Histone Deacetylase 3 Antagonizes Aspirin-Stimulated Endothelial Nitric Oxide Production by Reversing Aspirin-Induced Lysine Acetylation of Endothelial Nitric Oxide Synthase

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Rationale: Low-dose acetylsalicylic acid (aspirin) is widely used in the treatment and prevention of vascular atherothrombosis. Cardiovascular doses of aspirin also reduce systemic blood pressure and improve endothelium-dependent vasorelaxation in patients with atherosclerosis or risk factors for atherosclerosis. Aspirin can acetylate proteins, other than its pharmacological target cyclooxygenase, at lysine residues. The role of lysine acetylation in mediating the effects of low-dose aspirin on the endothelium is not known.

Objective: To determine the role of lysine acetylation of endothelial nitric oxide synthase (eNOS) in the regulation of endothelial NO production by low-dose aspirin and to examine whether the lysine deacetylase histone deacetylase (HDAC)3 antagonizes the effect of low-dose aspirin on endothelial NO production by reversing acetylation of functionally critical eNOS lysine residues.

Methods and Results: Low concentrations of aspirin induce lysine acetylation of eNOS, stimulating eNOS enzymatic activity and endothelial NO production in a cyclooxygenase-1–independent fashion. Low-dose aspirin in vivo also increases bioavailable vascular NO in an eNOS-dependent and cyclooxygenase-1–independent manner. Low-dose aspirin promotes the binding of eNOS to calmodulin. Lysine 609 in the calmodulin autoinhibitory domain of bovine eNOS mediates aspirin-stimulated binding of eNOS to calmodulin and eNOS-derived NO production. HDAC3 inhibits aspirin-stimulated (1) lysine acetylation of eNOS, (2) eNOS enzymatic activity, (3) eNOS-derived NO, and (4) binding of eNOS to calmodulin. Conversely, downregulation of HDAC3 promotes lysine acetylation of eNOS and endothelial NO generation.

Conclusions: Lysine acetylation of eNOS is a posttranslational protein modification supporting low-dose aspirin-induced vasoprotection. HDAC3, by deacetylating aspirin-acetylated eNOS, antagonizes aspirin-stimulated endothelial production of NO. (Circ Res. 2010;107:877-887.)

Key Words: aspirin ■ endothelial NOS ■ HDAC3 ■ lysine acetylation ■ calmodulin

Low-dose aspirin (81 to 325 mg/d) is a very useful tool in the armamentarium for the treatment of acute coronary syndromes, as well as for the secondary prevention of myocardial infarctions and stroke in high-risk patients. The antithrombotic effects of low-dose aspirin are principally attributed to acetylation of a serine residue in platelet cyclooxygenase (COX)-1, irreversibly inhibiting COX-1 in platelets, and limiting platelet aggregation because of prostanoids produced by COX-1. In addition to preventing thrombosis, low-dose aspirin also improves vasomotor function mediated by the endothelium in humans and in animal models of endothelial dysfunction. Although low-dose aspirin is not commonly used in the treatment of hypertension, it is efficacious, when given at bedtime, for reduction of blood pressure in individuals with mild hypertension or prehypertension. Suppression of vasoconstricting prostanoids produced by COX-1 could be one mechanism by which low-dose aspirin improves endothelial function and reduces mildly and bor-
Acetylation of Lysine Residues in Endothelial Nitric Oxide Synthase

Results and Discussion

We first determined whether eNOS is a target of lysine acetylation by low-dose aspirin. Within a concentration range that is acutely achieved in human plasma with oral administration of a 100-mg cardiovascular dose, aspirin rapidly and concentration-dependently acetylated eNOS expressed in HEK 293 cells (Figure 1A and 1B). A similar increase in lysine acetylation of endogenous eNOS in human umbilical vein endothelial cells (HUVECs) was observed with low concentrations of aspirin (Figure 1C). In parallel with the increase in lysine acetylation of eNOS, aspirin at low concentrations stimulated metabolites derived from NO produced by eNOS expressed in HEK 293 cells (Figure 1D), and by HUVECs (Figure 1E). In addition, low concentration of aspirin stimulated enzymatic activity of eNOS expressed in HEK 293 cells (Figure 1F). Because COX-1, a known target of low-dose aspirin, is constitutively expressed in many cells including endothelial cells, we then asked whether aspirin-stimulated NO in endothelial cells is dependent on COX-1. Knockdown of constitutive COX-1 expression with small interfering (si)RNA resulted in an increase in eNOS expression, but did not inhibit low-dose aspirin-stimulated NO production (Figure 1G). Similarly, treatment of HUVECs with sc-560, a COX-1 selective inhibitor, did not abolish aspirin-stimulated NO (Online Figure II). This reveals that clinically relevant concentrations of aspirin acetylate lysine residues of endogenous and ectopically expressed eNOS in cultured cells and suggests that acetylation mediates increased eNOS catalytic activity and NO production. Furthermore, this implicates a COX-1–independent mechanism for aspirin modulation of endothelial NO production.

We then asked whether eNOS is a direct target of lysine acetylation by aspirin. Aspirin acetylated recombinant eNOS on lysine residues in vitro (Figure 2A). Moreover, aspirin promoted the enzymatic activity of recombinant eNOS (Figure 2B). Increase in lysine acetylation and activation of recombinant eNOS was first evident at high micromolar concentrations, approximating the reported IC_{50} of aspirin for recombinant COX-1. However, to achieve a maximal effect on eNOS activity and lysine acetylation, millimolar concentrations of aspirin were required. The higher concentrations of aspirin required to achieve maximal lysine acetylation and activation of recombinant eNOS, when compared with eNOS expressed in cells, indicates that aspirin is a better acetylator of lysine residues of endogenous and recombinant eNOS in vitro. Such concentrations of aspirin are also required for in vitro lysine acetylation of other known target proteins, including hemoglobin and ubiquitin. The relative insensitivity of recombinant eNOS to aspirin may also be reflective of the fact that mechanisms that stimulate and are required for optimal eNOS activity, such as phosphorylation by Akt kinase and binding to hsp90, are present in intact cells but not in vitro. These findings show that eNOS is directly acetylated on lysine residues by aspirin, and this acetylation stimulates its enzymatic activity.

