P300/CBP Associated Factor Regulates Nitroglycerin-Dependent Arterial Relaxation by Nε-Lysine Acetylation of Contractile Proteins

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Objective—To address the role of epigenetic enzymes in the process of arterial vasorelaxation and nitrate tolerance, in vitro and in vivo experiments were performed in the presence or absence of glycyltrinitrate (GTN) or histone deacetylases/histone acetylases modulators.

Methods and Results—In vitro single GTN administration rapidly increased cGMP synthesis and protein Nε-lysine acetylation in rat smooth muscle cells, including myosin light chain and smooth muscle actin. This phenomenon determined a decrease in myosin light chain phosphorylation and actomyosin formation. These effects were abolished by prolonged exposure to GTN and rescued by treatment with trichostatin A. In vivo, adult male rats were treated for 72 hours with subcutaneous injections of GTN alone or in combination with the histone deacetylases inhibitors trichostatin A, suberoylanilide hydroxamic acid, MS-27–275, or valproic acid. Ex vivo experiments performed on aortic rings showed that the effect of tolerance was reversed by all proacetylation drugs, including the p300/CREB binding protein–associated factor activator pentadecylidenediamonolate 1b (SPV 106). Any response to GTN was abolished by anacardic acid, a potent histone acetylases inhibitor.

Conclusion—This study establishes the following points: (1) GTN treatment increases histone acetylases activity; (2) GTN–activated p300/CREB binding protein–associated factor increases protein Nε-lysine acetylation; (3) Nε-lysine acetylation of contractile proteins influences GTN–dependent vascular response. Hence, combination of epigenetic drugs and nitroglycerin may be envisaged as a novel treatment strategy for coronary artery disease symptoms and other cardiovascular accidents of ischemic origin. (Arterioscler Thromb Vasc Biol. 2012;32:2435-2443.)

Key Words: nitroglycerin ■ protein acetylation ■ histone deacetylases ■ histone acetylases ■ smooth muscle cells

Nitric oxide (NO) directly or indirectly, by S-nitrosylation or cGMP synthesis, regulates the function of epigenetic enzymes, such as histone deacetylases (HDACs), a family of molecules which recently emerged as important during endothelial cell activation and skeletal muscle precursor differentiation. HDACs and their functional counterpart, histone acetylases (HATs), are epigenetic enzymes regulating the number of lysine residues covalently modified by the addition of Nε-acetyl groups. This posttransduction modification alters protein structure and function and, besides the activity of a number of enzymes, it may modify chromatin accessibility to transcription factors determining changes in gene expression during the adaptive response to environmental signals. HDACs are 18 different molecules grouped in 4 classes; members of class I, II, and III (the latter are also named sirtuins) are the best characterized and catalyze the removal of the acetyl groups on lysine residues. HATs are a diverse set of enzymes that can be divided on the structural basis of their catalytic domains. Among them, members of the GCN5/p300/CBP associated factor (PCAF) family represent an emerging subset of molecules identified as able to add the acetyl groups to a growing number of nuclear and cytoplasm proteins with important functional consequences for cell function.

A large number of small molecules have been made recently available that regulate the activity of these enzymes. Specifically, HDAC inhibitors (deacetylase inhibitors [DIs]), HAT inhibitors, and HAT activators have been successfully synthesized and proven effective in different experimental settings. Among

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those, the class I/II DIs trichostatin A (TSA), suberoylanilide hydroxamic acid, MS-27–275, valproic acid and the HAT inhibitors anacardic acid (ANAC) have been the most studied and used so far. Other compounds, however, with more selective specificities, such as class IIA–restricted DIs, and the HAT activators CTPB and SPV106 have been recently synthesized and are objects of intense experimental investigation. These epigenetically active drugs (epi-drugs) have the common property of changing N\textsuperscript{-}lysine acetylation on a variety of histone and nonhistone proteins as demonstrated in the mouse heart.

Since the identification of NO as the endothelial–derived relaxing factor, its importance in the regulation of the majority of the physiological and physiopathological cardiovascular processes emerged clearly. Altered NO synthesis, in fact, has been implicated in the pathogenesis of endothelial dysfunction, atherosclerosis, angina, coronary spasms, cardiac ischemia, and arterial stiffness during aging. Hence, NO donors have been widely used for the treatment of many cardiovascular accidents. Among them, glyceryl trinitrate (GTN), the oldest donor to be synthesized, is also one of the most successful compounds applied into clinical practice. Despite the large number of studies addressing GTN effects and its wide clinical application, its mechanism of activation and function remains partially elusive. The evidence that, at clinically relevant concentrations, GTN does not require active release of NO points out that cGMP synthesis, elicited by GTN in the absence of detectable NO release, is an important step for GTN therapeutic effects and relevant for the epigenetic effect of nitrate donors as previously reported.

