Transcriptional Regulation of Endothelial Arginase 2 by Histone Deacetylase 2

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Objective—Arginase 2 (Arg2) is a critical target in atherosclerosis because it controls endothelial nitric oxide, proliferation, fibrosis, and inflammation. Regulators of Arg2 transcription in the endothelium have not been characterized. The goal of the current study is to determine the role of specific histone deacetylases (HDACs) in the regulation of endothelial Arg2 transcription and endothelial function.

Approach and Results—The HDAC inhibitor trichostatin A increased levels of Arg2 mRNA, protein, and activity in both human aortic endothelial cells and mouse aortic rings. These changes occurred in both time- and dose-dependent patterns and resulted in Arg2-dependent endothelial dysfunction. Trichostatin A and the atherogenic stimulus oxidized low-density lipoprotein enhanced the activity of common promoter regions of Arg2. HDAC inhibition with trichostatin A also decreased endothelial nitric oxide, and these effects were blunted by arginase inhibition. Nonselective class I HDAC inhibitors enhanced Arg2 expression, whereas the only selective inhibitor that increased Arg2 expression was mocetinostat, a selective inhibitor of HDACs 1 and 2. Additionally, mouse aortic rings preincubated with mocetinostat exhibited dysfunctional relaxation. Overexpression of HDAC2 (but not HDAC 1, 3, or 8) cDNA in human aortic endothelial cells suppressed Arg2 expression in a concentration-dependent manner, and siRNA knockdown of HDAC2 enhanced Arg2 expression. Chromatin immunoprecipitation indicated direct binding of HDAC2 to the Arg2 promoter, and HDAC2 overexpression in human aortic endothelial cells blocked oxidized low-density lipoprotein–mediated activation of the Arg2 promoter. Finally, overexpression of HDAC2 blocked oxidized low-density lipoprotein–mediated vascular dysfunction.

Conclusions—HDAC2 is a critical regulator of Arg2 expression and thereby endothelial nitric oxide and endothelial function. Overexpression or activation of HDAC2 represents a novel therapy for endothelial dysfunction and atherosclerosis.

Key Words: endothelial cells • epigenomics

Atherosclerotic cardiovascular disease is the most important cause of mortality in the Western world. Its pathobiology involves chronic inflammation of the vascular wall, resulting from endothelial dysfunction, adhesion molecule expression, and monocyte infiltration of the intima, ultimately leading to plaque development. It is well established that oxidized low-density lipoprotein (OxLDL) is one of the most important proatherosclerotic molecules and its effects are mediated by binding to the lectin-like OxLDL receptor (LOX-1) and thence by stimulation of proinflammatory gene expression, reactive oxygen species production, and down-regulation of endothelial protective nitric oxide (NO) production.1,2 Our group has previously demonstrated that exposure of endothelium to OxLDL induces the activation of arginase 2 (Arg2), resulting endothelial nitric oxide synthase (eNOS) uncoupling because of substrate -arginine depletion. This in turn leads to an increase in eNOS-dependent ROS generation and a decrease in NO production.2–4 Furthermore, we and others have demonstrated that both biochemical inhibition and genetic knockdown of endothelial Arg2 prevent eNOS uncoupling, endothelial dysfunction, and atherosclerotic plaque burden in atherogenic mice.4

Interestingly, our data suggest that the increase in endothelial Arg2 activity is dependent on 2 events, 1 of which is early and another that occurs later and is more long-lasting. The early process involves a posttranslational event: intracellular decompartmentalization from mitochondria where it resides in quiescent cells5 to the cytoplasm (unpublished data). The later regulatory process involves a transcriptional event that leads to an upregulation in Arg2 gene expression. Given the critical role of Arg2 in the regulation of endothelial function, its transcriptional regulation remains of great interest, but...
it remains incompletely defined. Some recent insights include upregulation of Arg2 by S6K and mTOR activation, and its transcriptional downregulation by pharmacological inhibition with rapamycin. Additionally, epigenetic modification such as methylation of the Arg2 promoter may regulate its transcription.

Interest in epigenetic mechanisms that regulate gene expression is growing. Histone modifications are known to be critical for transcriptional activity, and histone acetylases and deacetylases allow gene expression to be exquisitely regulated through chromatin remodeling. An increase in histone acetylation reduces DNA histone binding, and this allows greater access for DNA transcription factors. Deacetylation has the opposite effects. Although the role of histone deacetylases (HDACs) in tumorigenesis is well established and HDAC inhibitors are being tested as novel drugs for the treatment of cancer (for review), the role of HDACs in the regulation of endothelial proteins and function is less well established.

There are 18 different HDACs that are classified into 4 groups: Class I (HDACs 1, 2, 3, and 8), Class II (HDACs 4, 5, 6, 7, 9, and 10), Class III (SIRT1–7), and Class IV (HDAC11).

We tested the hypotheses that HDACs are critical regulators of endothelial Arg2 expression and that modulation of HDACs would impact endothelial function. Our data demonstrate that HDAC2 regulates Arg2. HDAC2 downregulation leads to endothelial dysfunction, and overexpression of HDAC2 improves endothelial function in an Arg2-dependent fashion.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
HDAC Inhibition Impairs Endothelial-Dependent Vascular Relaxation
Previous findings from the study by Rossig et al have shown that trichostatin A (TSA), a broad-spectrum HDAC inhibitor with promising effects on a variety of human cancer cells, may cause impairment of endothelium-mediated vascular relaxation. This response was attributed to a small decrease in eNOS expression, but the amount of this decrement raises the question of whether other genes that are regulated by HDACs may have contributed to the attenuated vascular relaxation responses to acetylcholine that Rossig’s group observed. Because Arg2 is a competitive inhibitor of eNOS that impairs endothelial function, we used Arg2 knockout mice and biochemical inhibitors to examine the possibility that Arg2 may mediate the diminished vascular relaxation caused by TSA.

Preincubation of aortic rings of wild-type mice with 200 nmol/L of TSA for 18 hours led to impairment of acetylcholine-mediated (endothelium-dependent) vasorelaxation compared with rings treated with vehicle alone (Emax 31.24±7.8 versus 81.9±6.2% for controls; P<0.001, n=6), but this TSA-mediated impairment of vasorelaxation was abolished in wild-type aortic rings that were pretreated with the arginase inhibitor amino-2-borono-6-hexanoic acid (ABH) (Emax 65.7±12.1%; P<0.05; n=6). Preincubation of aortic rings from Arg2−/− mice with TSA (200 nmol/L) showed no impairment of endothelial cell–dependent vasorelaxation compared with vehicle-treated vessels (Emax 66.3±7.54 versus 79.0±10.7% in controls; ns; n=6) (Figure 1A–1D). There was no significant difference in sodium nitroprusside (SNP)-mediated vasorelaxation (an endothelium-independent response because SNP is a NO donor) in aortas that were treated with TSA or MGC0103 versus the vehicle-only control. These findings strongly suggest that TSA-mediated impairment of vascular relaxation occurs through the upregulation of Arg2 in the intimal endothelium.

