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EBP50 Inhibits the Anti-Mitogenic Action of the Parathyroid Hormone Type 1 Receptor in Vascular Smooth Muscle Cells

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Abstract

Parathyroid hormone-related protein (PTHrP) and the parathyroid hormone type 1 receptor (PTH1R) are important regulators of vascular remodeling. PTHrP expression is associated to increased proliferation of vascular smooth muscle cells (VSMC). In contrast, signaling via the PTH1R inhibits cell growth. The mechanisms regulating the dual effect of PTHrP and PTH1R on VSMC proliferation are only partially understood. In this study we examined the role of the adaptor protein ezrin-radixin-moesin-binding phosphoprotein (EBP50) on PTH1R expression, trafficking, signaling and control of A10 cells proliferation. In normal rat vascular tissues, EBP50 was restricted to the endothelium with little expression in VSMC. EBP50 expression significantly increased in VSMC following angioplasty in parallel with PTHrP. Interestingly, PTHrP was able to induce EBP50 expression. In the clonal rat aortic smooth muscle cell line A10, EBP50 increased the recruitment of PTH1R to the cells membrane and delayed its internalization in response to PTHrP(1-36). This effect required an intact C-terminal motif in the PTH1R. In naive A10 cells, PTHrP(1-36) stimulated cAMP production but not intracellular calcium release. In contrast, PTHrP(1-36) induced both cAMP and calcium signaling in A10 cells over-expressing EBP50. Finally, EBP50 attenuated the induction of $p27^{kip1}$ and the antiproliferative effect of PTHrP(1-36). In summary, this study demonstrates the dynamic expression of EBP50 in vessels following injury and the effects of EBP50 on PTH1R function in VSMC. These finding highlight one of the mechanisms leading to increased VSMC proliferation and have important implication in the understanding of the molecular events leading to restenosis.

Keywords

Vascular smooth muscle cell; Parathyroid hormone-related protein; Parathyroid hormone type 1 receptor; Ezrin-radixin-moesin-binding phosphoprotein EBP50; Proliferation; Cyclic AMP; Calcium; Restenosis; Neointima

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1. Introduction

Restenosis, the reoccurrence of vascular narrowing following angioplasty, is a serious complication of this common procedure. Neointima formation depends on a number of events, including the initial inflammatory response and the change of vascular smooth muscle cells (VSMC) from a contractile to a secretory and migratory phenotype. However, the uncontrolled proliferation of VSMC is major contributing factor to restenosis. Parathyroid hormone-related protein (PTHrP) and the type 1 PTH/PTHrP receptor (PTH1R) are expressed in VSMC [1,2] and regulate VSMC proliferation [3]. PTHrP is expressed as a prepro-hormone that is post-translationally processed to generate a family of secreted peptides, including N-terminal fragments such as PTHrP(1-36), that are potent agonists for the PTH1R [3,4]. In addition, PTHrP possesses a nuclear localization sequence (NLS) that allows it to enter the nucleus and exert intracrine actions [5]. Elevated PTHrP levels are often associated with VSMC proliferation. In particular, after angioplasty PTHrP is up-regulated in the proliferating VSMC [6,7] and the development of restenosis is exacerbated by the local application of adenovirus expressing PTHrP [8]. This proliferative effect is, at least in part, intracrine and requires the presence of the NLS in the C-terminal region of the molecule. Indeed, adenoviral delivery of a secreted form of PTHrP (ΔNLS-PTHrP) lacking nuclear actions decreases VSMC proliferation *in vitro* and abrogates intima formation following arterial injury in rats [8]. Interestingly, overexpression of both PTHrP and ΔNLS-PTHrP in A10 cells results in higher secretion of biologically active N-terminal fragments of PTHrP (such as PTHrP(1-36)) [9] that act in an auto/paracrine fashion through the PTH1R. Since activation of the PTH1R by PTHrP (1-36) exerts anti-proliferative effects on VSMC, both *in vivo* and *in vitro* [5,10], the PTHrP/PTH1R system is a true regulator of vascular remodeling. Yet, the molecular events regulating PTH1R expression and function in VSMC have not been fully elucidated.

In many cells, including osteoblasts and tubule kidney cells, stimulation of the PTH1R by its cognate ligands activates at least two distinct intracellular signaling cascades: the Gs/adenylyl cyclase/cAMP and the Gq/protein lipase C/intracellular calcium pathways [11]. In contrast, in VSMC the PTH1R couples exclusively to Gs [12,13]. While the mechanism underlying this remarkable cell-specificity has not been fully elucidated, these observations suggest that factors controlling G protein selectivity of the PTH1R contribute to regulating the vascular actions of PTHrP. In 2002, Mahon, Segre and coworkers demonstrated that the PTH1R interacts with the PDZ-containing scaffolding protein EBP50 (also known as sodium-hydrogen exchanger regulatory factor 1 NHERF1) and that this interaction directs the specificity of G protein coupling: in the absence of EBP50 the PTH1R couples exclusively to Gs, whereas in the presence of EBP50 signaling occurs preferentially via PLC [14,15]. Therefore, we hypothesize that EBP50 may contribute to the signaling specificity of the PTH1R in VSMC and consequently to the effect of N-terminal PTHrP fragments on cell proliferation. In this study, we examined the expression of EBP50 in normal and restenotic vessels and determined the role of this scaffolding protein on PTH1R signaling, trafficking and regulation of cell growth. The experiments reported here show that EBP50 expression increases upon arterial injury causing an attenuation of the anti-proliferative effect of PTH1R agonists on VSMC.

2. Materials and Methods

2.1 Experimental animals

Balloon injury was performed as described previously [16]. Briefly, adult Sprague-Dawley male rats weighing 450 to 600 g anesthetized with intraperitoneal injections of ketamine (150 mg/kg body weight) and xylazine (15 mg/kg body weight). A 2F Fogarty balloon catheter (Baxter, Deerfield, IL) was inserted into the left common carotid artery, inflated with a calibrated inflation device to a pressure of 2 atm for 5 minutes, and passed back and forth 3 times. Two weeks after balloon injury, the control uninjured right and the balloon-injured left

carotid arteries were harvested, fixed in 4% paraformaldehyde for 48 hours at 4°C, embedded in paraffin blocks, sectioned (5 μm), and stained with EBP50 (Thermo Scientific, Rockford, IL) and PTHrP (Peninsula Lab, San Carlos, CA) antibodies as described below. All animal protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2. Peptide synthesis and radioligand preparation

The synthesis, purification, and characterization of PTHrP (1–36)-NH_2 [PTHrP (1–36)] and [Bpa^2, Ile^5, $Arg^{11, 13}$, Tyr^{36}]PTHrP (1–36)-NH_2 (Bpa^2-PTHrP) were carried out as previously described [17]. The pure products were characterized by analytical HPLC and electron spray mass spectrometry. Radioiodination and HPLC purification of PTH (1–34) was carried out as reported [17].

2.3. Cell culture and transfection

Monolayer cultures of A10 cells (ATCC, Manassas, VA) and human embryonic kidney HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) at 37 °C in a humidified 95% room air, 5% CO_2 incubator. Primary aortic VSMC were prepared and cultured as described [18]. Rat osteosarcoma ROS 17/2.8 cells were grown in DMEM/F12 medium supplemented with 10% FBS. Cells were transfected using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Cells were used for experiments 1–3 days after transfection. Small interfering RNA (siRNA) for EBP50 knockdown was generated by Dhamarcon (Thermo Scientific) as follows: 5′-GAAGGAGAGCAGCCGUGAAdTdT3′ (sense) and 5′-UUCACGGCUGCUCUCCUUCdTdT-3′ (antisense). As a control siRNA Accell Non-targeting siRNA (Dharmacon, Thermo Scientific) was used. A10 cells were plated onto 6-well plates and transfected with siEBP50 (200 nM) or control siRNA (200 nM) using DharmaFECT Duo (3 μl/ml) transfection reagent (Dharmacon, Thermo Scientific) in DMEM with 1% FBS in the absence of antibiotic. Cells were used for the experiment after 72 h of transfection.

2.4. Radioligand binding assay

Binding of radiolabeled PTH(1-34) was performed in confluent cells as described [19]. Briefly, cells plated in 24-well plates were incubated on ice for 2 h with 100,000 cpm of [^{125}I]-PTH (1–34)NH_2 in 250 μl of DMEM containing 10% fetal bovine serum. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), collected in 0.5 ml of 0.1 N NaOH, and bound [^{125}I]-PTH(1–34)NH_2 was assessed by γ spectrometry. Nonspecific binding was measured in parallel experiments carried out in the presence of 1 μM unlabeled PTH(1–34).