Non-standard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>Akt</td>
<td>serine/threonine protein kinase</td>
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<td>ASA</td>
<td>acetylsalicylic acid</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>HDAC</td>
<td>histone deacetylase</td>
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<td>hsp90</td>
<td>heat shock protein 90</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>L-NAME</td>
<td>N’-nitro-L-arginine methyl ester</td>
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<td>NOS</td>
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<td>SA</td>
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<td>WCL</td>
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Methods

An expanded Methods section detailing the materials, methods, and statistics implemented in this study is available in the Online Data Supplement at http://circres.ahajournals.org.

Endothelial NOS mice in a C57BL/6J background were purchased from The Jackson Laboratory. The C57BL/6J strain was used as a wild-type control strain. Mice were administered aspirin or salicylic acid (3 mg/kg) intravenously or by gastric gavage and euthanized after 45 to 60 minutes. The COX-1 inhibitor sc-560 (Cayman Chemicals) or vehicle control was injected intraperitoneally at 20 mg/kg 30 minutes before administration of aspirin or salicylic acid. All animals were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee.
Figure 1. Low concentrations of aspirin rapidly acetylate eNOS on lysine residues and stimulate eNOS enzymatic activity and NO production. A to C, Micromolar concentrations of aspirin rapidly stimulate lysine acetylation of eNOS. Representative immunoblots for acetylated lysine (Ac-Lys, Ac-K) in eNOS immunoprecipitated (from A to B) HEK 293 cells expressing ectopic eNOS and (C) HUVEC showing dose- and time-response to aspirin (ASA). Accompanying graphs show analysis by densitometry of Ac-K/total eNOS relative to no aspirin from independent blots. D to E, Low concentrations of aspirin increase NO metabolites (nitrite + nitrate) in conditioned media of (D) HEK 293 cells expressing ectopic eNOS and (E) HUVEC. F, Low concentrations of aspirin increase enzymatic activity (conversion of arginine to citrulline) of eNOS ectopically expressed in HEK 293 cells. (G) Knockdown of COX-1 does not suppress aspirin-stimulated NO (nitrite + nitrate) in conditioned media of HUVEC. *P<0.05 and **P<0.01 (n=3 to 4).
Aspirin directly acetylates eNOS on lysine residues, and acetylation of lysine 609 in eNOS mediates aspirin-stimulated eNOS NO production. A and B, Lysine acetylation and activation of eNOS by aspirin in vitro. In vitro concentration-dependent (A) lysine acetylation (Ac-Lys, Ac-K), and (B) enzymatic activity of recombinant eNOS by aspirin.

C, Conservation of the tandem histidine-lysine pair consisting of lysine 609 (in bovine eNOS) in the calmodulin auto-inhibitory domains of eNOS of different species.

D through G, Aspirin promotes eNOS-derived NO by specifically targeting lysine 609 in bovine eNOS. D and F, NO (nitrite+nitrate) measured in conditioned media, and E and G, lysine acetylation (Ac-Lys, Ac-K) of eNOS in lysates of HEK 293 cells expressing ectopic wild-type eNOS (eNOS WT), eNOS with lysine 609 mutated to nonacetylatable arginine (eNOS K609R or eNOS) nonacetylatable on lysines 631 and 633 (eNOS K631/633R) and treated with a low concentration of ASA. H, SIRT1 does not increase NO through deacetylation of lysine 609. NO (nitrite+nitrate) in conditioned media of HEK 293 cells expressing ectopic eNOS (WT) and eNOS (K609R), with and without SIRT1 overexpression. Representative immunoblots are shown with accompanying analysis by densitometry of Ac-K/total eNOS relative to no aspirin from independent blots. *P<0.05 and #P=NS (n=3 to 5).
Next, we examined the role of specific lysine residues in modulating NO production by low-dose aspirin. Lysine acetylation of ubiquitin by aspirin occurs by a transacetylation process in which a histidine residue, in proximity to the target lysine, is first acetylated by aspirin, and this acetylated histidine intermediate then transfers the acetyl group to the lysine residue. The occurrence of histidine in the catalytic microenvironment has been similarly proposed to play an important role in formation of lysine glycation adducts of alcohol dehydrogenase. Hypothesizing a similar catalytic role for histidine in facilitating the reactivity of lysine residues in eNOS to aspirin, we targeted lysines in proximity to histidine residues. Within bovine eNOS exists a single tandem histidine-lysine pair at position 608 to 609 (corresponding to positions 606 to 607 in human eNOS). This histidine–lysine pair is in the calmodulin autoinhibitory domain of eNOS, and is conserved across species (Figure 2C). We mutated lysine 609 to nonacetylatable arginine (eNOS [K609R]), and compared the stimulatory effect of low concentrations of aspirin on wild-type eNOS and eNOS (K609R). NO produced by eNOS (K609R) under resting conditions was similar to that of wild-type protein (Figure 2D). However, in contrast to its effect on wild-type eNOS aspirin did not stimulate NO produced by eNOS (K609R) (Figure 2D). Moreover, unlike wild-type eNOS, lysine acetylation of eNOS (K609R) was not stimulated by aspirin (Figure 2E). In addition to lysine 609, we also targeted other conserved lysine residues in the calmodulin autoinhibitory domain of eNOS. Because lysines 631 and 633 are in proximity to serine 635, phosphorylation of which stimulates eNOS activity, we asked whether acetylation of these lysine residues, by mimicking phosphorylation of serine 635, could mediate aspirin-stimulated NO production. However, similar to its effect on NO produced by wild-type eNOS, aspirin stimulated NO production by the acetylation-resistant eNOS (K631/633R) (Figure 2F). In addition, unlike the lack of effect of aspirin on lysine acetylation of eNOS (K609R), low concentrations of aspirin increased lysine acetylation of eNOS (K631/633R) (Figure 2G). Thus, aspirin stimulates eNOS-derived NO production by targeting specific lysine residues in the calmodulin autoinhibitory domain of eNOS for acetylation.

