GIT1 Mediates HDAC5 Activation by Angiotensin II in Vascular Smooth Muscle Cells

Jinjiang Pang, Chen Yan, Kanchana Natarajan, Megan E. Cavet, Michael P. Massett, Guoyong Yin, Bradford C. Berk

Objective—The G protein–coupled receptor (GPCR)-kinase2 interacting protein1 (GIT1) is a scaffold protein involved in angiotensin II (Ang II) signaling. Histone deacetylase-5 (HDAC5) has emerged as an important substrate of calcium/calmodulin-dependent protein kinase II (CamK II) in GPCR signaling. Here we investigated the hypothesis that Ang II–mediated vascular smooth muscle cell (VSMC) gene transcription involves GIT1-CamK II–dependent phosphorylation of HDAC5.

Methods and Results—Ang II rapidly stimulated phosphorylation of HDAC5 at Ser498 in VSMCs. Knockdown of GIT1 significantly decreased HDAC5 phosphorylation induced by Ang II. The involvement of Src, phospholipase γ (PLCγ), and CamK II in GIT1-mediated HDAC5 phosphorylation was demonstrated. The association of GIT1 and CamK II was constitutive but increased after stimulation with Ang II. Moreover, the interaction of GIT1 and CamK II through the ARF GTPase-activating protein (ARF-GAP) and coiled-coil domains of GIT1 was essential for the phosphorylation of HDAC5. Finally, knockdown of GIT1 decreased myocyte enhancer factor 2 transcriptional activity induced by Ang II.

Conclusions—This study identifies a novel function for GIT1 as a mediator of Ang II–induced VSMC gene transcription via a Src-PLCγ-CamK II-HDAC5 signaling pathway. (Arterioscler Thromb Vasc Biol 2008;28:892-898)

Key Words: G protein–coupled receptor-kinase interacting protein1 ■ GIT1 ■ angiotensin II ■ histone deacetylase 5 ■ Ca2+-calmodulin-dependent protein kinase II ■ VSMC

The importance of the renin angiotensin system (RAS) in cardiovascular disease has been dramatically shown by the beneficial effects of inhibiting the RAS with angiotensin-converting enzyme inhibitors and angiotensin II (Ang II) type 1 receptor (AT1R) blockers. The AT1R mediates VSMC migration, hypertrophy, proliferation, and vascular remodeling. The G protein–coupled receptor (GPCR)-kinase2 kinase 2 interacting protein-1 (GIT1) is a multi-domain scaffold protein involved in multiple GPCR signal pathways including endocytosis, cell adhesion, and migration. Our laboratory was the first to show that c-Src phosphorylates GIT1 in VSMCs after stimulation by Ang II. Furthermore, we showed that GIT1 binds key signaling mediators including PLCγ and MEK1. Conformational changes in GIT1 induced by tyrosine phosphorylation lead to the activation of PLCγ. Activation of PLCγ is responsible for the elevation of intracellular calcium, which results in the autophosphorylation (activation) of calcium/calmodulin-dependent protein kinase II (CamK II). Histone acetylation/deacetylation has emerged as a fundamental mechanism for the control of gene expression. Histone acetyltransferases stimulate transcription through acetylation of histones, resulting in relaxation of nucleosomes, and histone deacetylases (HDACs) antagonize this activity and repress transcription. Class II HDACs (HDACs 4, 5, 7, and 9) appear to be dedicated to the control of tissue growth and development. Phosphorylation of the amino termini of these HDACs from the nucleus to the cytoplasm, thereby derepressing HDAC target genes. HDAC5 acts as a negative regulator of cardiac growth. Transgenic mice lacking either HDAC5 or HDAC9 develop extremely enlarged hearts in response to pathological signals. CamK II plays an important role in HDAC5 phosphorylation induced by GPCR ligands. Based on these findings, we hypothesized that GIT1 mediates HDAC5 phosphorylation stimulated by Ang II via a pathway dependent on c-Src, PLCγ, and CamK II. Furthermore we propose that derepression of HDAC5 increases MEF2 transcriptional activity in VSMCs.