2.5. ELISA assay

Cells transiently expressing HA-PTH1R [18] were grown in 12-well plates, treated as indicated in the figure legends and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 min. For determination of surface receptor, cells were blocked with 5% milk for 20 min at room temperature followed by incubation with anti-HA antibody (Covance) at 1:2000. Cells were washed three times in PBS and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:3000, Cell signaling). Antibody binding was visualized by adding 0.2 ml substrate solution for horseradish peroxidase (BM-blue-POD solution, Roche, Indianapolis, IN). After incubation for 20 min at room temperature, the reaction was terminated by adding 0.2 ml of 10% sulfuric acid and the plate was read at 450 nm in a microplate reader.

2.6. Western blot analysis

Cells were solubilized by incubation for 10 min in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, 1 mM EDTA (pH 8.0) containing a protease inhibitor cocktail). The cell lysates were resolved on 12% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane, which was then subjected to two sequential 2 h incubations with appropriate antibodies at 1:2000 and horseradish peroxidase-conjugated anti-mouse IgG antibody at 1:2000. Immunoreactivity was detected by chemiluminescence. Quantitation of band intensity was performed with the Image J software (National Institutes of Health).

2.7. Immunostaining

Cells grown on 25-mm coverslips were transfected and treated as indicated in the figure legend and fixed for 20 min at room temperature with 4% paraformaldehyde in PBS. Cells were incubated with blocking buffer containing 5% goat serum and 0.2% Nonidet P-40 (NP-40) in PBS. Anti-HA antibody (Covance) or anti-EBP50 were applied at a dilution of 1:1000 and 1:500, respectively, in the same buffer overnight at 4 °C. Coverslips were washed with PBS, incubated with Alexa546-conjugated anti-rabbit secondary antibody (1:2000, Molecular Probe, Eugene, OR) for 3 h, and washed again. Coverslips were mounted for immunofluorescence microscopy, and analyzed with an Olympus Fluoview confocal laser-scanning microscope with a 63× oil immersion objective.

Immunofluorescence staining of vessels was performed as follows. Following standard deparaffinization with ethanol and xylene, sections were treated for 1 h with 1N HCl, incubated in 2.5% bovine serum albumin in PBS, and incubated with polyclonal antibodies for either PTHrP (1:500 dilution) or EBP50 (1:200) at 4°C overnight. After PBS washes, slides were then incubated with Alexa-546 or Fluorescein-conjugated anti-rabbit secondary antibody (1:1000) for 2 h at room temperature. Nuclei were visualized with DRAQ5 (Biostatus Limited, United Kingdom). The average fluorescence intensity in the media (defined as the area between the internal and external elastic laminae) and the neointima (defined as the region from the internal elastic lamina to the lumen) was determined with Image J software (National Institutes of Health).

2.8. Adenylyl Cyclase Activity and Intracellular Calcium Determinations

Cyclic AMP accumulation was determined as previously described [17]. Briefly, ligand-stimulated cAMP accumulation was measured in cells pre-incubated for 15 min with 3-isobutyl-1-methylxanthine (1 mM) and subsequently exposed to 100 nM PTHrP(1-36) for 30 min at 37 °C. Reactions were stopped with 1.2 M trichloroacetic acid, and cAMP was isolated by the two-column chromatographic method [20].

For the determination of intracellular calcium levels, cells plated on glass-bottom dishes were loaded with the calcium dye Fluo4 (4 μM) in Hank's Balanced Salt Solution (HBSS) containing 1.8 mM calcium and 20 mM HEPES at pH 7.4 for 30 min. Cells were washed with HBSS twice. The fluorescent intensity of cells were acquired every 3 sec on a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss, Thornwood, NY) with a 25× oil immersion objective.

2.9. Proliferation Assays

Cells on 24-well plates were transfected as indicated and cultured until 70-80% confluent. Cells were incubated with 1 μCi/ml [^{3}H]thymidine in the culture media for 18 hours at 37 °C, rinsed with PBS and exposed to 10% trichloroacetic acid (TCA) for 10 min. TCA was removed and the cell monolayers dissolved in 1N NaOH for the determination of radioactivity.

2.10. Statistical Analysis

Results from each experiment were averaged and expressed as mean ± S.E. Results were analyzed by ANOVA with Tukey's test or Student's t-test. P-values were considered statistically significant when lower than 0.05.

3. Results

3.1. Up-regulation of EBP50 following arterial angioplasty

The expression of EBP50 was determined by immunofluorescence in uninjured rat carotid arteries and two weeks following angioplasty. In normal vessels, EBP50 was predominantly expressed in the endothelium, with very low expression in smooth muscle cells (Fig. 1A, left panels). As expected, two weeks after angioplasty exuberant neointima formed (Fig 1A, middle panels). EBP50 expression significantly increased within the neointima of injured vessels (ratio intima/media 3.1 ± 1.1, Figure 1B). Interestingly, a similar increase (ratio intima/media 4.3 ± 0.6, Figure 1B) was observed for PTHrP. The expression of EBP50 and PTHrP in the media of untreated arteries was not different from that of restenotic vessels (Fig. 1B). To obtain a more precise definition of the cellular localization of PTHrP and EBP50 in the neointimal cells, immunofluorescence analysis was performed in sections following staining of the nuclei with DRAQ5. As shown in figure 1C, PTHrP was mostly localized in the cytoplasm, and nuclear localization was observed in exceedingly few cells (Fig. 1C arrow). EBP50 was exclusively localized in the cytoplasm with no evidence of nuclear staining. No staining was detected in vessels in which identical procedures were performed in the absence of primary antibody (Fig 1A, right panel) and increased fluorescence intensity was observed in the clonal rat aortic cell line A10 after overexpression of EBP50 (data not shown) and PTHrP confirming antibody specificity [18]. Next, the expression of EBP50 in primary VSMC and in A10 cells was compared to that of human embryonic kidney HEK293 cells (that are known to express EBP50 abundantly) and to the rat osteosarcoma cell line ROS as negative control. As shown in figure 1D, low expression of EBP50 was detected in both A10 and primary VSMC. Collectively, these findings indicate that VSMC express low levels of EBP50 and that its expression increases *in vivo* following balloon angioplasty.

3.2. Up-regulation of EBP50 by PTHrP in VSMC

The concomitant up-regulation of PTHrP and EBP50 in the neointima prompted us to examine whether the two events are related. To this end, PTHrP expression was increased in A10 cells by stable transfection [9] and the expression of EBP50 determined by Western Blot. A10 cells expressing green fluorescent protein (GFP) were used as control. As shown in figure 2, PTHrP transfection was sufficient to significantly augment EBP50 expression by 50% (left panels). Conversely, EBP50 expression was reduced in A10 cells stably expressing ΔNLS-PTHrP (right panels) suggesting that the intracrine action of PTHrP is important to increase EBP50 expression. These experiments indicate that the increased expression of PTHrP following injury may account, at least in part, for the concomitant increase of EBP50 expression in VSMC.

3.3. Effect of EBP50 on PTH1R expression

We next examined the effect of increasing EBP50 on PTH1R expression in VSMC. Receptor expression was determined by ELISA, immunoblotting and radioligand binding in A10 cells transiently transfected with HA-tagged PTH1R and either control plasmid (pcDNA3) or EBP50. In these experimental conditions EBP50 expression increased approximately 5-fold (Fig. 3C). Expression of EBP50 in A10 cells induced a corresponding increase in the number of PTH1R at the cell membrane, as shown by the higher binding of radioiodinated PTH (Fig. 3A). Similarly, increased PTH1R expression at the cell membrane was determined by ELISA

in non-permeabilized cells (Fig. 3B). Consistent with our previous findings [18], blocking PTH1R internalization with the antagonist Bpa^2-PTHrP increased PTH1R expression. Interestingly, the effect of EBP50 was additive to that of Bpa^2-PTHrP (Fig. 3B), suggesting that EBP50 increases the recruitment of PTH1R to the cell membrane. In addition, Western Blot analysis of whole cell lysates demonstrated an increase in total PTH1R in EBP50 transfected cells (Fig. 3C), consistent with the increased stability of the PTH1R at the cell membrane that prevents its degradation [18]. The specificity of these effects was confirmed by performing identical experiments with a PTH1R mutant that is unable to bind EBP50 (M593A-PTH1R) [21]. As shown in figure 3D and E, binding of radiolabeled PTH and antibody labeling of HA-tagged M593A-PTH1R were unaffected by EBP50. In addition, while as expected Bpa^2-PTHrP increased total expression of M593A-PTH1R, EBP50 had no effect (Fig. 3F). Collectively, these experiments show that EBP50 increases PTH1R expression and membrane localization in A10 cells, and these effects require the direct interaction between the receptor's C-terminal PDZ-binding sequence and EBP50.