After the long-term use of GTN, however, a severe nitrate tolerance develops and patients are at risk of losing GTN benefits. Several mechanisms have been reported as possible causes of GTN tolerance, including altered biotransformation, sympathetic activation, release of vasoconstrictors, plasma volume expansion, and increased oxidative stress. Remarkably, the role of epigenetic enzymes has never been explored in the general context of arterial relaxation and, specifically, in response to nitrates or in the presence of nitrate tolerance. In light of this consideration, the present work investigated whether epigenetic mechanisms could be involved in the regulation of the arterial adaptive response to GTN in vitro and in vivo. The results show that the HDAC/HAT activity balance, acting on the acetylation level of proteins involved in smooth muscle cell contraction, is important to preserve arterial responsiveness to GTN, preventing or reducing the extent of nitrate tolerance. Hence, epigenetic mechanisms may be envisaged at the basis of smooth muscle cell reaction in response to the GTN/cGMP axis activation.

Materials and Methods
A description of the materials and methods is presented in the online-only Data Supplement.

Results
GTN Treatment Alters HDAC/HAT Activity Balance in Rat Aortic Smooth Muscle Cells
Figure 1A shows that rat aortic smooth muscle cells (RASMC) produced a significant amount of cGMP measured at the 2-hour time point after GTN exposure (control vehicle cells [C-V] versus GTN-treated cells [G]). This treatment, repeated 24 hours later, failed to elicit the same effect as consequence of nitrate tolerance. At this later point, however, the addition of TSA, alone or in combination with GTN, stimulated cGMP production above basal and tolerant levels in tolerant (T/TSA), as well as normal control cells (TSA). Figure 1B shows that cGMP production, determined at 2 hours in whole rat aorta lysates, increased after the in vivo administration of GTN. A prolonged GTN treatment (72 hours), however, inhibited cGMP synthesis, as determined 2 hours after the last GTN administration. This negative effect was counteracted by the general HDAC inhibitor valproic acid (VPA) that administered together with GTN contributed to maintain cGMP production in tolerant animals (T/VPA) without influencing basal cGMP synthesis (VPA).

In this condition, systolic blood pressure revealed a significant reduction after the first injection of GTN. A phenomenon which disappeared after repeated injections (Figure IA in the online-only Data Supplement). Although VPA did not interfere with GTN response, a significant reduction of pressure was still detectable after repeated injection of GTN and VPA at the 72-hour time point (Figure IA in the online-only Data Supplement). This result suggests that the inhibition of HDAC activity could be important in preserving arterial and blood pressure responses to GTN. Total HDAC activity was measured in C-V-, G-, and tolerant (T)-RASMC, 2 hours after the first or second exposure to GTN and found significantly increased only after repeated exposures to the drug (T) (Figure 1C). In contrast, HAT activity was found increased 2 hours after the first GTN treatment (Figure 1D). In the presence of tolerance (T) the exposure to GTN for an additional 2 hours failed to elicit this effect (Figure 1D).

In order to verify whether cGMP production regulated HDAT activation in the presence of GTN a series of independent experiments were performed in which total HAT activity was measured in control cells, as well as cells treated with GTN or the NO donor diethyltriamine/nitric oxide in the presence or absence of the soluble guanylate cyclase inhibitor 1H-1,2,4 oxadiazolo[4,3-a]quinoxalin-1-1 (ODQ). Figure 1E shows that total HAT activity significantly increased in the presence of GTN (C-V versus G) or with the direct NO donor diethyltriamine/nitric oxide (D). This increment was efficiently counteracted by the soluble guanylate cyclase inhibitor ODQ that prevented HAT activation in both conditions. These results suggest that cGMP production is also important for HAT activation in smooth muscle cells.

To investigate whether the activation of HATS modified total N\textsuperscript{-}Lysine protein acetylation, a series of western blotting were performed on whole cell extracts obtained from RASMC cultured in control condition (C-V), treated for 2 hours with GTN (G) or made tolerant by repeated exposures to GTN (T). Figure 1F, in fact, shows in 3 independent experiments, a significant increase in total protein N\textsuperscript{-}lysine acetylation observed at the 2-hour time point after GTN treatment whereas the presence of tolerance avoided this modification, as confirmed by densitometry (Figure 1F, right).

