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Review Article

REVIEW ARTICLE

The 2023 Walter B. Cannon Award Lecture: Mechanisms Regulating Vascular Function and Blood Pressure by the PPARγ-RhoBTB1-CUL3 Pathway

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Abstract

Human genetic and clinical trial data suggest that peroxisome proliferator activated receptor γ (PPARγ), a nuclear receptor transcription factor plays an important role in the regulation of arterial blood pressure. The examination of a series of novel animal models, coupled with transcriptomic and proteomic analysis, has revealed that PPARγ and its target genes employ diverse pathways to regulate vascular function and blood pressure. In endothelium, PPARγ target genes promote an antioxidant state, stimulating both nitric oxide (NO) synthesis and bioavailability, essential components of endothelial-smooth muscle communication. In vascular smooth muscle, PPARγ induces the expression of a number of genes that promote an antiinflammatory state and tightly control the level of cGMP, thus promoting responsiveness to endothelial-derived NO. One of the PPARγ targets in smooth muscle, Rho related BTB domain containing 1 (RhoBTB1) acts as a substrate adaptor for proteins to be ubiquitinated by the E3 ubiquitin ligase Cullin-3 and targeted for proteasomal degradation. One of these proteins, phosphodiesterase 5 (PDE5) is a target of the Cullin-3/RhoBTB1 pathway. Phosphodiesterase 5 degrades cGMP to GMP and thus regulates the smooth muscle response to NO. Moreover, expression of RhoBTB1 under condition of RhoBTB1 deficiency reverses established arterial stiffness. In conclusion, the coordinated action of PPARγ in endothelium and smooth muscle is needed to maintain NO bioavailability and activity, is an essential regulator of vasodilator/vasoconstrictor balance, and regulates blood vessel structure and stiffness.

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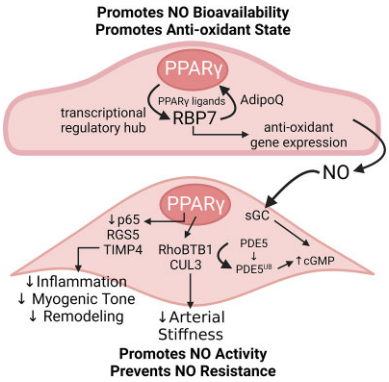

Key words: PPARγ; endothelium; smooth muscle; blood pressure; hypertension

Introduction

Peroxisome proliferator activated receptor γ (PPARγ) is a ligand-activated nuclear receptor transcription factor and a member of the large nuclear receptor superfamily.[1] Nuclear receptors are sensors that monitor the physiological environment and respond when a physiological stimulus or stress causes the production of ligands, which bind to and activate the receptor to mediate a reprogramming of gene expression. Peroxisome proliferator activated receptor γ is classified as an adopted orphan receptor because it is associated with at least one ligand (fatty acids and eicosanoids) but the precise endogenous ligands employed in vivo remain undefined.[2] Canonical nuclear receptors such as the glucocorticoid receptor translocate to the nucleus in response to ligand binding to induce transcriptional activity. Unlike those canonical nuclear receptors, PPARγ resides in the nucleus in its unliganded state and is complexed with its obligate heterodimeric partner, the retinoid X receptor (RXR) on chromatin. Transcription of PPARγ target genes is repressed because transcriptional co-repressors are recruited to PPARγ/RXR in the absence of ligand. In response to endogenous ligands or high-affinity pharmacologics that act as ligands, transcriptional co-repressors are dismissed, and transcriptional co-activators and chromatin-modulating enzymes such as histone acetyltransferases are recruited to the transcription complex, resulting in the expression of target genes. It was reported that PPARγ can bind to over 5000 genomic sites, but the actual number of targets genes is most likely substantially smaller and highly cell-specific.[3,4]

Peroxisome proliferator activated receptor γ is a potent anti-inflammatory and employs lessor appreciated mechanisms to inhibit the transcription of inflammatory genes. In the transrepression mechanism, posttranslational modification of the ligand binding domain of PPARγ promotes a co-repressor complex to be formed on the promoter of an inflammatory gene such as the gene encoding inducible nitric oxide synthase.[5] In other mechanisms, PPARγ binds to the p65 subunit of Nuclear factor kappa B (NK-κB) to either shuttle it out of the nucleus (where it can no longer promote transcription) or target it for degradation by the proteasome.[6,7]