HDACs Regulate Arg2 Expression and Activity
To define and quantify the nature of TSA modulation of Arg2 expression, human aortic endothelial cells (HAECs) were treated with 400 nmol/L TSA for 18 hours as suggested by the manufacturer (Cell Signaling), and arginase activity and protein expression were then measured. TSA increased both total arginase activity and Arg2 expression (Figure 2A and 2B). The effect of TSA on Arg2 protein expression was found to be dose dependent. HAECs were incubated with increasing concentrations of TSA (0, 0.1, 0.3, and 1 μmol/L). TSA treatment for 18 hours increased Arg2 in a dose-dependent fashion, with a maximum effect at 1 μmol/L (Figure 2C; Figure IA in the online-only Data Supplement). The effect was also time dependent with an increase in Arg2 protein expression observed as soon as 8 hours after TSA (1 μmol/L) exposure (Figure 2D; Figure IB in the online-only Data Supplement). TSA dose and incubation time were chosen to be 1 μmol/L and 18 hours, respectively, for subsequent experiments with HAECs. We next used adenoviral constructs of Arg1 and Arg2 shRNA to confirm that the increase in arginase activity is because of Arg2 rather than Arg1 upregulation. As shown in Figure 2E, Arg2 shRNA inhibited the TSA-mediated (1 μmol/L) increase in arginase activity, whereas Arg1 shRNA had no effect. To determine whether the TSA effects on increased Arg2 activity and protein expression resulted from changes at the transcriptional level, we examined Arg2 mRNA levels in HAECs and isolated mice aorta that were exposed to TSA using Arg2-specific primers (see Materials and Methods) for both RT-polymerase chain reaction (Figure 2F) and real-time polymerase chain reaction reactions. Arg2 mRNA was substantially upregulated in both cultured HAECs and mice aorta that had been preincubated with TSA (Figure 2F–2H). Incubation of isolated segments of mice aortas in TSA (200 nmol/L) also induced a robust increase in arginase activity (Figure 2I). This reduced...
concentration of TSA was used for experiments with aorta to protect their fragile intima from toxicity.

The NAD-dependent Sirtuin family (SIRT or Class III) of HDACs has been implicated in endothelial function. To determine whether SIRTs are involved in the regulation of Arg2 expression, we incubated HAECs in the presence of the SIRT inhibitor nicotinamide. We observed no changes in the Arg2 mRNA expression in HAECs after incubation with 0.1 or 0.2 mmol/L of nicotinamide for 18 hours (Figure 2F).

Interestingly, short-term inhibition (30 minutes) of SIRT with nicotinamide (5 mmol/L) had an activating effect on HAEC arginase activity, whereas TSA (1 μmol/L) had no effect in this time frame as shown in Figure 2J. This suggests that SIRTs might be important in the short-term regulation of Arg2 activity, and the time course of this effect implies a mechanism involving posttranslational modification.

HDAC Inhibition by TSA Does Not Affect Arg2 Ubiquitination

Posttranslational modification by ubiquitin subjects proteins to proteosomal degradation and, therefore, affects their abundance. Because both acetylation and ubiquitination target lysine residues of proteins, there is a significant cross talk between these 2 pathways. To determine whether Arg2 is ubiquitinated and whether increased acetylation resulting from exposure to HDAC inhibitors may compete with ubiquitination sites on Arg2 and, thus, increase Arg2 abundance by that mechanism, we immunoprecipitated FLAG from HAECs coexpressing FLAG epitope-tagged Arg2 and HA-tagged ubiquitin and immunoblotted for FLAG. As shown in Figure 3, bands migrating at higher molecular weights were observed in cells expressing both Arg2 and ubiquitin. However, preincubation with TSA had no effects on the ubiquitination of Arg2, and this excludes ubiquitin as a potential mechanism for TSA-mediated augmentation of Arg2 abundance. This is the first report to show that Arg2 is modified by ubiquitin.

HDAC Inhibition-Mediated Increases in Arg2 Expression Occur at the Transcriptional Level via Upregulation of Arg2 Promoter Activity

To determine whether TSA affects transcription of Arg2, HAECs were pretreated with actinomycin D and cycloheximide for 1 hour before treatment with TSA (1 μmol/L). TSA
Histone deacetylase (HDAC) inhibition upregulates arginase 2 (Arg2) activity, mRNA, and protein expression. Human aortic endothelial cells (HAECs) were incubated with 0.4 μM of trichostatin A (TSA) for 18 hours and (A) arginase activity was measured in cell lysates using urea production assay and (B) Arg2 protein levels were determined using Western blot (WB). C, Dose-dependent (0, 0.1, 0.3, and 1 μM/L and D) time-dependent (3, 2, 4, 8, and 18 hours) effects of TSA (1 μM/L) on endothelial Arg2 protein expression were determined using WB. E, HAECs were transduced with 15 MOI of adenoviruses encoding either nontargeted, Arg2 or Arg1 shRNA. Arg2 promoter activity was increased in cells treated with either agent (Figure 4A). Next we determined whether inhibition of HDAC affects Arg2 promoter activity. HAECs were transiently transfected with different fragments of a −1 kb region of the human Arg2 promoter (PI) that were cloned into the pGL3 luciferase (LUC) reporter plasmid. The following day, cells were treated with TSA (1 μM/L) for 8 hours, and luciferase activity was measured using a luciferase kit (Promega). As shown in Figure 4B, Arg2 promoter activity was increased in cells treated with TSA compared to control cells that were treated with vehicle alone. The −0.3-kb (PII) and −0.65-kb (PIII) fragments of the Arg2 promoter exhibited the highest activity after TSA exposure, whereas the fragment between −1 kb and −0.65 kb, PIV, was not responsive to TSA stimulation. The effect of TSA on the Arg2 promoter was also found to be dose dependent as shown in Figure 4C. These findings strongly suggest that TSA activates the Arg2 promoter.