3.4. Effect of EBP50 on PTH1R trafficking

The observation that blocking PTH1R internalization increases receptor levels ([18] and Fig. 3B and C), indicates that trafficking is a major mechanism determining PTH1R expression. Therefore, the effect of EBP50 on PTH1R internalization was determined by ELISA in non-permeabilized cells. In cells transfected with vector pcDNA3, PTHrP(1-36) caused 59.7±4.5% internalization within 30 min (Fig. 4A). EBP50 overexpression significantly delayed and reduced the extent of PTH1R internalization in response to PTHrP (44.3±3.5% receptor internalization within 30 min, Fig 4A).

In many cell types, PTH1R internalization is mediated by β-arrestins. To determine whether this mechanism is operational in VSMC, A10 cells were transfected with GFP-tagged β-arrestin2 (β-arr2-GFP) and HA-PTH1R and the cellular localization of both was determined by confocal microscopy before and after treatment with PTHrP(1-36). In unstimulated cells, β-arr2-GFP was uniformly distributed throughout the cytoplasm (Fig. 4B top left). Addition of PTHrP(1-36) (100 nM for 20 min) caused the redistribution of β-arr2-GFP to endosomal vesicles (Fig. 4B top middle) and clear co-localization of β-arr2-GFP and HA-tagged PTH1R was observed (Fig. 4B top right). In contrast, PTHrP(1-36) did not change the distribution of EBP50 (Fig. 4B, bottom left and middle) and no co-localization of EBP50 with β-arr2-GFP was observed (Fig. 4B, bottom right). Collectively, these studies demonstrate that PTH1R internalization in A10 cells involves β-arrestin2. In addition EBP50 does not traffic to endosomes in association with the PTH1R.

3.5. EBP50 effect on endogenous PTH1R signaling

To determine the effect of EBP50 on endogenous PTH1R signaling, A10 cells were transfected with vector (pcDNA3) or EBP50 and receptor activation was assessed by measuring cAMP production and intracellular calcium release. Consistent with previous studies [12], PTHrP (1-36) stimulated a three-fold increase in cAMP in A10 cells (Fig. 5A), but did not increase intracellular calcium (Fig. 5D). Transfection with EBP50 increased endogenous PTH1R expression (Fig. 5B) but did not change total cAMP production in response to PTHrP(1-36) (Fig. 5A). Thus, Gs-mediated signaling per receptor was decreased by EBP50 (Fig 5C). In addition, A10 cells expressing EBP50 responded to PTHrP(1-36) with a rapid and transient release of calcium (Fig. 5E).

3.6. EBP50 expression reduces the anti-mitogenic effect of PTH1R in VSMC

The anti-proliferative actions of N-terminal fragments of PTH and PTHrP in VSMC have been extensively documented [5,13,18,22]. We therefore sought to determine whether EBP50 affects the ability of PTH1R agonists to reduce proliferation of VSMC. To this end, tritiated

thymidine incorporation was measured in A10 cells transfected with either vector (pcDNA3) or EBP50 and maintained in 1% FBS. Proliferation of A10 cells was significantly inhibited by treatment with 100 nM PTHrP(1-36) for 16 h (66.9±3.4% compared to untreated cells, Fig. 6). Expression of EBP50 in A10 cells reduced the anti-proliferative effect of PTHrP(1-36) such as no differences in thymidine incorporation were observed in the presence or absence of PTHrP(1-36) (Fig. 6). However, treatment with the adenylyl cyclase activator forskolin (1 μM for 16 h) inhibited cells proliferation regardless of EBP50 expression (Fig. 6). Therefore, these data indicate that EBP50 specifically regulates the inhibition of A10 cells proliferation by PTH1R agonists.

PTH1R stimulation by PTHrP(1-36) increases $p27^{kip1}$ and, conversely, over-expression of PTHrP in A10 cells induces proteasomal degradation of $p27^{kip1}$ [23]. We therefore sought to determine if EBP50 influences the effects of PTHrP on the expression of $p27^{kip1}$. To this end $p27^{kip1}$ expression in A10 cells transfected with EBP50 and treated with PTHrP(1-36) was determined by Western Blot (Fig. 6B). Consistent with previous results [23], overnight stimulation with PTHrP(1-36) increased $p27^{kip1}$ expression by 50%. In contrast, the same treatment in A10 cells over-expressing EBP50 had no effect (Fig. 6B). In order to determine the effect of EBP50 on full-length PTHrP-stimulated degradation of $p27^{kip1}$, the expression of EBP50 in A10 cells stably expressing PTHrP was inhibited by siRNA. As shown in figure 6C, this treatment resulted in an 80% reduction of EBP50. However, $p27^{kip1}$ expression was not affected by inhibition of EBP50 expression. Collectively, these observations show that the ability of PTH1R agonists to increase $p27^{kip1}$ expression is inhibited by EBP50, whereas the autocrine effect of PTHrP on $p27^{kip1}$ stability is unaffected.

4. Discussion

Extensive studies showed that parathyroid hormone-related protein (PTHrP) and the parathyroid hormone type 1 receptor (PTH1R) are important regulators of vascular remodeling [3,24,25]. PTHrP is highly expressed in the neointima of injured vessels where it acts to regulate VSMC growth. PTHrP overexpression stimulates cell cycle progression by inducing proteasomal degradation of $p27^{kip1}$ [23], resulting in increased activity of cyclin E/cdk-2 kinase complex and phosphorylation of retinoblastoma protein (pRb) [23]. These effects require the presence of the NLS. In contrast activation of the PTH1R (either by endogenously produced N-terminal PTHrP fragments or by administration of PTHrP(1-36)) inhibits VSMC proliferation, an effect that is largely mediated by the activation of Gs/adenylyl cyclase/cAMP signaling [13,18,26]. Therefore, the PTHrP/PTH1R system generates both proliferative and anti-mitogenic signals and the balance of these opposing actions determines whether VSMC proliferation is accelerated or inhibited. Consequently, the identification of the factors that regulate PTH1R function in VSMC is important for the understanding of the molecular events leading to restenosis.

The expression of the PTH1R in VSMC is highly regulated. We have previously shown that endogenously secreted N-terminal fragments of PTHrP, such as PTHrP(1-36), control PTH1R expression by inducing its internalization and degradation. However, it has recently become apparent that other molecules play important roles on PTH1R expression and signaling. In particular the PDZ domain-containing protein EBP50 binds the PTH1R via a classical PDZ-binding motif at the receptor C-terminus (ETVM) [14,21]. This interaction directs the specificity of G protein coupling and affects PTH1R trafficking.

The role of EBP50 on vascular function is largely unknown. The studies reported here provide the first evidence that EBP50 is dynamically expressed in VSMC and regulates their proliferation. Our data show that: (1) in normal vascular tissues EBP50 expression is restricted to the endothelium with little or no expression in VSMC; (2) EBP50 expression increases in

parallel with PTHrP during neointima proliferation following angioplasty; (3) EBP50 increases PTH1R expression in A10 cells and induces calcium signaling by the PTH1R; and (4) EBP50 significantly attenuates the anti-proliferative effect of PTH1R agonists.

EBP50 expression in vessels

This study demonstrates that EBP50 expression in vessels changes dramatically upon injury. In particular after angioplasty, EBP50 expression increases in the cells forming the neointima. This is to the best of our knowledge the first evidence of dynamic regulation of EBP50 in tissues and suggests that this adaptor protein may participate in the molecular events leading to restenosis. It is also notable that the expression of PTHrP in both normal and injured vessels parallel that of EBP50. Indeed, the concomitant increase in both PTHrP and EBP50 suggests that they may be functionally related. In support of this idea, increasing PTHrP in A10 cells was sufficient to induce significant EBP50 expression. It is however likely that other (as yet unknown) stimuli contribute to EBP50 expression, since the magnitude of the effect of PTHrP was relatively small. To date, the only established activator of EBP50 expression in cell systems is estrogen [27] and more studies are required to fully understand the regulation of EBP50 in VSMC.

EBP50 effects on PTH1R function

As mentioned previously, PTHrP and the PTH1R exert important effects during vascular remodeling and their regulation is important in determining the response of VSMC to injury. The increased expression of EBP50 after angioplasty therefore raises the possibility that it may regulate the activity of the PTH1R. Our data strongly support this hypothesis, since increasing EBP50 expression in A10 cells profoundly affects PTH1R function.