Peroxisome proliferator activated receptor γ became interesting to us when genetic evidence was reported in 1999 that implicated its role in hypertension. This seminal (and career-transforming) study reported that patients carrying mutations in PPARγ exhibited severe hypertension in addition to insulin resistance and type II diabetes.[8] The mutations were present in a single copy, which would normally suggest a recessive genetic trait, but they caused a severe phenotype. Subsequent molecular analysis revealed that they acted in a dominant negative (DN) fashion. A DN mutation in PPARγ suggests that the mutant proteins are not only transcriptionally defective on their own, but they also inhibit the action of the wildtype allele.[9] Thus, instead of acting as a haploinsufficiency (where the activity would be approximately 50% of normal), the inhibitory effect of the DN PPARγ on wildtype PPARγ lowers total PPARγ activity below 50%, but probably not all the way to 0%. The preservation of some PPARγ activity likely accounts for the lack of embryonic lethality (but causes severe adult disease), as null mutations in PPARγ are embryonic lethal.[10] Other mutations in PPARγ were later discovered, which caused familial partial lipodystrophy associated with early-onset severe hypertension.[11] These mutations were reported to prevent the natural antagonism between PPARγ and the renin-angiotensin system, resulting in increased renin-angiotensin system activity.[12]

Amplifying the significance of the genetic studies were the results of clinical studies showing that exogenous activators of PPARγ, the thiazolidinediones (TZD), lowered blood pressure in hypertensive and diabetic subjects.[13,14] Larger clinical trials also confirmed the blood pressure-lowering actions of the TZDs.[15–17] Studies in a variety of hypertensive rodent models further supported the idea that PPARγ activation lowers blood pressure.[18–22] Admittedly, the antihypertensive effect of TZDs is not nearly as pronounced as classical antihypertensive drugs, including renin-angiotensin system blockers and diuretics. However, the clinical and genetic data were sufficient to strongly suggest that PPARγ plays a role in the regulation of arterial blood pressure. Indeed, this genetic and clinical trial data let us to conceptualize that PPARγ in the liver, muscle, and fat (among other tissues) is involved in glycemic control, whereas its expression in the endothelium and vascular smooth muscle controls blood pressure (Figure 1). The remainder of this review describes our efforts to understand the function of PPARγ in these two vascular cell types and how they regulate blood pressure.

Peroxisome Poliferator Activated Receptor γ in the Endothelium

The strongest evidence implicating endothelial PPARγ as an important regulator of blood pressure is derived from studies of genetically altered mouse models. One of the earliest studies suggested that endothelial-specific PPARγ deficiency

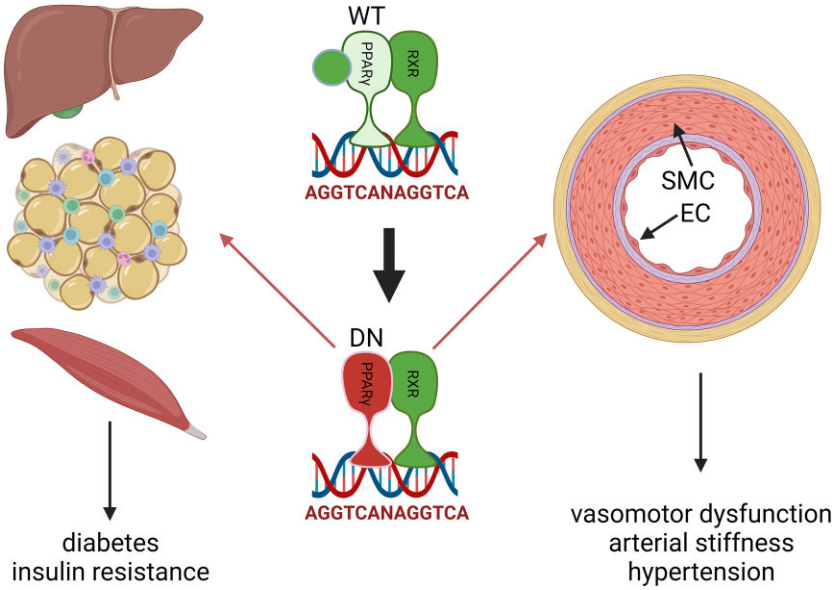

Figure 1. *Peroxisome proliferator activated receptor γ Hypothesis and Final Common Pathway.*
The PPARγ hypothesis states that interference with the action of PPARγ (by the DN mutant) in liver, adipose, and skeletal muscle leads to insulin resistance and diabetes, whereas its interference in vascular endothelium and smooth muscle leads to vasomotor dysfunction, arterial stiffness, and hypertension.