This evidence suggests that epigenetically–determined posttransduction modifications of cellular proteins, such as N\textsuperscript{-}lysine acetylation, may be the consequence of adaptive responses to GTN and cGMP.
Modulation of HDAC/HAT Activity Regulates Smooth Muscle Relaxation

To evaluate the effect of HDAC/HAT activity balance on smooth muscle cell contraction, a series of in vitro experiments were performed with RASMC embedded in a type I collagen lattice.\(^2\) Phase-contrast microscopy showed that in 24 hours control collagen–embedded smooth muscle cells formed a reticular structure that was lost after a short-term (2 hours)
GTN treatment (GTN) whereas it remained intact in the presence of tolerance (Tolerant; Figure 2A). The presence of a cellular network was paralleled by collagen contraction \(^{23}\) (control [C] in Figure 2B). Figure 2B and 2C depict representative pictures of collagen lattices in which the effect of TSA on RASMC contraction was explored in parallel to that of the pan-HAT inhibitor ANAC. In Figure 2B, RASMC, plated in collagen, were cultured with (G, T) or without (C) GTN and in the presence or absence of TSA (T/TSA, TSA). In this experiment, collagen gel diameter was significantly enlarged by GTN (G) compared with control cells (C) remaining, however, unchanged in the presence of tolerance (T). Remarkably, TSA reduced the effect of tolerance allowing collagen gel relaxation despite repeated exposure to GTN (T/TSA). TSA alone (TSA) did not significantly influence collagen gel contraction. Figure 2C shows a dose response curve to ANAC (4.25, 8.5, and 17 \( \mu \)mol/L, respectively) in the presence of GTN. The HAT inhibitor ANAC dose-dependently abolished gel relaxation associated with GTN treatment (Figure 2C, top and bottom). Both of these experiments suggest that the presence of active HATs or inactive HDACs recognizes a condition important for GTN to exert its function.

To further explore the properties of proacetylating epi-drugs as vasorelaxation modulators and antitolerant agents, a series of in vivo experiments were performed to evaluate the effect of selected epi-drugs on GTN–dependent arterial response. Figure 3 shows the result of a representative series of in vivo combined treatments and ex vivo aortic ring relaxation experiments in which animals were pretreated for 72 hours with repeated injections of GTN in the presence or absence of DIs that do not alter sirtuins (class III HDACs) function, such as TSA, MS-27–275, or the HAT inhibitors ANAC and the HAT activators SPV106. Figure 3A depicts the effect of solvent (EC\(_{50}\) treated/[EC\(_{50}\) control] = 1), GTN (EC\(_{50}\)T/EC\(_{50}\)C = 50), TSA (EC\(_{50}\)T/EC\(_{50}\)C = 1) or that of the combined treatment which rescued nitrate tolerance restoring GTN sensitivity (EC\(_{50}\)T/EC\(_{50}\)C = 3.3). A similar effect was obtained with the class I HDAC inhibitor MS-27–275 (EC\(_{50}\)T/EC\(_{50}\)C = 1.3; Figure 3B). Conversely, treatment of nontolerant animals with ANAC alone, which reduces global protein N\(\varepsilon\)-lysine acetylation, significantly limited response to GTN administered ex vivo (EC\(_{50}\)T/EC\(_{50}\)C = 17; Figure 3C). Remarkably, ANAC pretreatment did not limit response to nifedipine or sodium nitroprusside (not shown) suggesting specificity for GTN which, opposite from the other 2 inducers of rapid arterial relaxation, requires bioactivation through cytosolic aldehyde dehydrogenase-2.\(^{24}\) Further experiments are required to elucidate this important point. However, the compound SPV106, a selective GNC5/PCAF activator,\(^{14}\) administered daily with GTN prevented the development of tolerance (EC\(_{50}\)T/EC\(_{50}\)C = 0.9; Figure 3D).

Figure 2. Rat aortic smooth muscle cells (RASMC) cells were suspended in collagen gel and allowed to form reticular structures (A, top) that were lost after GTN treatment (A, lower left) while retained in tolerant cells (A, lower right). B. Contracted gels were treated 2 hours with GTN (G) or induced to tolerance (2 hours + 24 hours). The effect of trichostatin A (TSA) on relaxation, in normal and in tolerant condition, was evaluated by measuring gel areas. \(*p<0.05\) vs control. (n=5). C. The dose-dependent effect of anacardic acid (ANAC) on GTN–dependent collagen relaxation. The measurement of gel areas is shown in the bottom panel. \(*p<0.05\) vs control (n=4). T indicates tolerant; C-V, control-vehicle.
similar to TSA or MS-27–275. Similar results were obtained with deendothelialized aortic rings from animals exposed to the combined treatment of GTN and the proacetylation agents TSA or SPV106 (Figure 3E and 3F). This observation indicates that the presence of an intact endothelium is not required for the epigenetic regulation of smooth muscle cell relaxation in the presence of GTN. To ascertain that the epigenetic drug treatment was sufficient to induce protein lysine acetylation in aortic smooth muscle cells immunofluorescence analysis was performed in sections from intact aortic rings excised at the 72-hour time point after exposure to TSA or MS-27–275. Figure IB in the online-only Data Supplement shows that in these conditions a positive acetylation signal from histone H3 Lysine 9 is well detectable.
The complete list of compounds used in this study, their concentration and their effect on aortic ring relaxation, expressed as EC\textsubscript{50} value and EC\textsubscript{50 treated}/EC\textsubscript{50 control} ratio, is summarized in Table 1. Taken together, these results suggest that the regulation of N\textsuperscript{\epsilon}-lysine acetylation is important in the vascular adaptive response to GTN.