EBP50 increases both total and cell membrane PTH1R. This effect requires a direct interaction between these molecules, since EBP50 does not increase expression of a PTH1R carrying a C-terminal mutation (Met to Ala) that abolishes binding to EBP50 [21]. We have previously shown that PTH1R internalization and degradation occur constitutively in VSMC (expressing low EBP50 levels) in response to the endogenous secretion of N-terminal fragments of PTHrP [18]. In this study we show that PTH1R endocytosis in response to PTHrP(1-36) is mediated by β-arrestins and is delayed by EBP50, an observation that is consistent with previous studies in other cell systems [28-30]. In fact, EBP50 plays a critical role in regulating cellular trafficking of several GPCRs, including β2-adrenergic receptor [31], luteinizing hormone receptor (LHR) [32] and κ-opioid receptor [33]. However, the observation that EBP50 reduces internalization of PTH1R in A10 cells accounts only in part for the increased expression of PTH1R. Indeed, the effect of EBP50 was additive to that of the antagonist Bpa^2-PTHrP, that prevents PTH1R internalization [17,18], indicating that EBP50 increases the targeting of PTH1R to the cell membrane. This effect has been described for some ion transporters [34, 35]. In particular, the membrane localization of the type-2 sodium-phosphate transporter (Npt2a) is greatly perturbed in renal proximal tubules of EBP50-null mice [35] and deletion of the C-terminal PDZ-binding domain of CFTR alters its membrane distribution in epithelial cells [34].

EBP50 critically affects PTH1R signaling in A10 cells. In many cell types, including kidney and bone cells, the PTH1R couples to Gs and Gq to generate cAMP and calcium signals. However, Clemens and coworkers demonstrated that in A10 cells the PTH1R couples mostly, if not exclusively, to Gs [12,13]. Our studies show that in the presence of EBP50 the PTH1R (at endogenous levels) is able to induce calcium signaling in A10 cells, whereas in naïve cells (expressing low levels of EBP50) it does not. A recent study demonstrated the direct interaction of EBP50 with Gq and the increased coupling of the PTH1R to Gq in the presence of EBP50 [36]. Similarly, EBP50 directed PTH1R coupling to Gq but not to Gs [14]. Consistent with

these observations, EBP50 modulates calcium and PLC signaling by the PTH1R in opossum kidney cells [14,15] and in murine proximal kidney tubules [37].

The effect of EBP50 on PTH1R signaling has important consequences for the biological actions of PTHrP. Several studies have shown that stimulation of PTH1R results in inhibition of VSMC proliferation [5,22,38]. These effects are largely mediated by a cAMP-dependent pathway and indeed inhibition of VSMC proliferation can be potently induced by dibutyryl-cAMP, isoproterenol and forskolin [5]. We now show that increased EBP50 expression restores the ability of PTH1R to generate calcium signals, which are associated, directly and indirectly, to increased VSMC proliferation induced by GPCR such as the angiotensin II and the α1-adrenergic receptors [39,40]. The final result is the loss of anti-mitogenic effect of PTHrP (1-36). A possible link between the distinct activation of G protein-mediated signals and the control of the cell cycle emerged from the observations that increasing EBP50 levels in A10 cells affects the expression of the cycle inhibitor $p27^{kip1}$. A previous study, confirmed herein, indicated that relatively low doses of PTHrP(1-36) increase $p27^{kip1}$ expression in A10 cells, an effect that was similar to that of the cAMP analog 8Br-cAMP [8]. We observed that over-expression of EBP50 in A10 cells inhibited the effect of PTHrP(1-36) on $p27^{kip1}$. It is interesting to note that activation of calcium signals (that are promoted by EBP50) and the activity of calmodulin are associated to increased degradation of $p27^{kip1}$ in lymphocytes and smooth muscle cells [41,42]. The relation between EBP50 and PTH1R-mediated signals in controlling $p27^{kip1}$ expression is also suggested by the observation that EBP50 had no effect on the stability of $p27^{kip1}$ in A10 cells over-expressing full length PTHrP (Fig. 6C). In these cells, autocrine actions of PTHrP reduce the half-life of $p27^{kip1}$ independently from the activation of the PTH1R [8,16].

A summary of the current understanding of the regulation of PTH1R in VSMC is shown in figure 7. In resting VSMC, tonic release of low levels of N-terminal fragments of PTHrP causes continuous internalization and down-regulation of the PTH1R [18]. Therefore, modest stimulation of cell proliferation by the nuclear action of PTHrP and modest inhibition of mitogenesis through the PTH1R occur. Arterial injury increases PTHrP expression in VSMC. Consequently, EBP50 levels increase resulting in higher expression and membrane localization of PTH1R. By controlling PTH1R signaling, EBP50 reduces the anti-proliferative effect of PTH1R agonists, leaving the mitogenic action of nuclear PTHrP unopposed thus exacerbating uncontrolled VSMC growth.

While this study focuses on the effect of EBP50 on the regulation of PTH1R activities in A10 cells it is likely that this scaffolding protein affects other important mediators of VSMC growth as well as the responses of other cells (in particular endothelial cells) to vascular injury. Further studies are therefore required to fully characterize the role of EBP50 on vascular remodeling. In conclusion, the studies reported here show that the PDZ domain-containing protein EBP50, which is expressed in VSMC of injured vessels, regulates expression and function of the PTH1R causing an attenuation of the anti-proliferative effect of PTH1R agonists. As such, EBP50 likely contributes to the development of restenosis following angioplasty.

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References

1. Hongo T, Kupfer J, Enomoto H, Sharifi B, Giannella-Neto D, Forrester JS, et al. Abundant expression of parathyroid hormone-related protein in primary rat aortic smooth muscle cells accompanies serum-induced proliferation. J Clin Invest 1991 Dec;88(6):1841–7. [PubMed: 1752945]
2. Okano K, Wu S, Huang X, Pirola CJ, Juppner H, Abou-Samra AB, et al. Parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor and its messenger ribonucleic acid in rat aortic vascular smooth muscle cells and UMR osteoblast-like cells: cell-specific regulation by angiotensin-II and PTHrP. Endocrinology 1994 Sep;135(3):1093–9. [PubMed: 8070351]
3. Fiaschi-Taesch NM, Stewart AF. Minireview: parathyroid hormone-related protein as an intracrine factor--trafficking mechanisms and functional consequences. Endocrinology 2003 Feb;144(2):407–11. [PubMed: 12538599]
4. Orloff JJ, Reddy D, de Papp AE, Yang KH, Soifer NE, Stewart AF. Parathyroid hormone-related protein as a prohormone: posttranslational processing and receptor interactions. Endocr Rev 1994 Feb; 15(1):40–60. [PubMed: 8156938]
5. Massfelder T, Dann P, Wu TL, Vasavada R, Helwig JJ, Stewart AF. Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. Proc Natl Acad Sci U S A 1997 Dec 9;94(25):13630–5. [PubMed: 9391077]
6. Ozeki S, Ohtsuru A, Seto S, Takeshita S, Yano H, Nakayama T, et al. Evidence that implicates the parathyroid hormone-related peptide in vascular stenosis. Increased gene expression in the intima of injured carotid arteries and human restenotic coronary lesions. Arterioscler Thromb Vasc Biol 1996 Apr;16(4):565–75. [PubMed: 8624779]
7. Nakayama T, Ohtsuru A, Enomoto H, Namba H, Ozeki S, Shibata Y, et al. Coronary atherosclerotic smooth muscle cells overexpress human parathyroid hormone-related peptides. Biochem Biophys Res Commun 1994 Apr 29;200(2):1028–35. [PubMed: 8179578]
8. Fiaschi-Taesch N, Sicari B, Ubriani K, Cozar-Castellano I, Takane KK, Stewart AF. Mutant parathyroid hormone-related protein, devoid of the nuclear localization signal, markedly inhibits arterial smooth muscle cell cycle and neointima formation by coordinate up-regulation of p15Ink4b and p27kip1. Endocrinology 2009 Mar;150(3):1429–39. [PubMed: 18845646]
9. de Miguel F, Fiaschi-Taesch N, Lopez-Talavera JC, Takane KK, Massfelder T, Helwig JJ, et al. The C-terminal region of PTHrP, in addition to the nuclear localization signal, is essential for the intracrine stimulation of proliferation in vascular smooth muscle cells. Endocrinology 2001 Sep;142(9):4096–105. [PubMed: 11517189]
10. Ishikawa M, Akishita M, Kozaki K, Toba K, Namiki A, Yamaguchi T, et al. Expression of parathyroid hormone-related protein in human and experimental atherosclerotic lesions: functional role in arterial intimal thickening. Atherosclerosis 2000 Sep;152(1):97–105. [PubMed: 10996344]
11. Abou-Samra AB, Juppner H, Force T, Freeman MW, Kong XF, Schipani E, et al. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. Proc Natl Acad Sci U S A 1992 Apr 1;89(7):2732–6. [PubMed: 1313566]
12. Maeda S, Wu S, Juppner H, Green J, Aragay AM, Fagin JA, et al. Cell-specific signal transduction of parathyroid hormone (PTH)-related protein through stably expressed recombinant PTH/PTHrP receptors in vascular smooth muscle cells. Endocrinology 1996 Aug;137(8):3154–62. [PubMed: 8754733]
13. Wu S, Pirola CJ, Green J, Yamaguchi DT, Okano K, Jueppner H, et al. Effects of N-terminal, midregion, and C-terminal parathyroid hormone-related peptides on adenosine 3′,5′-monophosphate and cytoplasmic free calcium in rat aortic smooth muscle cells and UMR-106 osteoblast-like cells. Endocrinology 1993 Dec;133(6):2437–44. [PubMed: 8243262]
14. Mahon MJ, Donowitz M, Yun CC, Segre GV. Na(+)/H(+) exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. Nature 2002 Jun 20;417(6891):858–61. [PubMed: 12075354]
15. Mahon MJ, Segre GV. Stimulation by parathyroid hormone of a NHERF-1-assembled complex consisting of the parathyroid hormone I receptor, phospholipase Cbeta, and actin increases