Materials and Methods

Antibodies to glutathione S-transferase (GST) monoclonal, GIT1 polyclonal, CamK II polyclonal, 14-3-3 polyclonal, and ERK1 monoclonal were from Santa Cruz Biotechnology Inc. c-Src (for

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chicken) and PLCγ antibody was from BD Transduction Laboratories. FLAG M2 monoclonal antibody was from Sigma. Xpress monoclonal antibody was from Invitrogen. Phosphospecific-ERK1/2 was from Cell Signaling. Phospho-Ser498 HDAC5 and Ser498 HDAC5 antibodies were from Signal Antibody Technology. Ang II was from ICN Biomedicals. PP2, U73122, and KN93 were from Calbiochem. Other chemicals were purchased from Sigma.

**Cell Culture and Transfection**

VSMCs were obtained from rat aorta as described, and passages 6 to 10 were used in the experiments. HEK293 cells and VSMCs were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in 5% CO2. HEK293T cells were transfected by Lipofectamine/plus (Invitrogen) with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in 5% CO2. HEK293T cells were transfected by Lipofectamine/plus (Invitrogen). For cotransfection with ATIR, a ratio of 3:1 was used. After allowing protein expression for 24 hours, cells were serum-deprived for 16 hours and stimulated with 100 nmol/L Ang II. GIT1 siRNA (AAGGCTGGAAGAAGAAGCTAC) and control nonsilencing siRNA (AATTCTCCGACACGTGTCACTC) were designed as described and synthesized by Ambion. VSMCs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol as described previously. GIT1 siRNA were prepared and transfected at 1 μmol/L for 48 hours as previously described, then serum-deprived for 16 hours, and stimulated with 100 nmol/L Ang II.

**Plasmid Constructs**

The mGIT1 expressed sequence tag clone (GenBank accession number AI414223) was purchased and completely sequenced. Then, full-length mGIT1 (GIT1[WT]) was cloned into the NotI and XbaI sites of Xpress-pCDA3.1 vector (Invitrogen; Xpress-GIT1[WT]). Using polymerase chain reaction (PCR), GIT1(1 to 420aa), GIT1(420 to 770aa) were cloned into Xpress-pCDA3.1 vector (Xpress-GIT1[1 to 635aa], Xpress-GIT1[1 to 420aa], Xpress-GIT1[420 to 770aa]), pEBB-Flag-CamK II was a generous gift from Dr Richard A. Mauzer (Department of Cell and Developmental Biology, L215, Oregon Health & Science University, 3181 South West Sam Jackson Park Road, Portland, Oregon, 97239).

**Immunoprecipitation and Immunoblotting**

For immunoprecipitations, cells were lysed in RIPA buffer (150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mmol/L Tris-HCl, pH 8.0). Protein concentrations in the lysates were determined as described. The protein samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with appropriate primary antibodies. After incubating with fluorescence-conjugated secondary antibodies, immunoreactive proteins were visualized by an Odyssey infrared imaging system (LI-COR Biotechnology). Densitometric analysis of the blots was performed with Odyssey software (LI-COR Biotechnology). Results were normalized by arbitrarily setting the densitometry of control samples to 1.0.

**Immunofluorescence**

VSMCs cells were starved with serum-free DMEM overnight and then stimulated with Ang II in 0, 5, 10, 30, and 60 minutes. Cells were fixed with 4% formaldehyde for 10 minutes, washed with phosphate-buffered saline 3 times, permeabilized with 0.05% Triton for 5 minutes, and blocked with 10% normal goat serum for 1 hour. Cells were incubated with GIT1, and CamK II antibodies diluted in phosphate-buffered saline followed by Alexa Fluor 546 antirabbit IgG for red fluorescence or by Alexa Fluor 488 goat antimouse for green fluorescence (Molecular Probes Inc) in phosphate-buffered saline at a final concentration of 1.5 to 2 μg/mL each.