**GTN Promotes N\textsuperscript{\epsilon}-Lysine Acetylation of Actomyosin Subunits**

Recent evidence indicates that the HDAC/HAT activity balance, controlling cardiac protein acetylation,\textsuperscript{15} is important for cardiomyocyte contractility\textsuperscript{25} and heart function.\textsuperscript{15} Prompted by this observation and our experimental findings, we investigated whether the N\textsuperscript{\epsilon}-lysine acetylation of proteins important for smooth muscle cell contraction could be involved in the regulation of RASMC adaptation to GTN. In support of this possibility, sequence comparison indicated that smooth muscle actin (SMA) and myosin light chain (MLC) bear a number of potentially acetylatable lysine residues conserved across species, according to Phosphorylation Site Database (PHOSIDA) analysis (www.phosida.com; Figure II in the online-only Data Supplement). Figure 4A and 4B show the results of a series of immunoprecipitation experiments in which MLC and SMA were evaluated for the presence of N\textsuperscript{\epsilon}-lysine–acylated residues. Figure 4A, top, shows that MLC isolated by immunoprecipitation from RASMC cultured in control condition (C) or treated with GTN (G) and TSA (TSA and T/TSA) is recognized by an anti-acetyl lysine antibody and that this signal is reduced in tolerant cells (T) as indicated by densitometry analysis (Figure 4A, bottom). A similar result was obtained with SMA (Figure 4B, top and bottom) indicating that both of these proteins were hyperacetylated after GTN treatment and that this process was negatively regulated by tolerance, whereas the presence of TSA or that of other

![Figure 4. Immunoprecipitation (IP) experiments showing the level of acetylated myosin light chain (MLC) (A) or smooth muscle actin (SMA) (B) in rat aortic smooth muscle cells (RASMC) cells treated for 2 hours with GTN (G) or induced to tolerance (T), alone or in combination with trichostatin A (TSA) (T/TSA). *P<0.05 vs control (n=3). Densitometry is shown in the graph. C, Summarizes the results from (A) and (B) showing the variation in SMA and MLC acetylation. D, Evaluation of SMA-associated HAT activity in RASMC cells. *P<0.05 vs control; #P<0.05 vs tolerant (n=3). E, Western blotting analysis of MLC phosphorylation (serine 20) in control, GTN-treated, tolerant-induced, or TSA-treated tolerant and control RASMC. The bottom panel indicates the percentage of phosphorylation level of MLC. *P<0.05 vs control; #P<0.05 vs tolerant (n=3). F, Coimmunoprecipitation experiment showing the relative association level of MLC and SMA in control, GTN-treated, tolerant-induced or TSA-treated tolerant and control RASMC. Densitometry is shown in the bottom panel. *P<0.05 vs control (n=3). G, Summarizes the results of (E) and (F) showing the percentage of SMA/MLC association and of phospho-MLC (p-MLC) variation. C-V indicates control-vehicle; T, tolerant; acetyl-lys, α-acetyl-lysine.](http://arch.ahajournals.org/content/full/10/12/2440)
lysine proacetylation agents rescued the phenotype. The graph in Figure 4C reveals that MLC and SMA Nε-lysine acetylation follows a similar pattern. Consistently, in the presence of GTN (G), SMA immunoprecipitated with an associated HAT activity, which was downregulated in tolerant cells (T) and slightly but significantly rescued by TSA (T/TSA). As expected, this activity was strongly enhanced in the presence of TSA known to have a positive effect on HAT activity (Figure 4D). Figure 4E shows that either GTN or TSA significantly reduced MLC phosphorylation, which is important for actomyosin formation and smooth muscle cells contraction. This phenomenon was paralleled by a reduced MLC–SMA complex formation in the presence of GTN or TSA as indicated by coimmunoprecipitation experiments (Figure 4F). The coincident regulation of MLC phosphorylation and actomyosin formation is further displayed in Figure 4G. Taken together, these results suggest that the Nε-lysine acetylation of MLC influences its phosphorylation and association with SMA indicating this posttransduction modification as a new modification important for the adaptive vascular response to contraction/relaxation signals.

**PCAF Becomes Physically Associated With SMA**

In cardiomyocytes, the lysine acetylase PCAF is involved in the regulation of sarcomeres contraction, in the regulation of calcium sensitivity, and, as recently reported, in the appropriate localization of connexin 43 at gap junctions. In order to provide information about PCAF involvement in the regulation of smooth muscle cell contraction, a series of experiments were performed in which RASMC were treated with the PCAF activator SPV106. Figure 5A shows that SMA acetylation significantly increased at the 2-hour time point after SPV106 treatment.