intracellular calcium in opossum kidney cells. The Journal of biological chemistry 2004 May 28;279 (22):23550–8. [PubMed: 15037630]

16. Fiaschi-Taesch N, Takane KK, Masters S, Lopez-Talavera JC, Stewart AF. Parathyroid-hormone-related protein as a regulator of pRb and the cell cycle in arterial smooth muscle. Circulation 2004 Jul 13;110(2):177–85. [PubMed: 15210588]
17. Behar V, Bisello A, Bitan G, Rosenblatt M, Chorev M. Photoaffinity cross-linking identifies differences in the interactions of an agonist and an antagonist with the parathyroid hormone/ parathyroid hormone-related protein receptor. The Journal of biological chemistry 2000 Jan 7;275 (1):9–17. [PubMed: 10617579]
18. Song GJ, Fiaschi-Taesch N, Bisello A. Endogenous parathyroid hormone-related protein regulates the expression of PTH type 1 receptor and proliferation of vascular smooth muscle cells. Mol Endocrinol 2009 Oct;23(10):1681–90. [PubMed: 19574446]
19. Bisello A, Chorev M, Rosenblatt M, Monticelli L, Mierke DF, Ferrari SL. Selective ligand-induced stabilization of active and desensitized parathyroid hormone type 1 receptor conformations. The Journal of biological chemistry 2002 Oct 11;277(41):38524–30. [PubMed: 12107160]
20. Salomon Y, Londos C, Rodbell M. A highly sensitive adenylate cyclase assay. Anal Biochem 1974 Apr;58(2):541–8. [PubMed: 4827395]
21. Sneddon WB, Syme CA, Bisello A, Magyar CE, Rochdi MD, Parent JL, et al. Activation-independent parathyroid hormone receptor internalization is regulated by NHERF1 (EBP50). The Journal of biological chemistry 2003 Oct 31;278(44):43787–96. [PubMed: 12920119]
22. Ishikawa M, Akishita M, Kozaki K, Toba K, Namiki A, Yamaguchi T, et al. Amino-terminal fragment (1-34) of parathyroid hormone-related protein inhibits migration and proliferation of cultured vascular smooth muscle cells. Atherosclerosis 1998 Jan;136(1):59–66. [PubMed: 9544732]
23. Fiaschi-Taesch N, Sicari BM, Ubriani K, Bigatel T, Takane KK, Cozar-Castellano I, et al. Cellular mechanism through which parathyroid hormone-related protein induces proliferation in arterial smooth muscle cells: definition of an arterial smooth muscle PTHrP/p27kip1 pathway. Circ Res 2006 Oct 27;99(9):933–42. [PubMed: 17023675]
24. Clemens TL, Cormier S, Eichinger A, Endlich K, Fiaschi-Taesch N, Fischer E, et al. Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. Br J Pharmacol 2001 Nov;134(6):1113–36. [PubMed: 11704631]
25. Schluter KD, Piper HM. Cardiovascular actions of parathyroid hormone and parathyroid hormone-related peptide. Cardiovasc Res 1998 Jan;37(1):34–41. [PubMed: 9539855]
26. Koyama H, Bornfeldt KE, Fukumoto S, Nishizawa Y. Molecular pathways of cyclic nucleotide-induced inhibition of arterial smooth muscle cell proliferation. J Cell Physiol 2001 Jan;186(1):1–10. [PubMed: 11147803]
27. Ediger TR, Kraus WL, Weinman EJ, Katzenellenbogen BS. Estrogen receptor regulation of the Na +/H+ exchange regulatory factor. Endocrinology 1999 Jul;140(7):2976–82. [PubMed: 10385389]
28. Wang B, Yang Y, Abou-Samra AB, Friedman PA. NHERF1 regulates parathyroid hormone receptor desensitization: interference with beta-arrestin binding. Mol Pharmacol 2009 May;75(5):1189–97. [PubMed: 19188335]
29. Wang B, Bisello A, Yang Y, Romero GG, Friedman PA. NHERF1 regulates parathyroid hormone receptor membrane retention without affecting recycling. The Journal of biological chemistry 2007 Dec 14;282(50):36214–22. [PubMed: 17884816]
30. Wheeler D, Sneddon WB, Wang B, Friedman PA, Romero G. NHERF-1 and the cytoskeleton regulate the traffic and membrane dynamics of G protein-coupled receptors. The Journal of biological chemistry 2007 Aug 24;282(34):25076–87. [PubMed: 17599914]
31. Cao TT, Deacon HW, Reczek D, Bretscher A, von Zastrow M. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. Nature 1999 Sep 16;401 (6750):286–90. [PubMed: 10499588]
32. Kishi M, Liu X, Hirakawa T, Reczek D, Bretscher A, Ascoli M. Identification of two distinct structural motifs that, when added to the C-terminal tail of the rat LH receptor, redirect the internalized hormone-receptor complex from a degradation to a recycling pathway. Mol Endocrinol 2001 Sep; 15(9):1624–35. [PubMed: 11518811]

33. Li JG, Chen C, Liu-Chen LY. Ezrin-radixin-moesin-binding phosphoprotein-50/Na+/H+ exchanger regulatory factor (EBP50/NHERF) blocks U50,488H-induced down-regulation of the human kappa opioid receptor by enhancing its recycling rate. The Journal of biological chemistry 2002 Jul 26;277 (30):27545–52. [PubMed: 12004055]
34. Moyer BD, Denton J, Karlson KH, Reynolds D, Wang S, Mickle JE, et al. A PDZ-interacting domain in CFTR is an apical membrane polarization signal. J Clin Invest 1999 Nov;104(10):1353–61. [PubMed: 10562297]
35. Shenolikar S, Voltz JW, Minkoff CM, Wade JB, Weinman EJ. Targeted disruption of the mouse NHERF-1 gene promotes internalization of proximal tubule sodium-phosphate cotransporter type IIa and renal phosphate wasting. Proc Natl Acad Sci U S A 2002 Aug 20;99(17):11470–5. [PubMed: 12169661]
36. Wang B, Ardura JA, Romero G, Yang YS, Hall RA, Friedman PA. Na/H exchanger regulatory factors control PTH receptor signaling by facilitating differential activation of G{alpha} protein subunits. The Journal of biological chemistry. Jun 18; Epub ahead of print.
37. Capuano P, Bacic D, Roos M, Gisler SM, Stange G, Biber J, et al. Defective coupling of apical PTH receptors to phospholipase C prevents internalization of the Na+-phosphate cotransporter NaPi-IIa in Nherf1-deficient mice. Am J Physiol Cell Physiol 2007 Feb;292(2):C927–34. [PubMed: 16987995]
38. Stuart WD, Maeda S, Khera P, Fagin JA, Clemens TL. Parathyroid hormone-related protein induces G1 phase growth arrest of vascular smooth muscle cells. Am J Physiol Endocrinol Metab 2000 Jul; 279(1):E60–7. [PubMed: 10893323]
39. Berk BC, Corson MA. Angiotensin II signal transduction in vascular smooth muscle: role of tyrosine kinases. Circ Res 1997 May;80(5):607–16. [PubMed: 9130441]
40. Hu ZW, Shi XY, Lin RZ, Chen J, Hoffman BB. alpha1-Adrenergic receptor stimulation of mitogenesis in human vascular smooth muscle cells: role of tyrosine protein kinases and calcium in activation of mitogen-activated protein kinase. J Pharmacol Exp Ther 1999 Jul;290(1):28–37. [PubMed: 10381756]
41. Munoz U, Bartolome F, Bermejo F, Martin-Requero A. Enhanced proteasome-dependent degradation of the CDK inhibitor p27(kip1) in immortalized lymphocytes from Alzheimer's dementia patients. Neurobiology of aging 2008 Oct;29(10):1474–84. [PubMed: 17448572]
42. Hassan GS, Williams TM, Frank PG, Lisanti MP. Caveolin-1-deficient aortic smooth muscle cells show cell autonomous abnormalities in proliferation, migration, and endothelin-based signal transduction. American journal of physiology 2006 Jun;290(6):H2393–401. [PubMed: 16415072]