**Luciferase Reporter Assay**

To assess myocyte enhancer factor 2 (MEF2) transcriptional activation, we used 3× MEF2-dependent reporter gene [generous gift from Dr Joseph Miano (Aab Cardiovascular Research Institute, University of Rochester School of Medicine and Dentistry, Rochester, Rochester, NY 14642)] in which 3 tandem repeats of MEF2 sites were located upstream of the thymidine kinase gene promoter. VSMCs cultured in 24-well dishes were cotransfected with 3× MEF2 luciferase reporter gene, thymidine kinase-reilla-luciferase (an internal control from Promega), Myc-HDAC5-WT plasmid (gift from Dr Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, Texas) control siRNA, or GIT1 siRNA in each experiment using electroporation (Bio-Rad). At 32 hours posttransfection, cells were treated with Ang II for another 16 hours. The luciferase activities in cell lysates were determined using the Dual-Luciferase Reporter Assay kit (Promega) and Wallace 1420 multilabel counter (PerkinElmer).

**Statistical Analysis**

All values are expressed as means±SD from 3 to 6 independent experiments. The significance of the results was assessed by t test. A probability value <0.05 was considered statistically significant.

**Results**

**Ang II Stimulates Phosphorylation of HDAC5 in Rat VSMCs**

Because HDAC5 is phosphorylated by CamK II and we showed that GIT1 phosphorylation by Ang II is required for PLCγ-mediated calcium mobilization and CamK II activation, we studied HDAC5 phosphorylation in VSMCs in response to Ang II. Phosphorylation of HDAC5 was determined by phospho-HDAC5–specific antibody, which recognizes phosphorylated at Ser498. In response to 100 nmol/L Ang II, HDAC5 phosphorylation rapidly increased by 2.6-fold within 2 minutes, and reached a maximum at 5 minutes (3.3-fold; Figure 1A). HDAC5 phosphorylation returned to baseline after 30 minutes (Figure 1A). HDAC5 expression did not change during this time course.

**GIT1 Is Required for Phosphorylation of HDAC5 by Ang II**

To show a role for GIT1 in HDAC5 phosphorylation, we used rat GIT1 siRNA to decrease GIT1 expression in VSMCs as previously described. Rat control siRNA and rat GIT1siRNA were designed based on unique sequences and ability to inhibit mRNA expression. Transfection of GIT1 siRNA significantly decreased GIT1 protein expression at 48 hours, whereas HDAC5 expression was not altered (Figure 1B). Treatment with rat GIT1 siRNA significantly decreased HDAC5 phosphorylation induced by Ang II (80% inhibition), whereas control siRNA had no significant effect (Figure 1B).

**Ang II Stimulates HDAC5 Phosphorylation via Src-Dependent Pathway**

Previous data from our laboratory suggested an essential role for c-Src in AT1R signal transduction. To investigate the role of c-Src in HDAC5 phosphorylation, the c-Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d] pyrimidine (PP2) was administrated for 30 minutes in VSMCs, before stimulation with 100 nmol/L Ang II for 5 minutes. Phosphorylation of HDAC5 was significantly decreased by PP2 treatment (supplemental Figure IA). To further confirm the role of c-Src, the dominant negative (DN) chicken c-Src adenovirus (Ad.DN-Src) was used to infect VSMCs. Infection of VSMCs with Ad.DN-Src resulted in robust expression of chicken Src (supplemental Figure IB). Infection at MOI of 100 and 300 almost completely blocked...
PLCγ and CamK II Are Required for HDAC5 Phosphorylation by Ang II

c-Src phosphorylates GIT1, which is bound to PLCγ in a complex under basal conditions. Conformational changes in GIT1 induced by tyrosine phosphorylation lead to the activation of PLCγ which is required for elevation of intracellular calcium, and autophosphorylation of CamK II. To determine the involvement of PLCγ and CamK II in HDAC5 phosphorylation in VSMCs, the effects of the PLCγ inhibitor, U73122, and the CamK II inhibitor KN93 were investigated. Both U73122 and KN93 dose dependently inhibited the phosphorylation of HDAC5 (supplemental Figure IC and ID). These results suggest that PLCγ and CamK II play important roles in the Ang II–stimulated HDAC5 signaling pathway.

Association of CamK II, HDAC5, and 14-3-3 Is Increased by Ang II

Phosphorylation of HDAC5 creates binding sites for the 14-3-3 proteins, which escort p-HDAC5 from the nucleus to the cytoplasm, with consequent activation of HDAC5 target genes. To investigate the association of CamK II with HDAC5 and 14-3-3 after stimulation of Ang II in VSMCs, coimmunoprecipitation was performed. The interaction of CamK II with HDAC5 and 14-3-3 increased rapidly (within 1 minute, Figure 2A), and peaked at 5 minutes, similar to the peak phosphorylation of HDAC5.