lowered vascular nitric oxide (NO), caused impaired vasodilation to acetylcholine, an endothelial-dependent agonist, and was accompanied by oxidative stress.[23] Studies by a different research group found that endothelial-specific knockout of PPARγ caused a susceptibility to elevated blood pressure after a high-fat diet, suggesting that PPARγ may provide protection in response to physiological stressors.[24] Because PPARγ actively represses expression of its target genes in the absence of ligand, we reasoned that PPARγ-deficiency might relieve that repression and cause partial activation of PPARγ targets, which might mask some of the true phenotypes of PPARγ-deficiency. Based on this rationale, we designed an experimental approach specifically expressing one of the DN mutants of PPARγ (V290M), which causes hypertension in humans, under the control of an endothelial-specific promoter.[8] It was initially disappointing that transgenic mice with endothelial-specific expression of $PPAR\gamma^{V290M}$ (termed E-V290M) did not exhibit any strong phenotype at baseline. However, further analysis revealed they exhibited an augmented pressor response to either exogenously infused angiotensin-II (Ang-II) or in response to high-fat diet feeding.[25–27] The mice also exhibited endothelial dysfunction in response to both stressors, which mechanistically was caused by oxidative stress, as antioxidant treatment restored endothelial function. Gene expression profiling (at that time performed by the microarray) revealed significant alterations in the expression of the genes encoding both pro and antioxidant proteins.[4] Activation of the renin-angiotensin system (by low-salt diet) or inflammation (by infusion of IL-1β) pathways also caused worsened endothelial dysfunction in E-V290M mice.[28–30] These data are all consistent with the concept that in endothelium, PPARγ is a sensor that becomes activated and provides protection in response to a stressor. This is consistent with the notion that nuclear receptors are sensors designed to respond to a threat.

One of the key PPARγ target genes in endothelium linked to the oxidative stress phenotype in E-V290M mice was retinol-binding protein 7 (RBP7, Figure 2). RBP7 is a cellular retinol-binding protein and fatty acid-binding protein expressed in

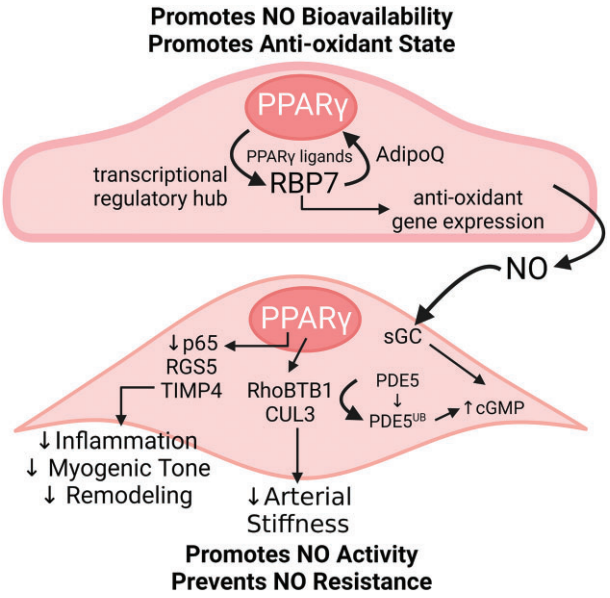

Figure 2. *Peroxisome proliferator activated receptor γ Final Common Pathway*
In the endothelium, PPARγ induces transcription of RBP7 and PPARγ/RBP7 together form a transcriptional regulatory hub promoting the expression of antioxidant genes, including adiponectin (AdipoQ), all of which promote an antioxidant environment, NO production, and NO bioavailability to the smooth muscle. In the vascular smooth muscle, endothelium-derived NO activates soluble guanylate cyclase to produce cGMP. To regulate cGMP levels in the cell, PPARγ induces the expression of Rho related BTB domain containing 1 (RhoBTB1), which acts as a substrate recognition protein delivering phosphodiesterase 5 (PDE5) to the Cullin-3 (CUL3) complex for ubiquitination and proteasomal degradation. The ensuing decrease in PDE5 activity promotes cGMP production and NO activity leading to vasodilation. Rho related BTB domain containing 1 also promotes a state of vessel distensibility reducing arterial stiffness. Other PPARγ target genes (RGS5 and TIMP4) control G protein receptor action, control myogenic tone, and reduce remodeling. Peroxisome proliferator activated receptor γ also acts as an antiinflammatory by shuttling p65 from the nucleus repressing expression of inflammatory genes.