![Image](http://atvb.ahajournals.org/)

**Figure 5.** A, Immunoprecipitation experiment to evaluate the acetylation level of smooth muscle actin (SMA) in rat aortic smooth muscle cells (RASMC) treated with the p300/CBP associated factor (PCAF) activator SPV106 (SPV) at 30, 60, and 120 minutes. *P<0.05 vs control (C-V, control vehicle; n=3). Densitometric analysis is shown in the graph. B, Coimmunoprecipitation experiment showing the relative association level of PCAF and SMA at 30 and 60 minutes after GTN administration. *P<0.05 vs control (n=3). Densitometric analysis is shown in the graph. C, Western blotting showing PCAF RNA interference performed using different doses of oligos. D, Analysis of the effect of PCAF knockdown on SMA acetylation in control and SPV106-treated cells (n=3). E, Western blotting showing PCAF overexpression (lane 1: control; lane 2: electroporation in PBS; lane 3: electroporation in complete medium). Acetylated tubulin is shown as control (left panel). Immunoprecipitation experiment showing the relative level of acetylated SMA and myosin light chain (MLC) in control RASMC culture in the presence or absence of SPV106 or overexpressing PCAF (middle panel). Densitometry is shown in the right panel. F, Confocal analysis showing PCAF and SMA localization in control and GTN-treated RASMC cells. The graph shows the percentage of PCAF (fluorescent labelled) and SMA (rhodamin labelled) colocalization in the cytoplasm. Magnification ×80. Representative details are shown in the right panels. *P<0.05 vs control (n=3). G, Western blotting of cytosolic cell fractions of control, GTN-treated or tolerant-induced RASMC showing PCAF expression. Tubulin was used as loading control. Band density analysis is shown in the bottom panel. *P<0.05 vs control (n=3). siRNA indicates small interfering RNA; Ac-lys, α-acetyl-lysine.
changes in the dystrophic mouse heart is an example of an antioxidant treatment may introduce important epigenetic alterations between the 2 processes. Our earlier evidence that oxidative stress, it is conceivable that common mechanisms to nitrates.

In Figure 5F, left confocal microscopy shows that in the presence of GTN, PCAF content was increased in the cytoplasm where it colocalized with SMA as indicated by the quantitative evaluation of colocalization shown in the right panel. Figure 5G documents the GTN-dependent enhancement of cytoplasm PCAF localization in smooth muscle cells as revealed by subcellular fractionation and western blotting analysis.

**Discussion**

GTN is one of the most commonly used therapies for the treatment of cardiovascular incidents of ischemic origin. Its mechanism of action is complex and implies an aldehyde dehydrogenase-2-dependent bioactivation. Nitrate tolerance could be an unfavorable consequence of the prolonged and repeated GTN treatment. The origin of nitrate tolerance is still debated, but the recent finding that the elevation of intracellular oxidative stress may have a causative role opens the possibility that other related molecular processes to which, until now, little consideration has been paid, could be important for a better understanding of the vascular response to nitrates.

Although our comprehension of the relationship between oxidative stress and epigenetics is still in its infancy and it is unclear how epigenetic drugs may regulate intracellular oxidative stress, it is conceivable that common mechanisms are shared between the 2 processes. Our earlier evidence that an antioxidant treatment may introduce important epigenetic changes in the dystrophic mouse heart is an example of the cross-regulation occurring between the 2 processes. Incidentally, we report here that the class I/II inhibitor TSA is able to reduce hypoxia inducible factor 1α expression and the content of oxidative stress in tolerant cells (Figure IV A and IVB in the online-only Data Supplement), suggesting these are important steps through which DIs, and possibly other epi-drugs, prevent or reduce tolerance (schema in Figure V in the online-only Data Supplement). This observation is in line with the general anti-inflammatory effect associated to this type of epigenetic drugs. It, however, helps to understand only limited aspects of the antitolerant properties of the epigenetic molecules investigated in this study. A major accent must be put on the new experimental evidence that GTN determines an increase in global N^ε-l lysine acetylation of smooth muscle cell proteins including those involved in the process of arterial wall contraction/relaxation. In cells or animals made tolerant to GTN, in fact, MLC and SMA acetylation was found reduced, a condition that was partially reversed in the presence of different DIs or by the HAT activators SPV106. Accordingly, the inhibition of the intracellular proacetylation function by ANAC determined a reduction in GTN reactivity either in smooth muscle cells than in aortic rings obtained from animals not previously exposed to nitrates further emphasizing the importance of acetylases in this process. Our findings suggesting that protein N^ε-l lysine acetylation is important for the vascular smooth muscle cell response to GTN shed new light on the contraction/relaxation mechanism of vascular structures inferring that not only phosphorylation, but other types of protein posttransduction modifications, such as N^ε-l lysine acetylation, may be relevant (schema in Figure V in the online-only Data Supplement). Although the consequences of N^ε-l lysine acetylation on protein folding, function, and localization are currently not well understood, we must consider that lysines are positively charged aminoacids targeted by multiple modifications, including acetylation, ubiquitination, and methylation, which change their original status with relevant structural and functional effects on protein stabilization, degradation, and complex formation. Hence, the activation and nuclear export of PCAF, occurring after smooth muscle cell treatment with GTN, indicate the presence of a GTN-/cGMP-dependent signaling finalized to the intracellular redistribution and activation of at least 1 important member of the HAT compartment. The possibility that alterations of this mechanism could play a physiopathological role during the adaptive response to GTN is clearly suggested.