Sirtuin (SIRT1) is a class III histone deacetylase. It targets many nonhistone proteins including eNOS, stimulating eNOS activity by deacetylating lysines in its calmodulin-binding domain. Although K609 is not in the calmodulin-binding domain, we nevertheless questioned whether SIRT1 plays any part in the regulation of eNOS-derived NO stimulated by low-dose aspirin. SIRT1 overexpression increased NO derived from both WT and K609R eNOS to the same extent (Figure 2H), indicating that lysine 609, which mediates aspirin-stimulated NO, is not a target of SIRT1.

The autoinhibitory domain of eNOS inhibits its activation by interfering with its binding to calmodulin. Because lysine 609 is in the autoinhibitory domain of eNOS, we hypothesized that its acetylation mediates aspirin-stimulated eNOS activation by promoting binding of eNOS to calmodulin. To investigate this possibility, we first examined the effect of low concentrations of aspirin on binding of calmodulin to eNOS. Aspirin rapidly stimulated the binding of eNOS and calmodulin expressed in HEK 293 cells (Figure 3A and 3B). A similar increase in the binding of endogenous eNOS to endogenous calmodulin was observed in endothelial cells exposed to aspirin (Figure 3C). In contrast, aspirin had no effect on binding of calmodulin to eNOS that is nonacetylatable on lysine 609 (K609R) (Figure 3D). These findings indicate that cardiovascular concentrations of aspirin stimulate eNOS activity by promoting its binding to calmodulin, and demonstrate the importance of lysine 609 in mediating aspirin-stimulated binding of eNOS to calmodulin.

Serine acetylation of platelet COX-1 by cardiovascular doses of aspirin is nonreversible and may reflect the limited complement of cellular proteins found in nonnucleated platelets. In nucleated cells, acetylation of lysines is a reversible posttranslational modification. Histone deacetylases (HDACs) remove acetyl residues from ε-amino-acetylated lysines in histones and nonhistone proteins, thereby modifying protein structure and function. We therefore asked whether lysine acetylation of eNOS is reversible by HDAC. Because eNOS is a cytosolic protein, we limited our search to HDAC that are expressed in the cytoplasm. HDAC3 belongs to class I HDAC and is expressed both in the nucleus and cytoplasm. In keeping with its presence in the cytoplasm, several nonhistone substrates of HDAC3 have been identified. In endothelial cells, HDAC3 mediates a prothrombotic and proinflammatory phenotype. We therefore focused our attention on HDAC3 as a potential endogenous antagonist to aspirin-stimulated lysine acetylation of eNOS.

We first asked whether HDAC3 and eNOS associate with each other. HDAC3 and eNOS expressed in HEK 293 cells coimmunoprecipitated with each other (Figure 4A). Endogenous HDAC3 and eNOS also coimmunoprecipitated in endothelial cells, and this association was stimulated by aspirin (Figure 4B). We next determined whether HDAC3 antagonizes aspirin-induced lysine acetylation of eNOS. Overexpression of HDAC3 in HEK293 cells abrogated the increase in lysine acetylation of eNOS (Figure 4C), and eNOS-derived NO (Figure 4D) stimulated by low concentrations of aspirin. Unlike HDAC3, the SIRT1 lysine deacetylase, which also binds to and deacetylates eNOS, did not inhibit aspirin-stimulated NO (Figure 4E). Conversely, knockdown of endogenous HDAC3 in endothelial cells increased lysine acetylation of endogenous eNOS, without affecting eNOS expression (Figure 4F). The increase in lysine acetylation of eNOS corresponded with an increase in NO produced by endothelial cells in which HDAC3 was knocked down (Figure 4G). Importantly, recombinant active HDAC3 suppressed lysine acetylation of recombinant eNOS by aspirin in vitro (Figure 4G), and decreased aspirin-stimulated eNOS enzymatic activity (Figure 4I). Because aspirin led to an increase in binding of eNOS to calmodulin (Figure 4A through 4C), we then asked whether HDAC3 modulates this binding. Aspirin-stimulated binding of eNOS to calmodulin was negated by overexpression of HDAC3 (Figure 4J). These findings show that HDAC3 targets lysine-acetylated eNOS for deacetylation, thereby antagonizing aspirin-stimulated binding of eNOS to calmodulin, and inhibiting aspirin-triggered increase in eNOS activity and NO production. The increase in resting lysine acetylation of eNOS and NO in...
endothelial cells with knockdown of HDAC3, independent of aspirin, also hints at the presence of an endogenous lysine acetyltransferase that targets eNOS for acetylation.