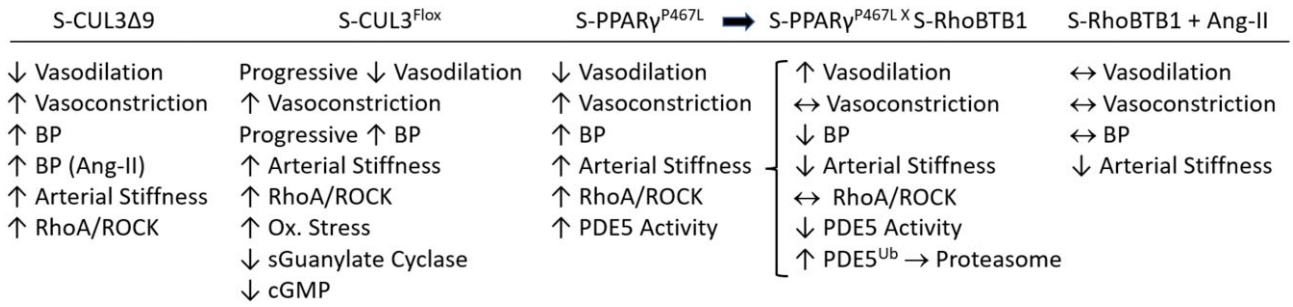

Figure 3. *Phenotypic Summary of Animal Models*
A summary of the main animal models discussed in the review is presented. S-CUL3Δ9 represents inducible expression of a dominant mutant of CUL3 that results in exon 9 skipping. S-CUL3^{Flox} represents the inducible smooth muscle knockout of CUL3. S-PPARγ^{P467L} also known as S-P467L represents smooth muscle-specific expression of a human mutant in PPARγ (P467L), which causes human hypertension. Although it is discussed early in the review, it is presented directly next to the S-PPARγ^{P467L} X S-RhoBTB1, which represents the inducible smooth muscle-specific re-expression of RhoBTB1 in the S-P467L model. S-RhoBTB1 + Ang-II is the inducible smooth muscle-specific expression of RhoBTB1 treated with a slow-pressor dose of Ang-II to induce hypertension and arterial stiffness. The arrows reflect the following comparisons: S-CUL3Δ9 versus control mice; S-CUL3^{Flox} versus control mice; S-PPARγ^{P467L} versus control mice; S-PPARγ^{P467L} X S-RhoBTB1 versus S-P467L mice; S-RhoBTB1 + Ang-II measures the effect of inducible RhoBTB1 after hypertension is fully established by Ang-II. In the latter, activation of RhoBTB1 did not alter vasodilation, vasoconstriction, or BP in response to Ang-II but relieved arterial stiffness.

the heart, muscle, fat, and endothelium.[31,32] RBP7-deficient mice exhibited endothelial dysfunction that could be reversed with an antioxidant.[33] Because RBP7-deficient and E-V290M mice appeared to exhibit similar phenotypes, we hypothesized they might be part of the same final common pathway in the endothelium. Interestingly, the antioxidant effects of RBP7 required adiponectin, another PPARγ target gene in the endothelium. Some members of the fatty acid-binding protein family act as nuclear receptor co-factors, shuttling ligands through the cell to interact with nuclear receptors.[34] Our data suggest that PPARγ and RBP7 support a regulatory hub in the endothelium promoting antioxidant gene expression.

Peroxisome Proliferator Activated Receptor γ in Vascular Smooth Muscle

A phenotypic summary of the models described in this section is presented in Figure 3. Like the experimental model defined above to study the role of PPARγ in endothelium, we generated mice similarly expressing a DN mutation in PPARγ (this time PPARγ^{P467L}) under the control of the smooth muscle myosin heavy chain promoter. Unlike E-V290M mice, the phenotype of S-P467L mice was striking even at baseline.[35] They exhibited elevated blood pressure and a severely impaired response to both acetylcholine (which induces the production of endothelium-derived NO) and sodium nitroprusside (which is an NO donor), suggesting a phenotype of NO resistance in the vascular SMC. The phenotype of S-P467L mice was not unlike SMC-specific PPARγ deficiency in that both exhibited elevated blood pressure and vascular dysfunction, but surprising the vascular dysfunction in S-P467L mice was not caused by oxidative stress.[36] Moreover, the impaired vascular response was not isolated to conduit vessels, as the vasodilator responses in the mesenteric and cerebral circulation were also impaired.[37,38] Vessels of all sizes exhibited augmented responses to vasoconstrictors such as endothelin-1 and exhibited enhanced myogenic tone (Figure 3).[38–40]