Inhibition of HDACs by TSA Attenuates NO Production

Next, we evaluated the functional consequences of HDAC inhibition on downstream endothelial cell signaling. Because eNOS-derived NO plays a central role in endothelial function, we assessed eNOS activity by measuring NO production using 3 different assays. DAF-DA fluorescence (Figure 5A and 5B) and the Siever’s NO analyzer (Figure 5C) were used to determine basal NO production from HAECs, and this failed to occur when cells were coincubated with TSA and ABH (1 μM/L). As shown in Figure 5A and 5B, inhibition of HDAC with TSA significantly decreased NO production in HAECs, and this failed to occur when cells were coincubated with TSA and ABH. A DCFH-DiOxyQ-based fluorescence assay (Figure 5C) was used to determine NO radical levels in a growth medium from 293 cells that were expressing calcium-insensitive eNOS that were treated with either

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TSA alone or together with ABH. Two hundred ninety-three cells lack native eNOS expression, but do produce abundant Arg2. Cell medium from 293 cells transfected with a control plasmid (LacZ) was used as a control background signal that was subtracted from all the groups containing calcium-insensitive endothelial nitric oxide synthase. TSA decreased NO production from 293 cells expressing calcium-insensitive endothelial nitric oxide synthase. In the presence of ABH, TSA failed to attenuate NO production from 293-calcium-insensitive endothelial nitric oxide synthase cells (Figure 5C). These findings support the hypothesis that the increase in Arg2 that is caused by HDAC inhibition is the major underlying mechanism that drives the diminished eNOS activity with TSA.

Arg2 Expression Is Specifically Regulated by HDAC2

To test whether augmented Arg2 expression was an effect limited to TSA, 2 other broad-spectrum HDAC inhibitors were tested together with TSA–SAHA and Scriptaid. We found that incubation of HAECs for 18 hours with 1 μmol/L of all 3 agents upregulated Arg2 expression to similar levels (Figure 6A). In contrast, these inhibitors had negligible effect on eNOS expression (Figure 6A). To determine the specific HDAC that regulates Arg2 expression, we first used class-specific commercially available pharmacological inhibitors (see Table I in the online-only Data Supplement for IC50 data), nicotinamide (5 mmol/L), a potent Sirtuin (Class III NAD-dependent HDACs) inhibitor, had no effect on Arg2 mRNA levels (Figure 2F) or Arg2 protein levels (Figure 6C and 6D), suggesting that this family of HDACs is not likely to regulate Arg2 expression. Class I–specific inhibitor that is selective for HDAC1 and HDAC3, entinostat (MS275, 2 μmol/L), Class I-specific and HDAC1-selective inhibitor, tacedinaline (CI994, 1 μmol/L), Class II-specific MC1568 (0.5 μmol/L), and the HDAC6/HDAC8 selective inhibitor-PC34051 (100 nmol/L) all had no substantial effects on endothelial Arg2 expression. In contrast, Class I specific inhibitor mocetinostat (MGCD0103, 1 μmol/L), which selectively inhibits both HDAC1 and HDAC2, did upregulate Arg2 expression (Figure 6B–6D). This screening suggested that HDAC2 was the most likely candidate for a direct role in Arg2 regulation.

To investigate whether MGCD0103 had similar effects on vascular relaxation as those seen with TSA, we incubated isolated mice aortic rings in MGCD0103 (1 μmol/L) for 18 hours. Vascular reactivity was then measured in the presence of either acetylcholine or SNP. MGCD0103 significantly attenuated endothelial-dependent vascular relaxation compared with vessels treated with vehicle alone (E max 42.7±8.5 versus 82.8±7.4%; P<0.001; n=6) as shown in Figure 6D. Endothelium-independent relaxation as assessed by SNP was unaffected by MGCD0103 (Figure 6E). To further determine the role of Arg2 in MGCD0103-mediated vascular dysfunction, we measured dose-dependent vascular relaxation in MGCD0103-treated aortas isolated from Arg2−/− mice treated...
with either acetylcholine or SNP and found no significant differences in endothelium-dependent relaxation compared with aortas from Arg2−/− that were treated with vehicle alone (E\textsubscript{max} 62.4±16.5 versus 79.0±10.7%; ns; n=6) (Figure 6F and 6G).

We next transfected HAECs with increasing concentrations of Class I HDAC (HDAC 1, 2, 3, and 8) cDNAs (0.3 and 1 μg). HDAC 1, 3, and 8 overexpression did not have any effect on Arg2 expression (Figure 7A–7C), whereas HDAC2 overexpression decreased Arg2 expression (Figure 7D; Figure VA in the online-only Data Supplement). Additionally, we did not observe any substantial change in Arg2 expression with increased expression of other HDACs that have been implicated in the regulation of endothelial cell function—HDAC 4, 5, 6, and 7 (Figure IIIA and IIB in the online-only Data Supplement). To further confirm the involvement of HDAC2 in Arg2 regulation, we silenced HDAC2 in HAECs using siRNA (Qiagen). Arg2 expression was significantly increased with HDAC2 siRNA knockdown (Figure 7E; Figure VB in the online-only Data Supplement). Because we observed a slight increase in Arg2 expression with PC34051, a specific HDAC8 inhibitor, we evaluated whether HDAC8 has an impact on Arg2 expression. We demonstrated that HDAC8 is expressed in HAECs by using RT-polymerase chain reaction (Figure IIA in the online-only Data Supplement). HDAC7, which is well known to be expressed by endothelial cells, was used as a positive control. Using siRNA-mediated knockdown and ectopic transfection-mediated overexpression of HDAC8 in HAECs, we showed that HDAC8 is not an important regulator of Arg2 expression (Figure IIA and IIB in the online-only Data Supplement; Figure 7C).

**HDAC2 Effects on Activation of the Arg2 Promoter by OxLDL**

We next examined whether the atherogenic stimulus OxLDL, which is known to increase Arg2 expression,\(^\text{14}\) (also demonstrated in Figure 8A) had any effect on Arg2 promoter activity. Arg2 promoter fragments were cloned into luciferase constructs as described in Figure 4B. Indeed, OxLDL robustly increased the activity of specific Arg2 promoter fragments (Figure 8B). Interestingly, similar to TSA, fragments between -1kb and 0.3kb were not responsive to OxLDL (Figure 8B). This suggested that common Arg2 promoter regions are regulated by atherogenic stimuli and HDACs. To determine whether OxLDL-mediated activation of the Arg2 promoter is a dose-dependent phenomenon, we transfected HAECs with Arg2 (−1 kb) promoter in a luciferase construct and stimulated cells with increasing concentrations of OxLDL (0, 25, 50, and 100 μg/mL). As shown in Figure 8C, OxLDL increased Arg2 promoter activity in HAECs in dose-dependent manner. Interestingly, the