The lysine acetylase PCAF is a GCN5 homolog, which has been discovered as a CBP/p300 cofactor. PCAF is often detected in the nucleus and in the cytoplasm where it has been found involved in the acetylation of a number of histone and nonhistone proteins. The presence of PCAF associated with the cardiac sarcomeres and its involvement in the regulation of cardiomyocyte contractility and electric impulse transmission, prompted us to investigate whether this molecule could be implicated in the regulation of smooth muscle cell contraction. Our findings suggest that PCAF increases MLC and SMA acetylation above the basal level reducing actomyosin formation. The positive effect of SPV106, a compound which activates PCAF function, or that of PCAF overexpression further points to HAT–mediated epigenetic processes as important events activated by GTN-dependent signals and capable of preventing/reducing nitrate tolerance (Figure V in the online-only Data Supplement). Although unlikely, according to our experimental evidence, it remains unclear whether the epigenetic mechanism described here is involved in the immediate/early response to GTN, which occurs very rapidly after its application. Nevertheless, the upregulation of lysine acetylation, which contrasts MLC phosphorylation on serine 20 and changes contractile proteins complex formation, may be important for the prolonged response to GTN and more, in general, for an optimal level of vascular relaxation.

The evidence that GTN responsiveness may be modulated by epi-drugs opens new perspectives for the development of innovative therapies aimed at controlling vascular wall contraction and relaxation. In this light, cardiovascular epigenetics deserves special attention as it represents a cutting
edge frontier from which new understanding and treatments for cardiovascular diseases may come.

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Disclosures
None.

References
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### Table 1

**EX VIVO AORTIC RING RESPONSIVENESS TO GTN EVALUATED AFTER IN VIVO TREATMENT (72 HOURS; n=6 each group; n=12 for control animals)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC₅₀ (nM)</th>
<th>EC₅₀ treated/EC₅₀ control</th>
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<td></td>
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<tr>
<td>Control</td>
<td>24</td>
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</tr>
<tr>
<td>GTN (50)</td>
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<td>GTN (50)</td>
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**Abbreviations:**

GTN: glyceryl trinitrate; TSA: trichostatin A; SAHA: suberoylanilide hydroxamic acid; VPA: valproic acid; VPM: valpromide; SPV106: pentadecylidenemalonate 1b.
Supplemental Materials and Methods

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standard error of the mean (SEM). The following antibodies were used: anti-MLC (1:1000, polyclonal; Abcam), anti-SMA (1:500, monoclonal; Santa Cruz), anti-PCAF (1:500; monoclonal; Santa Cruz), anti-acetylated lysine (1:1000, polyclonal; Abcam), anti-HIF1α (1:1500, rabbit monoclonal; Abcam), anti-H3 acetylated lysine 9 (1:1000, polyclonal; Abcam) and anti-tubulin (WB 1:4000, monoclonal; Sigma). Co-immunoprecipitation experiments were performed using 500 µg of extracts after lysis of samples in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% triton x-100, 2 mM MgCl₂ and 1% DOC supplemented with 1mM PMSF and protease inhibitor mix. Ademtech´s Bioadembeads paramagnetic beads system was used to immunoprecipitate the specific proteins.

**Evaluation of epigenetic enzymes activity**

Proteins were obtained from rat aortic rings or from RASMC and homogenized in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton x-100, 10% glycerol supplemented with 1 mM PMSF and protease inhibitor mix) and 100 µg of extract were used. HDAC activity (Upstate Biotechnology fluorescent kit) was performed according to the manufacturer’s instructions; samples were incubated with a standard HDAC substrate (1h at 30°C) to allow deacetylation of the fluorimetric substrate and after the addition of the activator, fluorescence was measured (excitation=360 nm; emission= 460 nm). HAT activity was determined by a colorimetric assay according to manufacturer’s instructions (Biovision colorimetric kit). Colour production was determined at 440 nm after incubation at 37 °C for 30’. Data are expressed in arbitrary units (A.U.).

**PCAF RNA interference**

PCAF knock-down was performed in RASMCs by siRNA (a combination of three oligos: S153734; S153735; S153736, Ambion) according to manufacturer’s instruction. Three concentrations of siRNA oligos (10, 30 and 100 nM) were used and the effect was compared
to that of scramble oligo in control cells after 72 hours post transfection. 30 nM was chosen for the analysis of SMA acetylation level in immunoprecipitation experiments.

