) Representative immunoblot showing the induction of MDA-protein adducts in high glucose-exposed cells and the regulatory effects of SAHA. $n = 4-5$, * $P < 0.05$, ** $P < 0.01$. P -values were taken in relation to 25 mM glucose condition.

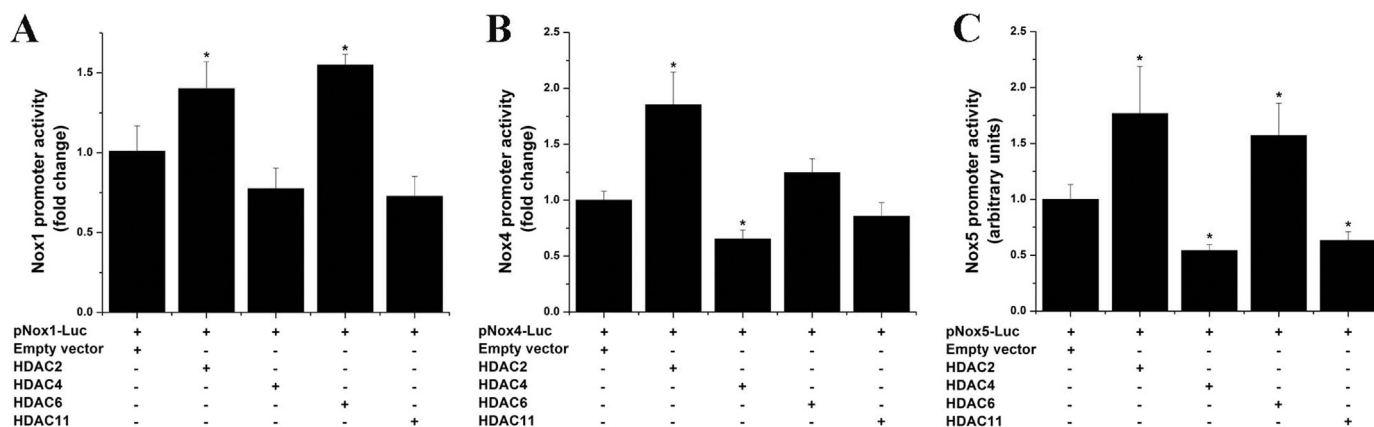


Fig. 8. Analysis of Nox1 (A), Nox4 (B), and Nox5 (C) gene promoter activities in cultured SMCs overexpressing HDAC2 (class I HDAC), HDAC4 (class IIa HDAC), HDAC6 (class IIb HDAC) or HDAC11 (class IV HDAC) proteins. $n = 4-5$, * $P < 0.05$. P -values were taken in relation to the corresponding empty vector control.

Nox4 in pulmonary arteries of PAH rats and the mRNA levels of Nox1, Nox2, Nox4, and Nox5 in various cell types including macrophages, lung microvascular endothelial cells, lung fibroblasts, and human epithelial colorectal adenocarcinoma cells [12]. Interestingly, it was found that *Anaplasma phagocytophilum*, a gram-negative bacterium, down-regulates the expression of gp91phox (Nox2, CYBB) via HDAC1-dependent mechanisms [39].

To investigate the implication of HDAC-dependent signaling in mediating vascular Nox overexpression in diabetes, we used SAHA (i.e., vorinostat, Zolinza™ as marketed by Merck), the first HDAC inhibitor that was approved (2006) by the U.S. Food and Drug Administration as

alternative treatment or in combination with other drugs for cutaneous T cell lymphoma. Mechanistically, SAHA binds to-, and inhibits Zn^{2+} -containing HDACs and typically targets class I, II, and IV HDAC [29].

Interestingly, the microarray studies showed that not all the genes are similarly regulated by HDAC inhibitors, some being up-regulated and other down-regulated. Thus, it has become evident that many pharmacological effects of HDAC inhibitors are, in some circumstances, independent of histone acetylation-induced chromatin conformational changes and most likely are driven by gene-specific regulators such as transcription factors and transcriptional co-regulators [40].