We next ascertained the role of eNOS in mediating the physiological effects of low-dose aspirin on the vasculature. Mice were administered a cardiovascular dose of aspirin intravenously or by gastric gavage, followed by measurements of eNOS acetylation, serum NO, and vascular bioavailable NO. A 3 mg/kg dose (corresponding to 300 mg given to an adult human) of aspirin given IV or by gastric gavage increased aortic bioavailable NO (Figure 5A), and serum NO (Figure 5B), respectively. The increase in serum and aortic bioavailable NO was accompanied by an increase in lysine acetylation of eNOS in hepatic tissue (Figure 5C and 5D). Importantly, aspirin did not increase aortic bioavailable NO in mice genetically deficient for eNOS (eNOS<sup>−/−</sup>) (Figure 5A). Because low-dose aspirin inhibits platelet COX-1 in vivo, we also determined the role of aspirin-induced COX-1 inhibition in mediating the increase in bioavailable vascular NO. Sc-560, the selective pharmacological inhibitor of COX-1, was delivered in vivo at a dose that inhibits COX-1-derived prostanoids. When compared with salicylic acid, low-dose aspirin led to an increase in bioavailable NO in aortas of mice pretreated with sc-560 just as it did in mice not

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**Figure 3.** Lysine 609 mediates aspirin-stimulated binding of eNOS to calmodulin. A through C, Aspirin rapidly stimulates binding of eNOS to calmodulin. Coimmunoprecipitation of (A) calmodulin (CaM) in eNOS immunoprecipitates and (B) eNOS in CaM immunoprecipitates from HEK 293 cells expressing ectopic eNOS and FLAG-CaM treated with a low concentration of ASA for the indicated times. C, Coimmunoprecipitation of endogenous CaM in eNOS immunoprecipitates and endogenous eNOS in CaM immunoprecipitates from HUVEC, with and without treatment with ASA. D, Lysine 609 in bovine eNOS mediates its aspirin-stimulated binding to CaM. Coimmunoprecipitation of FLAG-CaM with eNOS (WT) and eNOS (K609R) ectopically expressed in HEK 293 cells treated with ASA. Representative immunoblots are shown with accompanying densitometry of immunoprecipitated CaM/eNOS and eNOS/CaM relative to no aspirin from independent blots. *P<0.05, **P<0.01, and #P=NS (n=3 to 4).
Figure 4. HDAC3 binds to eNOS and antagonizes aspirin-stimulated lysine acetylation of eNOS, eNOS activity, and endothelial NO generation. A and B, HDAC3 binds to eNOS, and aspirin stimulates this binding. Coimmunoprecipitation of (A) FLAG-HDAC3 and eNOS expressed in HEK 293 cells and (B) endogenous HDAC3 and eNOS in HUVEC. The molecular weight of FLAG-HDAC3 overlaps with that of mouse immunoglobulin heavy chain. N-IgG-M, mouse nonimmune immunoglobulin; N-IgG-R, rabbit nonimmune immunoglobulin; WCL, whole cell lysate. C through J, HDAC3, but not SIRT1, antagonizes the effects of ASA on eNOS. C and D, Overexpression of HDAC3 inhibits aspirin-stimulated (C) lysine acetylation (Ac-Lys, Ac-K) in lysates and (D) NO (nitrite+nitrate) in conditioned media of HEK 293 cells expressing ectopic eNOS. E, Overexpression of SIRT1 promotes basal and does not inhibit aspirin-stimulated NO (nitrite+nitrate) in conditioned media of HEK 293 cells expressing ectopic eNOS. F and G, Knockdown of HDAC3 promotes (F) lysine acetylation of endogenous eNOS in lysates and (G) NO (nitrite+nitrate) in conditioned media of HUVEC. H and I, HDAC3 directly targets lysine-acetylated eNOS for deacetylation. HDAC3 reverses aspirin-stimulated (H) lysine acetylation (Ac-Lys) and (I) enzymatic activity of recombinant eNOS in vitro. J, HDAC3 inhibits aspirin-stimulated binding of eNOS to calmodulin. Coimmunoprecipitation of calmodulin (CaM) in eNOS immunoprecipitates and eNOS in CaM immunoprecipitates from HEK 293 cells expressing ectopic eNOS or nonacetylatable eNOS (K609R) and FLAG-CaM treated with ASA. Representative immunoblots are shown with accompanying analysis by densitometry of Ac-K/total eNOS, or immunoprecipitated CaM/eNOS and eNOS/CaM, relative to no aspirin, from independent blots. *P<0.05, **P<0.01, and #P=NS (n=3 to 4).
treated with sc-560 (Figure 5E). The lack of effect of COX-1 inhibition on aspirin-stimulated increase in vascular NO bioavailability was evident at all concentrations of phenylephrine (Figure 5F). These findings indicate that a cardiovascular dose of aspirin administered in vivo, via oral or parenteral route, promotes lysine acetylation of eNOS, and acutely increases vascular NO levels in a COX-1-independent fashion. A, Bioavailable NO in aortas of wild-type (eNOS+/+) and eNOS-deficient (eNOS−/−) mice 45 to 60 minutes after 3 mg/kg IV aspirin (ASA) or salicylic acid (SA) and nontreated animals. \( P^*<0.05, P^{**}<0.01, \) \( P^{{***}}<0.001 \) (n=3 to 4) compared with nontreated animals and animals treated with SA. B, Serum NO (nitrite+nitrate) 45 to 60 minutes after gastric gavage of wild-type (eNOS+/+) mice with ASA or SA (3 mg/kg). \( P^*<0.05 \) (n=3). C and D, Parenteral or oral low-dose aspirin increases lysine acetylation of eNOS in tissue. Lysine acetylation of eNOS immunoprecipitated from liver of wild-type (eNOS+/+) and eNOS−/− mice 45 to 60 minutes after administration of 3 mg/kg ASA or SA (C) IV and (D) by gastric gavage. Wildtype mice were used in D. N-kg-G-R, nonimmune rabbit immunoglobulin. Representative immunoblots are shown with accompanying analysis by densitometry of Ac-K/total eNOS relative to no aspirin from independent blots. \( P^*<0.05 \) (n=3 to 4). E and F, Aspirin-induced vascular bioavailable NO is independent of COX-1. A, Aortic bioavailable NO in wild-type mice 45–60 minutes after 3 mg/kg IV ASA or SA, pretreated with the COX-1 inhibitor sc-560. F, Increase in aortic bioavailable NO (difference between ASA and SA) in wild-type mice with and without pretreatment with sc-560. \( P^*<0.05, P^{{**}}<0.01, \) \( P^{{***}}<0.001, \) and \( \#P=NS \) (n=3 to 4).