Gene expression profiling studies combined with bioinformatic analysis were used to identify a series of PPARγ target genes (Figure 4). These genes were identified by first comparing gene expression patterns in the aorta comparing wildtype mice with those carrying a DN mutation in PPARγ. Next, this list of genes was compared with gene expression signatures of

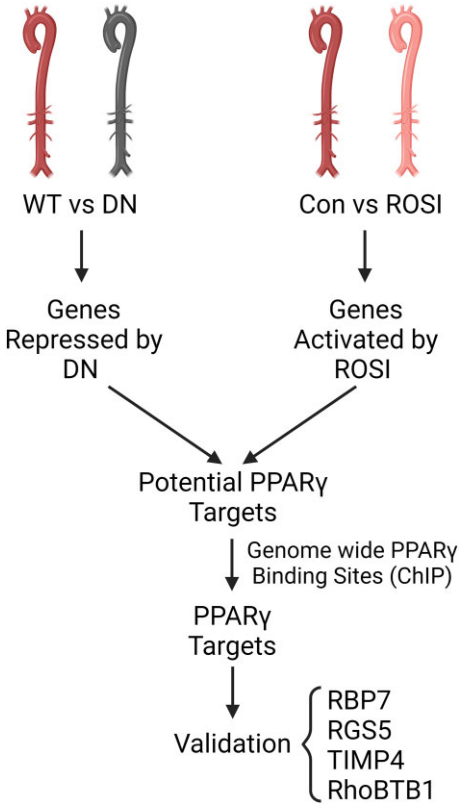

Figure 4. *Schema for the Identification of PPARγ Target Genes*
Gene expression profiling was performed comparing the list of genes repressed in the aorta in response to DN PPARγ with the list of genes induced by the PPARγ agonist ROSI. These potential PPARγ target genes were then screened using publicly available ChIP data for the presence of PPARγ binding sites around the gene identified above. Genes that passed all three criteria were considered PPARγ target genes and were separately validated. These include RBP7, RGS5, TIMP4, and RhoBTB1.

the aorta from untreated compared with rosiglitazone (ROSI) (a PPARγ ligand) treated mice. Finally, the intersection of the above gene lists was evaluated using publicly available genome-wide chromatin immunoprecipitation (ChIP) of PPARγ binding sites. Those genes that exhibited the opposite response to ROSI (increased expression) and DN mutants (decreased expression),

or vice versa, and identified to contain PPARγ binding sites in chromatin near the gene were prioritized for further validation and analysis.[4]

One of these target genes Regulator of G Protein Signaling 5 (RGS5), which controls G protein-coupled receptor activity, was found to be responsible for enhanced myogenic tone in the resistance vessels of S-P467L mice through a mechanism involving protein kinase C and the large-conductance calcium- and voltage-activated K + channel (BK_{Ca}).[38] Another PPARγ target gene, tissue inhibitor of metalloproteinase-4, was implicated in vascular remodeling in the deoxycorticosterone acetate-salt model of hypertension.[41] A third PPARγ target gene, RhoBTB1, has been the topic of investigation and will be the subject of the remainder of this review (Figure 2).