The Interaction of GIT1 and CamK II Is Increased by Ang II

Because GIT1 is a multidomain scaffold protein, we hypothesized that GIT1 functions as a scaffold for CamK II by binding to CamK II. The association of GIT1 and CamK II was assayed by immunoprecipitating CamK II from VSMC lysates. Whereas the binding of GIT1 and CamK II was constitutive (Figure 2B, time 0, supplemental Figure II), in response to Ang II binding rapidly increased and peaked at 5 minutes (2.6-fold increase, Figure 2B, supplemental Figure II), similar to the HDAC5 phosphorylation time course. The time course for GIT1-CamK II binding was similar to the time course for CamK II binding to HDAC5 and 14-3-3 (Figure 2A, supplemental Figure II), suggesting a multiprotein complex. To investigate further the potential interaction of HDAC5 and GIT1, we coexpressed them in HEK293 cells (which have low expression of CamK II). There was no basal interaction, nor increase in response to Ang II (supplemental Figure III).

GIT1 Recruited CamK II and PLCγ to Form a “Calciosome” Complex, Which Mediated HDAC5 Phosphorylation by Ang II

Because GIT1 can associate with both CamK II and PLCγ, we hypothesized that GIT1, CamK II, and PLCγ could form a complex required for phosphorylation of HDAC5, which we will term the “Calciosome.” The association of GIT1, CamK II, PLCγ, and HDAC5 was assayed by immunoprecipitating PLCγ and GIT1 from VSMC lysates. In VSMCs, GIT1, CamK II, and PLCγ were present in the same complex as shown by the findings that any one of these proteins coprecipitated the other two proteins (Figure 2C and 2D) if GIT1 was present. This presumed role of GIT1 as a scaffold molecule for CamK II and PLCγ was proved using 293 cells, which express no detectable GIT1. In 293 cells transfected with vector control (pcDNA), there was no interaction, nor increase in response to Ang II (supplemental Figure III).
HDAC5, 14-3-3, PLC increased by Ang II. The association of endogenous CamK II, were assayed by immunoprecipitation respectively with PLC and HDAC5 or the interaction of GIT1, CamK II, and HDAC5 were assayed by immunoprecipitation respectively with PLCγ antibody (C) or GIT1 antibody (D).

Figure 2. Association of GIT1, CamK II, and PLCγ was increased by Ang II. The association of endogenous CamK II, HDAC5, 14-3-3, PLCγ, and GIT1 in VSMCs was assayed by immunoprecipitation (IP) with CamK II antibody and probing for HDAC5 and 14-3-3 (A), GIT1 (B). The binding of CamK II, PLCγ, and HDAC5 or the interaction of GIT1, CamK II, and HDAC5 were assayed by immunoprecipitation respectively with PLCγ antibody (G) or GIT1 antibody (D).

Figure 3. Colocalization of GIT1 and CamK II by immunofluorescence. VSMCs were incubated with Ang II for the indicated times. The cells were washed, fixed, and stained using GIT1 and CamK II antibodies. GIT1 was visualized as red fluorescence, CamK II visualized as green fluorescence, and colocalization of GIT1 and CamK II as yellow fluorescence. Bar represents 25 μm.

Colocalization of GIT1 and CamK II
To further confirm the interaction of GIT1 and CamK II, immunofluorescence experiments were performed. After serum starvation for 24 hours, VSMCs were incubated with Ang II for up to 60 minutes. GIT1 and CamK II location was assayed by immunohistochemistry. In the absence of Ang II, GIT1 distributed across the entire cell (Figure 3A). The localization of CamK II was similar to GIT1 (Figure 3B). This is consistent with a previous report that CamK IIδ, locates in cytoplasm while CamK IIδ is nuclear.8,21 Dual immunofluorescent detection revealed that GIT1 colocalized with CamK II basally (Figure 3C). After administration of Ang II for 5 to 10 minutes, GIT1 translocated to the perinuclear and nuclear area (Figure 3D, G). CamK II also translocated to the perinuclear and nuclear areas (Figure 3E and 3H). GIT1 and CamK II were mostly colocalized during this period (Figure 3F and 3I), consistent with the immunoprecipitation results (Figure 2). From 30 to 60 minutes, both GIT1 and CamK II returned to the cytoplasmic compartment (Figure 3J through 3O). The similar translocation of GIT1 and CamK II suggests a significant functional interaction.