Figure 5. Cardiovascular dose of aspirin in vivo increases vascular bioavailable NO in an eNOS-dependent and COX-1-independent fashion. A and B, Parenteral or oral low-dose aspirin increases aortic bioavailable and serum NO in an eNOS-dependent manner. A, Bioavailable NO in aortas of wild-type (eNOS+/+) and eNOS-deficient (eNOS−/−) mice 45 to 60 minutes after 3 mg/kg IV aspirin (ASA) or salicylic acid (SA) and nontreated animals. \( P^*<0.05, P^{{**}}<0.01, \) \( P^{{***}}<0.001 \) (n=3 to 4) compared with nontreated animals and animals treated with SA. B, Serum NO (nitrite+nitrate) 45 to 60 minutes after gastric gavage of wild-type (eNOS+/+) mice with ASA or SA (3 mg/kg). \( P^*<0.05 \) (n=3). C and D, Parenteral or oral low-dose aspirin increases lysine acetylation of eNOS in tissue. Lysine acetylation of eNOS immunoprecipitated from liver of wild-type (eNOS+/+) and eNOS−/− mice 45 to 60 minutes after administration of 3 mg/kg ASA or SA (C) IV and (D) by gastric gavage. Wildtype mice were used in D. N-kg-G-R, nonimmune rabbit immunoglobulin. Representative immunoblots are shown with accompanying analysis by densitometry of Ac-K/total eNOS relative to no aspirin from independent blots. \( P^*<0.05 \) (n=3 to 4). E and F, Aspirin-induced vascular bioavailable NO is independent of COX-1. A, Aortic bioavailable NO in wild-type mice 45–60 minutes after 3 mg/kg IV ASA or SA, pretreated with the COX-1 inhibitor sc-560. F, Increase in aortic bioavailable NO (difference between ASA and SA) in wild-type mice with and without pretreatment with sc-560. \( P^*<0.05, P^{{**}}<0.01, \) \( P^{{***}}<0.001, \) and \( \#P=NS \) (n=3 to 4).

Antiinflammatory concentrations of aspirin acetylate eNOS on serines 765 and 771 (767 and 773 in bovine eNOS).\(^{10}\) The functional significance of serine acetylation in regulating eNOS has not been reported. Therefore, we examined the importance of these serine residues by measuring aspirin-stimulated NO produced by eNOS that is nonacetylatable on these residues (eNOS S767/773R). Low-dose aspirin increased NO derived from eNOS (S767/773R), although the magnitude of increase was smaller when compared with WT eNOS (Online Figure VI). Thus, acetylation of these serine residues by low-dose aspirin may play an important part in mediating the effects of aspirin on the endothelium. However, acetylation of these serine residues cannot fully account for aspirin’s stimulatory effect on eNOS.

Our work did not explore the effect of aspirin on endothelial NO in the context of vascular inflammation. It is known, however, that inducible NOS and eNOS mediate the antiinflammatory effects of high-dose aspirin, and this has been attributed to aspirin-triggered epi-lipoxin generation in inflamed tissues.\(^{33}\) In the inflammatory milieu, aspirin acetylates the active site of cyclooxygenase-2 (COX-2) in endothelial or epithelial cells resulting in conversion of arachidonic acid to the intermediary (15R) hydroxyeicosatetraenoic acid which is rapidly metabolized in a transcellular fashion by lipoxigenases in leukocytes to epi-lipoxins.\(^{34}\) Moreover, aspirin modulates COX-2 activity to generate omega-3 fatty acid derivatives such as docosahexanoic acid,\(^{35}\) which increases eNOS activity.\(^{36,37}\) COX-2 can be induced in endothelial cells,\(^{15}\) and is upregulated in blood vessels of individuals with cardiovascular risk factors.\(^{38}\) Thus, the effect of aspirin on endothelial NO production during vascular inflammation may also be dependent on COX-2 and be mediated by aspirin-triggered epi-lipoxins and keto-fatty acid derivatives.

Aspirin is not a potent vasodilator but does reduce systemic blood pressure in mild hypertension and prehypertension.\(^{6,8}\) This effect is consistent with observations that low-dose aspirin stimulates production of endothelial NO to a much lesser degree than vasodilators such as acetylcholine and bradykinin.\(^{8,39,40}\) In line with these observations, low-dose aspirin, when used alone, does not lead to vasodilation of preconstricted vascular rings.\(^{9}\) One limitation of our work is that we did not examine the effect of aspirin on vascular tone in vivo in a model of hypertension. However, we expect low-dose aspirin to have a
small impact, at best, on blood pressure in vivo. Despite this, low-dose aspirin is efficacious in improving vascular relaxation and endothelial function in the setting of endothelial dysfunction, such as seen with aging and hypercholesterolemia.\(^4,5\) In such settings where endothelial NO production is decreased, the increase in vascular NO stimulated by aspirin, albeit modest, may be sufficient to elicit an effect on vascular tone.

Lysine acetylation/deacetylation has recently been recognized as a novel posttranslational mechanism for the regulation of eNOS activity. The class III HDAC SIRT1 deacetylates and activates eNOS, and this activation is mediated by lysine residues in the calmodulin-binding domain of eNOS.\(^22\) The role of class I HDAC (to which HDAC3 belongs) and class II HDAC in targeting eNOS for deacetylation has hitherto not been reported. The stimulatory effect of SIRT1-mediated deacetylation of lysine residues in the calmodulin-binding domain, compared with the inhibitory role of HDAC3-mediated deacetylation of a lysine residue in the calmodulin autoinhibitory domain, speaks to a finely tuned acetylation-deacetylation mechanism for the regulation of eNOS enzymatic activity.