**PCAF overexpression**

Transient overexpression of CMV-PCAF (KAT2B; NM_003884, OriGene) was achieved in RASMC by electroporation either in PBS or in complete medium according to the following parameters: 10 μg of plasmid, capacitance 950 μF, voltage 0,2 V. After 48 hours the cells were lysed in immunoprecipitation buffer (Tris/HCl 20mM pH 7.4, EDTA 1mM, NaCl 150mM, NP40 1% plus protease inhibitors) and analyzed for SMA and MLC acetylation by immunoprecipitation assay. GFP- vector (PINCO) was used as transfection control.

**Statistical analysis**

Data are expressed as means ± SE from at least three experiments. Student's two-tailed t-test was applied to the experiments and a p<0.05 was considered statistically significant. In aortic ring relaxation assay the relaxation is expressed as percentage of decrease in tension developed after phenylephrine treatment. For each concentration-response curve, the concentration causing half-maximal relaxation was determined using the non-linear regression program, Graphpad Prism 5.0 and expressed as negative log molar concentration (EC$_{50}$). Data are presented as mean ± S.E.M. for EC$_{50}$ with 95% confidence intervals. The difference between independent aortic ring preparations was assessed using the F test. Significance was accepted at P < 0.01.

**References**

2. Colussi, C., Rosati, J., Straino, S., Spallotta, F., Berni, R., Stilli, D., Rossi, S., Musso, E., Macchi, E., Mai, A., Sbardella, G., Castellano, S., Chimenti, C., Frustaci, A., Nebbioso, A., Altucci,
Supplemental Figure I

Blood pressure and in vivo histone acetylation.

A) The graph shows the result of blood pressure measured in control male Swiss rats (n=5 each group) at day 1, 2 and 3 after the GTN administration alone or in combination with VPA 1mg/Kg. *p<0.05 vs control. B) The pictures show representative confocal immunofluorescence images depicting the level of histone H3 Lysine 9 acetylation (H3K9Ac) in sections obtained from aortas of rats kept in control condition or after treatment with TSA or MS275. Nuclei were counterstained with TOPRO3.

Supplemental figure II

SMA and MLC sequence comparison and acetylation sites prediction.

Sequence comparison and analysis of smooth muscle actin and myosin light chain reveals across species conserved putative acetylatable lysine residues according to PHOSIDA site.

Supplemental Figure III

Assessment of PCAF RNA interference and protein overexpression.

A) RASMC were transfected with increasing amounts of specific RNAi oligos as described in Methods. The picture (upper panel) shows a representative result of control western blotting experiments performed to evaluate interference efficiency. Band density analysis indicating reduction in PCAF expression is showed below. *p<0.05 vs control

B) A series of independent electroporation experiments were performed to induce PCAF overexpression in primary RASMC. The picture depicts the result of western blotting analysis showing PCAF levels in mock (1), phosphate buffered saline solution (PBS) (2) and complete medium (3) electropored cells. The level of acetylated tubulin-A (a known PCAF target) has
been evaluated as functional control (Ac-Tub). GAPDH normalization it is shown. Band density analysis is shown on the left. *p<0.05 vs control

**Supplemental Figure IV**

**HIF1α expression and oxidative stress determination.**

A) The upper panel shows the result of a western blot analysing revealing HIF1α expression in RASMC cells treated for 2 hours with GTN (G) or made tolerant upon repeated GTN treatment (24 hours+2 hours) alone (T) or in combination with TSA (T/TSA). TSA (TSA) and control vehicle treated cells (C-V) were also evaluated. The lower panel shows the relative band density plot. *p<0.05 vs control  

B) Representative confocal microscopy immunofluorescence images are shown depicting the intracellular content level of 8-oxoguanina determined during the experiment described in panel A. Fluorescence intensity plots and mean fluorescence intensity values (MFI) are reported for each condition.

**Supplemental Figure V**

**Schematic representation of the GTN-dependent mechanism leading to contractile proteins hyperacetylation and vasorelaxation.**

GTN administration induces release of nitric oxide species (NO, NOx) and/or elevation of the intracellular content of cGMP which results in PCAF activation. The PCAF-dependent acetylation of contractile proteins (MLC, SMA) contributes to actomyosin complex dissociation facilitating smooth muscle cell sarcomeres relaxation. Repeated administration of GTN determines oxidative stress and nitrate tolerance activating a HDAC-dependent signalling. Treatment with the HATa SPV106 compound, a PCAF activator, or different HDAC inhibitors (DIa), both conditions able to elevate protein Nε-lysine acetylation level, prevents tolerance increasing acetylation of contractile proteins and their dissociation.
Supplemental figure I

A

![Bar chart showing systolic pressure in millimeters of mercury (mmHg)]

- **C-V**
- **GTN**
- **GTN/VPA**

Day 1, Day 2, Day 3

GTN

VPA

B

RAT AORTA

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Supplemental Figure II : SMA and MLC Sequence comparison analyses and acetylation sites.