In our study we found that administration of SAHA reduced the

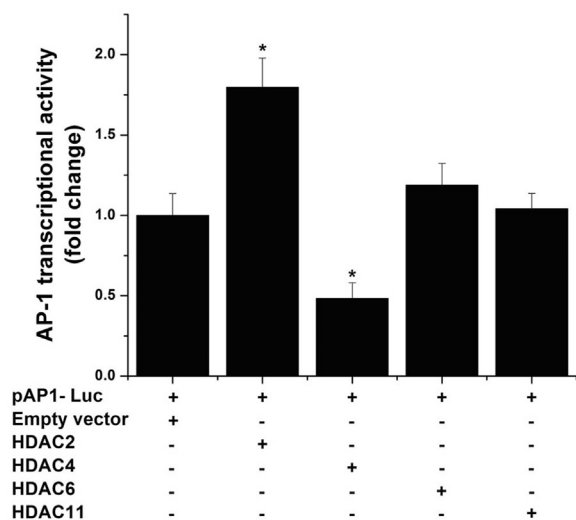


Fig. 9. Analysis of AP-1 transcription factor activation in cultured SMCs overexpressing HDAC2 (class I HDAC), HDAC4 (class IIa HDAC), HDAC6 (class IIb HDAC) or HDAC11 (class IV HDAC) proteins. $n = 4$, * $P < 0.05$. P -values were taken in relation to the corresponding empty vector control.

gene and protein expression of Nox1, Nox2, and Nox4 subtypes along with the NADPH-stimulated ROS formation in the aortas of diabetic mice. Likewise, SAHA down-regulated the transcript and protein levels of Nox1, Nox4, and Nox5 isoforms, and the formation of ROS and MDA-protein adducts in high-glucose exposed human aortic SMCs in vitro. Collectively, these data indicate that members of the HDAC superfamily mediate vascular Nox up-regulation and ROS formation in diabetic conditions. Our data are in agreement with a recent study demonstrating that inhibition/silencing of HDAC reduces ROS production, protein carbonylation and the expression levels of Nox2 and Nox4 in PC12 cells [41].

The specific assessment of Nox activity is challenging [26]. To determine the NADPH-dependent formation of ROS in the aortas of mice, lucigenin-enhanced chemiluminescence assay was employed. Notably, evidence exists that NADPH-stimulated lucigenin signal does not reflect Nox activity [42]. Thus, in the absence of data demonstrating the direct modulation of Nox-derived ROS we can not conclude that Nox activity is regulated by SAHA and therefore multiple sources of ROS should be considered including nitric oxide synthase or cytochrome P450 monooxygenase. Yet, it is important to note that whatever the molecular sources, the NADPH-stimulated production of ROS in the vessels of diabetic animals was significantly reduced following SAHA treatment. Our in vitro studies on cultured human SMCs provide additional support for this evidence and highlight the potential of HDAC inhibitors to reduce oxidative stress in diabetic conditions.

Previous mechanistic studies on β -cells have demonstrated that HDAC inhibitors display anti-inflammatory effects by negatively interfering in $\text{IFN}\gamma$ -induced Jak/STAT signaling in experimental diabetes and that overexpression of HDAC1, HDAC2, and HDAC3 enhanced STAT1-dependent transcriptional activity [43]. Since Jak/STAT pathway is implicated in the regulation of Nox expression and ROS production [24], one hypothesized that various HDACs may be directly or indirectly involved in the up-regulation of Nox in diabetic conditions via STAT-dependent mechanisms. Other than Jak/STAT, the transcriptional functions of NF- κ B and AP-1, key regulators of the Nox isoenzymes [28,44], are tightly controlled by acetylation/deacetylation via direct/indirect mechanisms [10,45,46].

Notably, it was recently demonstrated that HDAC inhibitors down-regulate Nox expression by inhibiting the recruitment of RNA polymerase II and p300 at the sites of active transcription, a condition that negatively affects the formation of transcriptional complexes within gene promoter regions of the Nox isoforms [12].