We did not observe a significant change in eNOS expression with knockdown of HDAC3 in endothelial cells. However, nonspecific inhibitors of class I and II HDAC lead to a change in eNOS expression. In nonendothelial cells in which eNOS is normally not expressed, inhibition of class I and II HDAC with trichostatin A (TSA), combined with inhibition of DNA methyltransferase activity with 5-aza-2'-deoxycytidine resulted in induction of eNOS transcript.\(^41\) In contrast, TSA and butyric acid, another nonspecific HDAC inhibitor, suppressed eNOS protein levels in endothelial cells, and prevented NO-dependent relaxation of coronary arteries.\(^42\) These somewhat contradictory findings hint at a complex role of class I and II HDAC in the regulation of eNOS expression and may reflect the use of nonspecific HDAC inhibitors. The added role of HDAC3 in posttranslational modification of eNOS injects another layer of regulation of eNOS by HDAC, and indicates that specific HDAC may have distinct and perhaps opposing effects on endothelium-dependent vascular functions.

Endothelium-derived NO is not only important in the regulation of vascular tone but also inhibits platelet aggregation and adhesion\(^43\) and suppresses smooth muscle cell proliferation and migration to form the neointima,\(^44\) all of which contribute to the pathological processes of atherosclerosis and thrombosis. Thus, the stimulatory effect of low-dose aspirin on endothelial NO production through lysine acetylation of eNOS, and its antagonism by HDAC3, is likely to have relevance not only to endothelial-dependent regulation of vascular tone but also to atherothrombosis, which results in tissue ischemia and infarction. Therefore, selective inhibition of HDACs may form the basis of adjunctive therapies aimed to improve the efficacy of aspirin in the prevention and treatment of cardiovascular disease and stroke. In addition, epidemiological data indicate that the use of low-dose aspirin also reduces the risk of proliferative disorders, including colorectal and prostate neoplasia.\(^45,46\) Because HDAC3 plays a part in the molecular pathogenesis of these disorders,\(^47,48\) aspirin and HDAC3 may also act in an antagonistic fashion to regulate the lysine acetylation status of targets that play a role in neoplastic development.

Although low-dose aspirin is widely used in the prevention and treatment of cardiovascular disease, a significant proportion of patients on aspirin experience atherothrombotic events. The pathophysiological and molecular mechanisms underlying this phenomenon, termed “aspirin resistance,” have not been clearly identified. There are likely multiple reasons for this phenomenon. Some that have been proposed include aspirin-insensitive thromboxane A\(_2\) synthesis in platelets, increased collagen sensitivity of platelets, and mutations/polymorphisms in genes involved in the mechanisms of action of aspirin.\(^49\) Suppression of aspirin-induced lysine acetylation and activation of eNOS, and consequent decreased endothelial NO production, could also be an endogenous mechanism responsible for the phenomenon of aspirin resistance. Importantly, HDAC inhibitors are presently under clinical trials for the treatment of solid and hematologic malignancies.\(^50,51\) raising the possibility that, if HDAC3 is an endogenous antagonist to the salutary effects of aspirin on the endothelium, then specific HDAC3 inhibitors might offer therapeutic benefit when used in combination with aspirin for the treatment and prevention of cardiovascular diseases.

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Disclosures
None.

References


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Novelty and Significance

**What Is Known?**
- Low-dose acetylsalicylic acid (aspirin) is widely used to treat and prevent acute coronary syndromes and strokes.
- Aspirin modifies cyclooxygenase-1 in platelets, via acetylation of a serine residue, thereby inhibiting platelet aggregation.
- Aspirin is also known to have beneficial effects outside of platelets, including the vasculature, where it stimulates nitric oxide (NO) production, thereby improving endothelium-dependent vascular function.

**What New Information Does This Article Contribute?**
- Low-dose aspirin stimulates endothelial nitric oxide synthase (eNOS) by inducing lysine acetylation of eNOS, thus providing a new mechanism for the salutary effect of aspirin on the endothelium.
- Lysine acetylation of eNOS by aspirin promotes binding of eNOS to its cofactor calmodulin.
- The lysine deacetylase histone deacetylase (HDAC)3, targets aspirin-acetylated eNOS for deacetylation, thereby antagonizing the effect of aspirin on endothelial NO production.

Aspirin improves endothelial function in individuals with atherosclerotic risk factors. The mechanisms underlying the beneficial effects of aspirin on the endothelium are not fully understood. We demonstrate that low-dose aspirin stimulates endothelial NO by direct lysine acetylation of eNOS. In addition, HDAC3 inhibits aspirin-induced endothelial NO by deacylating aspirin-acetylated eNOS. These findings identify eNOS as a biologically relevant target of lysine acetylation by low-dose aspirin and deacylation by HDAC3. eNOS-derived NO also inhibits platelet activation and aggregation. Because many patients on low-dose aspirin therapy continue to have thrombotic events, inhibiting HDAC3 may augment the therapeutic benefit of aspirin in such “aspirin resistant” patients.
Histone Deacetylase 3 Antagonizes Aspirin-Stimulated Endothelial Nitric Oxide Production by Reversing Aspirin-Induced Lysine Acetylation of Endothelial Nitric Oxide Synthase

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Methods

Cell culture, plasmids, siRNA, and transfections.
Human embryonic kidney (HEK 293) cells were purchased from American Type Culture Collection and cultured in DMEM supplemented with 5% FBS and antibiotics. Human umbilical vein endothelial cells were purchased from Clonetics and cultured in endothelial growth medium. The eNOS (K631/633R), eNOS (K609R) and eNOS (S767/773R) mutants were created in this template by site-directed mutagenesis using the QuickChange kit (Stratagene). All mutations were verified by sequencing. Small interfering RNA for HDAC3 (5’-AAGATGCTGAACCATGCACCT-3’), COX-1 (5’-TGAGTGGCTATTTTCTGCAGCTGAA-3’) and the appropriate control siRNA were purchased from Invitrogen. Cells were transfected with plasmids and siRNA using Lipofectamine2000 (Invitrogen) per recommendations of the manufacturer.