Peroxisome Proliferator Activated Receptor γ-RhoBTB1-CUL3 Pathway

Gene expression profiling first identified RhoBTB1 as a potential PPARγ target gene. We validated it as a PPARγ target because its expression was downregulated in the aorta from S-P467L mice, upregulated in mice overexpressing wildtype PPARγ in vascular SMC (S-WT mice), and bound both PPARγ and RXR by ChIP.[40] Rho related BTB domain containing 1 was first identified as a tumor suppressor gene reported to regulate the Golgi apparatus integrity.[42–45] The protein consists of a number of domains: an atypical GTPase domain (which may not exhibit GTPase activity), a proline-rich domain that may play a role in cytoskeletal or organelle organization, two BTB domains, and a C-terminal domain.[46] Proteins carrying BTB domains (bric-à-brac, tramtrack, and broad complex, sometimes called POZ domains) are interesting because they function as adaptor proteins for the E3 Ring Ubiquitin Ligase CUL3. They act as substrate recognition proteins, binding substrate proteins and delivering them to the CUL3 complex (called the CUL3-RING ubiquitin ligases, CRL3) complex), where they are ubiquitinated and targeted for proteasomal degradation.[47] Indeed, RhoBTB1 binds to CUL3 through its first BTB domain.[40,48] The interaction between RhoBTB1 and CUL3 is interesting because like PPARγ, mutations in CUL3 (which also act dominantly) cause hypertension through the combined effects of renal and vascular impairment.[49–52] Moreover, RhoBTB1 was reported to be associated with diastolic BP and was identified as an interacting locus in a large hypertension genome-wide association study of more than a million people.[53,54] The interaction of RhoBTB1 and CUL3 suggested two lines of investigation in which we separately examined the relative roles of CUL3 and RhoBTB1 in vascular SMC.

We first reasoned that interference with CUL3 or CUL3-deficiency would cause a severe phenotype, as RhoBTB1 is only one of many substrate recognition proteins that deliver substrates to the CUL3 complex for degradation. Indeed, SMC-specific expression of a human DN hypertension-causing mutation in CUL3, which causes skipping of exon 9 (CUL3Δ9) caused impaired vasodilation, hypercontractile response to agonists, elevated blood pressure at baseline and in response to Ang-II, and arterial stiffness.[50] The impaired vasodilation was caused by increased RhoA and Rho kinase (ROCK) activity. Other studies revealed that the CUL3Δ9 acted dominantly and impaired the association with substrate recognition proteins.[51,55]

Like DN mutations in PPARγ, similar mutations in CUL3 likely preserved some level of activity. For this reason, we created an inducible model of CUL3-deficiency.[56] Induction of SMC-specific Cre-recombinase with tamoxifen caused a progressive decline in vasodilation, a marked hypercontraction, severe hypertension, and arterial stiffness. Defects in the production of cGMP appeared to be mechanistically associated with the decline in vasomotor function.

Based on our observation that S-P467L mice exhibited increased RhoA and ROCK activity, that CUL3 was reported to regulate RhoA, that mutations in CUL3 impair RhoA ubiquitination, and the increased RhoA/ROCK in CUL3Δ9 mice, we tested the hypothesis that RhoBTB1 is a substrate recognition protein for RhoA.[55,57] However, we could not validate the binding of RhoBTB1 to RhoA. Indeed, another BTB-domain-containing protein termed BACURD was shown to be the substrate recognition protein for RhoA.[57] This suggested either RhoBTB1 was not playing an important role in the regulation of vascular function or blood pressure or it has a different unidentified substrate.

To test the importance of RhoBTB1 as a regulator of vascular function and blood pressure, we created a transgene in which expression of RhoBTB1 could be induced by Cre-recombinase using a loxP-STOP-loxP signal. Cell-specific expression of Cre-recombinase removed the STOP signal and induced expression of RhoBTB1 and a tandemly expressed tdTomato reporter.[58] Double transgenic mice expressing smooth muscle-specific Cre ($Myh11^{CRE}$) and the inducible RhoBTB1 construct were bred to S-P467L mice to ask if re-expression of RhoBTB1 (which was downregulated by DN PPARγ) could complement or reverse the hypertension and vascular dysfunction in S-P467L mice. Inducible expression of RhoBTB1 lowered blood pressure, improved vascular function, and reversed arterial stiffness in the model. This data suggested that RhoBTB1 is an important regulator of blood pressure and vasomotor function and may be the PPARγ target responsible for its protective function in vascular SMC. Interestingly, whereas restoration of RhoBTB1 improved the vasodilator response to acetylcholine and SNP, hypercontraction to agonists such as endothelin-1 was preserved. Thus, it remains fascinating that RhoBTB1 can regress arterial stiffness during a state of preserved vasoconstriction. A combination of pharmacological and biochemical experiments ultimately revealed that the RhoBTB1 substrate responsible for this phenotype was phosphodiesterase 5 (PDE5). Phosphodiesterase 5 is responsible for converting cGMP to GMP. Thus, loss of RhoBTB1 decreased proteasomal turnover of PDE5, increasing its activity thus, limiting cGMP and inducing a state of NO resistance (Figure 2).