Based on the fact that SAHA inhibits multiple HDAC isoforms, we questioned whether different classes of HDACs differentially affect the transcription of the Nox subtypes. To address this issue, we performed co-transfection assays employing Nox promoter-luciferase constructs and overexpression vectors for class I, IIa, IIb, and IV of the HDAC superfamily. We have demonstrated that transient overexpression of typically SAHA-targeted HDACs (e.g., HDAC2 - class I, HDAC6 - class IIb) up-regulated the promoter activities of Nox1, Nox4, and Nox5 genes. Conversely, overexpression of HDAC4 (class IIa) or HDAC11 (class IV) decreased the luciferase level directed by the Nox1 and Nox5 promoters. Collectively, these data suggest that HDAC isoforms may induce opposite effects on Nox transcription. Notably, unlike highly organized genomic DNA, the promoter-luciferase constructs lack complex chromatin-like histone packing and networking with other transcriptional regulators. Thus, the transactivation of specific Nox subtype in response of HDAC overexpression in vitro may not reflect the biological activity of endogenous Nox1, Nox4, and Nox5 gene promoters.

To investigate the potential involvement of HDACs in mediating transcription factor activation we performed co-transfection experiments using a control plasmid carrying highly conserved AP-1 binding sites cloned upstream to the luciferase reporter gene and the aforementioned HDAC expression vectors. The results showed that transient overexpression of HDAC2 resulted in a marked up-regulation of luciferase level directed by AP-1 elements whereas HDAC4 induced the down-regulation of AP-1 transcriptional activity. These evidences indicate the different effects of HDACs in relation to AP-1 function. Our data are on line and extend previous studies examining the effects of HDAC on AP-1 expression and activity [10,47].

To gain additional mechanistic insights on Nox gene promoter – HDAC interactions in cultured human aortic SMCs we performed ChIP assays. We found that HDAC1 and HDAC2 proteins are constitutively located at the sites of active transcription within Nox1, Nox4, and Nox5 proximal promoter regions as indicated by the presence of the RNA polymerase II (POLR2A) and epigenetic markers of active gene expression namely, H3K27ac and H3K9ac. Interestingly, ChIP assays showed that p300 (HAT) is also present within Nox1, Nox4, and Nox5 proximal promoter regions. Moreover, real-time PCR analysis indicated that high glucose induces histone H3K27ac enrichment at the promoters of Nox genes in cultured SMCs. Collectively, our data suggest that high glucose activates HAT- and HDAC-dependent pathways that are coordinately involved in the up-regulation of Nox. The overall effect consists in a global increase in histone acetylation, a condition that may contribute to chromatin relaxation thereby facilitating the access of specific transcription factors (e.g., AP-1) to their consensus DNA elements in the genome. In this context, members of the HDAC superfamily regulate the activity of specific transcription factors via direct or indirect mechanisms rather than histone deacetylation. Our data are in good agreement with previous studies on mouse mammary tumor virus promoter (MMTV) indicating that HDAC1 is constitutively present and positively regulates MMTV promoter activity by histone acetylation-independent mechanisms [48,49]. Thus, one can safely assume that HDAC isoforms induce Nox transcription via histone modification-independent mechanisms, most likely by acting on non-histone proteins such as activation of specific transcription factors or repression of negative regulators of Nox expression. The up-regulation of AP-1 transcription factor activity, an important regulator of Nox, in response to HDAC2 overexpression further supports this hypothesis.

Collectively, the data of this study indicate the existence of a mechanistic link among induction of HDAC expression, up-regulation of Nox, and ROS overproduction in vascular cells in diabetes. Further studies are required to uncover the precise cross-communications between HDAC, HAT, transcription factors, nuclear receptors, and different signaling systems that control Nox expression and function in diabetes.

In-depth exploration of the novel “diabetes-HDAC-Nox-ROS axis” as an alternative molecular mechanism regulating ROS formation may be