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**Figure 5.** Attenuated nitric oxide production in trichostatin A (TSA)-treated human aortic endothelial cells (HAECs) was restored by arginase inhibition. HAECs were incubated with either TSA (1 μmol/L) alone or together with amino-2-borono-6-hexanoic acid (ABH; 1 mmol/L). A, Representative epifluorescence images and (B) quantitative analysis of DAF-DA fluorescence were used to assess nitric oxide (NO) production. C, A NO analyzer from Siever was used to measure byproducts of NO, mainly nitrite, in HAEC cell media that were incubated with either TSA alone or coincubated with TSA and ABH. D, Two hundred ninety-three cells that do not express native endothelial nitric oxide synthase (eNOS) were expressed with constitutively active eNOS constructs and incubated with either TSA alone or together with ABH. The DCFH-DioxyQ-based fluorescent assay from Cell Biolabs was used to determine NO radicals in cell culture media. *P<0.05 vs control, **P<0.05 vs TSA alone, $P<0.05 vs TSA+ABH. DAF fluorescence images are representative of 3 independent experiments. CleNOS indicates calcium-insensitive endothelial nitric oxide synthase; RFU, XXX; and RNS, XXX.
activating effect of 50 μg/mL of OxLDL on Arg2 promoter activity was completely abolished in HAECs transduced with adenovirus encoding HDAC2. Further, we were able to amplify the Arg2 promoter bound to immunoprecipitated HDAC2 from HAEC DNA/protein complexes (Figure 8D) using a ChIP assay. To further determine whether OxLDL modulates HDAC2 abundance, we exposed HAECs to increasing doses of OxLDL (0, 25, 50, and 100 μg/mL) and measured HDAC2 expression. HDAC2 expression was progressively decreased by exposure to 50 and 100 μg/mL of OxLDL (Figure 8E; Figure VC in the online-only Data Supplement). These cells simultaneously exhibited a dose-dependent increase in Arg2 expression, suggesting a reciprocal regulation of Arg2 by HDAC2 (Figure 8E, second panel). We further demonstrated that OxLDL-mediated increases in HAEC Arg2 expression (Figure 8F) were abolished by HDAC2 overexpression (Figure 8D; Figure VD in the online-only Data Supplement). This finding is further supported by recent observations that link reduced HDAC2 expression with OxLDL exposure.9 These results strongly suggest that decreased HDAC2 abundance is a critical downstream mechanism for OxLDL-mediated increase in Arg2 expression during atherogenesis. It is possible that other transcriptional regulators may also modulate Arg2 expression under conditions of cellular stress. The Arg2 promoter sequence, candidate regulators, and their potential targets in the Arg2 promoter sequence are summarized in Tables II and III in the online-only Data Supplement.

Additionally, we showed that increased expression of a histone acetyl transferase, CBP, which causes global cell acetylation, has no effect on the endothelial Arg2 expression (Figure IV in the online-only Data Supplement). This further supports the notion that the transcriptional effect on Arg2 expression involves HDAC2 interaction with the Arg2 promoter, and this effect is not occurring simply by enhanced transcriptional accessibility because of increased global acetylation.
OxLDL-Mediated Impairment of Vascular Relaxation Is Ameliorated by HDAC2 Overexpression

We next determined whether HDAC2 overexpression could be used as a strategy for reversing vascular dysfunction because of atherogenic stimuli such as OxLDL. Isolated mouse aortic rings were transduced with either HDAC2 or GFP (control) adenoviruses and incubated with or without OxLDL (50 μg/mL) for 48 hours. As shown in Figure 9A, OxLDL significantly impaired endothelial-dependent vascular relaxation (acetylcholine study) compared with the untreated controls ($E_{\text{max}}$ 52.1±3.1% versus 93.0±2.8%; $P<0.0001$; $n=6$), suggesting that endothelial function is compromised in this group. The dose–response effect of SNP on OxLDL-incubated aortic rings was not significantly different from that in the untreated controls, suggesting that OxLDL does not affect the endothelial-independent component of blood vessel reactivity. Furthermore, mice aortic rings overexpressing HDAC2 (Figure 9C; Figure VE in the online-only Data Supplement) exhibited significantly improved acetylcholine-dependent vascular relaxation despite OxLDL exposure compared with GFP-overexpressing aortic rings exposed to OxLDL ($E_{\text{max}}$ 78.4±5.2% versus 52.1±3.1%; $P<0.0001$; $n=4–6$) (Figure 9A). There was no significant difference in SNP-induced vascular relaxation between these groups (Figure 9B). Consistent with studies in cultured HAECs, Arg2 expression was significantly attenuated in mice aortas overexpressing HDAC2 (Figure 9C, second panel). Increased expression of HDAC2 in HAECs also decreased the total arginase activity, and we have found this activity to be predominantly due to Arg2 (data not shown). We next determined whether increased expression of Arg2 as observed with knockdown of HDAC2 affects NO production in HAECs. Indeed, HDAC2 shRNA significantly attenuated NO production as determined with DAF-FM DA fluorescence, and this was rescued above the control levels in the presence of Arg2 shRNA (Figure 9E). NO production was also significantly augmented in HAECs where only Arg2 was knocked down. The effectiveness of Arg2 and HDAC2 shRNAs in HAECs is demonstrated in Figure VIA and VIB in the online-only Data Supplement.

Discussion

Work on this current report of the role of HDAC2 in the regulation of Arg2 expression is inspired by our previous observation that OxLDL activation of Arg2 in HAECs has a bimodal time line. Increases in arginase activity occurring as early as minutes to hours are attributable to posttranslational modifications of Arg2 and resulting changes in its subcellular compartmentalization14 (unpublished data). We also noted that Arg2 mRNA levels rose as early as 4 hours after OxLDL exposure, a delayed peak in Arg2 protein levels occurred beginning at 12 hours, and these changes were ablated by Actinomycin D and cycloheximide, respectively. Data presented here provide a mechanism for the second and longer term of these 2 effects, whereby OxLDL triggers the release of the Arg2 promoter from the negative epigenetic transcriptional regulation exerted by HDAC2. This leads to an increase in Arg2 transcription and translation and is followed by substantial increments in Arg2 uncoupling of eNOS that begin 12 to 18 hours later, and may have a much longer duration. Precedents for these effects may be found in the work of others. Thus, HDAC1 regulation of Arg1 has been reported in macrophages (albeit in an activating manner), and several groups have reported a mixture of up- and downregulation by HDACs in genes with promoters containing cAMP response elements.15,16 Fass et al16 proposed a model for the constraint of NUR77 gene transcription by HDAC in which binding of the promoter to the preinitiation complex is prevented solely by HDAC activity; TSA and other HDAC inhibitors, therefore, trigger NUR77 transcription by inhibiting HDAC activity by either direct or indirect means.
Our findings that TSA and OxLDL interact with the same regions of the Arg2 promoter (Figures 4B, 4C, 8B, and 8C) and that the Arg2 promoter eluted with immunoprecipitated HDAC2 in a ChIP assay (Figure 8D) suggest that the transcriptional activation of Arg2 by OxLDL occurs via HDAC2 inhibition. Indeed, there is evidence from the work of others that show that atherogenic stimuli such as OxLDL and flow modulate HDAC2 expression.9,17 These findings are consistent with the current study in which we observe dose-dependent decreases in HDAC2 expression and simultaneous increases in Arg2 expression with increasing concentrations of OxLDL (Figure 8E). It is possible that OxLDL may also inhibit HDAC2 activity and thereby remove the block that HDAC2 activity presents for Arg2 promoter binding to its preinitiation complex. This hypothetical scenario gains credence from recent precision mapping of RNA polymerase, which indicates that promoter initiation complexes can extend up to 30 base pairs from the transcription start site and physically contact pausing complexes until these tethers are released.18 Indeed, we found a dose-dependent relationship between OxLDL concentrations and activation of the Arg2 promoter (Figure 8C).