Recent studies showed that induction of RhoBTB1 could also rapidly regress established arterial stiffness induced by Ang-II through a mechanism involving the regulation of actin polymerization.[59] Like the preservation of hypercontractile activity in S-P467L mice, the regression of arterial stiffness in the Ang-II model occurred with a concomitant preservation of Ang-II-induced hypertension and decreased vasodilation. It is highly significant that the expression of a single protein, RhoBTB1, can rapidly regress established arterial stiffness even with worsening hypertension and suggests we must revisit the relationship between arterial stiffness and hypertension.

Identification of Novel RhoBTB1 Target Proteins

The data comparing the protective effects of RhoBTB1 in the S-P467L and Ang-II models suggest that RhoBTB1 may be engaging differing substrate proteins in each model, PDE5 (and perhaps others) in the S-P467L model, and unidentified RhoBTB1 target proteins in the Ang-II model. Thus, we hypothesized

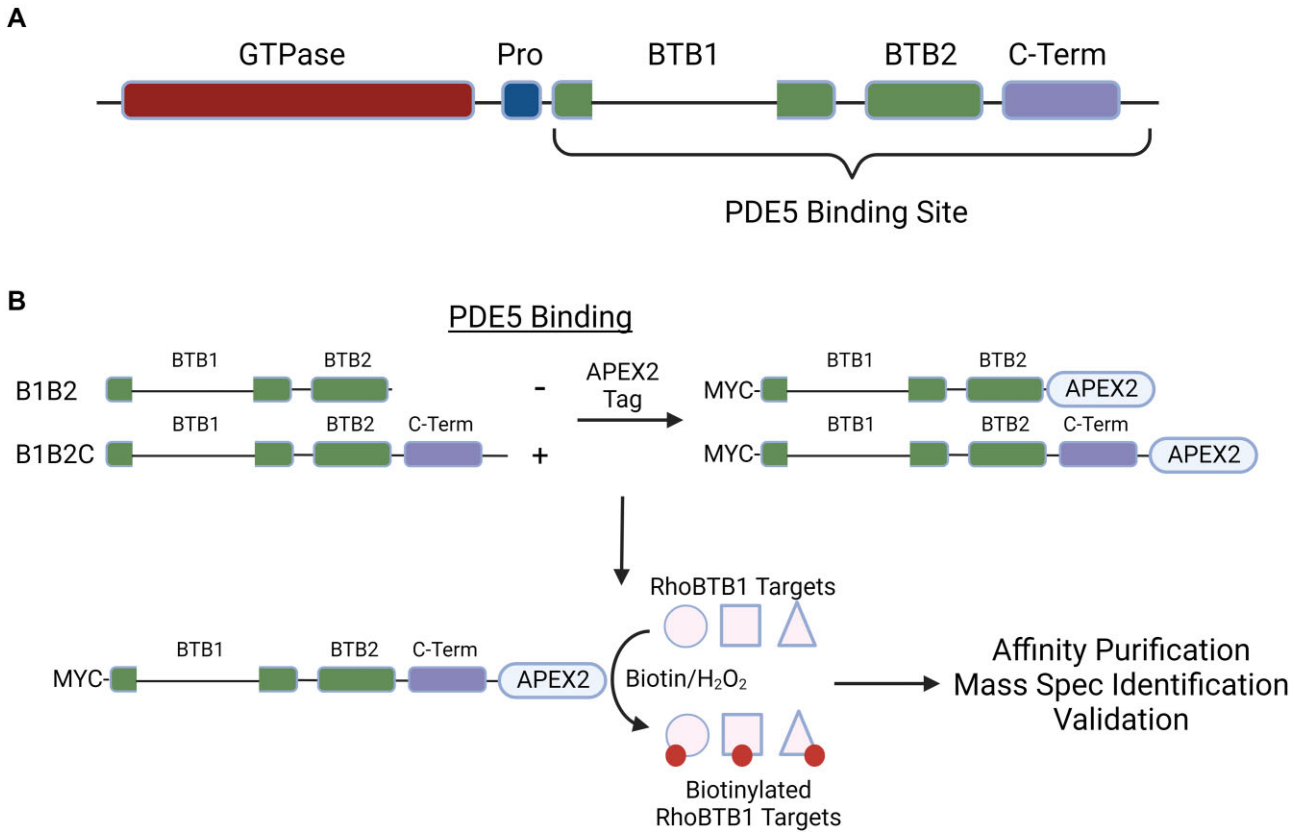

Figure 5. *Structure and Targeted Proteomics of RhoBTB1*
(A) Domain structure of RhoBTB1 showing the relative position of the GTPase domain, the proline rich domain (Pro), the two BTB domains, and the C-terminal domain, in some papers referred to as the BACK domain. The first BTB domain (BTB1) is interrupted by a spacer. The minimal PDE5 binding site is shown. (B) The B1B2 and B1B2C domains were separately cloned, Myc-epitope tagged, and the APEX2 was added to the C-terminal. A brief pulse of biotin, phenol, and H_2O_2 results in the biotinylation of interacting proteins and proteins in close proximity of the APEX2 tag. Biotinylated proteins are affinity-purified with streptavidin and subjected to mass spectroscopy. Proteins that bound B1B2C greater than B1B2 were separately validated to be RhoBTB1-binding proteins and subject to RhoBTB1-CUL3 ubiquitination and proteasomal degradation.

that RhoBTB1 might act as a substrate recognition protein for other substrates, which required us to develop a targeted proteomics approach. To accomplish this, we first mapped the minimal binding site on RhoBTB1 for PDE5. We did this based on the admittedly naive hypothesis that the binding site for PDE5 might be the same as those for other substrates. Our strategy was to express individual and combinations of domains of RhoBTB1 as Myc-fusion proteins and assess their ability to co-immunoprecipitate with His-tagged PDE5 (Figure 5A).