Abbreviations

A10	clonal rat aortic smooth muscle cells
β-arr2-GFP	β-arrestin2-GFP
EBP50	ezrin-radixin-moesin-binding phosphoprotein
GFP	green fluorescent protein
PTHrP	parathyroid hormone-related protein
PTH1R	parathyroid hormone type 1 receptor
VSMC	vascular smooth muscle cell

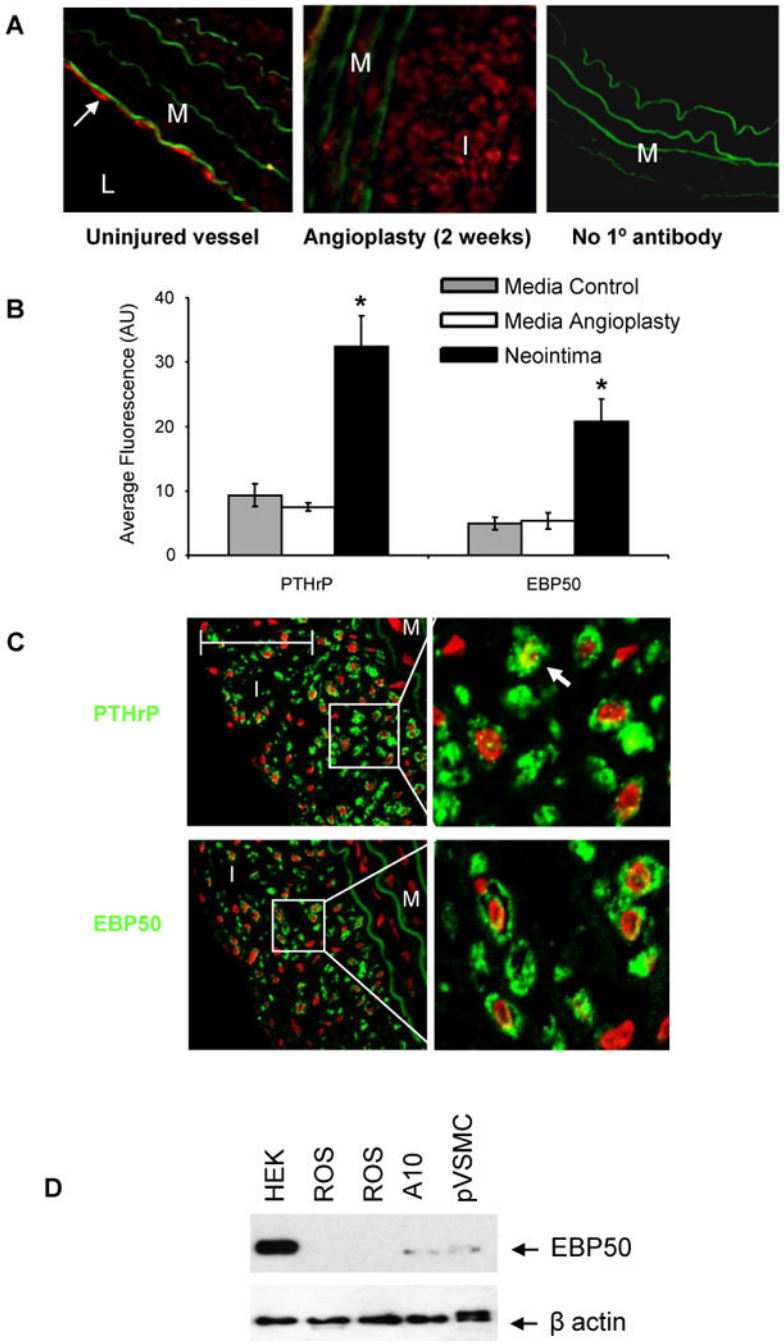

Figure 1. EBP50 up-regulation in the neointima following arterial angioplasty

(A) Expression of EBP50 in rat carotid arteries. Sections from uninjured arteries or 2 weeks after angioplasty were fixed and immunostained for EBP50 (in red). Shown in green are auto-fluorescent elastin fibers. In control vessels EBP50 is localized in endothelial cells (left panel, indicated by the arrow) with very limited staining in the media (M). In contrast, 2 weeks after angioplasty EBP50 is strongly expressed in the neointima (I) (middle panel). No staining was detected in arteries processed in the absence of primary antibody (right panel). **(B)** Quantification of EBP50 and PTHrP expression in carotid arteries. Graphs show the average fluorescence intensity (± SE) of EBP50 and PTHrP in the media and intima determined from sections immunostained as described above. Intimal EBP50 p=0.015 (N=3) and intimal PTHrP

p=0.002 (N=4) versus media. **(C)** Expression of PTHrP and EBP50 in restenotic carotid arteries. Sections from rat carotid arteries 2 weeks after angioplasty were fixed and immunostained for PTHrP (upper panels) or EBP50 (lower panels) (in green). Nuclei were visualized with DRAQ5 (in red). M and I indicate the media and the neointima, respectively. Scale bars, 50 μm. **(D)** Expression of EBP50 in HEK293, ROS, primary VSMC and A10 cells. Equal amounts of proteins (30 μg) were analyzed by Western Blot.

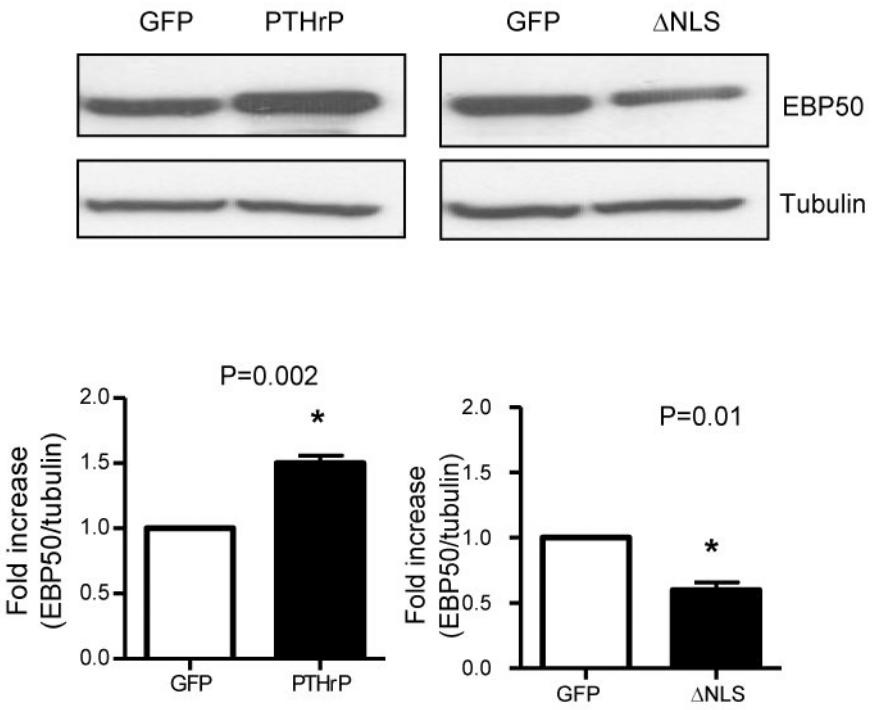

Figure 2. PTHrP regulates EBP50 expression in A10 cells

EBP50 expression in A10 cells stably expressing either PTHrP (left panels) or ΔNLS-PTHrP (right panels) was determined by Western Blot. A10 cells expressing GFP were used as control. Graphs show the expression of EBP50 relative to tubulin (n=7 for PTHrP and 3 for ΔNLS-PTHrP).