Furthermore, OxLDL has been implicated as a potent inhibitor of Class III HDAC SIRT1 in vascular smooth muscle cells found in atherosclerotic lesions and has been shown to regulate both SIRT3 and aldehyde dehydrogenase 2 activity in HAECs.19,20 A similar pattern of oxidative influence over epigenetic transcriptional regulation is seen in redox modulation of the HDAC control over lineage commitment events in neural progenitor cells.21

The notable finding and a major impetus for the current study is that the attenuated endothelial-dependent vascular relaxation, caused by the broad-spectrum HDAC inhibitor TSA, is almost completely rescued by pharmacological inhibition or genetic knockout of Arg (Figure 1). Aortic rings preincubated with Class I specific HDAC inhibitor, MGCD0103 (which is highly specific for HDAC1 and HDAC2), also exhibited significantly attenuated vascular relaxation, a pathophysiologic consequence that is highly consistent with the molecular findings in the current study.

Figure 8. Increased endothelial arginase 2 (Arg2) expression in response to oxidized low-density lipoprotein (OxLDL) involves promoter activation and histone deacetylase (HDAC) 2. A, Human aortic endothelial cells (HAECs) were exposed to 50 μg/mL of OxLDL for 18 hours and subjected to Western blot (WB) using Arg2 antibody. B, Luciferase activity was determined in HAECs that were transfected with pGL3 luciferase (LUC) reporter plasmids consisting of either −1 kb human ARG2 promoter or 1 of 3 different promoter fragments (as illustrated above the graph in Figure 4B) and incubated with 50 μg/mL of OxLDL for 18 hours. C, HAECs were transfected with an LUC reporter plasmid containing the −1-kb human ARG2 promoter, and cells were exposed to increasing doses of OxLDL (0, 25, 50, and 100 μg/mL) for 18 hours. Firefly luciferase activity was measured and normalized to Renilla luciferase. GFP and HDAC2 adenoviruses were added 8 hours after transfection with the luciferase constructs. D, Binding of HDAC2 to the Arg2 promoter was quantified using a ChIP assay. E, HAECs were exposed to increasing doses of OxLDL (0, 25, 50, and 100 μg/mL) for 18 hours and immunoblotted for HDAC2 and Arg2. F, HAECs with adenoviral-mediated HDAC2 overexpression or controls (Ad-GFP) were exposed to OxLDL, and expression levels of Arg2, HDAC2, and GAPDH (loading control) were determined with WB. *P<0.05 vs control. WB results are representatives of 3 to 4 independent experiments. IB indicates XXX; and RLU, XXX.
The demonstration of HDAC8 expression in vascular endothelial cells (Figure IIA in the online-only Data Supplement) is the first report of its kind that we know of. HDAC8 has been implicated in many biological contexts including tumorigenesis, myocardial hypertrophy, and gene silencing during craniofacial development. Its role in the regulation of endothelial transcription has yet to be determined.

Targeted drug development for atherogenesis has included both preventive and remedial strategies and has largely focused on decreasing plaque burden. Our data and wider experience in preclinical studies in cancer biology indicate that transcriptional regulators such as HDAC2 may offer a safer and more effective way forward, provided that specific agents can be developed. A small molecule strategy that maintained the inhibition of Arg2 transcription by upregulating/activating HDAC2 activity in the specific context of the Arg2 promoter would be an ideal reagent. Indeed, in the context of COPD, resistance to the anti-inflammatory response to corticosteroids may be explained by a reduction in oxidative and nitrosylation stress-induced reduction in HDAC2 expression. Low-dose theophylline increases HDAC2 activity and expression in alveolar macrophages and restores steroid responsiveness. Although more selective small molecule strategies are under development, translational testing of HDAC2 enhancers that are already widely available, such as methylxanthines, is an intriguing possibility.

**Summary**

Our data indicate that HDAC2 tonically limits the availability of the eNOS competitor Arg2 in vascular endothelium by constraining Arg2 transcription in baseline homeostatic conditions, and that this brake on the Arg2 population in HAECs is released by oxidative injury. Precise details of molecular interactions between HDAC2, OxLDL, and the Arg2 promoter have yet to be elucidated, but this line of inquiry may lead to therapeutic interventions that restore and preserve vascular endothelial health against the epidemic onslaught of oxidative injury and atherogenesis.

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Disclosures

D.E. Berkowitz is a scientific founder and consultant for Corridor Pharmaceuticals Inc, a biotechnology company dedicated to the development of therapeutics targeting arginase in diseases in which endothelial dysfunction is an important contributing factor. The other authors report no conflicts.

References


Significance

Despite the emphasis on lifestyle risk management and the advent of statins, atherosclerotic cardiovascular disease remains the leading cause of morbidity and mortality in the West. HDACs that control the expression of various genes by removing an acetyl group from histones are emerging as potential targets in cancer therapeutics and cardiovascular disease. HDAC2 has been recently implicated in atherosclerosis, although the mechanism remains elusive. We demonstrate that inhibition of HDAC2 leads to upregulation of Arg2 and reciprocal impairment in endothelial nitric oxide production and endothelial dysfunction. The demonstration that HDAC inhibition leads to profound endothelial dysfunction offers a potential note of caution regarding the use of nonspecific HDACs as cancer therapeutics. Furthermore, the identification of a single HDAC, HDAC2, as a transcriptional regulator of endothelial Arg2 and vascular homeostasis, suggests that this enzyme may be a critical node in the modulation of endothelial health.
Transcriptional Regulation of Endothelial Arginase 2 by Histone Deacetylase 2
Deepesh Pandey, Gautam Sikka, Yehudit Bergman, Jae Hyung Kim, Sungwoo Ryoo, Lewis Romer and Dan Berkowitz

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Detailed Material and Methods

Animals and reagents
All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at The Johns Hopkins University School of Medicine. C57BL6 mice were purchased from Jackson Laboratory. Ox-LDL that were prepared by the reaction of LDL and CuSO₄ was purchased from Intracel Co (Frederick, MD). All HDAC inhibitors were purchased from Selleckchem (Houston, Tx) except TSA, which was purchased from Cell Signaling. Unless otherwise stated, all reagents were obtained from Sigma. HDAC2, HDAC8 siRNA was purchased from Qiagen. HDAC2 antibody was purchased from ABGENT. Arginase 2 antibody was purchased from Sigma (St. Louis, Mo).