[48] As published in the journal *Function* in July 2023, we identified that the C-terminal half of the protein, consisting of the two BTB domains, and the C-terminal domain (termed B1B2C) are required to bind PDE5. Removal of the C-terminal domain eliminated binding to PDE5, and the C-terminal on its own was insufficient to bind PDE5. Interestingly, the B1B2C domains, which lack the GTPase and proline-rich domains in the N terminus of RhoBTB1, were sufficient on their own to ubiquitinate PDE5 and target it for proteasomal degradation. We next employed the B1B2C domain in a targeted proteomic screen of RhoBTB1-binding proteins in HEK293 cells by biotinylating proteins that either directly bound to B1B2C or were in close proximity to B1B2C by creating a fusion between B1B2C and ascorbate peroxidase 2 (APEX2, Figure 5B).[60] The biotinylated proteins were then affinity-purified and identified by mass spectroscopy. To reduce what we expected to be a high background, we also resolved proteins that bound to the B1B2 domains (which lacked the C terminal) and then chose proteins that bound 1.5-fold better to B1B2C than B1B2 and were statistically significant. Among the pathways identified, cytoskeletal remodeling was prominent. This was interesting because we showed that RhoBTB1 reversed arterial stiffness in two different models of hypertension.[58,59] We selected 5 proteins (SETD2, ANXA6, CAMKK2, CAMSAP1, and TRAPPC9) and showed they all co-immunoprecipitated with B1B2C to a much greater extent than with B1B2. Another protein (HMGB1) that was detected in the proteomic assay to bind B1B2 better than B1B2C was also confirmed by co-immunoprecipitation, illustrating the surprising fidelity of the assay. Finally, we chose SETD2 to explore further. The level of SETD2 protein in HEK203 cells was markedly increased after treatment with MG132 (a proteasome inhibitor) and MLN4924 (a pan-Cullin inhibitor). Moreover, the level of SETD2 was markedly increased in HEK293 cells engineered to be CUL3-deficient by CRISPR-Cas9 and in cells after RhoBTB1 inhibition with siRNA. Thus, the targeted proteomic approach using APEX2 has high fidelity to identify RhoBTB1-binding proteins, which are targets of RhoBTB1-CUL3-mediated ubiquitination and proteasomal regulation. Identifying RhoBTB1-binding proteins in vascular smooth muscle cells and in other cell types identified by single-cell sequencing to express RhoBTB1 (vascular endothelial cells, resident macrophages in vasculature and

adipose tissue, proximal tubule cells in the kidney, and syncytiotrophoblast cells of the placenta) may help illuminate fundamental cellular mechanisms, including mechanisms of vascular function, blood pressure, and arterial stiffness, among others.

Acknowledgments

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Conflict of Interest

None declared.

Data Availability

All data referred to in this review has been previously published and cited. Nevertheless, the data underlying this article will be shared on reasonable request to the corresponding author.

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