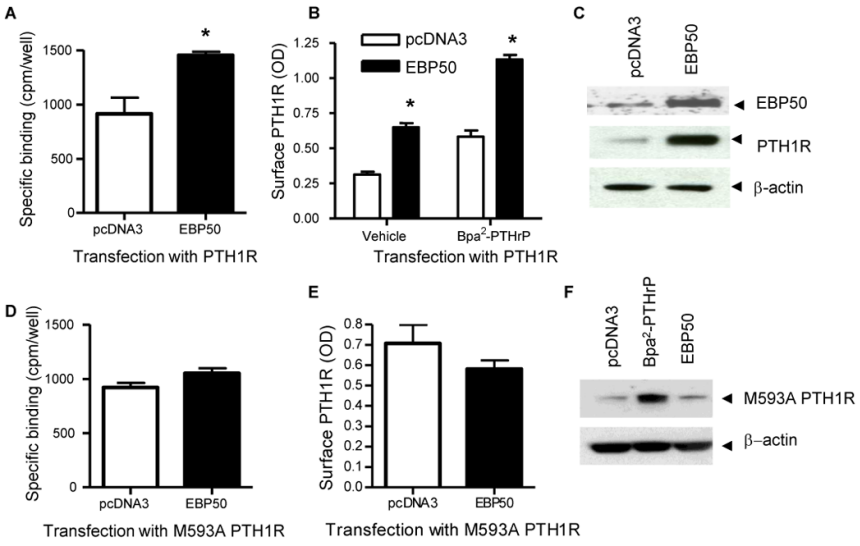

Figure 3. EBP50 increases PTH1R expression in A10 cells

PTH1R expression at the cell membrane was determined by radioligand binding **(A** and **D**) and ELISA (**B** and **E**) in A10 cells expressing either the wild type HA-PTH1R or the mutant M593A (that does not bind EBP50) and transfected with either control plasmid (pcDNA3) or EBP50. Total Binding (TB) and Nonspecific Binding (NS) data for graph **A** were: pcDNA3 TB=1294±148, NS=380±11; EBP50 TB=1984±32, NS=532±64. For graph **D** data were: pcDNA3 TB=1178±43, NS=258±15; EBP50 TB=1288±45, NS=236±10. Graphs show the Specific Binding ±SE of triplicate determinations. Background values for the ELISA assays in **B** and **E** were 0.068 and 0.113, respectively, and were subtracted from the corresponding values. Total PTH1R expression **(C** and **F)** was determined by Western Blot in A10 cells expressing either the wild type HA-PTH1R or the mutant M593A and transfected with either control plasmid (pcDNA3) or EBP50. Where indicated, cells were treated with 1 μM Bpa^2-PTHrP for 48 h. *, $p<0.05$.

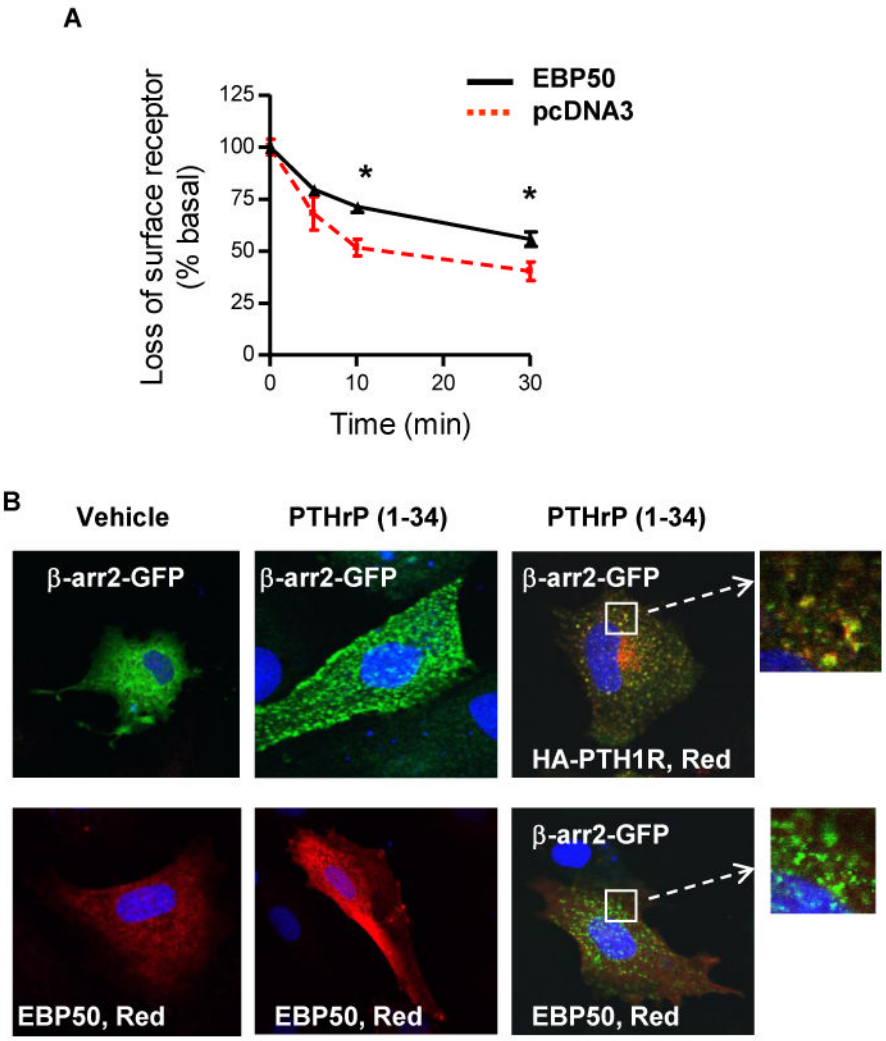

Figure 4. EBP50 reduces internalization of PTH1R in A10 cells

(A) A10 cells were transfected with HA-PTH1R and either control plasmid (pcDNA3) or EBP50. To measure internalization, cells were treated with 100 nM PTHrP(1-36) in DMEM/10% FBS for the indicated times after which PTH1R was measured by ELISA in non-permeabilized cells. **(B)** A10 cells were transfected with β-arr2-GFP and HA-PTH1R. After treatment with vehicle or PTHrP (100 nM for 20 min), cells were fixed and stained for HA-PTH1R or EBP50 (in red), as indicated. β-arr2-GFP was uniformly distributed in the cytoplasm in untreated cells (top left panel). Stimulation with PTHrP(1-36) caused a redistribution of β-arr2-GFP into endosomes (top middle panel) which contained HA-PTH1R (top right panel). The cellular distribution of EBP50 did not change upon PTHrP(1-36) stimulation (bottom panels) and no co-localization of EBP50 with β-arr2-GFP was detected (bottom right panel).