Cell culture
HAEC were maintained in ECM culture medium (Science Cell Research Laboratories, Carlsbad, CA) according to the supplier’s protocol. 293 cells were transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer’s protocol and HAEC were transfected using Amaxa transfection system (Lonza).

RTPCR and real time PCR:
Total RNA was extracted from segments of mice aortas and HAEC using Trizol and PureLink (Invitrogen). RNA was then reverse transcribed with oligo dT primers to obtain cDNA and quantitative real time PCR (Applied Biosystems) was performed using SYBR Green Supermix mix (Applied Biosystems) whereas semiquantitative RTPCR was performed using conventional Biorad PCR machine and the following primer sets: Mouse Arg2: Forward: 5’-GGG CCC TGA AGG CTG TAG-3’, Reverse: 5’-AAT GGA GCC ACT GCC ATC-3’, Human Arg2: Forward: 5’-GGG CCC TGA AGG CTG TAG-3’, Reverse: 5’-AAT GGA GCC ACT GCC ATC-3’, Human HDAC8: Forward: 5’-AAC ACG GCT CGA TGC TGG-3’, Reverse, 5’-CCA GCT GCC ACT TGA TGC-3’; Human HDAC7 Forward 5’-CCC AGC AAA CCT TCT ACC A-3’; 5’-AAG CAG CCA GGT ACT CAG G-3’.
Real Time PCR data are expressed as “fold change”, which is calculated as 2-ΔΔCt. This is derived as follows: Delta Ct (ΔCt) denotes the difference in Ct values between the gene of interest (ARG2 in our case) and the housekeeping gene 18s; Delta Delta Ct (ΔΔCt) denotes the difference in “Delta Ct” values between data from samples that are untreated and those that are treated groups with TSA, and this is the ‘fold change’.

DNA constructs and Adenoviruses: FLAG-tagged HDAC2 was constructed by PCR using untagged HDAC2 purchased from the Arizona State University plasmid repository as a template using and following primers: Forward 5’-CAC CAT GGA TTA CAA GGA TGA CGA CGA TAA GG-3’; Reverse, 5’-TTA CAA GGG GTT GCT GAG CTG TTC TGA TTT GGT TCC-3’. The PCR product was cloned into pcDNA3.1 using directional topo cloning (Invitrogen) Adenoviruses encoding FLAG-HDAC2 was constructed by subcloning into entry PENTR1a vector using BamH1 and EcoRv restriction enzymes followed by LR recombination with destination vector PDEST. The HDAC2-PDEST DNA was digested with Pael, ethanol precipitated and transfected into 293 HEK cells. After cytopathic effect (CPE), adenoviruses were collected and purified via three freeze-thaw cycles and a Millipore adenovirus purification Kit.
Adenovirus encoding shRNA

Ad-shNontargeted, Ad-shArg1 and Ad-shArg2 encoded viruses were generated using a pAdBLOCK-iT kit (Life Sciences). Briefly, oligonucleotides targeting 2 different regions of Human Arg1 and Arg2 and non-targeted sequence were designed with proprietary software from Life Sciences and cloned into pU6-ENTR. Sequences used were as follows.

Non targeted: Top, 5’-CAC CGA TGG ATT GCA CGC AGG TTC TCG AAA GAA CCT GCG TGC AAT CCA TC-3’; Bottom, 5’-AAA AGA TGG ATT GCA CGC AGG TTC TTT GCA AAT CCT GCG TGC AAT CCA TC-3’.

Arg1sh#A: Top, 5’-CAC CGG GAT TAT TGG AGC TCC TTT CCG AAG AAA GGA GCT CCA ATA ATC CC-3’; Arg1sh#B: top, 5’-CAC CGG AGA CAA AGC TAC CAT GTC TCG AAA CAT GTG GTA GCT TTG TCT CC-3’; bottom, 5’-AAA AGG AGA CAA AGC TAC CAT GTC TTT GGA CAT GTG GTA GCT TTG TCT CC-3’.

Arg2sh#A: top, 5’-CAC CGC AGA AGG AGG TTC TTT AGC TGT CAC TTA GCG AAC TAA GTG ACA GCT AAA GAA CC; Arg2sh#B: top, 5’-CAC CGC ATT CCA TCC TGA AGA AAT CCG AAT TCG TCA GGA GAT TTA ATG TCG AAA CAT TAA GAA GCT AAA GAA CC-3’; bottom, 5’-AAA AGG TTC TTT AGC TGT CAC TTA GTT CGC TAA GTG ACA GCT AAA GAA CC; Arg2sh#C: top, 5’-CAC CGG TTC TTT AGC TGT CAC TTA GCG AAC TAA GTG ACA GCT AAA GAA CC-3’; bottom, 5’-AAA AGG AGA AGG AGG TTC TTT AGC TGT CAC TTA GCG AAC TAA GTG ACA GCT AAA GAA CC-3’; Arg2sh#D: top, 5’-CAC CGG TTC TTT AGC TGT CAC TTA GCG AAC TAA GTG ACA GCT AAA GAA CC-3’; bottom, 5’-AAA AGG AGA AGG AGG TTC TTT AGC TGT CAC TTA GCG AAC TAA GTG ACA GCT AAA GAA CC-3’.

HDAC2sh #A: top, 5’-CAC CGC AGA AGG AGG TTC TTT AGC TGT CAC TTA GCG AAG TAA ATC TCT GAA TATCT GC-3’; bottom, 5’-AAA AGC AGA AGG AGG TTC TTT AGC TGT CAC TTA GCG AAG TAA ATC TCT GAA TATCT GC-3’.

The resulting pU6-sh-Nontargeted, pU6-Arg1shRNA and pU6-Arg2shRNA plasmids were tested for function in transient transfection experiments with 293A cells. The constructs showing the greatest inhibition were LR recombined with pAD/BLOCK-iDEST (Invitrogen) to generate pAd-shArg1 and pAd-shArg2. Viruses were amplified and purified/concentrated using a Millipore Kit.