Immunoblotting

Antibodies against acetyl-lysine (Cell Signaling), eNOS (Santa Cruz Biotechnology), HDAC3 (Santa Cruz Biotechnology), FLAG epitope (Santa Cruz Biotechnology), calmodulin (Cell signaling) and COX-1 (Cell signaling) were purchased. Lysine acetylation of eNOS was detected by immunoblotting of immunoprecipitated eNOS with acetyl-lysine (Ac-K) antibody. Immunoprecipitations of eNOS were carried out by incubating 2 μg of antibody with 1 mg of cell lysate or tissue homogenate overnight, followed by 30 μl of proteinA-sepharose slurry (Amersham) for 4 h. After washing, immunoprecipitates were boiled in SDS-PAGE gel loading buffer, subjected to SDS-PAGE, transferred to nitrocellulose filter, and probed with eNOS and Ac-K antibodies and the appropriate peroxidase-conjugated secondary antibodies (Santa Cruz Biotech). Western blotting of 50 μg of whole cell lysates or tissue homogenate was similarly performed using appropriate primary and secondary antibodies.

Co-immunoprecipitation

HEK 293 cells were transfected with FLAG-HDAC3 and eNOS. Cell pellet was collected by brief centrifugation. The pellet was suspended in IP lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 1x protease inhibitor cocktail (Roche Applied Science), 10 mM nicotinamide (NAM), 5 μM trichostatin A (TSA)). After centrifugation (14,000g, 15 min, 4°C), cell lysates were collected and protein concentration was determined by Bio-Rad Protein Assay reagent (Bio-Rad). Immunoprecipitations were carried out with rabbit (eNOS) and mouse (FLAG) antibodies, followed by immunoblotting with the same primary antibodies. SIRT1 (Santa Cruz Biotechnology), phosphor-eNOS (Thr495) (Santa Cruz Biotechnology), phospho-eNOS (Ser1177) (Santa Cruz Biotechnology) and β-actin (Santa Cruz Biotechnology) and were purchased. Non-immune mouse IgG (N-IgG-M) and rabbit IgG (N-IgG-R) were used for control immunoprecipitates. Co-immunoprecipitation of HDAC3 and eNOS in human umbilical vein endothelial cells were performed with and without treatment with low-dose aspirin. Immunoprecipitations were carried out mouse (HDAC3) antibody, followed by immunoblotting with rabbit (eNOS) and mouse (HDAC3) primary antibodies. Non-immune mouse IgG (N-IgG-M) was used for control immunoprecipitates. Cell lysates
were incubated with 2 μg of antibody for 16–20 h at 4°C followed by the addition of 30 μl of Protein-A or -G Sepharose beads (50% slurry). After further 3 h incubation at 4°C, beads were washed three times with IP lysis buffer with high salt (300mM NaCl). Samples were mixed with 2x SDS sample buffer and subjected to SDS–PAGE, followed by immunoblotting using the specified primary antibody and the appropriate secondary antibody. Because HDAC3 co-migrates with IgG heavy chain, peroxidase-conjugated Protein A (GE Healthcare Biosciences) was substituted for secondary antibody to detect HDAC3 immunoprecipitated from HUVEC lysates. Chemiluminescent signal was developed using Super Signal West Pico or Femto substrate (Pierce), and blots imaged with a Gel Doc 2000 Chemi Doc system (BioRad).

Nitrate and nitrite measurements. The NO-derived metabolites nitrite and nitrate were used as surrogate measures of NO. Phenol red-free conditioned medium or mouse serum was de-proteinized with 10-kDa cutoff filter (Millipore). The medium or serum was then processed for the measurement of the NO metabolites nitrite (NO\(_2^–\)) and nitrate (NO\(_3^–\)), the stable breakdown products of NO, using a commercially available Nitrate/Nitrite Fluorometric Assay kit (Cayman Chemicals) according to the manufacturer's recommendations. After subtraction of background fluorescence, values were normalized for total cell number (protein amounts).

NOS enzymatic activity. NOS activity was measured with a commercially available kit NOS Detect Assay kit (Stratagene). In brief, the conversion of L-[\(^{14}\)C]arginine to L-[\(^{14}\)C]citrulline was used to determine NOS activity in 30 μg whole-cell lysates, or 5 μg GST-eNOS, with addition of co-factors, according to the manufacturer's recommendations.

In vitro eNOS acetylation-deacetylation. The cDNA of full-length bovine eNOS was cloned into pGEX4T-2 (Amersham) bacterial expression plasmid. Protein was expressed and induced with isopropyl β-thiogalactoside (IPTG) (0.1 mM) in BL21-Gold (DE3) pLysS (Stratagene) bacterial host strain. Expressed eNOS was purified by using glutathione Sepharose beads (Amersham Biosciences) following the batch purification protocol recommended by the manufacturer. Purity of the eluted fractions was determined by SDS/PAGE and Coomassie staining. Acetylation was performed on purified GST-tagged recombinant eNOS that was desalted and buffer exchanged with acetylation buffer (10 mM Tris-HCl, pH 7.4). Five micrograms of GST-eNOS was incubated with aspirin (pH7.4) at 37°C for 30 min with shaking. De-acetylation was carried out by incubation with 300 ng of human recombinant HDAC3/N-CoR protein complex (BIOMOL) at 37°C for 30 min. NOS enzymatic activity of the reaction mixtures was measured, and total and lysine acetylated eNOS activity determined by immunoblotting.