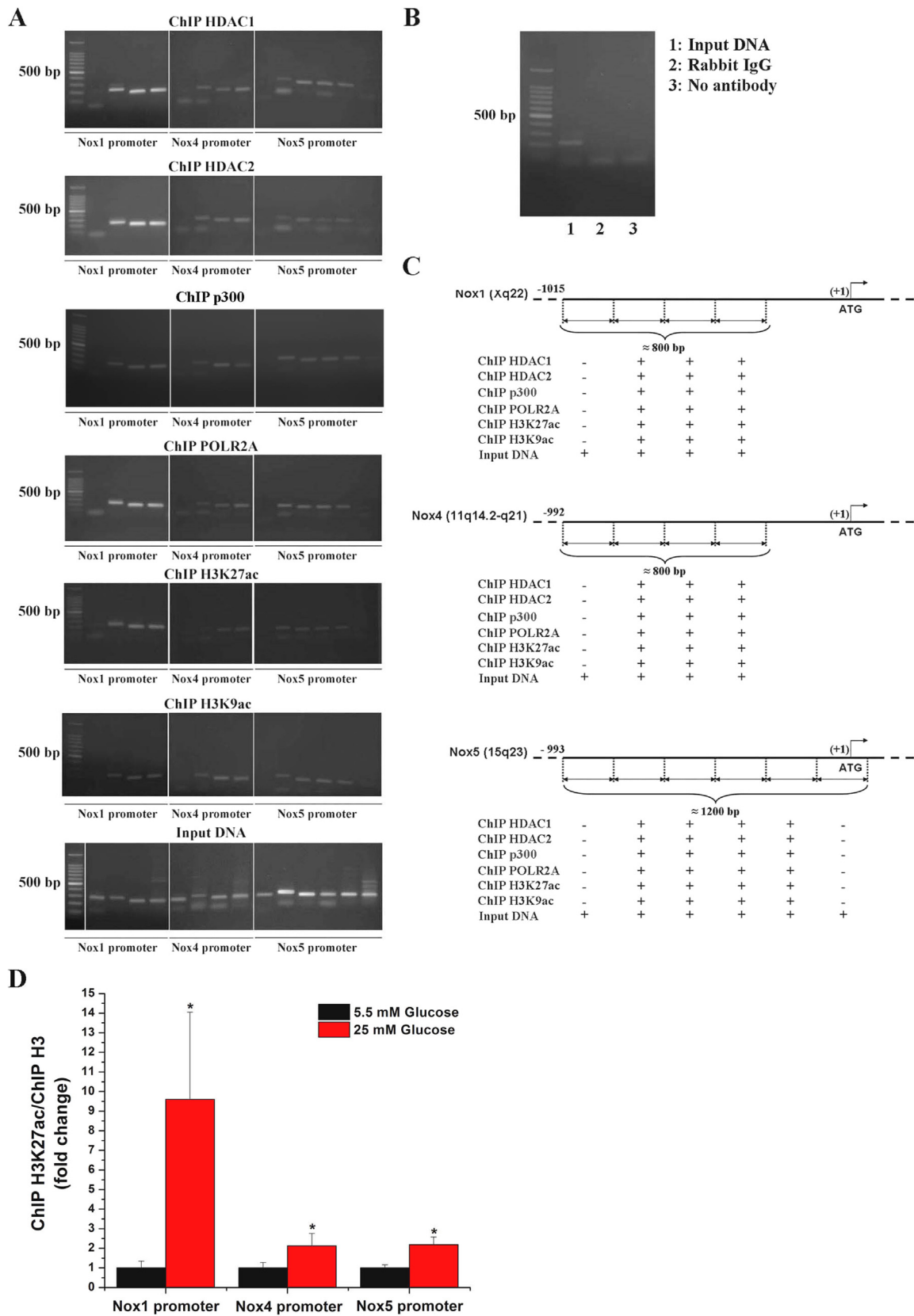


Fig. 10. ChIP analysis of HDAC/HAT recruitment and histone modification (acetylation) at the proximal promoter regions of Nox genes in cultured human aortic SMCs. (A) Representative agarose gel electrophoresis illustrating the physical interaction of HDAC1, HDAC2, and p300 proteins with Nox1–5 promoters at the sites of active transcription (i.e., presence of POLR2A, H3K27ac, H3K9ac). (B) Rabbit IgG and “No-antibody” negative controls. (C) Schematic drawing of Nox1 (chromosome Xq22), Nox4 (chromosome 11q14.2-q21), and Nox5 (chromosome 15q23) gene promoters depicting the relative positions of the primers sets. The positive or negative DNA – protein interactions is represented by +/- marks. (D) Assessment of H3K27ac enrichment at the level of Nox1, Nox4, and Nox5 gene promoters in SMCs exposed to normal (5.5 mM) or high (25 mM) concentrations of glucose. n = 3–4, * P < 0.05. P-values were taken in relation to 5.5 mM glucose condition.

the foundation of new oxidative stress-oriented therapeutic approaches in diabetes and associated cardiovascular disorders.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.redox.2018.03.011>.

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