Force Tension Myography

Mouse aorta was isolated and cleaned in ice-cold Krebs-Ringer-bicarbonate solution containing the following (in mM): 118.3 NaCl, 4.7 KCl, 1.6 CaCl₂, 1.2 KH₂PO₄, 25 NaHCO₃, 1.2 MgSO₄, and 11.1 dextrose. The aorta was immersed in a bath filled with constantly oxygenated Krebs buffer at 37°C. Equal size thoracic aortic rings (2mm) were mounted using a microscope, ensuring no damage to the smooth muscle or endothelium. One end of the aortic rings was connected to a transducer, and the other to a micromanipulator. Aorta was passively stretched to an optimal resting tension using the micromanipulator, after which a dose of 60 mM KCl was administered, and repeated after a wash with Krebs buffer. After these washes, all vessels were allowed to equilibrate for 20–30 min in the presence of indomethacin (3µM). Phenylephrine (1 µM) was administered to induce vasoconstriction. A dose-dependent response (1nM to 10 µM), with the muscarinic agonist, ACH or nitric oxide donor, SNP, was then performed as necessary. The responses were repeated in the presence of inhibitors. Relaxation responses were calculated as a percentage of tension following pre-constriction. Sigmoidal dose-response curves were fitted to data with the minimum constrained to 0. Two to four rings were isolated from each animal and the number of animals in each group (n) was 6.
**Human Arg2 promoter assay using luciferase activity**

Chromosomal DNA was prepared from HAECs using Trizol reagent as per manufacturer’s protocol (Life sciences). Promoter fragments of Arg2 were amplified by PCR using specific primer sets as follows.

PI Forward 5’-GGG CTC GAG TGA GGT GAA ATA AAT TTC AGG AGT TTA-3’;
Reverse 5’-GCC AAG CTT AGC CGG GAG TGA GCG CCA CCG CCC-3’, 1 Kb;
PII Forward 5’-GGG CTC GAG ATA ACC AGC GCT CCC GTT ATT CAG-3’;
Reverse 5’-GCC AAG CTT AGC CGG GAG TGA GCG CCA CCG CCC-3’, 0.3 Kb;
PIII Forward 5’-GGG CTC GAG GTT CAG GAA CCT GGC ATG GGC CGG-3’;
Reverse 5’-GCC AAG CTT AGC CGG GAG TGA GCG CCA CCG CCC-3’, 0.65 Kb;
PIV Forward 5’-GGG CTC GAG TGA GGT GAA ATA AAT TTC AGG AGT TTA-3’;
Reverse 5’-GCC AAG CTT AGC CGG GAG TGA GCG CCA CCG CCC-3’, 0.3 kb.
These were cloned into the restriction sites for XhoI and HindIII on the pGL3-enhancer vector (Promega Co.). HAECs were transfected with the plasmids using Fugene 6 reagent (Roche Co.) and luciferase activity was measured by Dual-luciferase reporter assay system (Promega Co.). The luciferase activity was reported as relative luciferase units by dividing firefly luciferase activity by Renilla luciferase activity.

Transcription factor (TF) binding consensus sequences in Arg2 promoter sequence were analyzed by using Ensembl, TRANSFAC database (http://www.gene-regulation.com/pub/databases.html#transfac) and MacVector software (Accelrys, San Diego, CA).

**Immunoprecipitation and Western blotting**

After 48hrs of HAEC cells transfection, cells were lysed in ice cold RIPA lysis buffer consisting of 20 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 1% sodium deoxycholate, 1 mM Na3VO4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 μg/mL leupeptin, and a 1:1000 diluted protease inhibitor cocktail (Sigma). For immunoprecipitation studies, whole cell lysate lysates were centrifuged at 14,000 x g and supernatants were pre cleared by incubation with Protein A/G-agarose beads for 2 h at 4º C with rocking. Agarose beads were then pelleted by centrifugation at 1,000 x g. FLAG-Arg2 in pre cleared lysates were immunoprecipitated by incubation overnight at 4º C with rocking following addition of anti-FLAG antibody (10 μl). Immune complexes were eluted in 2x SDS sample buffer by boiling for 5 minutes before loading into SDS–PAGE. Western blotting analysis was performed by transferring the SDS gel onto a PVDF membrane and visualized using secondary antibodies conjugated to alkaline phosphatases.

**Arginase activity assay**. HAEC were lysed using RIPA buffer (50 mM of Tris.HCl, pH 7.5, 0.1 mM EDTA, 0.1% Triton X-100, and protease inhibitor), and centrifuged for 30 min at 14,000 g at 4ºC. 50 μL(50-100μg) of supernatant was added to 75μl of 10 mM MnCl2 made in Tris-HCl (50 mM, pH 7.5) and heat activated by incubating at 55-60°C for 10 min. 50 μL of L-Arginine that was prepared in 300mM Tris-HCL, pH 9.7 were then added to the activated cell lysates to achieve final concentrations of 150mM and incubated at 37°C for 3 hrs with shaking. The reaction was stopped by adding 400 μL of an acid solution (H2SO4: H3PO4: H2O=1:3:7). For colorimetric determination of urea, α-isonitrosopropiophenone (25 μL, 9% in ethanol) was added and further heated at 100°C for 45 min. Samples were allowed to cool at room temperature in the dark for 10 min and urea concentration was determined by measuring absorbance at 550nm using a spectrophotometer.
**Chromatin Immunoprecipitation (ChIP).** ChIP was performed using Magna ChIP kit from Millipore as per manufacturer’s protocol. Briefly, Confluent HAEC were incubated with 1% formaldehyde at 37°C for 10 minutes to crosslink Histones and DNA. The crosslinked complexes were subjected to shearing with sonication and immunoprecipitated with anti-HDAC2 antibody. After reverse crosslinking, purified and immunoprecipitated DNA was amplified using Arg2 promoter specific primers as shown in methods for Arg2 promoter assay.

**Measurements of Nitric Oxide production**
Nitric Oxide levels in HAEC were determined using DAF-DA as per manufacture instructions (Life Sciences). Briefly, cells were loaded with DAF-DA (5 µM) for 20 min and washed 3 times with PBS and incubated for another 30 min in phenol-free media. Fluorescence was determined using a Nikon TE-200 epifluorescence microscope. Images were captured with a Rolera EMC2 camera (Q-Imaging, BC, Canada) with Velocity software (PerkinElmer, Lexington, MA). NO production in the cell culture media was also determined using by measuring Nitrite levels using a Siever’s NO analyzer. NO radicals were measured in cell media collected after 24 hours of treatment using the Oxiselect assay (Cell Biolabs, San Diego, CA).