**Smooth muscle actin sequences**

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**Smooth muscle actin acetylation prediction sites (by Phosida)**

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Myosin light chain-2 sequences

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>gi|117558477|gb|AA126065.1| Myl2 protein [Rattus norvegicus]
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>gi|148224893|ref|NP_001087308.1| myosin, light chain 2, regulatory, cardiac, slow [Xenopus laevis]
MAPKKVTLNFGELGAKGAPDTELNAAFVPGDQIEVNNKAYRMLTQAERFSEKKEIQMFAPFDPVGTNLKQNLWIIHGGEDK

Myosin light chain acetylation prediction sites (by Phosida)

GI|49456869 |Homo sapiens ... K62 (86%), K89 (89%), K153 (89%)
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GI|50978373 |Canis lupus familiaris ... K62 (86%), K89 (89%), K153 (89%)
GI|17933672 |Drosophila melanogaster ... K62 (85%), K89 (88%), K109 (88%), K157 (86%), K180 (89%)
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standard error of the mean (SEM). The following antibodies were used: anti-MLC (1:1000, polyclonal; Abcam), anti-SMA (1:500, monoclonal; Santa Cruz), anti-PCAF (1:500; monoclonal; Santa Cruz), anti-acetylated lysine (1:1000, polyclonal; Abcam), anti-HIF1α (1:1500, rabbit monoclonal; Abcam), anti-H3 acetylated lysine 9 (1:1000, polyclonal; Abcam) and anti-tubulin (WB 1:4000, monoclonal; Sigma). Co-immunoprecipitation experiments were performed using 500 μg of extracts after lysis of samples in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% triton x-100, 2 mM MgCl₂ and 1% DOC supplemented with 1mM PMSF and protease inhibitor mix. Ademtech’s Bioadembeads paramagnetic beads system was used to immunoprecipitate the specific proteins.

**Evaluation of epigenetic enzymes activity**

Proteins were obtained from rat aortic rings or from RASMC and homogenized in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton x-100, 10% glycerol supplemented with 1 mM PMSF and protease inhibitor mix) and 100 μg of extract were used. HDAC activity (Upstate Biotechnology fluorescent kit) was performed according to the manufacturer’s instructions; samples were incubated with a standard HDAC substrate (1h at 30°C) to allow deacetylation of the fluorimetric substrate and after the addition of the activator, fluorescence was measured (excitation=360 nm; emission= 460 nm). HAT activity was determined by a colorimetric assay according to manufacturer’s instructions (Biovision colorimetric kit). Colour production was determined at 440 nm after incubation at 37 °C for 30’. Data are expressed in arbitrary units (A.U.).

**PCAF RNA interference**

PCAF knock-down was performed in RASMCs by siRNA (a combination of three oligos: S153734; S153735; S153736, Ambion) according to manufacturer’s instruction. Three concentrations of siRNA oligos (10, 30 and 100 nM) were used and the effect was compared
to that of scramble oligo in control cells after 72 hours post transfection. 30 nM was chosen for the analysis of SMA acetylation level in immunoprecipitation experiments.

**PCAF overexpression**

Transient overexpression of CMV-PCAF (KAT2B; NM_003884, OriGene) was achieved in RASMC by electroporation either in PBS or in complete medium according to the following parameters: 10 μg of plasmid, capacitance 950 μF, voltage 0.2 V. After 48 hours the cells were lysed in immunoprecipitation buffer (Tris/HCl 20mM pH 7.4, EDTA 1mM, NaCl 150mM, NP40 1% plus protease inhibitors) and analyzed for SMA and MLC acetylation by immunoprecipitation assay. GFP- vector (PINCO) was used as transfection control.

**Statistical analysis**

Data are expressed as means ± SE from at least three experiments. Student's two-tailed t-test was applied to the experiments and a p<0.05 was considered statistically significant. In aortic ring relaxation assay the relaxation is expressed as percentage of decrease in tension developed after phenylephrine treatment. For each concentration-response curve, the concentration causing half-maximal relaxation was determined using the non-linear regression program, Graphpad Prism 5.0 and expressed as negative log molar concentration ($EC_{50}$). Data are presented as mean ± S.E.M. for $EC_{50}$ with 95% confidence intervals. The difference between independent aortic ring preparations was assessed using the F test. Significance was accepted at P < 0.01.

**References**

2 Colussi, C., Rosati, J., Straino, S., Spallotta, F., Berni, R., Stilli, D., Rossi, S., Musso, E., Macchi, E., Mai, A., Sbardella, G., Castellano, S., Chimenti, C., Frustaci, A., Nebbioso, A., Altucci,