Domains of GIT1 That Mediate Interaction With CamK II
GIT1 is composed of an ARF GAP domain, an ankyrin repeat region, 2 carboxyl paxillin-binding subdomains, a SpaI homology domain (SHD), synaptic localization domain (SLD), and 3 putative coiled-coil (CC) domains (Figure 4A). To define the domains responsible for the GIT1-CamK II interaction, we transfected HEK 293 cells with Flag-CamK II and the GIT1 deletion mutants described in methods. Immunoprecipitation of CamK II with anti-Flag antibody coprecipitated GIT1(1–770), GIT1(1–635), but not GIT1(1–420), GIT1(250–770), or GIT1(420–770; Figure 4B and 4C). These results suggest that both the ARF-GAP and CC2 domains are required for GIT1-CamK II interaction because the only GIT1(1–635) has both the ARF-GAP domain and the CC2 domain, whereas the other mutants lack one of these domains. GIT1 functions as a scaffold for CamK II by binding to CamK II, which is essential for phosphorylation of HDAC5.

To determine the functional significance of GIT1 binding with CamK II, HDAC5 phosphorylation induced by Ang II was studied in 293 cells transfected with Flag-CamK II, Xpress-GIT1 or both (note the AT1R was also cotransfected; Figure 5). Overexpression of GIT1 or CamK II alone had no significant effect on HDAC5 phosphorylation (Figure 5A and 5B). However, when both were overexpressed, HDAC5 phosphorylation significantly increased (2.8-fold, Figure 5A and 5B). Based on the finding that the ARF GAP domain and CC2 domain are essential for GIT1 and CamK II interaction, we determined the ability of GIT1 mutants to increase phosphorylation of HDAC5.
As shown in Figure 5C and 5D, GIT1 mutants lacking the ARF-GAP domain (eg, GIT1[420–770]) or CC2 domain (eg, GIT1[1–420]) had significantly less effect on phosphorylation of HDAC5 compared with WT GIT1 (P<0.05, Figure 5C and 5D), but still substantial effect on phosphorylation of HDAC5 compared to pcDNA group (P<0.05, Figure 5C and 5D). However in Figure 4 we showed that these mutants could not bind to CamK II. How can the mutants that do not bind to CamK II still induce HDAC5 phosphorylation? Our hypothesis is that these 2 mutants may bind to CamK II through 1 binding site. However, with loss of 1 required binding domain, this binding is much weaker than the binding of GIT1 (WT) or GIT1[1–635] (including required 2 binding domain) to CamK II. This weak interaction is difficult to detect by immunoprecipitation. All these data suggest that GIT1 functions as a scaffold for CamK II, thereby increasing phosphorylation of HDAC5.

**GIT1 Is Required for Ang II–Induced MEF2 Transcriptional Activity in VSMCs**

In the nucleus, HDAC5 associates with the myocyte enhancer factor–2 (MEF2), which represses MEF2 transcriptional activity.22,23 To determine the role of GIT1 in HDAC5–mediated regulation of MEF2 transcriptional activation in VSMCs, we transfected VSMCs with a 3× MEF2-luciferase reporter plasmid and myc-HDAC5 WT. We then determined the effect of decreasing GIT1 expression with GIT1 siRNA on MEF2 activity. Ang II significantly increased MEF2 transcriptional activity in VSMCs (Figure 6), which was decreased by knockdown of GIT1 (Figure 6).

**Discussion**

The major finding of this study is that GIT1 is a novel mediator of Ang II–mediated VSMC gene transcription. Specifically we show that GIT1 participates in an Ang II signaling pathway that involves phosphorylation of HDAC5 and activation of MEF2, downstream of a pathway that requires Src, PLCγ, and CamK II (supplemental Figure V). Our results suggest that GIT1, via its multi-domain scaffolding function, coordinates Ang II signaling events that control calcium-dependent signaling (PLCγ and CamK II).