Intracellular calcium imaging
HUVECs were incubated with the fluo 3-acetoxymethyl ester (AM; 5 μM) in Tyrode buffer (in mmol/ml): 140 NaCl, 4 KCl, 1.1 MgCl\(_2\), 10 HEPES, 10 glucose, 1.8 CaCl\(_2\); pH=7.4) for 30 min at 25 °C. After being washed twice with the buffer, the cells were again incubated for 20 min at 25 °C HUVECs loaded with fluo 3-AM were incubated
with aspirin (30 uM) or the calcium ionophore A23187 (10 µM; Sigma) for 10 min. Measurements of calcium imaging were obtained with a fluorescence microscope (excitation at 485 nm, emission at 520 nm). Intracellular fluo 3-AM fluorescence intensity was measured in 40 random cells in a filed using Adobe Photoshop. Mean intracellular fluorescence from 10 independent fields was calculated.

Mouse aortic ring reparations and vascular bioavailable NO measurements. 8-12 week old male mice were anesthetized and euthanized by rapid cardiac excision. The thoracic aorta was carefully dissected, and placed in ice-cold oxygenated Krebs-Ringer bicarbonate solution. The vessel was carefully cleared of loose connective tissue, and cut into 5-10 1.5 mm rings. Each ring was suspended between two wire stirrups (150 µm) in one of the 25-ml organ chambers of the four-chamber myograph system (DMT Instruments) in 5 ml Krebs-Ringer (95% O2-5% CO2, pH 7.4, 37°C). One stirrup was connected to a three-dimensional micromanipulator, and the other to a force transducer. The mechanical force signal is amplified, digitalized, and recorded (PowerLab 8/30). All concentration-effect curves were performed on arterial rings beginning at their optimum resting tone. This was determined by stretching arterial rings at 10 min intervals in increments of 100 mg to reach optimal tone (~500mg). One dosage of KCl (60mM) was administered to verify vascular smooth muscle viability. Bioavailable NO was measured physiologically by determining the increase in contractile response to NOS inhibition (L-NAME 10^-4M) in rings pre-constricted with phenylephrine (10^-9-10^-5 M). Values for bioavailable NO are expressed as percent of KCl-induced contraction.

Statistical analysis. All data are expressed as mean ± SEM. Endpoints included densitometric values or ratios from immunoblots, nitrite and nitrate concentrations in medium, 3H-citrulline counts, absolute or change in bioavailable NO, intracellular Fluo-3 AM fluorescence, and PE-induced contraction (expressed as % KCl-induced contraction). In time course experiments (0 min, 15 min, 30 min, 1h, 3h, 6h), the sample at each time point was independent form the other time points. Statistical analysis between two independent groups at a time was performed using SigmaStat. The two-sample unpaired t-test was employed for parametric data, and the Mann Whitney rank sum test for non-parametric data. P value <0.05 was considered statistically significant.
Online Figure I. Lysine acetylation of eNOS in HUVEC by aspirin. eNOS or lysine acetylated proteins were immunoprecipitated, followed by immunoblotting with eNOS and Ac-Lys antibodies. WCL: whole cell lysate. Immunoblots are representative of three independent experiments.
Online Figure II

Online Figure II. Inhibition of COX-1 does not abrogate low-dose aspirin-stimulated NO in HUVEC. NO metabolites (nitrite + nitrate) were measured in conditioned media of HUVEC treated with the COX-1 selective inhibitor sc-560. *p<0.05 (n=3). Representative immunoblots are shown.
Online Figure III. Low-dose aspirin does not affect SIRT1 expression. SIRT1 protein expression in HUVEC treated with aspirin. Representative immunoblots are shown with densitometric analysis of SIRT1/β-actin relative to no aspirin from independent blots. #p=NS (n=3).
Online Figure IV

Online Figure IV. Low-dose aspirin does not affect intracellular calcium in HUVEC.
a) Intracellular calcium, detected by Fluo-3 AM fluorescence in HUVEC treated with aspirin or the calcium ionophore A23187 for 10 min. Mean intracellular fluorescence relative to control (no aspirin) is shown. **P<0.001, and #p=NS (n=10). b) Calcium influx does not change lysine acetylation of eNOS. Lysine acetylation of eNOS immunoprecipitated from HUVEC treated with ASA or A23187. Representative immunoblots are shown with densitometric analysis of Ac-Lys/total eNOS relative to no aspirin. *p<0.05 (n=3).
Online Figure V. Aspirin stimulates NO produced by nonacetylatable eNOS (S767/773R). Low-dose aspirin stimulated NO (nitrite + nitrate) in conditioned media of HEK 293 cells expressing wildtype eNOS (eNOS (WT)) or eNOS with serine 767 and 773 mutated to non-acetylatable arginine (eNOS (S767/773R)). *p<0.05 (n=3). Representative immunoblots are shown.
Supplementary Figure VI. Aspirin does not change phosphorylation of eNOS. Threonine 497 (T497) and serine 1179 (S1179) phosphorylation of eNOS in HUVEC treated with aspirin. Representative immunoblots are shown.
Online Figure VII. Aspirin lysine acetylates eNOS expressed in HEK 293 cells. Whole immunoblots showing a) dose and b) time dependency of lysine acetylation of eNOS expressed in HEK 293 cells by aspirin.
Online Figure VIII. Acetylcholine-stimulated vascular relaxation is not affected by low-dose aspirin. Acetylcholine-stimulated, endothelium-dependent vasorelaxation of aortas of C57/Bl6 mice administered 3 mg/kg IV aspirin (▲) or salicylic acid (Δ) (n= 3).
Online Figure IX. Effect of low-dose aspirin on phenylephrine-induced vasoconstriction. Phenylephrine-induced vasoconstriction in the (a) absence, and (b) presence of L-NAME (10^{-4}M) of aortas of C57/Bl6 mice administered 3 mg/kg IV aspirin (▲) or salicylic acid (Δ). *p<0.05 (n= 3) for all data.