**Statistics:** All statistical analyses were performed using Prism 5 for Mac by GraphPad Software Inc. and Microsoft Excel version 14.1.3 statistical analysis software. The results were expressed as mean and standard error (mean ± SEM). One-way analysis of ANOVA and the Bonferroni post-hoc test for multiple-comparison were used to compare all experimental data sets groups and pairs of data sets, respectively. A value of p < 0.05 was considered statistically significant.
Supplemental Materials

Transcriptional Regulation of Endothelial Arginase 2 by HDAC2

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*denotes equal contribution

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*denotes equal contribution
SUPPLEMENTAL FIGURES:

Table I. IC50 for HDAC inhibitors used in current study:

<table>
<thead>
<tr>
<th>HDAC Inhibitors</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA</td>
<td>1.8nM (do not block HDAC8 and SIRT)</td>
</tr>
<tr>
<td>Scriptaid</td>
<td>HDAC1: IC50 = 0.6 µM; HDAC3: IC50 = 0.6 µM; HDAC8: IC50 = 1 µM</td>
</tr>
<tr>
<td>SAHA (Suberoylanilide Hydroxamic Acid)</td>
<td>10nM ; block Class I and Class II HDACs</td>
</tr>
<tr>
<td>MGCD0103 (Mocetinostat)</td>
<td>HDAC1 with IC50 of 0.15 µM, 2 to 10 fold selectivity against HDAC2, 3, and 11, and no activity to HDAC4, 5, 6, 7, and 8.</td>
</tr>
<tr>
<td>CI994 (Tacedinaline)</td>
<td>Inhibit HDAC1 with IC50 of 0.57 µM</td>
</tr>
<tr>
<td>MS275 (Entinostat)</td>
<td>Selective to HDAC1 and HDAC3 with IC50 of 0.51 µM and 1.7 µM</td>
</tr>
<tr>
<td>MC1658</td>
<td>Selective to HDAC1A with IC50 of 100 nM</td>
</tr>
<tr>
<td>PCI-34051</td>
<td>Specific HDAC8 inhibitor with IC50 of 10 nM</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>IC50&lt;50µM</td>
</tr>
</tbody>
</table>
Table II. Arginase (-1 kb) promoter sequence.

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-100</td>
<td>TAGATTTCCTGGCATGCCCAGGAAGGAGTTTATGTGATAAAAAATTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>101-200</td>
<td>TAGATTTCCTGGCATGCCCAGGAAGGAGTTTATGTGATAAAAAATTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>201-300</td>
<td>TAGATTTCCTGGCATGCCCAGGAAGGAGTTTATGTGATAAAAAATTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>301-400</td>
<td>TAGATTTCCTGGCATGCCCAGGAAGGAGTTTATGTGATAAAAAATTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>401-500</td>
<td>TAGATTTCCTGGCATGCCCAGGAAGGAGTTTATGTGATAAAAAATTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
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</table>

Table III. Transcription factors binding consensus sequences in Arg2 promoter

<table>
<thead>
<tr>
<th>Factor name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGIY</td>
<td>gaaatAAATTCAGGAGTTTATGTGATAAAAAATTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>Fre-ac3</td>
<td>tttatGTAAAAGAACTACTATTTACTTTTACGTCATTCTTGGCATAGATCACAAACGAGGAGTTTATGTGATAAAAAATTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>ATF2</td>
<td>TTATGtaa</td>
</tr>
<tr>
<td>HNF1</td>
<td>ttatgtaaaaaATTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>SOX10</td>
<td>cTTTTGtaa</td>
</tr>
<tr>
<td>CDX-2</td>
<td>TTTATa</td>
</tr>
<tr>
<td>HIF-1alpha</td>
<td>ACATG</td>
</tr>
<tr>
<td>ZNF333</td>
<td>ATTAT</td>
</tr>
<tr>
<td>p53</td>
<td>tgaCTAGGcagggccagacc</td>
</tr>
<tr>
<td>BEN</td>
<td>CAGCCGcagggccagacc</td>
</tr>
<tr>
<td>CPBP</td>
<td>caGGGGCCGcagggccagacc</td>
</tr>
<tr>
<td>SREBP</td>
<td>gaggGcagtGTCATG</td>
</tr>
<tr>
<td>CREB1</td>
<td>ggtgacGTCGtcacg</td>
</tr>
<tr>
<td>ATF2</td>
<td>TGAGGTGAATTTTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>Sp1</td>
<td>tggGGCCGcagggccagacc</td>
</tr>
<tr>
<td>Egr-1</td>
<td>gcGCGGGGcagggccagacc</td>
</tr>
<tr>
<td>RNF96</td>
<td>ggcgcGCGGGGcagggccagacc</td>
</tr>
<tr>
<td>CPBP</td>
<td>gcGCGGGGcagggccagacc</td>
</tr>
<tr>
<td>CREB1</td>
<td>ggtgacGTCGtcacg</td>
</tr>
<tr>
<td>CREB1</td>
<td>ggtGAGGTGAATTTTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
<tr>
<td>ATF2</td>
<td>TGAGGTGAATTTTAATTTTTGTTGAGAAATAATGTC</td>
</tr>
</tbody>
</table>
S.Figure I. Densitometric analysis of Arg2 expression. A. Dose-dependence, and B. Time-dependence of the effects of TSA on Arg 2 expression were quantified using densitometry and normalized to GAPDH expression.

S.Figure II. HDAC8 is expressed in HAEC but does not regulate Arg2 expression. A) Total RNA was extracted from using trizol reagent followed by RNA purification. cDNA was synthesized using total RNA as a template and using oligodT and reverse transcriptase. Primers specific to HDAC7 and HDAC8 were used to detect the expression of HDAC7 and HDAC8. B) Increasing dose of HDAC8 siRNA (0, 5, 10 and 15nM) was transfected in HAEC and post 72 hours, cell lysates were subjected to immunoblotting with HDAC8 and Arg2 antibody.
**S. Figure III. HDAC4-7 do not regulate Arg2 expression.** A) Flag-tagged cDNA encoding HDAC4-7 in pcDNA3.1 was expressed in HAEC and cell lysates were subjected to immunoblotting using Arg2, FLAG and GAPDH antibody. B) Flag-tagged HDAC7 was expressed in HAEC in increasing concentration (0, 100, 300 and 1000ng) and cell lysates were subjected to immunoblotting using Arg2, FLAG and GAPDH antibodies.

**S. Figure IV. Increased global cell acetylation with CBP does not influence Arg2 expression.** Increasing concentrations of HA-CBP (0, 300ng and 1µg) were transiently expressed in HAEC, and cell lysates were subjected to immunoblotting for Arg2, HA, and GAPDH.
S.Figure V. Densitometry analysis of HDAC2 and Arg2 expression. Immunoblots from Figure 7D, 7E, 8E, 8F and 9C (in order) were subjected to densitometry analysis of Arg2 and HDAC2 expression and each value was normalized to GAPDH expression for the respective sample.
S.Figure VI. Validation of Adenoviral HDAC2 and Arg2 shRNA in HAEC. HAEC were transduced with non-targeted shRNA (30MOI), HDAC2 shRNA (5, 10 and 30 MOI), and Arg2 shRNA (30MOI), and lysates were subjected to immunoblotting with antibodies against HDAC2 or Arg2, respectively, and with GAPDH antibodies.