The focus of the present study is on CamK II which is a family of cytosolic serine/threonine protein kinases that exist as multimers consisting of α, β, δ, or γ subunits, each encoded by a different gene.24,25 Whereas CamK Iα and β are mainly expressed in neuronal tissues, CaMKIIδ, and CaMKIIγ are abundant in the heart.6,21,26 and VSMCs.27 CaMKII can phosphorylate type II HDACs.11 These HDACs (HDAC 4, 5, 7, and 9) are mainly expressed in neuronal tissues, CaMKII can phosphorylate type II HDACs.11 This HDAC5 results in marked cardiac hypertrophy.14,15 Ang II is an important cardiovascular hormone that induces myocardyocyte and VSMC hypertrophy.3,29,30

Here we investigated the role of HDAC5 phosphorylation in MEF2 activation induced by Ang II. Ang II rapidly induces phosphorylation of HDAC5 in rat VSMCs, with a peak at 5 minutes. In VSMCs, Ang II binding to the AT1R activates c-Src. c-Src phosphorylates GIT1, which is bound to PLCγ in a complex under basal conditions. Conformational changes in GIT1 induced by Src-dependent tyrosine phosphorylation lead to the activation of PLCγ. Activation of PLCγ is responsible for elevation of intracellular calcium, which results in the autophosphorylation of CamK II. Activation of CamK II causes HDAC5 phosphorylation and changes in target gene expression. Data to support this pathway include the finding that knockdown of GIT1 by siRNA significantly inhibited phosphorylation of HDAC5. Furthermore, DN-Src adenosivirus infection, Src inhibitor PP2, PLCγ inhibition, and CamK II inhibition decreased phosphorylation of HDAC5 induced by Ang II. These data show that GIT1 plays an important role in HDAC5–dependent signaling by regulating the activation of PLCγ.

We previously showed that GIT1 acted as a scaffold protein for the MEK1-ERK1/2 pathway. Specifically GIT1 exhibits 4 scaffold characteristics4,31 including (1) assembly of specific modules; (2) excluding (or insulating) other molecules; (3) promoting sequential activation of enzymes by physical interaction; and (4) providing feedback regulation of cell surface receptors. Here we show that GIT1 acts as a scaffold protein for CamK II. The interaction of GIT1 and CamK II was constitutive and increased after Ang II stimulation. The interaction of GIT1 and CamK II was further confirmed by immunofluorescence colocalization. Both GIT1 and CamK II (CamK IIβ or CamK IIδ) translocated from cytoplasm to nucleus in a similar manner after stimulation with Ang II. In contrast, there was no direct interaction between GIT1 and HDAC5, nor between PLCγ and CamK II. Rather GIT1 acts as a scaffold protein to assemble a “calcium signaling complex” (eg, “Calciosome”), which includes
PLCγ as well as CamK II. This complex facilitates phosphorylation of HDAC5 by CamK II. Although it has been reported that PLCγ translocates to the nucleus in VSMCs, it is unclear how this complex could translocate from cytoplasm to nucleus.

GIT1 is composed of an ARF GAP domain, an ankyrin repeat region, 2 carboxyl paxillin-binding subdomains, a SpaII homology domain (SHD), and 3 putative coiled-coil (CC) domains. The ARF-GAP domain has been shown to be functionally important for endocytosis. The CC2 region is important for interactions with PAK, and the CC3 region (including residues 646 to 770) is essential for interactions with paxillin, and the SHD region overlaps with binding sites defined for FAK and PIX. The domains of GIT1 required for CamK II interaction are located in the N-terminal ARF GAP domain and CC2 domains. Although binding of CamK II to GIT1 is constitutive, it appears that specific conformational changes occur in GIT1 or CamK II in response to tyrosine phosphorylation of GIT1 that enable GIT1 (and CamK II) translocation to the nucleus. It is likely that dephosphorylation contributes to export from the nucleus.