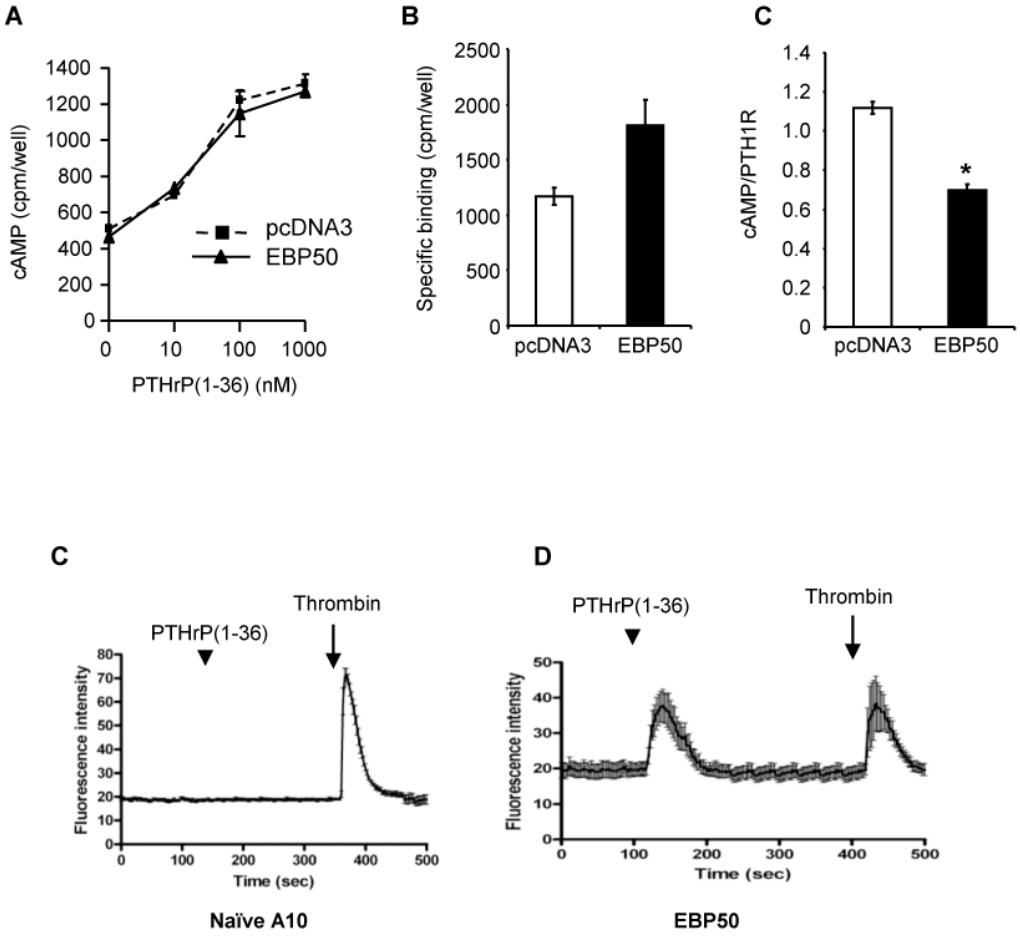

Figure 5. EBP50 regulates PTH1R signaling in A10 cells

(A) Cyclic AMP production in response to PTHrP(1-36). A10 cells were transfected with control plasmid (pcDNA3, dotted line) or EBP50 (solid line), incubated with the indicated concentrations of PTHrP(1-36) at 37 °C for 30 min in the presence of 1 μM IBMX. Results are presented as means ± S.E. in one out of three experiments performed in triplicates. **(B)** PTH1R expression in A10 cells. A10 cells were transfected with control plasmid (pcDNA3, white bar) or EBP50 (black bar) and PTH1R expression was measured by radioligand binding. Results are presented as mean Specific Binding ± S.E. of triplicate determinations. Total Binding (TB) and Non-specific Binding (NS) data were: pcDNA3 TB=1547±181, NS=380 ±11; EBP50 TB=2472±357, NS=661±65. **(C)** Maximal cAMP levels relative to PTH1R expression. Bars represent the ratio between the maximal cAMP (shown in A) and the specific binding of radiolabeled PTH (shown in B) determined in A10 cells transfected with control plasmid (pcDNA3, white bar) or EBP50 (black bar). **(D) and (E)** Real-time recordings of intracellular [Ca^{2+}]. A10 cells transfected with control plasmid (D) or EBP50 (E) were loaded with Fluo4 and treated with 100 nM PTHrP(1-36) and thrombin (10 U/ml) at the times indicated. Fluorescence intensity was measured every three seconds for 500 seconds. Similar results were obtained in three different experiments.

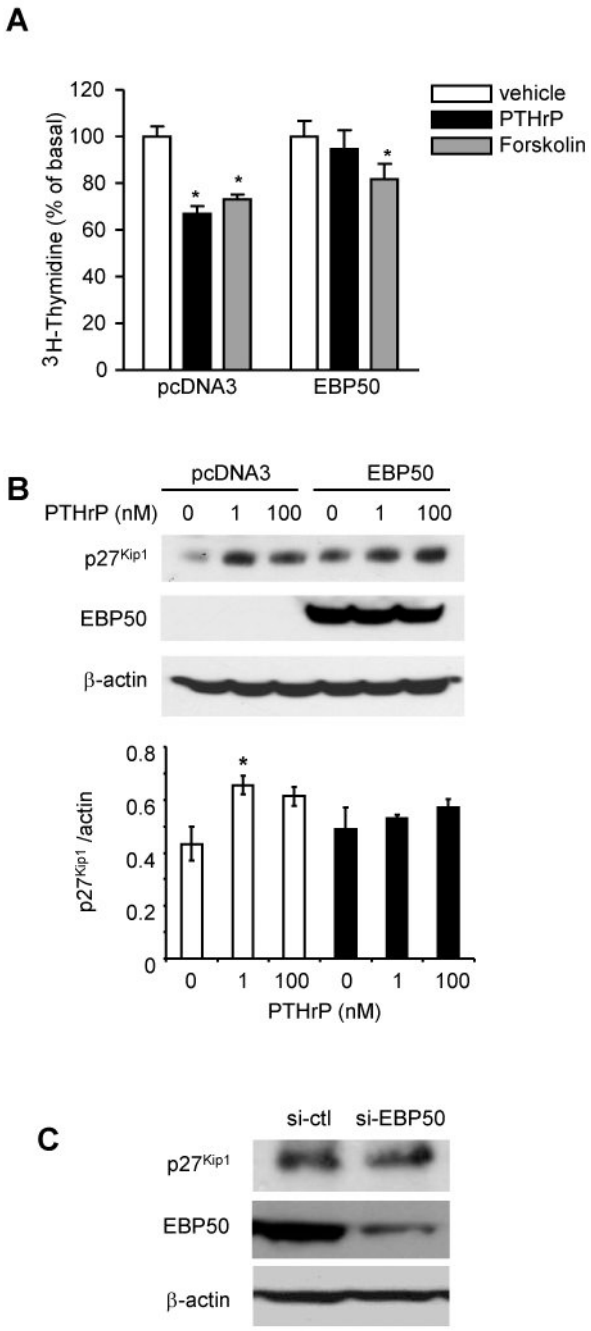

Figure 6. EBP50 expression reduces the anti-proliferative effect of PTHrP(1-36) in A10 cells

(A) Proliferation of A10 cells transfected with control plasmid (pcDNA3) or EBP50, as indicated, and maintained in 1% FBS was determined by measuring ^{3}H-thymidine incorporation either in untreated cells (*white bars*) or in the continuous presence of 100 nM PTHrP(1-36) (*black bars*) or 1 μM forskolin (*gray bars*) for 18 h. EBP50 expression completely inhibited the anti-proliferative effect of PTHrP(1-36). *, $p<0.001$ vs. basal (n=6). **(B)** Effect of EBP50 on PTHrP(1-36)-stimulated $p27^{kip1}$ expression. A10 cells transfected as in (A) were treated with PTHrP(1-36) at the indicated concentrations for 18 h. Expression of EBP50 and $p27^{kip1}$ expression was determined by Western Blot. β-actin was used as loading control. Graph shows $p27^{kip1}$ expression normalized by β-actin (mean ± range) from two independent

experiments. **(C)** Effect of EBP50 on $p27^{kip1}$ expression. A10 cells stably expressing PTHrP were treated with control siRNA (si-ctl) or siRNA against EBP50 (si-EBP50). Three days later, expression of EBP50 and $p27^{kip1}$ was determined by Western Blot. β-actin was used as loading control.

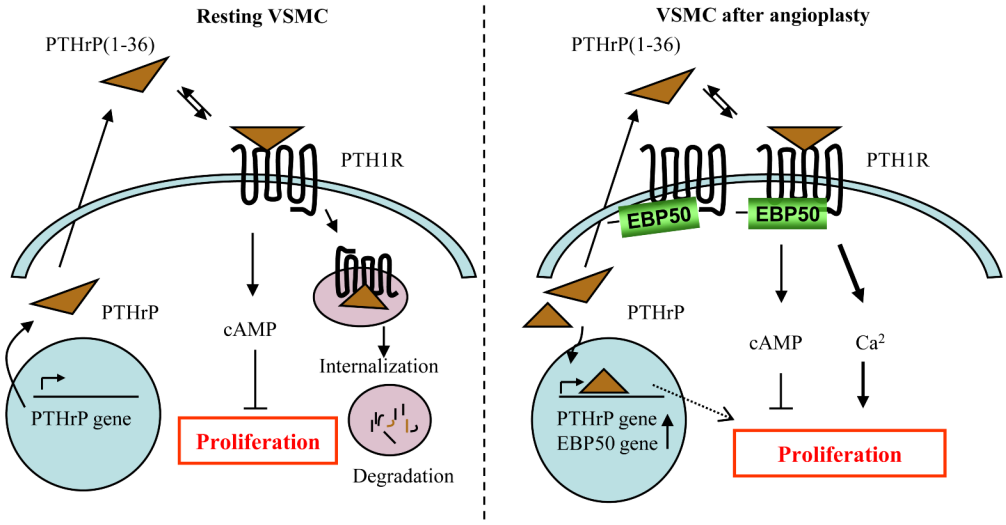

Figure 7. Schematic representation of PTH1R regulation in VSMC

Left panel. In resting VSMC, tonic release of low levels of N-terminal fragments of PTHrP causes continuous internalization and down-regulation of the PTH1R. **Right panel.** Arterial injury increases vascular remodeling that is accompanied by increased expression of PTHrP and EBP50 in VSMC. This results in increased expression and recruitment of PTH1R to the cell membrane. By controlling PTH1R signaling EBP50 reduces the anti-proliferative effect of PTH1R agonists, leaving the mitogenic action of nuclear PTHrP unopposed.