Recently it was reported that Ang II–dependent phosphorylation of HDAC5 in VSMCs is through a PKC and PKD pathway that is resistant to the calcium chelator BAPTA/AM as well as CaMK inhibitors KN93 and KN62. Our data suggest that GIT1 and CamK II play an essential role in phosphorylation of HDAC5, possibly independent of the

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Figure 5. Binding of GIT1 and CamK II is essential for phosphorylation of HDAC5. A–B, HEK293 cells were transfected with pcDNA, Xpress-GIT1(wt), Flag-CamKII, or Xpress-GIT1(wt)/H11001 Flag-CamKII and stimulated with Ang II for 5 minutes. C–D, HEK293 cells were cotransfected with Xpress-GIT1WT or GIT1 mutants with Flag-CamK II and stimulated with Ang II for 5 minutes. Lysates was immunoblotted for p-HDAC5, Actin, anti-Xpress, and anti-Flag antibody B and D, Relative increase of HDAC5 phosphorylation compared to pcDNA group or WT group. *P<0.05 compared with pcDNA group; #P<0.05 compared with WT group. (means±SD; n=3).

Figure 6. GIT1 altered Ang II–stimulated MEF2 transcriptional activity. Luciferase activity was determined in VSMCs transfected with MEF2 reporter gene and control or GIT1 siRNA. The graphs represent averaged data (means±SD, n=6). (*P<0.05 vs control siRNA without Ang II; #P<0.05 vs control siRNA and Ang II group.)
PKC-PKD signal pathway. The discrepancy regarding the role of calcium and CamK II could be attributable to different cell conditions and experimental procedures.

In summary, we have demonstrated that HDAC5 is phosphorylated in rat VSMCs by Ang II via a pathway that involves PLCγ and CamK II. We also found that GIT1 and HDAC5 are involved in Ang II–stimulated MEF2 transcriptional activity (supplemental Figure V). These findings suggest novel roles for GIT1 and HDAC5 in Ang II signaling in VSMCs.

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Disclosures
None.

References
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Supplemental Data

Supplemental Figure1. c-Src, PLCγ and CamkII are required for HDAC5 phosphorylation
VSMC were incubated with PP2(A), U73122(C) or KN93(D) for 30 min, then stimulated with AngII for 5 min. In (B) Ad.DN-Src or Lac-Z were infected, then the VSMCs were stimulated with AngII for 5 min. HDAC5, ERK1/2 were detected.

Supplemental Figure2. Quantitation of the binding of GIT1, CamKII, PLCγ and HDAC5 in VSMC in figure2
Ratio of GIT1 to CamKII(A); HDAC5 or 14-3-3 to CamKII(B); CamKII or HDAC5 to PLCγ(C); HDAC5 or CamKII to GIT1(D) of different time point compared to time 0 group.

Supplemental Figure3. GIT1 didn’t associate with HDAC5 in HEK293 cells.
HEK293 cells were transfected with Xpress-GIT1 and Myc-HDAC5 and stimulated with AngII for 5min. The association of GIT1 and HDAC5 was assayed by immunoprecipitation with Xpress antibody. Western blot of total cell lysis showed the equal expression of HDAC5 and GIT1.

Supplemental Figure4. PLCγ didn’t directly interact with CamKII.
HEK293 cells were transfected with Flag-CamKII and Xpress vector or Flag-CamKII and Xpress-GIT1 (WT). The association of PLCγ and CamKII was assayed by immunoprecipitation (IP) with PLCγ or CamKII antibody. Lower panel showed the expression of GIT1, PLCγ and CamKII.

Supplemental Figure5. Model for GIT1-mediated HDAC5 signaling.
AngII activates c-Src, PLCγ and CamKII by AT1R. GIT1 assembles CamKII and PLCγ to form a “Calciosome”, which is required for phosphorylation of HDAC5. Phosphorylated HDAC5 is
exported via 14-3-3, thereby increasing MEF2 transcriptional activity, derepressing HDAC5 target genes induced by AngII.
Supplemental Fig. 1

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Supplemental Fig. 2
HEK293+AT1R

AngII

- + - +

IB: Myc-HDAC5

IB: Xpress-GIT1

IP: Xpress-GIT1 Total Lysis

Supplemental Fig. 3
Supplemental Fig. 4

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<th>Xpress-GIT1</th>
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<th>Total Lysis</th>
<th>IP: CamKII</th>
<th>Total Lysis</th>
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HEK293 cell

**IB:** Flag-CaMKII

**IB:** Xpress-GIT1

**IB:** PLCγ

**IP:** PLCγ

**Total Lysis**

**IP:** CamKII

**Total Lysis**